

Christian E.W. Steinberg

Aquatic Animal Nutrition

Organic Macro- and Micro-Nutrients



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Preface

When I started planning *Aquatic Animal Nutrition* as a one-volume book, I have not been aware of the exploding numbers of papers, often good to excellent ones, on this issue in recent years. Many of these contributions originate from China due to simple but effective mechanisms: Chinese researchers may increase their salary by publications in top journals of their discipline, and if they do so and if they are granted highly ranked projects, their staff will be reinforced with good young faculties and good students by their academic institution. And very soon, these laboratories reach the size of small to medium academic departments at Western universities. Nevertheless, it has been effort and pleasure at the same time to sort out intriguing from mediocre papers and to figure out gaps and potential future research directions.

Therefore, *Aquatic Animal Nutrition* (AAN) will be extended. It will now comprise three volumes:

AAN I—A Mechanistic Perspective from Individuals to Generations

AAN II—Organic Macro- and Micro-Nutrients

AAN III—Alternative Feeds and Antinutritional Factors

I thank Springer Nature Switzerland for this courtesy. My sincere appreciation goes to “my Springer ladies,” namely Alexandrine Cheronet and Judith Terpos, who were always very supportive. Particularly warm regards go to Éva Lörinczi who has always been understanding and helpful, even in critical phases of this volume.

The extent of *Aquatic Animal Nutrition* has changed, but my primary interest remains constant. My wish has not been to discuss higher productivity in the aquaculture industries or review recipes for more effective functional aquafeeds in order to increase survival, reproduction, or productivity of farmed animals. Instead, I am interested in answering the question of how certain dietary ingredients influence the life history traits not only of the consumers but also of their succeeding generations. And as an ecologist, my preference is to understand how the aquatic superorganisms function: What is the contribution of the intestinal microbiota to the well-being of the host and, on the other side, how does domestication and farming modify the originally wild animals?

I am thankful to all the photographers and artists who allowed me to use their wonderful images and artworks free of charge. Doubtless, they contribute to an attractive appearance of this book. We all agree that good illustrations can often explain complex ideas much better than thousands of words.

This book is dedicated to my wife, Anette S.

Berlin, Germany

Christian E.W. Steinberg

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Chapter 1

Aquatic Animal Nutrition: Organic Macro- and Micronutrients—‘Do Blind Men and Their Elephant Get Wet Feet?’



Abstract This introductory chapter to organic macro- and micronutrients in aquatic animal nutrition points out recent highlights in identifying nutrient-triggered pathways. It stresses that, in the entire book, particular emphasis will put on the differentiation between esoteric and innovative research in the Kuhnian sense. Innovative research in aquatic animal nutrition has to go beyond production performance and consider aquatic animals as entire, individual ecosystems with complex controlling pathways, in which host-microbiota interactions are central. Therefore, this introduction eventually refers to the classical superorganism concept as guiding principle, recently specified to fishes as hologenomics concept.

“Do blind men and their elephant get wet feet?” What a strange introductory question! Frankly, it may sound strange or even crazy to pure aquaculturists. Other scientists surely claim that it is not scientific at all. At least, it is provocative—and this actually is intended, because since the concerns by, for instance, Hua and Bureau (2012) raised one decade ago and referred to below, the study design and publication practice have not significantly changed for better. In contrast to the emergence of “predatory journals,” which publish papers due to paid publication charges rather than based on scientific contents, the numbers of weak, questionable, or even faked studies have quasi exploded.¹

Turning the table round, let’s continue our bossiness (or is it only a friendly discussion?) at the end of this book. If then the critiques continue, I promise improvement in the next volume of *Aquatic Animal Nutrition* and the answer to the introductory question would be “No!”

“A group of six blind men endeavor to describe an elephant (Fig. 1.1); however, their attempts are thwarted as none of them are able to describe the entire animal.

¹It goes without saying that papers published in predatory journals did not find entry into *Aquatic Animal Nutrition*, provided that these journals have been identified as such. However, there are also journals, still listed in “The Directory of Open Access Journals,” who practice unbelievable short intervals between submission and acceptance of papers (often some 10 days) and still pretend to subject submissions to rigorous reviews. As long as these critical journals are listed in this directory, these papers have not been removed from this book.

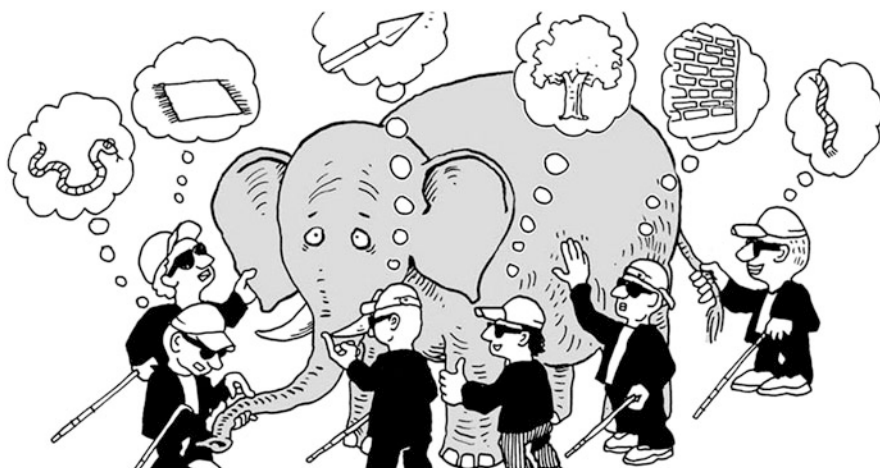


Fig. 1.1 Blind men examining an elephant. (Credit Hans Moller, Amdur)

They then describe the elephant based on their limited experience and their descriptions of the elephant are different from each other. The moral of the parable is that humans have the tendency to claim absolute truth based on their limited, subjective experience as they ignore other people's limited, subjective experiences which may be equally true" (Snyder et al. 1987).

To continue more seriously after this jam: There is an astonishingly huge discrepancy between the numbers of published papers in peer-reviewed journals and the scientific progress in aquatic animal nutrition. Repetitive application of existing approaches and recipes to new aquatic species or transfer to new geographic regions as well as minor variations of existing approaches and recipes applied to well-studied animals is common practice. A striking feature of doing such research is the aim to discover what is known in advance—the basic character of esoteric research (Kuhn 1970). Researchers only find what they want to find; they are biased. They only have, if at all, one hypothesis in mind to test or to refine (Kuhn 1970). Many of the approaches in aquatic animal nutrition are food production-oriented, rather than science-oriented. This implies that most researchers are not open-minded enough and willing to challenge their own expectations. Or as Kuhn has phrased it: "Normal science, the activity in which most scientists inevitably spend almost all their time, is predicated on the assumption that the scientific community knows what the world is like. Much of the success of the enterprise derives from the community's willingness to defend that assumption, if necessary at considerable cost. Normal science, for example, often suppresses fundamental novelties because they are necessarily subversive of its basic commitments. Nevertheless, so long as those commitments retain an element of the arbitrary, the very nature of normal research ensures that novelty shall not be suppressed for very long." Overall, it is the dialectic relationship between normal (esoteric) and innovative research in the Kuhnian sense.

More practically orientated and less based on science history, Hua and Bureau (2012) express their discomfort with many empirical approaches in aquatic animal nutrition. Reviewing fishmeal replacement, the authors meta-analyzed flaws and misconceptions in common (esoteric) research of aquatic animals nutrition. Their concerns, however, are not restricted to fishmeal replacement studies; rather, they generally apply. “It has been common practice to evaluate plant protein ingredients through experimentation and simple inferential statistical analysis. However, there are several weaknesses associated with this approach. Firstly, only a limited number of experimental diets can be investigated per experiment, thus only a limited number of factors can be evaluated per experiment. Secondly, significant variations exist across studies with regard to dietary nutrient compositions (and/or anti-nutritional factors) and nutrient digestibility as well as differences in fish strains, life stage and body weight, body composition, feed intake, growth, feed efficiency, rearing conditions, etc. Thirdly, using fishmeal control diets as a comparison basis across studies may not be valid since the composition and nutritive value of fishmeal is known to vary according to many factors, such as fish species, raw material type (whole fish vs. processing by-product), seasons, and processing temperature... Fourthly, results have been presented and evaluated in different ways of expression. For example, the growth response of experimental fish is expressed as weight gain, specific growth rate, thermal growth coefficient, feed efficiency, and feed conversion ratio. Finally, interpretation and inference of experimental results overly rely on null hypothesis testing of the absence of effect of the experimental diets from the fishmeal control diet. A typical experimental design consists of a control diet with high fishmeal levels and a series of experimental diets formulated with plant protein ingredients replacing either % fishmeal or % protein of fishmeal. Under these conditions, the evaluation of the nutritive value of the test ingredients is not sufficiently robust or specific. Same level of test ingredients may or may not support the optimal growth performance, depending on nutrient composition of the diets” (Hua and Bureau (2012), extract taken with permission from Elsevier).

Politely and well phrased; however, one major reason has to be emphasized more strongly: In most papers of a given species under study, one miracle occurs: worldwide, the species is identical. Acclimation to specific environmental conditions, genetic variations and epimutations do obviously not take place. “A fish is a fish; and a salmon is a salmon”; that simple, stupid! We shall revisit this aspect in the concluding Chap. 40.

One small step into the right direction, Zhang et al. (2020) discuss precision nutritional regulation in aquaculture, which means that the nutrient contents of feed should meet the requirements for an animal’s optimal production performance and achieve minimum fodder waste at the same time. Therefore, the authors propose that precision nutritional intervention needs to be carried out from the perspectives of “precision feeding,” “precision metabolism,” “precision output,” and “precision support” simultaneously. However, production performance is still the major control variable and the other ‘precision’ variables have serving functions.

More biology-based: most considerations of the evolution of diet breadth and associated foraging behaviors have focused on foods, while the emphasis in

explaining the functions and mechanisms of diet choice has been placed on particular chemical characteristics of foods, including the balance and concentrations of nutrients, secondary metabolites, and plant structural compounds. More recently, the application of Nutritional Geometry, a multidimensional approach that focusses on mixtures of nutrients and other food constituents, has shown that neither foods alone nor individual nutrients (e.g., energy or protein content) alone adequately explain foraging, nutritional choices, or demographic responses. Diets are complex meta-mixtures comprising combinations of foods, each of which is itself a mixture of many nutrients and other constituents. The nutrients and chemical constituents of foods interact in sometimes complex ways, and consequently animals have evolved to select and ingest foods to balance the gain of several nutrients and other chemicals. Species, and even individuals within species, have their own specific nutritional requirements in terms of the amount and blend of nutrients that will optimize fitness and these requirements may vary according to traits such as growth rate, mass, sex, age, life history stage, or reproductive status. Long-term studies on model organisms, as well as quantitative syntheses, show that both the amounts and ratios of nutrients in the diet, particularly macronutrients, can have substantial effects on behavior and life history traits such as longevity and reproduction. Additionally, observational studies indicate that some animals foraging in the wild combine foods in their diet to tightly modulate the ingested balance of nutrients (from Machovsky-Capuska et al. (2016) with references therein). We shall return to this, in aquatic animal nutrition, almost completely overlooked approach of Nutritional Geometry several times, since, for instance, elevated cannibalism in farmed carnivores should be considered as inappropriate provision of proteinaceous feeds.

The majority of nutritional papers, study either the host animals or the intestinal microbiota. This simplified approach, however, ignores that food sources act as major environmental determinant of intestine morphology and intestine microbiota (→AAN I (Steinberg 2018)), and these, in turn, determine life history traits of the aquatic animals, such as growth, immune response, or reproduction as well as the epigenome.

Recently, Perry et al. (2020) stressed the role of the gut microbiome in sustainable teleost aquaculture. The authors summarize that current findings show that the teleost gut microbiome plays an important role in aquaculture; however, a knowledge gap exists between ascertaining the composition of the microbiome and understanding its function, partly due to the complexity and variability in the ecology of teleost gastrointestinal tracts and unknown bacterial taxa. More specifically, however, it has been caused by the lack of synthesis between multiple cutting-edge molecular techniques. Progression in teleost gut microbiome research will depend on combining function (RNA sequencing), composition (metabarcoding and metagenomics), and spatial distribution (fluorescence in situ hybridization). Understanding host genetic diversity (population genomics) and expression (RNA sequencing) of that diversity, all while incorporating environmental variation, will also be vital (Perry et al. 2020).

And even more recently, Infante-Villamil et al. (2021) drew the attention to microbiome diversity and dysbiosis in aquaculture. There is an association between

productivity and microbial diversity in aquaculture systems, as changes in bacterial microbiomes are implicated in animal performance, in disease development associated with both bacterial and viral origin, and in dysbiosis triggered by environmental stressors or diet choice. Development of management strategies toward preserving the microbial balance, including maintaining or increasing diversity in the host, is critical for the health of cultured aquatic animals and will likely be critical for the expansion of aquaculture (Infante-Villamil et al. 2021).

Food sources have such a strong impact on consumers that apparently mandatory carnivores, such as salmonids, show symptoms of omnivory. Cherished paradigms are challenged and going to be reconsidered. To illustrate this topic with only one example, Marandel et al. (2018) reassessed the carnivorous habit of brown trouts and found that hepatic glucokinase is expressed in wild fishes in water bodies on Kerguelen Islands indicating the ability to metabolize carbohydrates. This ability may evolve quickly in wild populations submitted to strong environmental change. And Marandel et al. (2018) did not suppress fundamental novelties; they did not defend the paradigm of salmonids being strictly carnivorous. Rather, their paper is comparable to one rock in the “Garden of Empty Nothing” (Fig. 1.2).

One picture tells much more than 1000 words; therefore, we are visiting the Ryōan-ji temple in Kyoto, Japan, with its “Garden of Empty Nothing” (Fig. 1.2). Out of a sweep of smooth pebbles (equivalent to normal papers of esoteric science), a few distinctive larger rock formations stand out. These rock formations are the scientific innovations that cannot be suppressed—only four or five in a plethora of minor pebbles (papers).



Fig. 1.2 “Garden of empty nothing,” Ryōan-ji temple in Kyoto, Japan. (Credit Cquest, Wikimedia)

Consequently, the book *Aquatic Animal Nutrition—Organic Macro- and Micro-Nutrients* is, after stocktaking of each individual organic nutrient, going to put particular emphasis on such rocks or islands looking out of the “sea of empty nothing.”

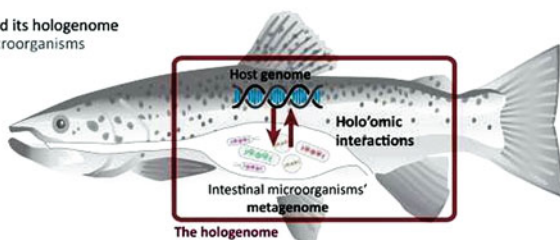
Considerable efforts have already been directed toward the integration of biomolecular tools in aquaculture, which have undergone a revolution over the past decade owing to the rapid development of DNA sequencing techniques (Abdelrahman et al. 2017). So far, such tools have mainly been used to sectorally explore how phenotypic traits related to growth, health and immunity, or fecundity are associated with variation in either (I) the fish genome—very occasionally including also the epigenome—or, more recently, (II) its gut microbiota. Sectoral means that studies devoted to growth performance of the candidate animals focus on biomolecular pathways regulating growth and feed efficiency and do not necessarily consider also whether, and if how, the studied nutrients may affect immunity or fecundity. However, hosts are not isolated from their intestinal (mutualistic, commensal, and pathogenic) microorganisms, and these are instead interconnected and coregulated systems that condition host phenotypes (Limborg et al. 2018).

The classical superorganism concept revived by Wilson and Sober (1989) seems to reemerge. Is this concept more than a poetic metaphor in scientific guise? Sure it is; the superorganism is a multispecies community that functions as an organizational unit and needs a multidisciplinary approach. And, as Limborg et al. (2018) argue, the corresponding methodology is now advanced to cover this complex issue (Fig. 1.3).

This concept and its improvement by Limborg et al. (2018) may serve as long-term goal to aim at. Let’s now return to the Garden of Empty Nothing in the Ryōan-ji temple and figure out some more existing rocks.

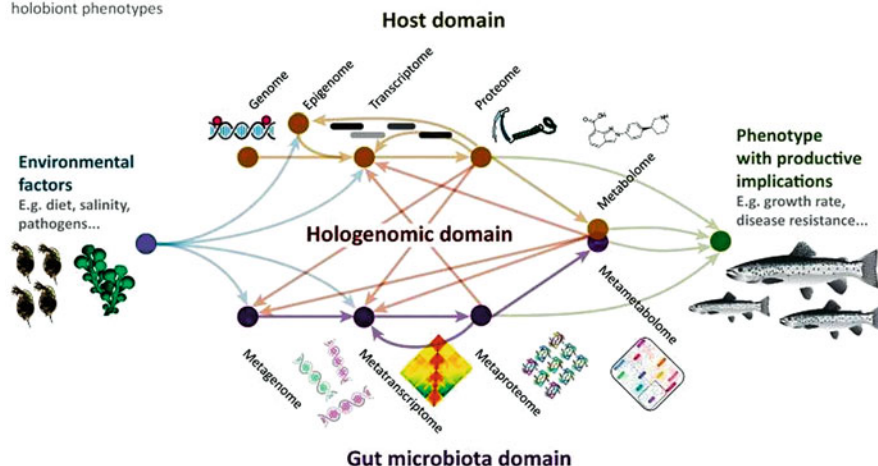
- Emerging rocks that are viewed from all possible sides are the studies in young grass carps which focus exclusively on immunity and health modulated by macro- and micronutrients as well as antinutrients. These studies are published by a consortium at the Sichuan Agricultural University (China). Comparably, the papers on dietary regular or excess carbohydrates and lipids in young Wuchang bream by a consortium at the Nanjing Agricultural University (China) stand out.
- With already a little moss on top, but still a clearly visible rock, the comprehensive study of the European seabass answers the question of whether peptides or amino acids are preferentially taken up, published by collaboration between the Universities of Insubria and Salento, Italy.
- Furthermore, also the self-selection approaches of certain feed qualities by fishes of different feeding habits, such as rainbow trout, European seabass, Senegalese sole, Nile tilapia, goldfish, or tambaqui as well as the underlying circadian rhythmicity (among others, groups at the University of Murcia, Spain, and the Federal University of Bahia, Brazil) deserve mention.
- The emerging functional metabolomics elegantly shows that, under pathogen challenges, even considered worthless metabolites are identified as crucial for

(A) The holobiont and its hologenome
Host + intestinal microorganisms



(B) Holo'omic interactions

Biomolecular interactions between hosts and symbiotic microorganisms triggered by environmental factors yield different holobiont phenotypes



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Fig. 1.3 Arrows indicate the directionality of the effect. Blue arrows indicate environmental effects, which might affect metagenomic composition, gene expression of both the host and its intestinal microorganisms, and also introduce epigenomic variation. Brown arrows show molecular interactions within the host domain, while purple arrows show molecular interactions within the gut microbiota domain. Red arrows highlight holo-omic interactions, in other words potential reciprocal effects between the host and its intestinal microorganisms at different omic levels. Finally, green arrows link all these interactions with host phenotypes. Note the overlapping circles of the host metabolome and the microbiota metametabolome; this indicates that the source of metabolites often cannot be assigned either to the host or the microbiota but is the result of the combination of both domains. (From Limborg et al. (2018), with permission from Elsevier)

improved pathogen resistance in fishes and crustaceans. This applies, for instance, to aspartic acid, phenylalanine, threonine, leucine, proline, taurine, malate, α -ketoglutarate, α -linolenic acid, cellobiose, and even glucose, saturated fatty acids, and likely also oligosaccharides (groups at Sun Yat-sen University, Guangzhou, or Nanjing Normal University, China). There are indications that even the nonprotein amino acids (ornithine and citrulline), so far considered antinutritional, might have comparable controlling functions as recently reported by a laboratory from the Tokyo University of Marine Science and Technology, Japan.

- Several laboratories—pioneered by the Norwegian Institute of Water Research, Oslo—contribute to the impressive reduction of stress symptoms and even cannibalism by appropriate dietary tryptophan supply as found in, for instance, brown trout, rainbow trout, meagre, European seabass, Indian major carp, butter catfish, Atlantic salmon, Senegalese sole, or totoaba.
- Dietary methionine bears great potential as epigenetic modulator and means of offspring nutritional programming as shown by a laboratory from the French National Institute for Agricultural Research (INRA), St-Pée-sur-Nivelle.
- Doubtless, a well-visible huge rock in the temple garden is comprised by the approaches of a laboratory from the Universidade de Vigo, Spain, to identify nutrient sensing in fishes enabling nutrient and metabolic homeostasis.
- Probiotics are beginning to be understood as means to reduce appetite and glucose level in fishes and to overcome adverse effects of high-carbohydrate diets. This temple garden rock points out that probiotics have been overlooked as control variable too long. The Università Politecnica delle Marche, Ancona, Italy, started to fill this gap.
- Unexpectedly, the next rock in the temple garden identifies that glucose transporter may be a double-sided sword. A laboratory from the National Taiwan Ocean University described that a glucose transporter facilitates also the entry of the white spot syndrome virus in giant tiger prawn.
- One in the near term emerging, but currently still submerged, rock should be the introduction of the hormesis concept in aquatic animal nutrition to differentiate between real anti-nutritional only apparently anti-nutritional dietary compounds. The latter exert a beneficial effect if supplied in low doses. Fibers and arachidonic acid have recently been shown to have such biphasic dose-response curves.
- One young, but huge temple garden rock is comprised by epigenetic studies and particularly those of microRNAs in the action of dietary nutrients with the leading laboratory now at the University of Ottawa, Canada. The current stock-taking promises a high variety of unexpected, but fascinating new results. The same applies to epimutations, for instance, in developmental genes as studied by the Consejo Superior de Investigaciones Científicas, Barcelona, Spain. Due to epimutations, domestication can take place even within the first generation isolated from the wild.
- With little moss on its top, the next temple garden rock clearly shows that dietary carbohydrates can affect transcription factor expression that regulates skeletal muscle myogenesis in rainbow trout reported by a laboratory at the University of Idaho, USA. Currently, it cannot be figured out why this finding did not initiate further studies.
- Small, but fascinating rocks that have to prove their applicability in aquaculture are the trophic improvement of fatty acid profiles or increasing the bioavailability of sterols by parasites or saprophytes with free-living zoospore stages, developed in Dutch, German, and Canadian limnologic laboratories. These approaches appear to have the potential of application in biofloc systems in order to extend and even improve the food sources.

- A trilateral collaboration (Universidade do Algarve, Portugal, Sorbonne University, Paris, France, and IRTA–SCR, Spain) has created the next temple garden rock: the action of several vitamins in the ontogenetic development, particularly of flatfishes, which resulted also in intriguing educational graphs.
- The next temple garden stone has the potential to request a revision of dear paradigms. Can water soluble vitamins really be supplied in clear excess without any adverse effect? Intriguing crustacean studies headed by the Bharathiar University, Tamil Nadu, India, demand detailed future studies with improved graded dietary supplies.
- Owing to the collaboration between laboratories at the University of Stirling, Scotland, UK, and University of Porto, Portugal, the discovery of ω x desaturases in a huge variety of invertebrates comprises one of the hugest rocks in the Garden of Empty Nothing. This discovery will lead to the revision of several paradigms about worldwide production and trophic transfer of polyunsaturated fatty acids.
- In the long term, the inclusion of the intestinal microbiota and the life performance of aquatic animals and its dietary modulation will emerge as huge future temple garden rock—aiming at the hologenomics (Limborg et al. 2018). Encouraging beginnings have been carried out, for instance, with starch in Chinese perch (Huazhong Agricultural University, Wuhan, China), lipids and fatty acids in Atlantic salmon (Norwegian University of Science and Technology, Trondheim), zebrafish (Duke University School of Medicine, Durham, United States, or Università Politecnica delle Marche, Ancona, Italy, or Chinese Academy of Agricultural Sciences, Beijing, China), or gilthead seabream (University of Insubria, Varese, Italy).

Finally, keeping in mind the Kuhnian definition of a paradigm, namely: “to be accepted as a paradigm, a theory must seem better than its competitors, but it need not, and in fact never does, explain all the facts with which it can be confronted” (Kuhn 1970), this book wants to encourage studies which challenge existing paradigms rather than those which carry out only esoteric research. Real scientific progress can only be expected beyond or offside esoteric research.

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Chapter 2

Protein Requirement—‘*Only Meat Makes You Strong*’



Abstract Proteins represent the dominant biomass of aquatic animals; consequently, proteins are significant nutrients and energy sources with digestive efficiencies between 60 and almost 100%. For most aquatic animals, the quantity of prey available is typically the nutritional bottleneck. A deficiency of dietary protein or amino acids has long been known to impair immune function and increase the susceptibility of animals to infectious disease. In addition to function as energy source, free amino acids can act as osmolytes. The average dietary protein requirement of fishes is 42%; that of invertebrates appears to be below this value. Protein requirement depends on environmental factors, such as salinity and temperature, as well as trophic level and content of the other macronutrients. Interactions with other macronutrients, however, are not yet adequately considered. Adverse effects occur in animals fed deficient or excess proteinaceous diets. Biomolecular modes of action of hyperproteic diets are beginning to be understood; impairment of the immune system is central. Finally, this chapter points out gaps of protein nutrition in aquatic animals.

From a quantitative perspective, amino acids (AAs), either free or combined in macromolecules (peptides, proteins), represent the dominant dry mass component of most living systems. In general, aquatic animals consume nearly every type of food ranging from sediment, detritus, fruits, algae, or wood to slime, scales, blood, mollusks, echinoderms, arthropods, mammals, and other fishes in order to meet their needs.

Fishes efficiently digest protein from most feedstuffs with high efficiency of some 60% to almost 100% (Table 2.1). Considering that 65–75% of their carcass dry mass is protein, fishes need to absorb protein efficiently from food, if for nothing else, tissue maintenance and growth (although many carnivorous fishes metabolize AAs for energy). Noteworthy, the dietary protein requirement of aquatic invertebrates lies in the same range (Table 2.2).

Table 2.1 Summarized digestive efficiency of protein in selected fishes of different trophic levels consuming artificial feeds. (From German (2011), with permission from Elsevier)

Species name, common name	Trophic level (feeding habit)	Efficiency, %
<i>Cyprinus carpio</i> , Common carp	Omnivorous/benthivorous	80–93
<i>Dicentrarchus labrax</i> , European seabass	Carnivorous	62–97
<i>Ictalurus punctatus</i> , Channel catfish	Carnivorous/omnivorous	66–87
<i>Oncorhynchus mykiss</i> , Rainbow trout	Carnivorous	68–96
<i>Oreochromis niloticus</i> , Nile tilapia	Omnivorous/detritivorous	88–96
<i>Pagrus major</i> , Red seabream	Carnivorous	88–96

Animal prey is richer in proteins and more digestible than plant or detrital materials (Fig. 2.1). However, the quantity of prey available is typically the bottle-neck. It is common for fishes to ingest well below 5% of body mass per day when feeding on invertebrate prey. This is consistent with the notion that morphology and behavior of fishes feeding extensively on invertebrates are adapted to optimize energy gain (Bowen et al. 1995). Algae, aquatic macrophytes, and detritus are all inferior to fishes and aquatic invertebrates as sources of protein and energy. Furthermore, protein supply is the major constraint for detritivores.

Dietary protein is the major and most expensive component of formulated aquafeeds; fishmeal is generally considered to be the most ideal protein source for aquatic animals (Li et al. 2009). A deficiency of dietary protein or AAs has long been known to impair immune function and increase the susceptibility of animals to infectious disease (Li et al. 2007). Aquatic invertebrates, fish eggs, embryos, and larvae are no exception, and several studies point out the importance of AAs for their catabolic and anabolic energy metabolism. To demonstrate this importance, we refer to hybrid striped bass and zebrafish: glutamate (Glu) and glutamine (Gln) are more actively oxidized in the proximal intestine, liver, and kidney of fish than glucose and palmitate. Glu provides more energy than Gln in all tissues except in the liver where Gln serves as the main metabolic fuel. In the skeletal muscles, Glu is the preferred nutrient to generate adenosine triphosphate (ATP). Together, Glu plus Gln (plus leucine, Leu) contributes to about 80% of ATP production in the fish tissues (Jia et al. 2017).

In addition to the function of AAs as energy source, free AAs (fAAs), individually or in concert, can act as osmolytes in habitats of varying salinity as recently shown in Amazon river prawn *Macrobrachium amazonicum* (Mazzarelli et al. 2015) and San Francisco brine shrimp *Artemia franciscana* (Zadehmohseni et al. 2020).

Table 2.2 Typical crude protein requirement in selected aquatic animals

Species	Requirement, % dry matter	References
<i>Scientific name, common name</i>		
<i>Invertebrates</i>		
<i>Astacus leptodactylus</i> , Narrow clawed crayfish	30	Ghiasvand et al. (2012)
<i>Babylonia areolate</i> , Ivory shell	45	Zhou et al. (2007)
<i>Cherax quadricarinatus</i> , Red claw crayfish	25	Pavasovic et al. (2007)
<i>Eriocheir sinensis</i> , Chinese mitten crab	35–50	Pan et al. (2005)
<i>Farfantepenaeus aztecus</i> , Northern brown shrimp	40–51	In Shiau (1998)
<i>F. californiensis</i> , Yellow leg shrimp	35	
<i>Fenneropenaeus indicus</i> , Indian prawn	43	
<i>F. merguensis</i> , Banana shrimp	34–42	Sedgwick (1979)
<i>F. chinensis</i> , Chinese white shrimp	45	Wu and Dong (2002)
<i>Haliotis asinina</i> , Ass’s-ear abalone	27	Bautista-Teruel et al. (2003)
<i>H. discus hannai</i> , Disk abalone	30	Ma et al. (2020)
<i>H. fulgens</i> , Green abalone	40.4–44.1	Gómez-Montes et al. (2003)
<i>H. iris</i> , Black-footed abalone	38.8–42.3	Tung and Alfaro (2011)
<i>H. laevigata</i> , Greenlip abalone	24–~35 ^a	Stone et al. (2013)
<i>H. midae</i> , South African abalone	22–26	Green et al. (2011)
<i>Jasus edwardsii</i> , Southern rock lobster	29–31	Ward et al. (2003)
<i>Limulus polyphemus</i> , Atlantic horse-shoe crabs	8.7 ^b	Tzafrir-Prag et al. (2010)
<i>Litopenaeus setiferus</i> , Northern white shrimp	28–44	Andrews et al. (1972), Guzman et al. (2001)
<i>L. stylirostris</i> , Western blue shrimp	27–40	Rosas et al. (2001), Martinez-Cordova et al. (2003)
<i>L. vannamei</i> , Pacific white shrimp	25–48	Smith et al. (1985)
	79–86	
<i>Lytechinus variegatus</i> , Green sea urchin	~20	Hammer et al. (2006)
<i>Macrobrachium americanum</i> , Cauque river prawn	37	Méndez-Martínez et al. (2017)
<i>M.idea</i> , River prawn	27–40	Sethuramalingam and Gideon (2003)
<i>M. malcolmsonii</i> , Monsoon river prawn	35	Thirumurugan and Subramanian (2004)
<i>M. nipponense</i> , Oriental river prawn	33	Zhang et al. (2017a)
@ 20°C	38	Lv et al. (2021)
@ 25°C	42	
@ 30°C	34	
<i>M. rosenbergii</i> , Giant river prawn	13–40	Gomez et al. (1988), Felix and Prince Jeyaseelan (2006)

(continued)

Table 2.2 (continued)

Species	Requirement, % dry matter	References
<i>Marsupenaeus japonicus</i> , Kuruma shrimp	34–57	Deshimaru and Yone (1978), Koshio et al. (1993)
<i>Penaeus monodon</i> , Black tiger shrimp	33–50	Alava and Lim (1983), Syama Dayal et al. (2003)
<i>Pomacea urceus</i> , Edible apple snail	20	Ramnarine (2004)
<i>Portunus trituberculatus</i> , Swimming crab	52	Jin et al. (2013)
<i>Procambarus clarkii</i> , Red swamp crayfish	30	Hubbard et al. (1986)
<i>Scylla serrata</i> , Giant mud crab	32–45	Catacutan (2002), Unnikrishnan and Paulraj (2010)
<i>Sipunculus nudus</i> , Peanut worm	43	Zhang et al. (2015)
<i>Fishes</i>		
<i>Acanthopagrus macrocephalus</i> , Blackhead bream	41	Zhang et al. (2010)
<i>Acipenser baerii</i> ♀ × <i>A. gueldenstaedtii</i> ♂, Hybrid sturgeon	34–37	Guo et al. (2012)
<i>Anabaris grahami</i> , Fuxian minnow	37	Deng et al. (2013)
<i>Anabas testudineus</i> , Climbing perch	40	Hossain et al. (2012)
<i>Anguilla anguilla</i> , European eel	36–45	De La Higuera et al. (1989)
<i>A. japonica</i> , Japanese eel	44	Okorie et al. (2007)
<i>A. rostrata</i> , American eel	47	Tibbetts et al. (2000)
<i>Arapaima gigas</i> , Pirarucu	~49	Rodrigues et al. (2019)
<i>Barbonymus altus</i> , Red tailed tinfoil	40	Elangovan and Shim (1997)
<i>B. gonionotus</i> , Silver barb	32	Mohanta et al. (2008)
<i>Bidyanus bidyanus</i> , Silver perch	37	Yang et al. (2002)
<i>Brachymystax lenok</i> , Manchurian trout	~44	Lee et al. (2001)
<i>Brycon orbignyanus</i> , Piracanjuba	~31	De Borba et al. (2003)
<i>Candidia barbata</i> , Lake Candidus dace	32	Shyong et al. (1998)
<i>Carassius auratus</i> , Goldfish	29	Lochmann and Phillips (1994)
<i>C. gibelio</i> , Gibel carp	juveniles	Ye et al. (2017b)
	pre-adults	
<i>Gibelion (Catla) catla</i> , Major Indian carp, catla	30	Renukaradhya and Varghese (1986)
<i>Cebidichthys violaceus</i> , Monkeyface eel	19	Fris and Horn (1993)
<i>Centropristis striata</i> , Black seabass	53	Shah Alam et al. (2008)
<i>Channa micropeltes</i> , Indonesian snakehead	52	Wee and Tacon (1982)
<i>C. punctatus</i> , Spotted snakehead	~47	Zehra and Khan (2012)
<i>C. striata</i> , Striped snakehead	55	Mohanty and Samantaray (1996)

(continued)

Table 2.2 (continued)

Species		Requirement, % dry matter	References
<i>C. maculata</i> ♀ × <i>C. argus</i> ♂, Hybrid snakehead		51	Zhang et al. (2017b)
<i>Chanos chanos</i> , Milkfish		~40	Jana et al. (2006)
<i>Chelon ramada</i> , Thinlip grey mullet		24	Papaparaskeva-Papoutsoglou and Alexis (1986)
<i>Cichlasoma urophthalmus</i> , Mexican mojarra		33	Martínez-Palacios et al. (1996)
<i>Chirostoma estor</i> , Mexican (pike) silverside		41	Martínez-Palacios et al. (2007)
<i>Cirrhinus mrigala</i> , Mrigal carp		32	Singh et al. (2008)
<i>Clarias batrachus</i> , Walking catfish		40	Jindal (2011)
<i>C. nieuhoftii</i> , Slender walking catfish		40	Kiriratnikom and Kiriratnikom (2012)
<i>Coptodon zillii</i> , Redbelly tilapia		35	Mazid et al. (1979)
<i>Ctenopharyngodon idella</i> , Grass carp		41–43	Dabrowski (1977)
<i>Culter alburnus</i> , Topmouth culter		40	Zhang et al. (2016)
<i>Cyprinus carpio</i> , Common carp	adult	25–50	Murai et al. (1985), Kaushik (1995)
	Fry	43–47	Nose (1979)
	Fingerling and grower	34–37	
	Adult	28–32	
<i>C. carpio</i> var. Jian, Jian carp		34.1	Liu et al. (2009)
<i>Dascyllus aruanus</i> , Striped damsel		36	Vijayagopal et al. (2008)
<i>Dicentrarchus labrax</i> , European seabass		32–56	Peres and Oliva-Teles (1999)
<i>Diplodus vulgaris</i> , Two-banded seabream		35	Ozório et al. (2009)
<i>Eleginops maclovinus</i> , Patagonian blennie		35	Sa et al. (2014)
<i>Epinephelus akaara</i> , Red-spotted grouper		50.5	Wang et al. (2016)
<i>E. coioides</i> , Orange-spotted grouper grow-out stage (~100 g) large size (275 g)		48.0	Luo et al. (2004)
		46.7	Yan et al. (2020b)
		43.8	Yan et al. (2020a)
<i>E. fuscoguttatus</i> ♀ × <i>E. lanceolatus</i> ♂, Hybrid grouper		45–54	Jiang et al. (2015)
<i>E. malabaricus</i> , Malabar grouper		42	Chen and Tsai (1994)
<i>Gadus morhua</i> , Atlantic cod		~55	Pérez-Casanova et al. (2009)
<i>G. morhua</i>	40–107 g	47–52	Árnason et al. (2010)
	400–900 g	36	
<i>Gobiocypris rarus</i> , Chinese rare minnow		35	Wu et al. (2016)
<i>Hemibarbus maculatus</i> , Spotted barbel		35	Chen et al. (2010)
<i>H. nemurus</i> , Asian redbtail catfish		42	Khan et al. (1996)

(continued)

Table 2.2 (continued)

Species		Requirement, % dry matter	References
<i>H. wyckioides</i> , Asian red-tailed catfish		44	Deng et al. (2011)
<i>Heteropneustes fossilis</i> , Stinging catfish		40	Khan and Abidi (2012)
<i>Hippoglossus hippoglossus</i> , Atlantic halibut		48–62	Grisdale-Helland and Helland (1998)
<i>Hypophthalmichthys nobilis</i> , Bighead carp		30	Santiago and Reyes (1991)
<i>Ictalurus punctatus</i> , Channel catfish		24	Garling and Wilson (1976)
<i>Ictiobus niger</i> , Black buffalo		41	Guy et al. (2018)
<i>Labeo fimbriatus</i> , Fringed-lipped carp		26–27 (dp)	Jena et al. (2012)
<i>L. rohita</i> , Rohu, roho labeo		30–40	Renukaradhya and Varghese (1986), Singh et al. (2005)
<i>Liza haematocheila</i> , Redlip mullet	1-year 2-year	40–45 35	Yoshimatsu et al. (1992)
<i>L. ramada</i> , Thinlip grey mullet		24	Papaparaskeva-Papoutsoglou and Alexis (1986)
<i>Lates calcarifer</i> , Asian seabass, barramundi		40–53	Bermudes et al. (2010)
<i>Lepomis macrochirus</i> , Bluegill sunfish		42	Yang et al. (2016)
<i>Lutjanus argentimaculatus</i> , Mangrove red snapper		40–43	Abbas et al. (2011)
<i>Megalobrama terminalis</i> , Triangular bream		44	Yang et al. (2017)
<i>Melanogrammus aeglefinus</i> , Haddock		~55	Kim and Lall (2001)
<i>Metynnis hypsauchen</i> , Silver dollar		35	Singh et al. (2007)
<i>Mugil cephalus</i> , Flathead grey mullet		30	De et al. (2012)
<i>Nibea coibor</i> , Chu’s croaker		44–48	Huang et al. (2017)
<i>Notemigonus crysoleucas</i> , Golden shiners		29	Lochmann and Phillips (1994)
<i>Oncorhynchus mykiss</i> , Rainbow trout		24–35	Mahmud et al. (1996), Kim (1997)
<i>Oplegnathus fasciatus</i> , Parrot fish		45	Kim et al. (2017)
<i>Oreochromis aureus</i> , Blue tilapia		34	Winfree and Stickney (1981)
<i>O. mossambicus</i> , Mozambique tilapia		40	Jauncey (1982)
<i>O. niloticus</i> , Nile tilapia		29–40	Siddiqui et al. (1988), Kpundeh et al. (2015)
<i>O. niloticus</i> , Genetically Improved Farmed Tilapia		29	Liu et al. (2017)
<i>O. niloticus</i> × <i>O. aureus</i> , Hybrid tilapia		24	Shiau and Huang (1989)
<i>Pampus argenteus</i> , Silver pomfret		49	Arshad Hossain et al. (2010)
<i>Parachanna obscura</i> , Obscure snakehead		45–56	Kpogue et al. (2013)
<i>P. aetnarius</i> , Cortez flounder		47	González-Félix et al. (2014)
<i>P. lethostigma</i> , Southern flounder		51	González et al. (2005)

(continued)

Table 2.2 (continued)

Species	Requirement, % dry matter	References
<i>P. olivaceus</i> , Japanese or olive flounder	32–37 45–51	Kim et al. (2002) Lee et al. (2002)
<i>Piaractus mesopotamicus</i> (<i>Colossoma mitrei</i>), Pacu	35	Merola (1988)
<i>Platichthys stellatus</i> , Starry flounder	50	Lee et al. (2006)
<i>Pleuronectes platessa</i> , European plaice	70	In Oliva Teles et al. (2020)
<i>Poecilia reticulata</i> , Trinidad guppy	55	Kithsiri et al. (2010)
<i>Protonibea diacanthus</i> , Blackspotted croaker	49	Li et al. (2016)
<i>Pseudoplatystoma reticulatum</i> , Cachara catfish	49	Gomes Cornélio et al. (2014)
<i>Rhamdia quelen</i> , Black catfish	37	Salhi et al. (2004)
<i>Rutilus kutum</i> , Kutum	41.6	Ebrahimi and Ouraji (2012)
<i>Salmo salar</i> , Atlantic salmon smolt	58	Nordgarden et al. (2002)
<i>Sciaenops ocellatus</i> , Red drum	20–25 (db)	McGoogan and Gatlin (1998)
<i>Scophthalmus maximus</i> , Turbot	55–70	Liu et al. (2014), Caceres-Martinez et al. (1984)
<i>Sebastes schlegelii</i> , Rockfish	50	Cho et al. (2015)
<i>Seriola lalandi</i> , Yellowtail kingfish	63	Miegel et al. (2010)
<i>S. quinqueriata</i> , Japanese yellowtail	38	Morimoto Kofuji et al. (2006)
<i>Siganus canaliculatus</i> , White-spotted spinefoot	36	Yousif et al. (1996)
<i>S. guttatus</i> , Goldlined spinefoot	30	Parazo (1990)
<i>S. rivulatus</i> , Spinefoot rabbitfish	~40	El-Dakar et al. (2011)
<i>Silurus asotus</i> , Amur catfish	45	Kim et al. (2014)
<i>Sinocyclocheilus grahami</i> , Dianchi golden-line barbell	~40	Deng et al. (2014)
<i>Sparus auratus</i> , Gilthead seabream	47	Ibarz et al. (2007)
<i>Sperata seenghala</i> , Giant river catfish	35	Ramzan Ali et al. (2014)
<i>Spinibarbus hollandi</i> , Taiwanese cyprinid	31	Yang et al. (2003)
<i>Symphysodon</i> spp., Discus	45–50	Chong et al. (2000)
<i>Takifugu obscurus</i> , Obscure puffer	42	Ye et al. (2017a)
<i>T. rubripes</i> , Tiger puffer	41	Kim and Lee (2009)
<i>Tor putitora</i> , Himalayan mahseer	40–45	Islam and Tanaka (2004)
<i>T. tambroides</i> , Malaysian mahseer	48	Ng et al. (2008)
<i>Totoaba macdonaldi</i> , Totoaba or totuava	~50	Minjarez-Osorio et al. (2012)
<i>Trachinotus carolinus</i> , Florida pompano	45	Lazo et al. (1998)
<i>Umbrina cirrosa</i> , Shi drum	51	Akpinar et al. (2012)

db digestible protein

^aTemperature dependent^bmg of digestible protein for maintenance per day per gram body mass

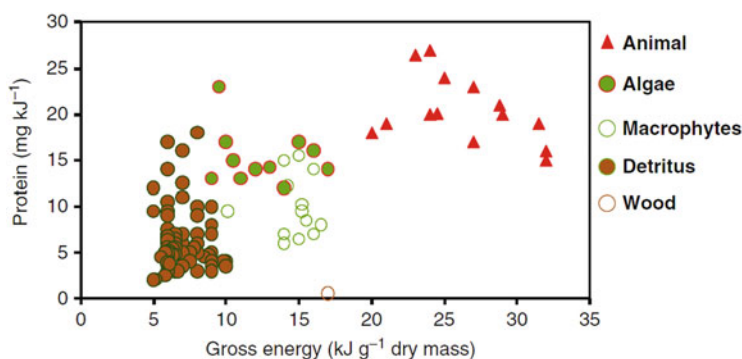


Fig. 2.1 Protein and gross energy in food resources available to aquatic consumers. (From German (2011) after Bowen et al. (1995), with permission from Elsevier)

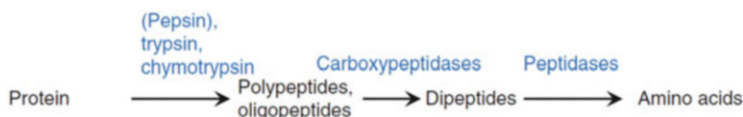


Fig. 2.2 Protein and the endogenous digestive enzymes fishes produce to hydrolyze them. The digestion of proteins requires multiple steps, beginning with initial hydrolysis in the lumen by extracellular enzymes and followed by membrane digestion by intestinal mucosal enzymes. The enzyme pepsin is listed in parentheses because agastric species (i.e., those that lack an acidic stomach) generally lack this. (From German (2011), with permission from Elsevier)

2.1 Requirement

Estimates of fish dietary protein requirements from many taxa are 2–4 times greater than the amount needed by other vertebrates (German 2011). Most fish species are carnivorous and are adapted to use protein as a preferred energy source over carbohydrate, and thus require high levels of dietary protein (20→70%: *Cebidichthys violaceus*→*Scophthalmus maximus*, *Pleuronectes platessa*) with an average of 42% (Oliva Teles et al. 2020). Figure 2.2 shows schematically the steps of protein digestion.

Some invertebrates appear to have lower protein requirements. The few papers, which studied protein requirement during the ontogenetic development, indicate that it decreases with increasing age (Table 2.2). In *Litopenaeus vannamei*, Smith et al. (1985) detected that the growth of small individuals is influenced more by protein level than protein source whereas growth of medium and large ones is more influenced by protein source than protein level. Various decapod crustaceans have a wide range of quantitative optimal dietary protein requirements strongly depending on the protein quality.

Early life stages of fishes can acquire exogenous proteinaceous nutrients as AAs or peptides, rather than intact proteins. In fact, the composition of the dietary proteins triggers life history traits. Nevertheless, even recent studies address gross protein requirements of fishes or aquatic invertebrates (Table 2.2) and continue to disclose that protein requirements follow an optimum dose-response curve.

Traditional in the experimental approach, Guy et al. (2018) evaluated the effects of dietary protein levels on growth and body composition of juveniles of the threatened omnivorous black buffalo (*Ictiobus niger*). Therefore, artificial propagation and culture are frequently a part of native species recovery plans, and developing formulated diets is a critical component of these plans. This study shows that a diet with 41% crude protein produces optimal growth for juvenile black buffalo.

Several data about protein requirement from different papers mutually agree well, others deviate considerably. Such discrepancies within one species are not astonishing, if one considers

- The high diversity of feed qualities applied (→Chap. 1, Hua and Bureau (2012))
- The missing standardization of larval age or developmental stages of juveniles used
- The feeding history of offspring and parental individuals (→AAN III “Nutritional Programming”)
- The genetic and epigenetic diversity within one species (→Chap. 40)

Abalone species are generally characterized by slow and heterogeneous growth rates. Therefore, artificial feed for abalone must contain sufficient protein and essential AAs (EAAs) in order to satisfy their nutrient requirements. To optimize aquaculture of the intrinsically herbivorous animals, artificial diets have to be amended with animal proteins. Bautista-Teruel et al. (2003) fed *Haliotis asinina* with fishmeal, shrimp meal, defatted soybean meal, and *Arthrospira* (*Spirulina*) sp. Highest weight gain (WG) is attained with a combination of fishmeal, shrimp meal, and defatted soybean meal. Astonishingly, abalone on pure plant protein diets (soybean, spirulina) shows lower WG than on mixed protein sources: The plant diets have relatively low methionine contents; therefore, the AA pattern is not appropriate and, obviously, the main reason for the low abalone growth on the pure plant diet.

Several papers demonstrate adverse effects in animals, if diets are deficient or in excess of proteins. Such effects are well documented in *Babylonia areolate*, *Eriocheir sinensis*, *Haliotis discus hannai*, *H. midae*, *H. iris*, *Jasus edwardsii*, *Penaeus monodon*, *Procambarus clarkia*, *Scylla serrata*, *Acipenser baerii* ♀ × *A. gueldenstaedtii* ♂, *Anguilla anguilla*, *Barbonymus altus*, *Gibelion catla*, *Diplodus vulgaris*, *Epinephelus malabaricus*, *Hippoglossus hippoglossus*, *Hypophthalmichthys nobilis*, *Labeo fimbriatus*, *L. rohita*, *Mystus nemurus*, *Oreochromis niloticus*, *Platichthys stellatus*, *Protonibea diacanthus*, *Barbonymus gonionotus*, *Scophthalmus maximus*, *Coptodon zillii*, or *Totoaba macdonaldi* (references in Table 2.2).

The mechanism by which excess of dietary protein adversely affects life history traits has to be discussed. Due to the limited capacity of digestive enzymes, excess dietary protein can be excreted as nitrogenous waste into the environment (Burford and Williams 2001). In an elaborate study, it has been demonstrated that particularly trypsin sets the physiological limit on growth rate and feed conversion (Torrisen et al. 1994; Lemieux et al. 1999). This is one of the classical modes of action.

In addition to limited trypsin capacity, hyperproteic diet proteins can cause oxidative damage on pancreas functions (Gu and Xu 2010) and acute hepatocellular injury in small mammals (Oarada et al. 2012). Furthermore, hyperproteic diet can reduce weight (Andriamihaja et al. 2010; Camiletti-Móiron et al. 2015). Whether this weight reduction is combined with oxidative stress is discussed controversially, since several studies report decreased internal oxidative stress upon hyperproteic diet (Lacroix et al. 2004; Machín et al. 2004). Therefore, more than one mechanism appears to be responsible for reduced growth or WG upon excess dietary protein supply. Illustrative biomolecular studies are sketched below.

Since many fishes are usually cultured outside their optimal temperature range, it is important to identify the nutritional requirements under these non-optimal thermal conditions. A few years ago, Bowyer et al. (2013) showed that the protein level can be reduced in diets for most marine species, but it is the quality and digestibility of nutrients in the diet that matters when fishes are cultured at non-optimal temperatures. At cooler temperatures, fish metabolism is reduced that, in turn, lowers the gut-transit time, digestibility, digestive enzyme activity and affects the uptake and absorption of nutrients required for energy and growth. Therefore, high-quality protein and low-lipid diets are necessary during cool-water periods.

In the same line of evidence, Oliva Teles et al. (2020) show in a meta-analysis that dietary protein requirements are directly related to fish trophic position and water salinity and slightly, but significantly inversely related to rearing temperature (Fig. 2.3).

Recent biomolecular studies shed some more light on underlying mechanisms of increased immunity. In juvenile big head carps, consumption of optimal dietary protein amounts improves liver immune responses (lysozyme, immunoglobulin heavy chain, alkaline phosphatase activity) (Sun et al. 2019). Transcriptome analysis identifies differentially expressed genes (DEGs) in the liver in response to different dietary protein levels, and bioinformatics links many DEGs to immune responses, inflammatory responses, and energy metabolism (Fig. 2.4). Moreover, abnormal serum biochemical indices are apparent in the high protein group, indicating that consuming excess protein aggravates liver metabolic burden.

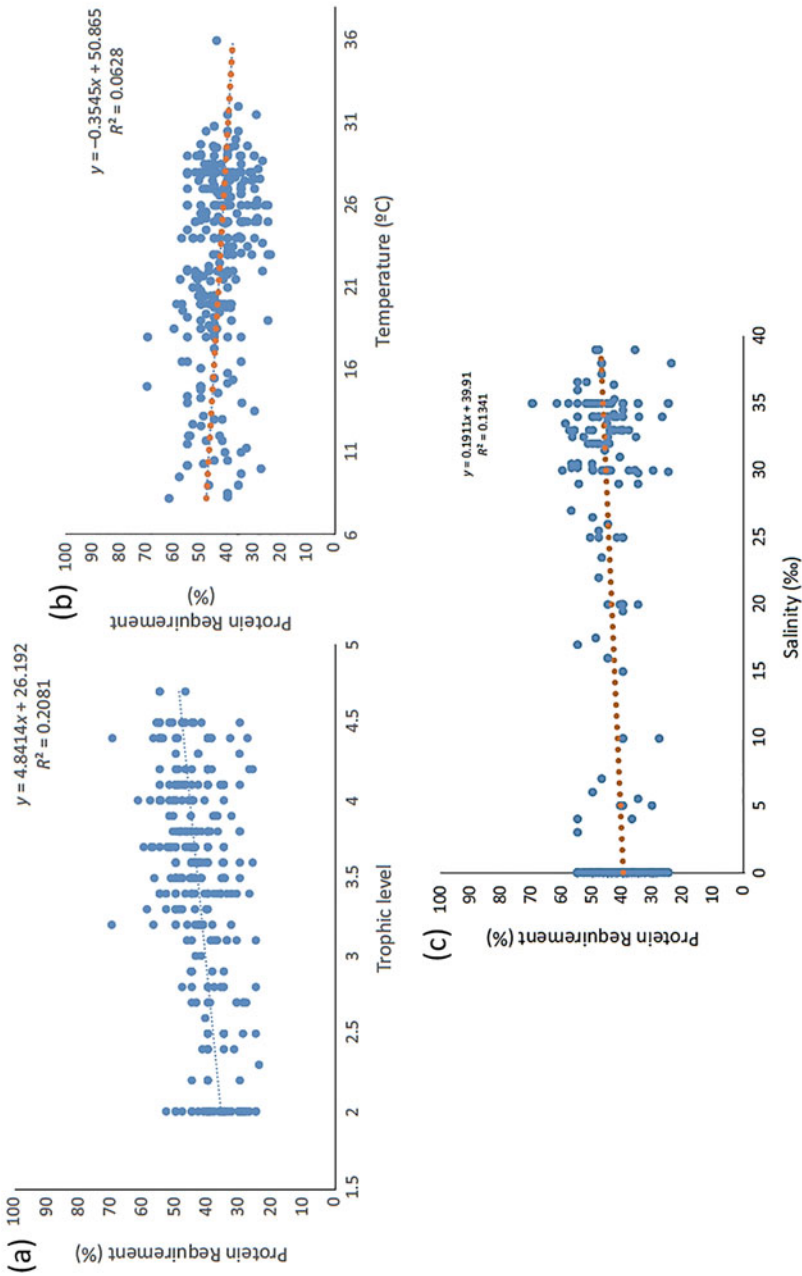


Fig. 2.3 Dietary protein requirement (%) of fishes as function of trophic levels (a), temperatures (b), and salinities (c). (From Oliva Teles et al. (2020), with permission from Wiley)

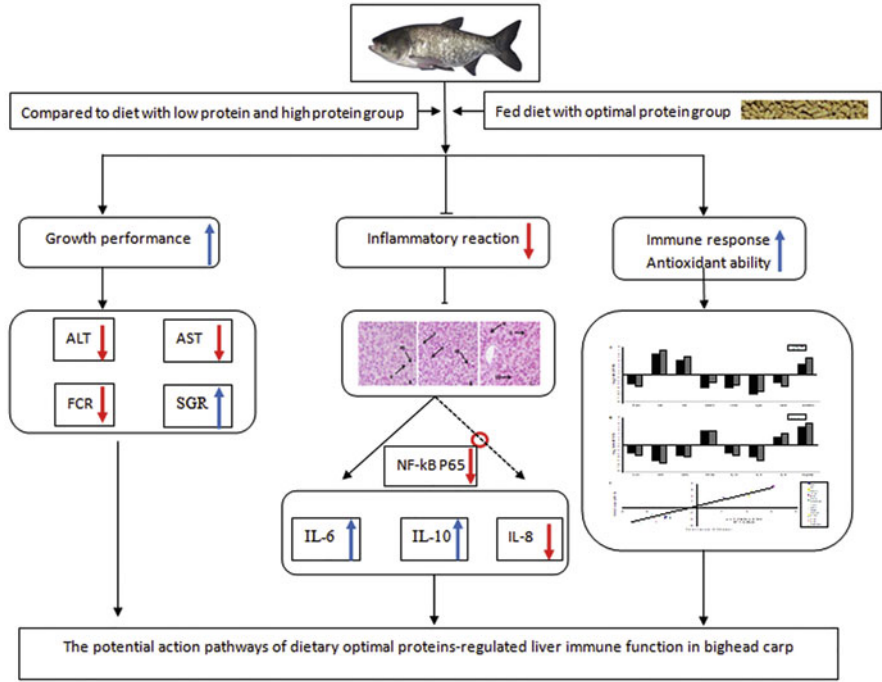


Fig. 2.4 Potential dietary protein-regulated pathways related to liver immune functions in juvenile bighead carp. *Blue* upregulated; *Red* downregulated. (From Sun et al. (2019), with permission from Elsevier)

Box 2.1 The Complement System

The complement system, also known as complement cascade, is a part of the immune system that enhances (complements) the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promote inflammation, and attack the pathogen’s cell membrane. It is part of the innate immune system (Janeway et al. 2001), which is not adaptable and does not change during an individual’s lifetime. The complement system can be recruited and brought into action by antibodies generated by the adaptive immune system.

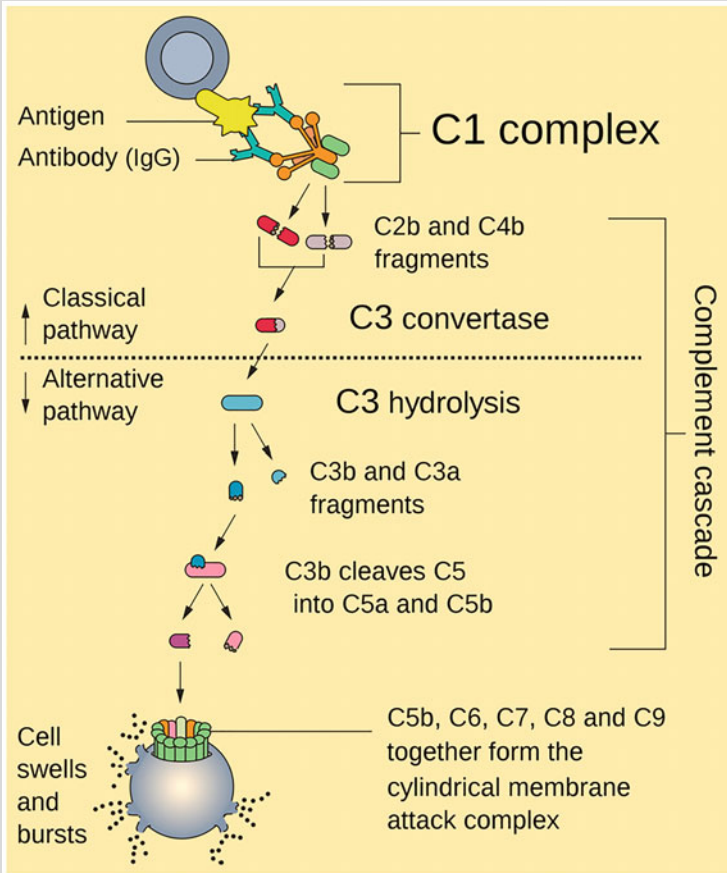
The complement system consists of a number of small proteins that are synthesized by the liver and circulate in the blood as inactive precursors. When stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The result of this complement activation (or complement fixation cascade) is stimulation of phagocytes to clear foreign and damaged material, inflammation to attract additional phagocytes, and activation of the cell-killing membrane attack complex. Over 30 proteins and protein fragments make up

(continued)

Box 2.1 (continued)

the complement system, including serum proteins and cell membrane receptors.

In brief, three biochemical pathways activate the complement system: the classical complement pathway, the alternative complement pathway, and the lectin pathway (Ricklin et al. 2017) Box Fig. 1.



Box Fig. 1 Scheme of the complement system. The complement system is made up of about 30 proteins that work together to “complement” the action of antibodies in destroying bacteria. The term “complement” was coined by Nobel Laureate Paul Ehrlich (Chaplin 2005). Complement proteins circulate in the blood in an inactive form. When the first protein in the complement series is activated—typically by antibody that has locked onto an antigen—it sets in motion a domino effect. Each component takes its turn in a precise chain of steps known as the complement cascade. The product is a cylinder inserted into—and puncturing a hole in—the cell’s wall. With fluids and molecules flowing in and out, the cell swells and bursts (credit: DO11.10, Wikimedia)

The most closely linked metabolic pathways include glycolysis/gluconeogenesis, followed by pyruvate metabolism, the citrate cycle, and nitrogen metabolism. Some pathways associated with the immune system are also identified, including cell adhesion molecules, the PI3K-Akt signaling pathway,¹ complement and coagulation cascades, the Toll-like receptor signaling pathway,² and the NF- κ B signaling pathway. Many genes associated with the biosynthesis of immunity-related signaling pathways are detected in the LP vs. OP and HP vs. OP comparisons, including immune responses (lysozyme, immunoglobulin, alkaline phosphatase, etc.), inflammatory reactions (nuclear factor kappa B, interleukin-8, interleukin-10, etc.), and antioxidant ability (catalase, glutathione transferase, glutathione peroxidase, etc.). The discovery of immune-related pathways and unigenes provides one significant theoretical basis for understanding the molecular mechanisms of dietary protein regulation of liver function.

In one invertebrate equivalent *Macrobrachium nipponense*, Lv et al. (2021) showed that the interaction of dietary protein supply and rearing temperature affects alanine aminotransferase (ALT) and complement component 4 (C4). In contrast to bighead carp, immunity of this shrimp does not significantly improve solely due to modulation of dietary protein contents. In a marine counterpart, Ma et al. (2020) identified that deficient or excessive dietary protein levels depress the growth, health, and anti-stress capacity of abalone (*H. discus hannai*). 17.6% or 43.3% (but 30%, Table 2.2) of dietary protein contents are not recommended (Fig. 2.5): on non-appropriate diet, pro-inflammatory cytokine *tnf- α* is upregulated (Fig. 2.5a), and *nrf2*, a major transcription factor of cytoprotective responses to oxidative stress is downregulated (Fig. 2.5b). Consequently, animals are increasingly susceptible to heat stress (Fig. 2.5c).

2.2 Amino Acid Landscapes

Proteins differ from each another. It can easily be predicted that different AA patterns (“landscapes”) translate into higher trophic levels along the food chain. Consequently, a central challenge is to understand how alterations of community structure transmit into food web processes (Hooper et al. 2005). Herein, macroinvertebrates are a critical trophic link between basal energy sources and higher-order consumers. If macroinvertebrate taxa differ in the quality and quantity

¹Intracellular signaling pathway is important in regulating the cell cycle, directly related to cellular quiescence, proliferation, cancer, and longevity. PI3K activation phosphorylates and activates AKT (protein kinase B), localizing it in the plasma membrane (King et al. 2015).

²Toll-like receptor signaling pathways play crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns derived from various microbes. Toll-like receptors signal through the recruitment of specific adaptor molecules, leading to activation of the transcription factors NF- κ B and IRFs, which dictate the outcome of innate immune responses (Kawasaki and Kawai 2014).

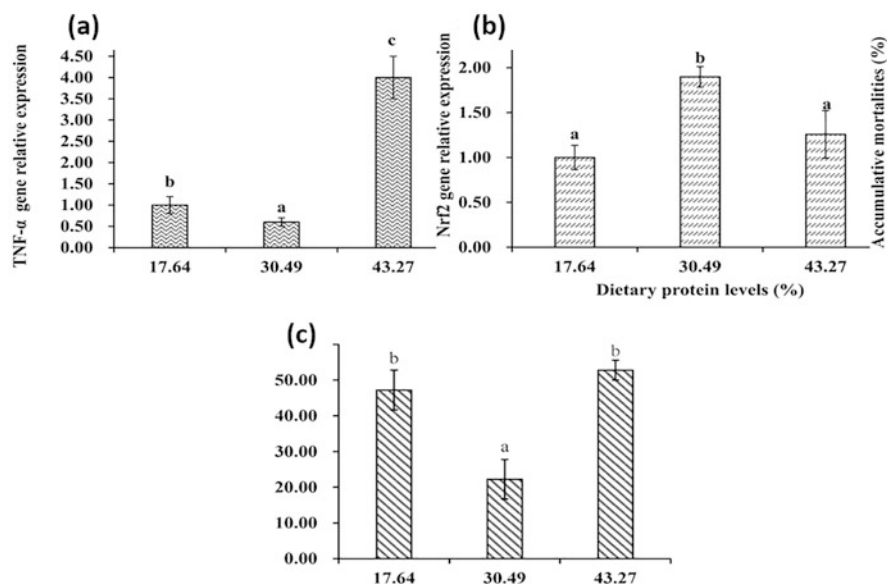


Fig. 2.5 Expressions of the immune-related genes (*tnf- α* (a) and *nrf2* (b)) in the hepatopancreas and accumulative mortalities (c) of abalone *H. discus hannai* after the heat challenge test. Abalones were fed diets with different protein levels. All data are mean \pm SE of three replicates. Different letters indicate significant differences ($P < 0.05$). (From Ma et al. (2020), with permission from Elsevier)

of nutrients they contain, then alteration of macroinvertebrate community composition will affect predator fitness (Dwyer et al. 2018). These authors showed that AA composition vary significantly among taxa; simulated deterioration of macroinvertebrate communities changes the AA landscape, resulting in lower availability of essential AAs, particularly threonine, phenylalanine, proline, and tyrosine to carnivores.

In a succeeding paper, Dwyer et al. (2020) verified that changes in AA landscapes affect the growth of individuals of higher trophic levels and the success of these populations. They determined whether AA composition of animal prey alone affects protein synthesis efficiency and N wastage of a freshwater carnivore. River blackfish (*Gadopsis marmoratus*) were fed two diets differing only in AA composition: the first diet matches the composition of the fish themselves, representing a balanced “ideal protein,” whereas the second diet matches the composition of a major prey (*Macrobrachium australiense*). By measuring the postprandial increase in metabolic rate and ammonia excretion, it turns out that the AA composition of the fish diet is associated with an increase in protein synthesis, whereas the shrimp diet doubles the amount of dietary AAs directed to catabolic energy production and N wastage.

In a subsequent study, Shakya et al. (2021) show that the AA profile of freshwater macroinvertebrates is not only taxon-specific but can vary temporally (between seasons) and spatially between sites depending on taxa. A change in the community

composition of macroinvertebrates can therefore potentially alter the nutritional landscape available to higher-order consumers within riverine environments. How the influence of seasonal and spatial variation in AAs due to inter- and intraspecies differences translates into the growth and metabolism of top predators and across the food web deserves future studies and may be almost as significant as the biochemical bottom-up effect of dietary polyunsaturated fatty acids (→Chap. 30). Therefore, in the following chapters, the controlling effects of individual AAs in aquafeeds will be demonstrated.

2.3 Nutrient-Specific Foraging

How does an animal identify which macronutrients are essential and which may have been deficient in previous feedings? Furthermore, how does it understand or feel that compensatory feeding is overdue? This is an ultimate goal in nutritional ecology.

In nature, many herbivores and omnivores adjust their food selection behavior to regulate the intake of multiple nutrients. Carnivores, however, are generally assumed to optimize the rate of prey capture rather than select prey according to nutrient composition. In an intriguing study, however, Mayntz et al. (2005) showed experimentally that invertebrate predators can forage selectively for protein and lipids to redress specific nutritional imbalances. This selection can take place at different stages of prey handling: The predator may select among foods of different nutritional composition, eat more of a prey if it is rich in nutrients that the predator is deficient in, or extract specific nutrients from a single prey item. This applies particularly to an environment where there is a high probability of encountering nutritionally heterogeneous foods.

There is a growing awareness that nutritional phenotypes are best understood in a multidimensional context, where foraging is viewed as a process of balancing the intake and use of multiple nutrients to satisfy complex and dynamic nutrient needs (Raubenheimer et al. 2009). Simpson and Raubenheimer (1993) coined the term “geometry of nutritional decisions” of an animal, and Mayntz et al. (2005), for instance, reported about nutrient-specific foraging in invertebrate predators.

In details, a common approach has been to assume that a single component dominates these interactions, with the focus usually on energy, nitrogen, or allelochemicals. However, a substantial body of data exists for a number of systems showing that this simplification is not in general warranted because consumer–food relations are usually dominated by the simultaneous effects of several dietary components. Numerous laboratory studies have shown that this view can yield novel insights into unresolved questions and provide a framework for generating new hypotheses (Raubenheimer 2011). Comparably controlled conditions as in laboratory studies do also exist in aquaculture so that this approach is feasible for application in this discipline, since it has already been successfully applied to companion animals (Raubenheimer et al. 2015). Raubenheimer (2011) proposed mixture triangles, which enable an n -dimensional problem to be visualized in an $n-1$

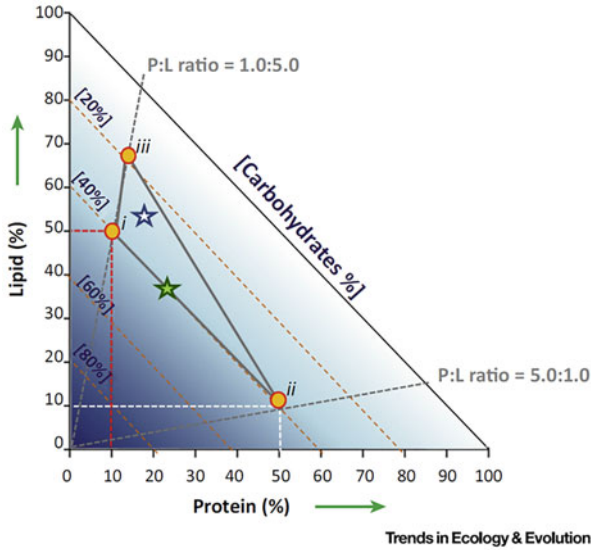


Fig. 2.6 Right-Angled Mixture Triangles (RMTs) provide a platform to plot three components in 2D graphs using proportional data (Raubenheimer et al. 2009). Here Machovsky-Capuska et al. (2016b) model foods, each with a different mixture of protein (%P), lipid (%L), and carbohydrate (%C). Carbohydrate concentration increases with density of blue shading (i.e., across the negative diagonal carbohydrate isolines). Hypothetical food **i** contains 10% P, 50% L, and 40% C, whereas food **ii** contains 50% P, 10% L, and 40% C. The model also illustrates that if an animal mixes its intake of two foods (e.g., **i** and **ii**), the resulting diet composition is constrained to lie on the line connecting the foods (e.g., green star). By mixing its intake of three foods (e.g., **i**, **ii**, and **s**), the accessible space expands to a triangle connecting these foods (e.g., blue star). (From Machovsky-Capuska et al. (2016b), with permission from Elsevier)

dimensional space. A recently developed tool from nutritional geometry is the Right-Angled Mixture Triangle (RMT) (Fig. 2.6). Each point represents a mixture of protein (P), lipid (L), and carbohydrate (C). %P and %L increase along the x- and y-axes, respectively, and the P:L ratio of a mixture is given by the slope of the radial that connects the point to the origin. %C of a point is determined as the difference between 100% and the value at which a negatively sloped diagonal through the point intersects with the two axes (Fig. 2.6).

The application of RMTs is shown in a recent example. To understand the nutritional requirements of species and predict their response to environmental changes, Rowe et al. (2018) combined nutritional geometry and metabolic performance. The authors provide evidence that the Indo-Pacific damselfishes (*Abudefduf vaigiensis*) adjust their energy intake and select specific macronutrients in their diets, thereby reducing the effects of important environmental conditions such as thermal variation on critical metrics of performance when stressed.

In particular, the proportional macronutrient intakes by *A. vaigiensis* in different diet and temperature treatments are shown in Fig. 2.7, together with the macronutrient composition of the experimental foods and an estimate of the natural diet. A first point to note is that the fishes feed non-randomly. This is evident in Fig. 2.7 as

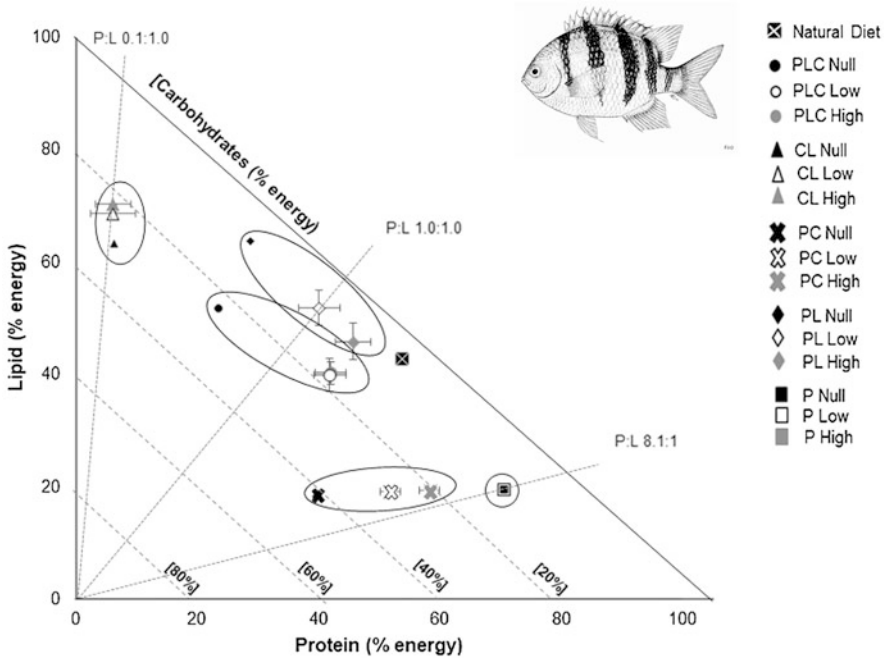


Fig. 2.7 Right-Angled Mixture Triangle showing foraging choices of damselfish. Each diet represents a proportional mixture of protein (P), lipid (L) and carbohydrate (C) (by energy). To geometrically define diets in an RMT, % P is plotted against % L. Considering that the three macronutrients in the mixture sum to 100%, plotting % P (first axis) and % L (second axis) will automatically reflect the value of % C in the third axis (Raubenheimer 2011). Diets are presented as mean (\pm SE) for each free choice (PLC) and restricted treatments (CL, PC, and PL) and compared with a null hypothesis that damselfish consume equal proportions of foods (solid black) at low (hollow black) and high temperatures (solid grey). Rowe et al. (2018) also included in the RMT the 98% energetic dietary intake (black square with white cross) of *A. vaigiensis* in the wild (estimated from Fr  d  rich et al. (2009)). The grey dotted lines given by the slope of the radial that connects the point to the origin represent the P:L ratios of the highest and lowest P:L treatments offered. (From Rowe et al. (2018), with permission from Springer Nature; image credit FAO)

the proportions of macronutrients eaten by fishes differed from the null hypothesis that *A. vaigiensis* consume equal proportions of foods.

There is considerable spread in macronutrient intake because of the different nutritional treatments, and there is a strong effect of temperature, but it varied depending on the treatment (Fig. 2.7). Specifically, high temperatures increase the proportional energy intake on the different nutritional treatments. The animals tend to adjust the macronutrient intake closer to their natural diet, especially by increasing protein consumption in the high-temperature treatment (Fig. 2.7). Such nutrient-specific diet selection is consistent with the ecology of marine organisms, which forage in nutritionally complex and fluctuating marine environments that vary spatially and temporally (Tait et al. 2014; Machovsky-Capuska et al. 2016a, 2018).

In nature, dietary choices can have profound effects on growth, survival, and reproduction, and the mechanisms that determine what fish choose to eat have been

strongly molded by natural selection. Fish bring these mechanisms with them into culture, where they can cause problems, in terms of production, welfare, and environmental impact (Raubenheimer et al. 2012). Long-established cultured species that are farmed intensively, such as salmonids, are usually provided with feeds formulated to contain sufficient nutrients to meet all their known requirements. In theory, fish cultured in this way have neither the need nor the opportunity to be selective about their food. However, there are reasons why an ideal situation is rarely achieved. In the first place, even for well-established species, knowledge about requirements is incomplete. Second, devising optimal feeds for fish of all ages raised in different environments (with respect to temperature, light regimes, and water quality, for example) and that differ in status with respect to maturation, and disease may simply not be possible. In addition, the capacity to provide what is known to be ideal may be compromised by problems of the sustainable supply of potential feed ingredients. For all these reasons, cultured fish may well be given food that is imperfect, with the feed formulation representing a compromise involving nutritional requirements, processing and economic and environmental constraints (Raubenheimer et al. (2012) and references therein). Choosing to feed on smaller conspecifics is a natural aspect of diet selection and a means to overcome protein deficiencies, since smaller conspecifics represent high-quality prey for piscivores, at least in terms of provision of all nutrients needed for growth; cannibalism is therefore a natural feeding strategy in some fishes that are cultured (Jobling et al. 2012). Moreover, elevated cannibalism in farmed carnivores should be considered as inappropriate provision of proteinaceous feeds.

2.4 Concluding Remarks

There is an increasing catalog of studies figuring out the dietary protein requirement in farmed aquatic animals. It is obvious that proteins are significant nutrients and energy sources. However, most approaches follow the traditional format of production studies: feeding → weighing → calculating losses by excretion. Since most often the protein sources vary, the resulting productive traits become almost incomparable. This concern applies to studies of most macro- and micronutrients and is an immanent weakness of studies of productive traits. Furthermore, interactions of macronutrients and their influence on protein requirements are well documented from ecological studies, but considered rather scarcely in aquaculture. It can be predicted that the application of the sketched RMT approach has the potential to improve aquafeeds' quality. Moreover, in the age of highly developed biomolecular and bioinformatics techniques, the weakness of the classical aquaculture production approach has to be overcome. In brief:

- The studied strains of aquatic species have to be characterized including hints of potential specific life history traits as discussed in depth in Chap. 40. This information should be deposited in central aquaculture data base.

- The effect of proteinaceous nutrients on various traits has to be traced back to the “omics” levels to identify general regulatory pathways and to provide a means to translate from studied population to the other one and from one species to the other one. One encouraging example is shown above with the affected immunity traits in bighead carp.
- Feedback mechanisms of the intestinal microbiota have to be elucidated.
- Of particular interest is further the identification of biomolecular (genetic, epigenetic) pathways of excess nutrition: Which traits are affected when aquatic animals are fed proteinaceous feeds in excess, and how does this interact with other macro- and micronutrients?
- Almost all papers neglect the circadian rhythmicity of the animals. They consider creatures more or less as simple cybernetic systems. However, in AAN I “Chrononutrition” (Steinberg 2018), we have seen that all animals studied so far, from invertebrates up to mammals, are subject to a circadian rhythmicity with distinct ups and lows of metabolic activity. The unstudied aquatic animals are no exception to this rule.

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Chapter 3

Utilization of Proteinaceous Nutrients— *‘Becoming Strong with Meat’*



Abstract This chapter focuses on the utilization of proteinaceous nutrients in various aquatic animals. In invertebrates, carnivores tend to have the highest and herbivores the lowest protease content and activity. In many fishes, the capability of self-selection of the appropriate diet in terms of quality and quantity can be observed. In a brief inventory, it is shown that most fishes match the hypothesis that the ontogeny of the digestive system is a genetically programmed process where digestive enzymes follow a spatiotemporal pattern of gene transcription during larval development. So far, it appears that only in a few species, the digestive enzymes are translationally regulated. During early fish ontogeny, AAs are important fuel molecules, signaling factors, and major substrates for the synthesis of a wide range of bioactive molecules and proteins. Consequently, feeding protocols based on different proteinaceous sources and preparations are increasingly developed for farmed fishes. Dietary protein formulation of microdiets should be adapted to each developmental stage. Dietary protein excess might have adverse effects. Finally, this chapter points out knowledge gaps of protein utilization by aquatic animals.

Dietary protein is an essential, but the most expensive nutrient in the diets of aquatic animals that directly affects individual growth, health, and feed intake. As depicted in Chap. 2, insufficient dietary protein amounts cause reduced growth and immunity and unregulated feed intake, while its excessive amounts make the diet composition unbalanced and may result in growth reduction and impaired immunity, extra feed costs, nitrogen excretion, and aquatic pollution. Therefore, this chapter tries to improve the understanding of uptake mechanisms and effects of protein consumption in invertebrates and fishes.

3.1 Invertebrates

3.1.1 Crustaceans

In crustaceans, protein digestion is usually high, and, in general, the higher the protein content, the higher the digestive efficiency. This efficiency depends on protein quality and amount of nonprotein energy available such as lipids and, to a lesser degree, carbohydrates in the meal. These substances have a sparing effect and proteins do not have to be catabolized as energy source (McGaw and Curtis 2013).

Ontogenetic stage, age, and size of an animal influence the absorption of nutrients. Digestive efficiencies increase during larval development as shown in European lobster (*Homarus gammarus*), Florida stone crab (*Menippe mercenaria*), western blue shrimp (*Litopenaeus stylirostris*), and through juvenile stages (V–XII) of the American lobster (*H. americanus*). Small *Litopenaeus vannamei* tend to process proteins more efficiently and grow faster than their medium or large counterparts. Differences in digestive efficiency related to sex have also been noted; female galatheid crabs (*Munida gregaria*) are more efficient at processing nutrients than male crabs. This is likely related to the extra nutrients required for egg production and maintenance (McGaw and Curtis (2013) and references therein).

Carnivorous crustaceans have a wide range of proteolytic enzymes at high concentrations, consistent with their ability to hydrolyze high levels of dietary protein, whereas omnivores and herbivores have a wide range and higher level of carbohydrases, consistent with their ability to hydrolyze plant and animal dietary carbohydrates. Comparing digestive enzyme activities among growth stages of the southern rock lobster (*Jasus edwardsii*), it occurs that the importance of carbohydrates as an energy substrate is relatively high in smaller juveniles and decreases as the lobsters grow (Johnston 2003; Simon 2009).

Dietary protein regulates trypsin enzymes on the biomolecular and biochemical levels, as found in the Caribbean spiny lobster (*Panulirus argus*) (Perera and Simon 2015). This complies well with other crustaceans and many other aquatic animals. Contrary to secretion, trypsin transcription in lobster is mediated by the appearance of free amino acids (AAs), the products of protein digestion, indicating a stepwise regulation of trypsin during digestion (Perera and Simon 2015).

As opportunistic feeders, crabs have a wide range of digestive enzymes. Nevertheless, the specific enzymes dominant within each species are consistent with their particular diets. Johnston and Freeman (2005) characterized the nutritional niches of six crabs. Significant trypsin and total protease activities break down the high-protein diet of the speedy crab (*Guinusia (Plagusia) chabrus*)—it is carnivorous. In contrast to the other species, the green crab (*Carcinus maenas*) does not have a dominant enzyme indicating that it is a generalist feeder utilizing a broad range of dietary items. This low specialization helps to explain its incredible success in a range of diverse habitats. Overall, specific enzyme patterns indicate specific feeding behavior and dietary preference and different strategies of resource use.

3.1.2 Mollusks

Well in line with the study of crustaceans, Bansemer et al. (2016) demonstrated that trypsin activity in greenlip abalone (*Haliotis laevis*) is influenced differently by age and dietary protein levels: By 53%, the activity of a 2-year-old abalone is lower than that of a 1-year-old one. In addition to the ontogenetical digestive enzyme development, Garcia-Esquivel and Felbeck (2006) studied their activities in different gut section in the red abalone (*Haliotis rufescens*). Two main digestion regions along the gut of juvenile *H. rufescens* occur: (1) stomach-digestive gland region and (2) mouth-intestine region. The stomach appears to be the main site of chymotrypsin secretion. Trypsin activity is barely detected in the foregut and rectum. Significant dietary effects are observed in the activity of enzymes, especially in the digestive gland. Garcia-Esquivel and Felbeck (2006) conclude that, although the digestive system of *H. rufescens* is naturally adapted to feed primarily on polysaccharide-rich diets, this species can adjust its enzymes in order to maximize the acquisition of dietary proteins and carbohydrates.

3.1.3 Echinoderms

Several protein utilization studies have been carried out mainly in sea urchins.

3.1.3.1 Sea Urchins

Protein utilization of sea urchins varies depending on species tested and feed source used. Based on growth and feed efficiency, a dietary protein level of 20% seems to be optimal in *Pseudocentrotus depressus* (Akiyama et al. 2001). In contrast, McBride et al. (1998) found in *Strongylocentrotus franciscanus* that growth is best on 40% dietary protein. The authors used kelp, krill, soybean, and gluten as main protein sources.

Nutrient Interactions

Besides different experimental set-up and different genetic material used, discrepancies observed in protein requirements can result from interactions with other major nutrients. Heflin et al. (2012) fed adult *Lytechinus variegatus* eight formulated diets with different protein and carbohydrate levels (Fig. 3.1). Dietary protein level and protein:energy ratio are the predictors of total weight gain (WG). Protein efficiency ratio decreases with increasing dietary protein level, more so at higher carbohydrate levels. Dietary protein level is the best predictor of gonad WG. Specifically, the

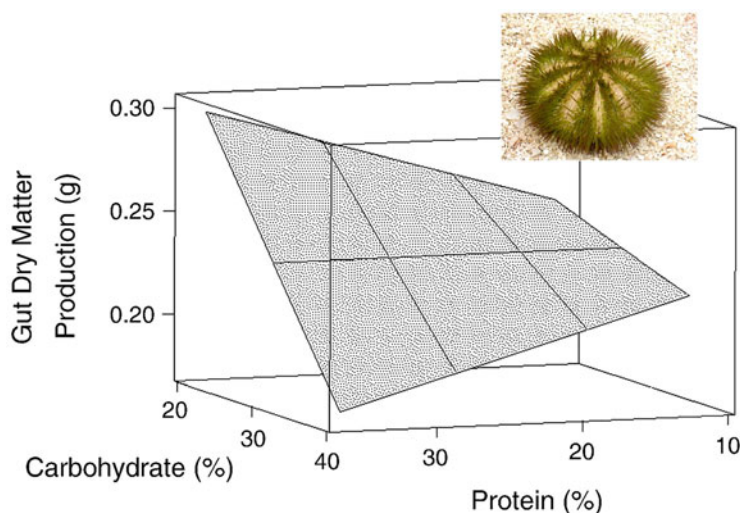


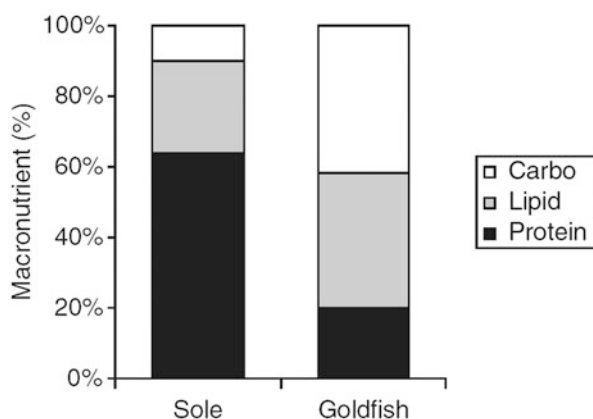
Fig. 3.1 Relationship between gut dry matter production (g) and protein+carbohydrate \pm (protein \times carbohydrate) of individual *Lytechinus variegatus* fed one of eight semipurified diets for 9 weeks. (From Heflin et al. (2012), with permission from Elsevier; image of *L. variegatus* credit L. Holly Sweat, Smithsonian Marine Station at Fort Pierce)

protein \times carbohydrate interaction is the best predictor of gut dry matter production (Fig. 3.1).

Compared to *L. variegatus*, the northern sea urchin (*Strongylocentrotus droebachiensis*) responds differently: diets with low protein levels (16–23% protein) and high kelp carbohydrate levels (>40% carbohydrate) produce the fastest growth (Eddy et al. 2012). The authors conclude that at least two diet formulations may be required to farm northern sea urchin from settlement to harvest: a diet that promotes fast somatic growth during the juvenile stages and a finishing diet to enhance gonad quality prior to harvest. The relatively low protein requirement is in good compliance with an earlier study on small *L. variegatus* reporting a requirement of >15% protein (Hammer et al. 2004).

In adult purple sea urchins (*S. purpuratus*), the interplay between carbohydrates and proteins facilitates an optimal survival (Cuesta-Gomez and Sánchez-Saavedra 2017): Urchins on high-protein–low-carbohydrate diet experience decreased survival. The survival is best on medium- to low-protein–medium- to high-carbohydrate diet. In terms of gonad growth, no differences are found. Interestingly, urchins that are fed high carbohydrate levels utilize protein more efficiently. Overall, diets that contain protein levels of 17–23% with carbohydrate levels of 50–58% serve life history traits of *S. purpuratus* best.

Fig. 3.2 Self-selection of macronutrient diets in fish. The percentage of energy taken in as carbohydrate (carbo), lipid, and protein by Senegalese sole (*Solea senegalensis*) and goldfish (*Carassius auratus*) using self-feeders delivering these three macronutrients in pure form. (From Raubenheimer et al. (2012), with permission from Wiley-Blackwell)



3.2 Fishes

The ability of fishes to select nutritionally appropriate diets is based on previous experience and particularly on protein-specific foraging to meet the requirements (→Chap. 2). It can easily be shown in self-selection studies. When offered three separate diets, each providing a single macronutrient (protein, lipid, carbohydrate), various fish species soon learn to compose a diet that reflects their natural feeding habits and the actual needs. Carnivorous species, such as rainbow trout (*Oncorhynchus mykiss*) and European seabass (*Dicentrarchus labrax*), select diets composed predominantly of protein (Sánchez-Vázquez et al. 1999; Aranda et al. 2000). Senegalese soles (*Solea senegalensis*), too, prefer protein-rich diets (Fig. 3.2) (Rubio et al. 2009). In contrast, goldfishes select a diet comprising 22% protein, 32% lipid, and 46% carbohydrate, reflecting their omnivorous feeding habit (Fig. 3.2) (Raubenheimer et al. 2012). This result is in compliance with studies in other omnivorous fishes, such as common carp and Nile tilapia (Yamamoto et al. 2003; Fortes-Silva et al. 2011).

Moreover, carnivorous Senegalese sole and omnivorous sharp snout seabream (*Diplodus puntazzo*) are able to adjust their diet to compensate for protein dilution. The latter species responds to dietary protein dilution (diluted with 50% of cellulose) by consuming double the amount of the diluted diet. In this way, they sustain both energy intake and the relative proportions of macronutrients. As long as fishes are given the chance to discriminate between different single macronutrients, dietary adjustments can be made on the basis of post-ingestional cues even in the absence of oral stimuli (Raubenheimer et al. (2012) and references therein). Later, this laboratory confirmed an active selection of the dietary macronutrient balance composition in the Indo-Pacific damselfish (*Abudefduf vaigiensis*) suggesting the generalizability of this property (Rowe et al. 2018).¹

¹The Right-Angled Mixture Triangles to identify nutrient deficiencies is described in Chap. 2.

3.2.1 Protein Consumption During Ontogenesis

The origin of AA pools and their subsequent degradation during ontogeny are receiving increasing attention. Studies reveal that AA metabolism in form of yolk formation and degradation is intractably linked to lipid and water transport in oocytes, embryos, and larvae (Wright and Fyhn 2001). Teleost larvae are extremely well adapted for nutrient utilization and have correspondingly fast growth rates with extreme rates found in common carp and an African catfish. Such growth rates, however, contradict the claim that larval fishes are “morphologically and functionally incomplete” (Dabrowski et al. 2003).

Despite this contradiction, it is well understood that, during this period of rapid growth, fish rely on proteinaceous substrates as a source of structural units for tissue proteins, for energy, and for synthesis of other macromolecules such as nucleic acids (Dabrowski 1986). After hatch, the quantity of initial proteinaceous substrates as fuel varies; however, free AAs play a paramount role, particularly in species with eggs that lack oil globules. This aspect is going to be exemplified with selected studies.

Necessary for rapid larval growth, the cumulative needs for protein deposition, protein turnover, and AA catabolism dictate a high AA requirement. The ontogenetic changes in digestive tract development during the larval–juvenile transition can be categorized into three types:

1. Stomachless fishes with an increase in complexity of the coiling pattern (cyprinids)
2. “Stomachless” larvae which develop a stomach structure after ingestion of feed (coregonids, silurids, serrasalמידs), and
3. Alevin and/or juvenile stages of fishes capable of ingesting the first feed when the stomach is present as a distinguished feature (salmonids, cichlids) (Dabrowski and Portella (2005) and references therein; for more information, →AAN I “Diets and Digestive Tracts” (Steinberg 2018).

Evidence based on comparison of free AA diets simulating AA profiles of proteins led to an unequivocal acceptance of the superiority of dietary free AAs over dietary proteins. Losses of dietary free AA are taking place through gill and urinary excretion. However, common carp and other species are unable to utilize exclusively dietary free AA or peptides compared with stomach-possessing fishes, such as rainbow trout. In the latter species, synthetic dipeptide-based diets sustain high growth rates (Dabrowski et al. 2010).

In stomachless fishes, no acidic content is released into the proximal intestine, and its intestinal lumen might be alkaline under normal physiological conditions (due to pancreas and gallbladder secretions into the intestinal lumen). To figure out how oligopeptide transporters function in stomachless fish, Verri et al. (2003) identified a oligopeptide transporter in zebrafish embryos, ortholog to mammalian and avian Peptide Transporter 1 (PEPT1, →Chap. 4). It is abundantly expressed in larval zebrafish prior to digestive tract differentiation, 4 days after fertilization in the

proximal intestine. Zebrafish PEPT1 is a low-affinity/high-capacity system. However, in contrast to higher vertebrate counterparts, the maximal transport rates of zebrafish PEPT1 increase at alkaline extracellular pH. This indicates that these stomachless teleost larvae are completely adapted to a high-capacity transport system of dipeptides at alkaline pH at the time of first exogenous feeding (Verri et al. 2003).

To complete this picture, the basolateral (serosal) transporters of dipeptides in tilapia are distinctly different from mucosal transporters. The former are characterized by inhibition of Gly-Sar transport by several other dipeptides; they are shared transporters (Dabrowski and Portella (2005) and references therein). Moreover, Chen et al. (2016) showed that feeding young Jian carps Ala-Gln can increase the transcription of glucocorticoid receptors. These receptors reside in the cytosol complexed with a variety of proteins including heat shock proteins (HSPs). Due to these HSPs, dietary Ala-Gln has the capability to enhance crowding stress and disease resistance in young Jian carps.

Recently, Orozco et al. (2017, 2018) investigated genes encoding one oligopeptide transporter (PEPT1; *slc15a1a*²) and two neutral AA transporters (*slc6a18* and *slc6a19*) in the intestine of Mozambique tilapia under fasting and refeeding conditions. *Slc6a18* and *slc6a19* have different roles in the intestine; they act coordinately and contribute to an efficient absorption of neutral AA. The *slc6a19a* responds to changes in nutrient condition and appears as the major neutral AA transporter in the anterior intestine. In contrast, *slc6a18* stays stable during fasting and refeeding, and is likely responsible in the absorption and recycling of AAs in the posterior intestine.

Supporting the notion of a coordinated action, Orozco et al. (2017) demonstrated that *slc15a1a* is primarily expressed in the hepatic loop (HL) and the proximal major coil (PMC) of the anterior intestine of Mozambique tilapia, and poorly expressed in other segments of the intestinal epithelium. The difference in the response of *slc15a1a* between the HL and PMC points out possible spatial characteristics of this transporter and the regulation of the absorptive capacity to adapt to changes in nutritional status. The findings of the two papers by Orozco et al. contribute not only to a better understanding of the complex mechanisms underlying the absorption of peptides and AAs but also to the development of possible means to manipulate the absorptive processes for the improvement of growth and other metabolic and physiological conditions.

Relatively little work has been conducted on nitrogen (N) metabolism during the early development of teleosts. A detailed picture is drawn by Terjesen et al. (1997) studying the larvae of the African catfish (*Clarias gariepinus*). Ammonia is the dominant product from the nitrogen metabolism not only in most adult teleosts but appears to be also the dominant product during the embryonic and yolk sac stage. Terjesen et al. (1997) established a nitrogen budget for the unfed *C. gariepinus* larvae. A continuous loss in dry weight, protein, and energy content throughout the yolk sac and starved larval stages occurs (Fig. 3.3). The larvae lose 1500 nmol N

²*slc* = solute carrier; *slc15* encodes oligopeptide transporter.

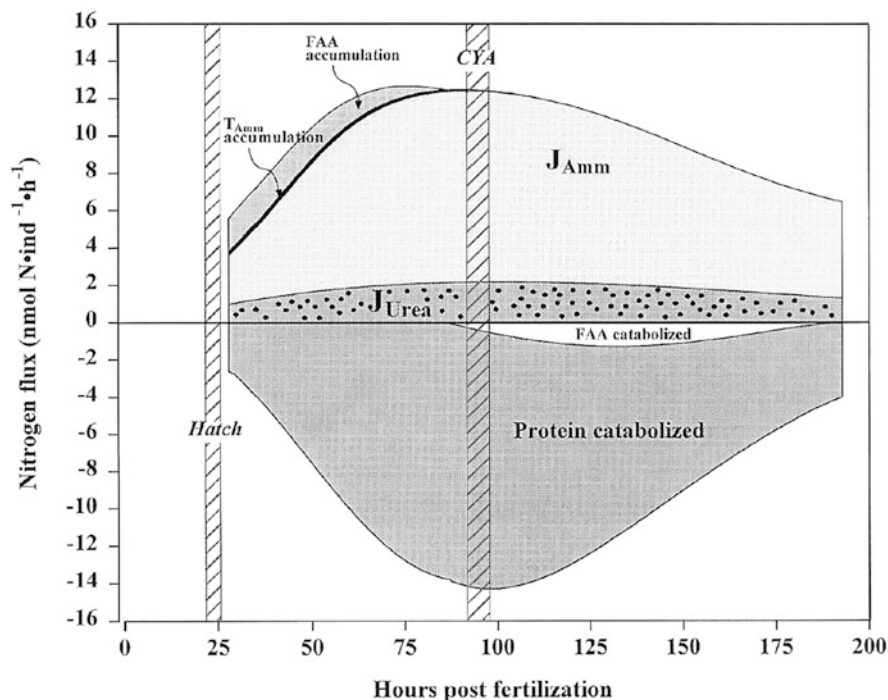


Fig. 3.3 Nitrogen flux in yolk sac and starved larvae of *Clarias gariepinus*. Upper half: the rate of metabolite formation. Lower half: rate of protein and free amino acid (fAA) catabolism. CYA complete yolk absorption. J_{Amm} and J_{Urea} denote ammonia and urea excretion, respectively, while T_{Amm} refers to the rate of ammonia accumulation. (From Terjesen et al. (1997), with permission from Springer Netherland)

Ind^{-1} from degradation of AAs during the period between 28 and 193 hpf. The accumulation and subsequent loss of free AAs have an insignificant effect on the balance. Concurrently, the individual larva produces a total of $1600 \text{ nmol N Ind}^{-1}$ as nitrogenous products, measured as excretion (sum of J_{Amm} and J_{Urea}) plus internal accumulation (T_{Amm}). Assuming that urea is produced from AA catabolism, the nitrogen budget balances within 6%.

Studies of fish larval digestive ontogeny have traditionally focused on changes in the morpho-anatomy and histological organization of the digestive organs as well as digestive enzyme activities. Meanwhile, these classical approaches are supplemented by biomolecular studies that provide insight into both temporal and spatial transcription patterns of genes involved in the development and functionality of the digestive system during early ontogeny (Zambonino Infante et al. 2008). Instantaneous growth rates of Chinese loach (*Paramisgurnus dabryanus*) larvae and juveniles are shown in Fig. 3.4a (Zhang et al. 2015). The growth rates decline from 2 to 5 days past hatch (dph) and increase up to the highest value at 10 dph. Changes in RNA-DNA ratio follow this trend (Fig. 3.4b): it decreases from a peak at 2–5 dph,

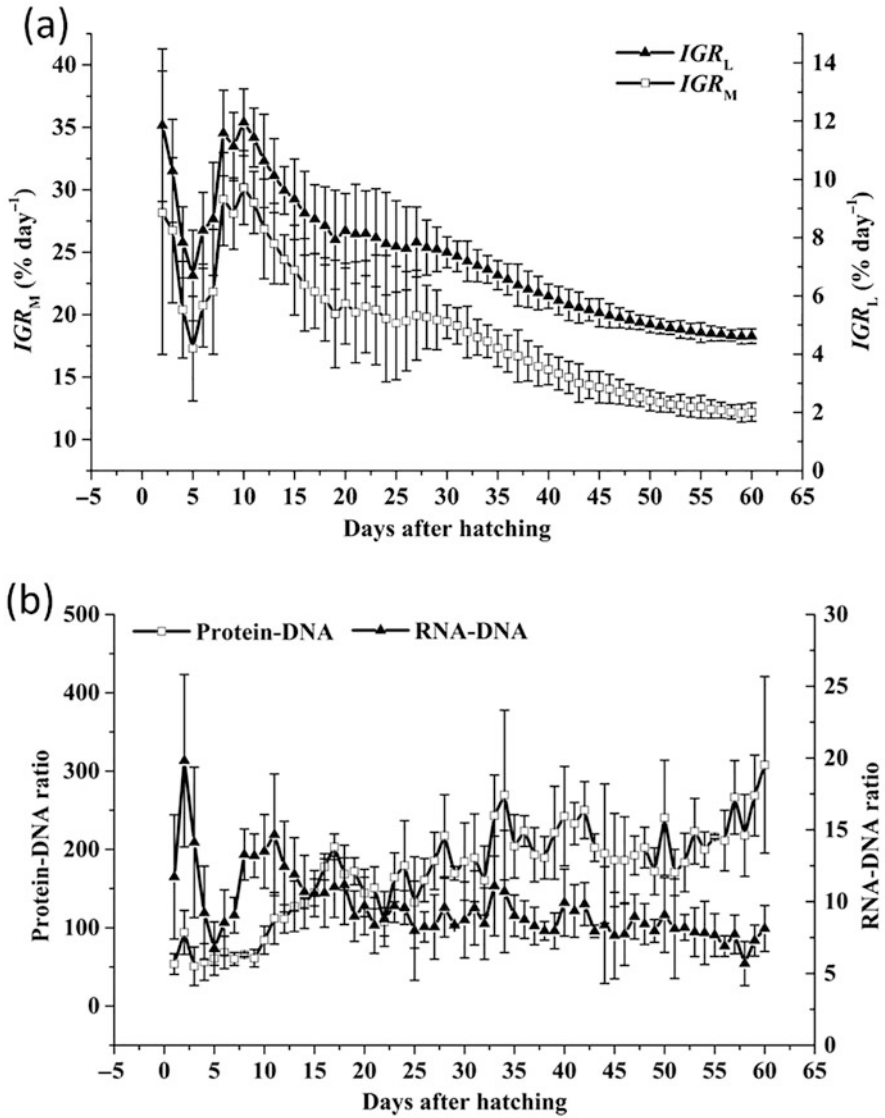


Fig. 3.4 Instantaneous growth rates of body mass (IGR_M) and total length (IGR_L) (a) and RNA-DNA ratio, and protein-DNA ratio (b) of *Paramisgurnus dabryanus* during larval and juvenile development related to days post-hatch at $24.4 \pm 0^\circ\text{C}$. Values are means \pm SD. (From Zhang et al. (2015), with permission from Wiley-Blackwell)

increases until 11 dph, and then declines to a stable level. The protein-DNA ratio maintains a steady level until 9 dph, increases markedly until 16 dph, and thereafter fluctuates at a relatively high steady level. The protein-DNA ratio reflects cell mass or cell size (Fig. 3.4b) and shows a stable level before 9 dph, and then increases

dramatically until 16 dph, fluctuating at a high level after 16 dph (Fig. 3.4b), revealing the cell enlargement (hypertrophy) after 9 dph. These results show that growth at the cellular level in Chinese loach is characterized by rapid hyperplasia from hatching through completion of the yolk sac stage followed by continued rapid hyperplasia combined with increasing hypertrophy after feeding commences. Larval somatic growth occurs chiefly through hypertrophy resulting from active protein synthesis. The same pattern is found in the metamorphosis stage of other fishes, such as turbot, Pacific bluefin tuna, thick lipped grey mullet, or Japanese flounder (Zouiten et al. 2008; Tanaka et al. 1996, 2007; Tong et al. 2010).

The regulation of digestive enzyme expression is important for larval growth and nutrient digestive capacity and processes. In fishes, expression of digestive enzymes is regulated by at least two complex physiological signals: hormones and diet (O'Connor and O'Moráin 2014). In line with this notice,

- Pancreatic trypsin, chymotrypsin, elastase, carboxypeptidase A and B, and lipase of Japanese flounder are expressed at first feeding.
- Composition and quantity of the diet control the adaptation of trypsin and amylase in European seabass larvae. The regulation of trypsin synthesis does not occur at the transcriptional level and the regulation of amylase synthesis is primarily translational (Wang et al. 2006).

Moreover, Wang et al. (2006) described the expression of pepsin, trypsin, and amylase genes of yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*) larvae fed formulated diets varying in protein level from first feeding. Since there is still a dispute of whether this species is carnivorous or omnivorous, the authors chose two key protease and amylase as target enzymes to identify their roles during larval development. Diets have no effect on amylase activity and mRNA level, likely due to unchanged carbohydrate contents in the diets. Pepsin and trypsin can be detected at hatch and first feeding, indicating that these enzymes are not induced by food. Trypsin activity found at hatch may come from the hatching glands in mother fish (Wang et al. 2006). From hatch to 26 dph, pepsin and trypsin activity shows first increase and then decrease—because of growth, development of new organs and tissues, and an increase in tissue proteins. The diet with the highest protein content of the three formulated diets leads to the highest pepsin activity and mRNA amount (Fig. 3.5) indicating that the dietary protein level induces the pepsin gene expression. This species is likely carnivorous.

The pattern, found in *T. fulvidraco*, does not generally apply to carnivorous species. In European seabass larvae, trypsin synthesis from 20 to 40 dph appears to be regulated at the translational level. Trypsin activity increases with dietary protein level, while trypsin transcription remains at a constant level until an advanced developmental stage, indicating that there exists a possible stabilization of trypsin mRNA or a regulation of trypsin expression at the translational level by the dietary protein content (Péres et al. 1998).

In Atlantic cod, too, the ontogeny of digestive capability and its hormonal components are tied directly to the type and quality of initial and early dietary constituents (Kortner et al. 2011). It has previously been proposed that genes coding

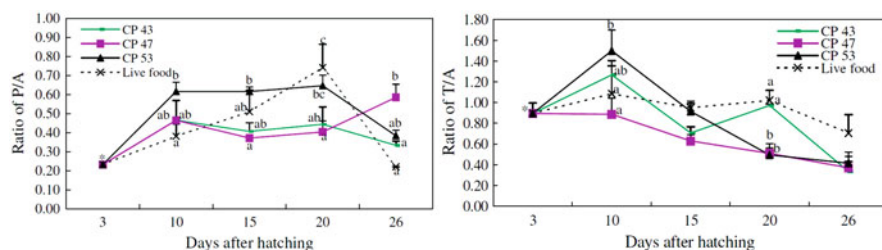


Fig. 3.5 Level of mRNA coding for pepsin (total pepsin mRNA/total actin mRNA, P/A) and trypsin (total trypsin mRNA/total actin mRNA, T/A) during ontogeny for the four treatment groups of *Tachysurus (Pelteobagrus) fulvidraco* larvae. Live food (newly hatched, unenriched *Artemia*) is a control. * is initial data. (From Wang et al. (2006), with permission from Elsevier). **CP 43** 42.8%; **CP 47** 47.3%; **CP 53** 52.8% crude protein

digestive enzymes can be transcribed independently of the external diet (i.e., being genetically or epigenetically programmed, →AAA III “Nutritional Programming”) in early stages of marine fish larvae, since the mRNA is already detected before first feeding, or even at hatching (Zambonino Infante and Cahu 2001; Cahu et al. 2004; Lazo et al. 2011). This assumption is supported by Kortner et al. (2011), who found no diet-specific alterations in gene expression at 5 dph. In addition, digestive enzyme mRNA levels do not seem to be highly susceptible to the external diet at 8 dph, whereas significant diet-specific modulations in gene expression take place at 16 and 29 dph.

Also the study of a complete data set on gene expression and activity levels of the most important digestive enzymes during development in *Lates calcarifer* larvae supports the generally accepted theory that ontogeny of the digestive system is a genetically programmed process where digestive enzymes follow a spatiotemporal pattern of gene transcription during larval development (Srichanun et al. 2013). Supporting evidence comes also from a recent study of the spotted rose snapper (*Lutjanus guttatus*): transcripts of the assayed digestive enzymes (trypsinogen, chymotrypsinogen, α -amylase, lipoprotein lipase, phospholipase A, and pepsinogen), with the exception of pepsinogen and the orexigenic and anorexigenic peptide hormones, are already present in the hatching stage (Moguel-Hernández et al. 2016).

We have to draw a general picture.

During early fish ontogeny, AAs are important fuel molecules, signaling factors, and major substrates for the synthesis of a wide range of bioactive molecules and proteins. Because the majority of fish eggs are closed free-living systems following ovulation and activation, early development of fishes depends on the maternal provision of AAs during oogenesis. While more than 600 proteins have been identified in the growing oocytes of fishes, the major vehicles for supplying AAs to the growing oocyte before ovulation are the vitellogenins, of which many genes and multiple forms are known (Finn and Fyhn 2010).

In the sequence of catabolic substrate oxidation, two types of fuel selection prevail:

- Type I strategy for species that develop from eggs without globules
- Type II strategy for those with oil globules

Both types of embryos initially rely on cytosolic glycogen and then catabolize AAs from protein (benthophils) or free AAs (pelagophils). AAs are co-catabolized with lipids, initially polar lipids from phosphatidylcholine and later neutral lipids from triacylglycerols in type I eggs or predominantly neutral lipids from triacylglycerols and wax esters in type II eggs. In the latter case, an intense period of lipid mobilization occurs following hatch when the oil globule is resorbed. The post-yolk sac ontogenetic dependence on AAs as fuel molecules is demonstrated in Atlantic cod and yellowtail kingfish (*Seriola lalandi*) larvae over several orders of magnitude during short-term starvation (Finn et al. 2002). During the early phases of starvation, a switch to glucose and lipids occurs not only in this species but also in fishes general (Finn and Fyhn 2010).

In order to overcome major problems in the larviculture of Senegalese sole, such as the difficult early adaptation to inert diets and highly variable growth rates, Canada et al. (2019) created three educational schemes on optimized proteinaceous diets for larvae and juveniles (Fig. 3.6). Different diet formulation strategies are used to improve Senegalese sole larvae capacity to utilize and deposit protein throughout metamorphosis and to maximize its growth potential:

1. Increasing essential AA (EAA) content
2. Meeting the ideal EAA profile by adjusting the dietary AA profile to the larval body AA profile
3. Decreasing the complexity of dietary protein to increase its digestibility

Either manipulating the quality or the complexity of dietary protein has effects on the larval capacity to utilize protein for growth (Fig. 3.6): Sole larvae are able to adapt their digestive functions and metabolic capacity to dietary protein (Fig. 3.6 above). Moreover, increasing the dietary EAA/NEAA³ ratio (Fig. 3.6 center) and changing the degree of hydrolysis of dietary protein affected the expression pattern of muscle growth-related genes, with consequences on muscle cellularity and potential for growth. The expression of DNA methyltransferases is altered in response to changes in dietary protein (Fig. 3.6 below; →AAN III “Nutritional Programming”). The novelty of this information will trigger further studies on the effect of dietary protein on the epigenetic regulation of growth (Canada et al. 2019). In other words, such educational schemes appear indispensable for all major farmed fish species.

³NEAA (non-essential AA), which can be synthesized de novo from α -keto acids in the TCA cycle or through transamination.

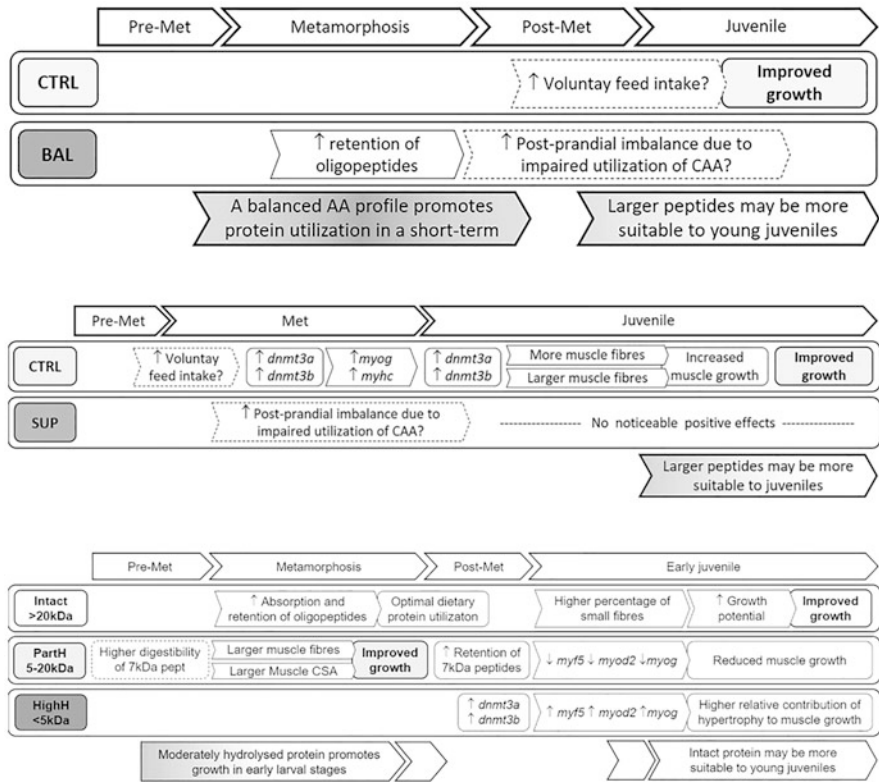


Fig. 3.6 *Above* General effects of a balanced dietary AA profile according to the ideal protein concept on protein metabolism and somatic growth in Senegalese sole. *Center* General effects of a balanced dietary AA profile with an increased IAA/DAA ratio on the regulation of muscle growth and somatic growth in Senegalese sole. *Below* General effects of dietary protein complexity on protein metabolism and the regulation of muscle growth and somatic growth in Senegalese sole. (From Canada et al. (2019), with permission from Elsevier). *BAL* balanced diet, *SUP* supplementation of a microdiet with 8% crystalline essential AAs, *Intact* non-hydrolyzed protein, *PartH* partly hydrolyzed protein, *HighH* highly hydrolyzed protein

For Senegalese sole, the required optimal protein quality changes during larval development. Whereas the inclusion of a moderately hydrolyzed protein comes up as a promising way to improve growth in early larval stages, larger peptides and intact protein seem to be more suitable to sole post-larvae and young juveniles. Therefore, these results indicate that dietary protein formulation of microdiets for Senegalese sole should be adapted to each developmental stage, which has important consequences for practical larval microdiet formulation and feeding protocols (Canada et al. 2019).

3.2.2 Dietary Protein Excess

Dietary protein levels control appetite, lipid, and glucose metabolism (Li et al. 2017). Surprisingly, the authors show in juvenile grass carps that high dietary protein levels have adverse effects on larval growth. The authors provided diets with three protein levels at P1 200, P2 ~300, and P3 ~440 g kg⁻¹. Grass carp larvae fed with protein levels at ~300 g kg⁻¹ show highest WG. Dietary surplus (~440 g kg⁻¹) induces the lowest protein efficiency ratio, body and liver lipid, and inhibits glucose and lipid metabolism so that growth depression occurs.

Li et al. (2017) trace these phenotypic results back to biomolecular controls. The appetite gene expressions of individuals fed with optimal dietary protein show downregulation of orexigenic *neuropeptide Y* (*npv*) and one of its receptors (*y8b*). Genes involved in glycolysis (*gk*, *pk*) and *g6pase*, an important gene involved in gluconeogenesis (→Chap. 18), decrease at optimal dietary protein levels. *Pepck*, also a key gluconeogenesis gene, decreases with increasing dietary protein level. Gene transcription involved in fatty acid synthesis decreases with increasing dietary protein level. This indicates that increasing dietary protein levels elevate the AA concentrations, which result in high insulin secretion. In contrast, genes encoding FA oxidation are increasingly expressed when fishes feed surplus proteins. Growth depressions due to surplus dietary protein levels are also observed in juveniles of Wuchang bream (*Megalobrama amblycephala*) (Habte-Tsion et al. 2013), rabbitfish (*Siganus guttatus*) (Parazo 1990), or olive flounder (*Paralichthys olivaceus*) (Lee et al. 2000) and may be valid in general.

3.3 Concluding Remarks

Different proteinaceous nutrients are required during larval development of fishes and invertebrates. This requirement appears to be type- and/or species-specific and changes during larval ontogenesis. However, the exact catalog of required nutrients is available only for a few fish species. With invertebrates, even less and less sophisticated information is available. For applied purposes, however, this catalog has to be enlarged in order to develop better feeds for larval and post-larval stages and, in turn, to reduce larval mortality. Furthermore, it is not well understood, whether optimal and development-specific diets with proteinaceous nutrients have the potential to increase the resistance to pathogens; however, this can be hypothesized (Martin and Król 2017). There are first, but robust, indications that underlying mechanisms involve epigenetic modes of action (Dhanasiri et al. 2020).

As demonstrated in recent studies, the utilization of proteinaceous nutrients is more than the provision of easily utilized energy. There is a plethora of feedbacks with appetite regulation, transcriptional and translational regulation of digestive enzymes not only of proteinaceous nutrients but also of the other macronutrients (lipids and carbohydrates). We are just at the beginning to understand these

feedbacks. Nevertheless, it can easily be predicted that the regulatory feedbacks by dietary proteins comprise the effectiveness of micronutrients, enzymes, as well as anti-nutritional factors.

To have a common platform for generalization, regulation by proteinaceous nutrients and their feedbacks have to be evaluated on the biomolecular level by application of various “omics” techniques. They have to include the action of the intestinal microbiota as well as epigenetic pathways, such as DNA methylation, histone modification, as well as the translational action of various non-coding RNAs, rather than extending the existing catalog of simple production studies.

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Chapter 4

Peptides or Amino Acids?—‘*The Smaller, the Better?*’



Abstract This chapter addresses the question of whether amino acids or oligopeptides are preferably taken up in animal cells. Evidence is growing that peptides are preferred over free amino acids. However, the knowledge about the role of AAs and oligopeptides in the nutrition of aquatic invertebrates remains fragmentary; the situation of fishes appears to be gradually better than that of invertebrates. It can be expected that in-depth knowledge will enable nutritionists to design more appropriate aquafeeds, particularly because recent studies indicate the involvement of oligopeptides in strengthening the innate immunity. Finally, we point out epigenetics in connection with oligopeptide transporters in human cancer studies as incentive to adept this biomolecular tool also for amino acid and peptide nutrition in fishes and aquatic invertebrates.

Which are the more effective nutrients, amino acids (AAs) or peptides? Which are preferably taken up? One would expect that the smaller the molecule, the better it could be transported and metabolized. In several relatively early papers, Dabrowski's laboratory pointed out the nutritional paradox: Stomach-less fish species, such as common carps, are unable to utilize exclusively free AA-(fAA)-based diets (Zhang et al. 2006); similarly, in stomach-possessing fishes, such as rainbow trouts, synthetic dipeptide-based diets sustain high growth rates (Terjesen et al. 2006).

Meanwhile, it is obvious that peptides, rather than fAAs, are preferably taken up. Two examples:

- Rahimnejad and Lee (2014) compared the utilization efficiency of dietary free Lys and dipeptide Lys-Gly in terms of growth performance of juvenile olive flounders (*Paralichthys olivaceus*): for protein synthesis, juveniles utilize Lys-Gly more efficiently than free Lys.
- In Pacific white shrimp (*Litopenaeus vannamei*), a Met-Met dipeptide supports growth better than the equivalent of free Met (Niu et al. 2018).

AA uptake from dipeptides appears to be faster than from the equivalent of fAAs; this issue will be revisited.

4.1 Amino Acid and Peptide Sensing

Since sensing and responding to fluctuations in nutrient levels are a requisite for life, it is not surprising that different organisms are able to detect extracellular and intracellular levels of sugars, AAs, and lipids. Both fishes and crustaceans use their olfactory and gustatory systems to detect AAs, amines, and nucleotides, among many other compounds, while fish olfactory systems also detect mixtures of sex steroids and prostaglandins with high specificity and sensitivity. In both fishes and crustaceans, the olfactory system is especially adept at mixture discrimination, while gustation is well suited to facilitate precise localization and ingestion of food (Derby and Sorensen 2008).

The chemical nature of food cues is best understood. AAs appear to be universally used by fishes in this regard, likely because most are predators and have high protein requirements, and thus, AAs are good indicators of high-quality food. Laboratory studies consistently demonstrate that specific mixtures of L-AAs attract many fish species and that single AAs sometimes trigger reflexive snapping and biting behaviors, which at least on occasion are linked with the gustatory. Electrophysiological recordings demonstrate that fish olfactory systems detect all primary L-AAs with high (nanomolar) sensitivity and specificity (Derby and Sorensen (2008) and references therein). For instance, studies in the Japanese rice fish (medaka, *Oryzias latipes*) show that the receptors react to L-Arg, L-Pro, L-Ser, or L-Tyr, but not to D-Arg (Oike et al. 2007).

In crustaceans, too, AAs (and nucleotides) are major sets of attractant molecules. Once near the food, ingestion is based on input from their gustatory systems on legs and mouthparts. Food selection and ingestion are influenced by the blend of attractive and deterrent compounds, although we know more about the former than the latter. Some crustaceans can learn to avoid food associated with gastric malaise (Derby and Sorensen 2008).

Evidence obtained in recent years in a few species, especially rainbow trout, show that the increase of specific branched-chain AAs (BCAAs), such as Leu, inhibits food intake. This process occurs through activation of AA sensing systems mediated by activation of mammalian target of rapamycin (mTOR, a “metabolic sensor”) and/or inhibition of AMP-activated protein kinase (AMPK) signaling¹ or via activation of BCAA metabolism. Furthermore, deficiency in essential AAs (including BCAAs) elicits an increase in food intake through AA sensing systems (Conde-Sieira and Soengas 2017). In fact, Chen et al. (2021) identified in apex predatory Chinese perch (*Siniperca chuatsi*) that intracerebroventricular administration of Leu results in inhibition of food intake and stimulation of TOR signaling; administration of Val stimulates food intake and inhibition of TOR signaling; and administration of Ile decreases TOR signaling with no modulation of food intake.

Diets high in AAs/protein inhibit food intake in teleost fishes, whereas poor diets stimulate it (Soengas et al. 2018). Considering the high importance of AAs for fish

¹→Box: Yin and Yang of Energy Regulation in Chap. 5.

metabolism, the existence of fish-specific unknown features of AA sensing is a reasonable hypothesis to test in studies to come (Soengas et al. 2018).

Recent biomolecular progress of taste reception reveals that in mammals, the receptor T1R1²–T1R3 responds to AAs. In teleosts and their larvae (Ueberschär et al. 2018), these receptors are also present. Fishes, too, have three types of T1Rs, of which T1R1 and T1R3 have the highest degree of identity to mammalian orthologs. Furthermore, similarly to mammals, fish T1R1 and T1R2 are expressed in different subsets of taste bud cells (Yasuoka and Abe 2009; Morais 2017).

In the gastrointestinal track, AAs are absorbed more efficiently as small peptides than as fAAs indicating a potential role of the Peptide Transporter 1³ (PepT1) (Wang et al. 2017). Furthermore, PepT1 has been hypothesized to be an intestinal peptide chemosensor (termed as “transceptor”). The spatio-temporal expression of *pept1* mRNA has been analyzed in, among others, zebrafish, Atlantic cod, and grass carp during ontogeny.

European seabass is one of the first fish species in which the distribution of the *pept1* mRNA expression is documented (Terova et al. 2009) (Fig. 4.1). High levels of expression take place in segments 1–3 (the first 3 cm) of the proximal intestine, lower levels in pyloric ceca and intestinal segments 4–10, and very low levels in gastroesophageal junction, stomach fundus, pyloric antrum, most distal intestinal region (segments 9–10), and rectum (Fig. 4.1a, b). In other tissues, the expression of seabass *pept1* is 100-fold less than that found along the intestine. Nutritional status significantly influences PepT1 mRNA copy numbers in the proximal intestine, inducing a downregulation during prolonged fasting and an upregulation during the recovery from fasting (Terova et al. 2009).

4.2 Amino Acids Versus Oligopeptides

Since long, sodium-dependent transepithelial AA transport is well understood in vertebrates (Schultz and Curran 1970). In invertebrates, the transmembrane net entry of fAAs can occur against concentration gradients from external AA concentrations as low as 125 nM in echinoderm larvae and blue mussels (*Mytilus edulis*) (Davis et al. 1985). These authors presented a detailed kinetic analysis of the effects of Na⁺ on the transmembrane transport of neutral AAs by bacteria-free larvae of the purple sea urchin (*Strongylocentrotus purpuratus*) and showed that the translocation of AAs is electrogenic. AAs taken up from external solution are distributed rapidly to the internal tissues of the animal as shown in the Pacific oyster (Rice and Stephens

²T1R1 is the taste receptor type 1 member 1, a G protein-coupled receptor (Behrens and Meyerhof (2016), and part of the heteromeric AA receptor T1R1–T1R3. This receptor is formed as a dimer of the T1R1 and T1R3 proteins. Moreover, the T1R1 protein is not functional outside of formation of the 1 + 3 heterodimer (Nelson et al. 2001; Nuemket et al. 2017).

³Also known as solute carrier family 15 member 1 (SLC15A1) (Liang et al. 1995).

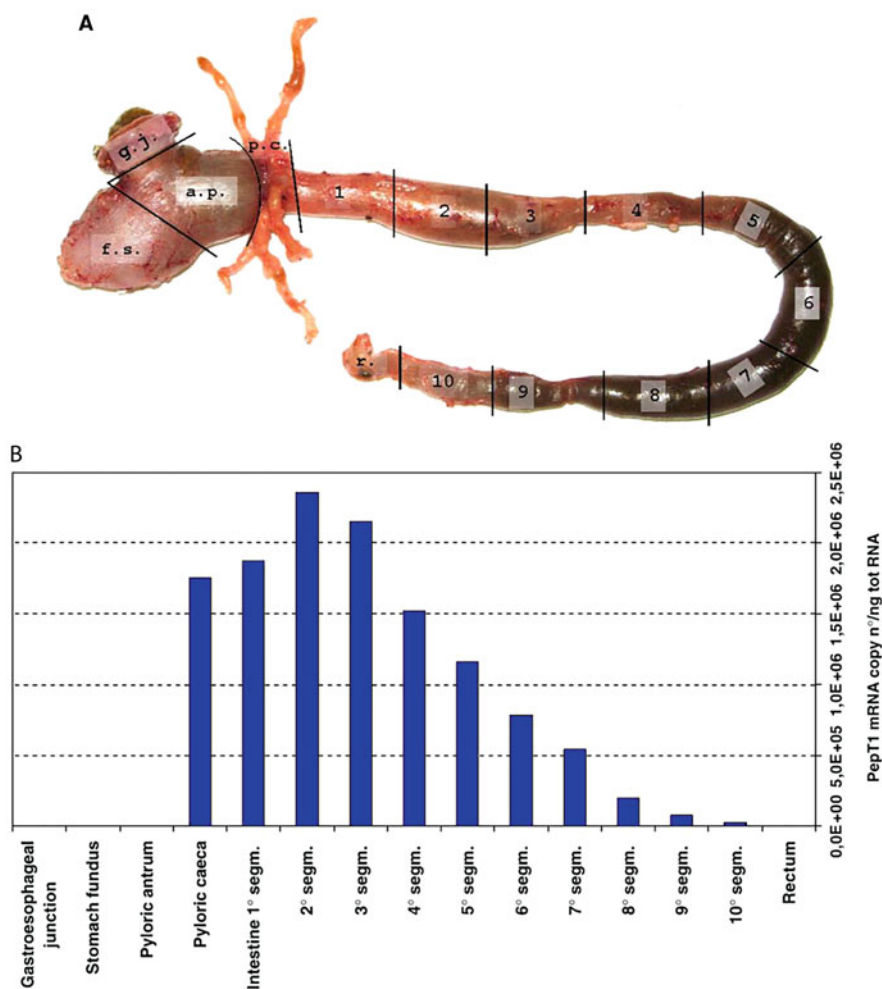


Fig. 4.1 Distribution of the seabass (*Dicentrarchus labrax*) *pept1* transcripts in the digestive tract. (a) Image of seabass digestive tract: gastroesophageal junction (g.j.), fundus stomach (f.s.), pyloric antrum (p.a.), pyloric caeca (p.c.), ten adjacent intestinal segments starting after the pyloric area (1–10), and rectum (r.). (b) Relative expression of PepT1 in different segments of the seabass digestive tract. *PepT1* mRNA copy number was normalized as a ratio to 100 ng total RNA. (From Terova et al. (2009), with permission from Elsevier)

1987). Entry of acidic and basic AAs appears to be Na^+ -independent. Recent papers refine this picture.

As mentioned above, evidence is accumulating that small peptides are absorbed more efficiently than fAAs. Already, Abe (1995) pointed out the existence of specific dipeptides, the His-related dipeptides (\rightarrow Chaps. 3 and 5), and puts forward several hypotheses of their physiological functions. Most likely, these dipeptides are intracellular proton buffers in vertebrate fast-twitch glycolytic muscles adapted for

anaerobic performance such as burst swimming in fishes (Abe 2000). Although slow, fishes are able to biosynthesize His-related dipeptides and do not have a dietary requirement of these compounds. Despite the potential immunostimulatory effect of small peptides (as shown in sea cucumber (*Apostichopus japonicus*) (Wang et al. 2013)), early life stages of aquatic animals can acquire exogenous AAs through prior hydrolysis of proteins and peptides or direct absorption of AAs in the free form. Studies show that environmentally induced species- and life stage-specific differences exist, in which some of these AA sources are nutritionally preferred. The use of low molecular weight peptides in larval diets has received increasing attention and is successfully applied (Bogé et al. 1981; Dabrowski et al. 2003; Rojas-García and Rønnestad 2003).

4.2.1 Invertebrates

Studies of oligopeptide transporters in invertebrates focus almost exclusively on model animals (*Caenorhabditis elegans*, *Drosophila melanogaster*) (Meredith and Boyd 2000). Reports on aquatic invertebrates are lacking, except of a few on crustaceans, such as Antarctic krill (*Euphausia superba*) (Seear et al. 2010), American lobster (*Homarus americanus*) (Obi et al. 2011; Peterson et al. 2015), and Atlantic white shrimp (*Litopenaeus setiferus*) (Peterson 2014).

In contrast to most fishes which possess dipeptide transporters stimulated by acidic pH, the transport of Gly-Sar across the intestine of American lobster increases by almost three times, when the luminal pH rises from pH 5.5 to pH 8.5. The lobster hepatopancreas has a slightly acidic lumen and an apical, electrogenic, $2\text{Na}^+/\text{H}^+$ antiporter in vesicles of the brush border membrane, which is able to provide the required symport substrate for dipeptide accumulation. The stimulation of intestinal Gly-Sar (dipeptide from sarcosine, a nonproteinaceous AA) transport in lobster by luminal alkalization shows that the two absorptive organs in this invertebrate have different pH values during absorptive activities. A similar enhancing effect of luminal alkaline pH on dipeptide transport occurs in zebrafish (Verri et al. 2003), and these authors assumed that no Na^+/H^+ antiporter may be present to power dipeptide accumulation. At present, it is unclear whether lobster and zebrafish intestines share a similar alkaline-dependent dipeptide transport mechanism, and if so, what pH regulatory characteristics it displays (Peterson et al. 2015). In Atlantic white shrimp (*Litopenaeus setiferus*), the transport of dipeptides appears to be similar to American lobster (Peterson 2014).

Supporting the notion that dipeptides are more effective nutrients than fAAs, Xie et al. (2018) showed in Pacific white shrimps that the same growth performance can be achieved with diets containing reduced fishmeal contents if the feed is supplemented with 3.0 g kg^{-1} DL-Met or 1.0 g kg^{-1} Met-Met. Even more pronounced, Guo et al. (2020a) found a higher peak of Met over time in the hemolymph of this shrimp with the Met-Met supplemented diet than in the DL-Met supplemented

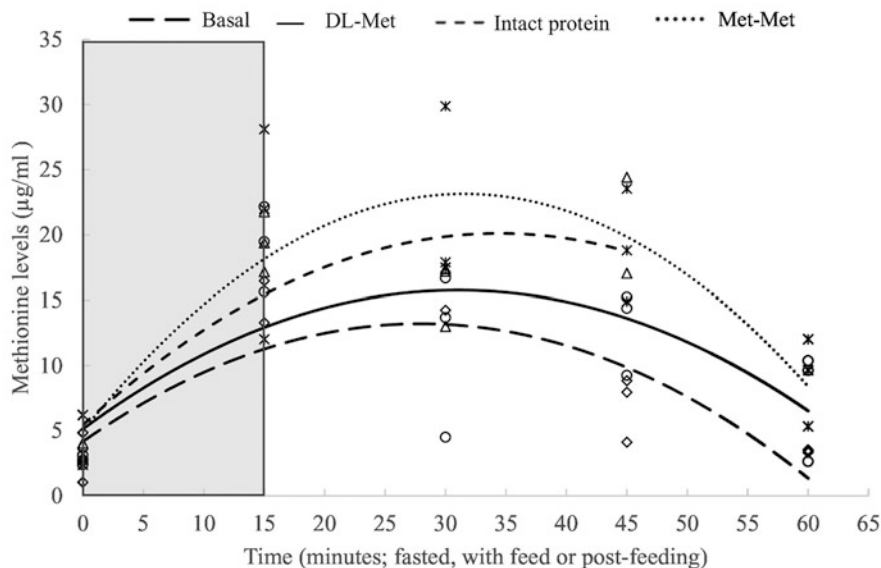


Fig. 4.2 Methionine levels of hemolymph from Pacific white shrimps fed four experimental diets (Basal, DL-Met, Met-Met, Intact Protein). Shrimps were fasted overnight and fed for 15 min (gray area) and then post feeding fasted for 45 min. (From Guo et al. (2020a), with permission from Elsevier)

diet (Fig. 4.2), providing another evidence for the high biological efficiency of a dipeptide.

Overall, the knowledge about the role of AAs and oligopeptides in the nutrition of aquatic invertebrates remains fragmentary; the situation of fishes appears to be gradually better. However, it can be expected that an improvement of this situation will enable aquaculturists to design more appropriate aquafeeds.

4.2.2 Fishes

Bogé et al. (1981) appear to be the first to show that, as in mammals, the AA uptake in rainbow trout is more rapid from dipeptides than from the equivalent of fAAs. This issue has intriguingly been confirmed in yellow perch (*Perca flavescens*) (Kwasek et al. 2012). Individuals subjected to free Lys treatment show a small increase in *pept1* transcripts. The dipeptide (Lys-Gly) diet, however, induces a significant increase in *pept1* transcription, compared with both free Lys and no Lys treatments (Fig. 4.3).

In fishes, oligopeptide transporters have first been identified in Mozambique tilapia (Reshkin and Ahearn 1991); and, in 2003, the first peptide transporter from

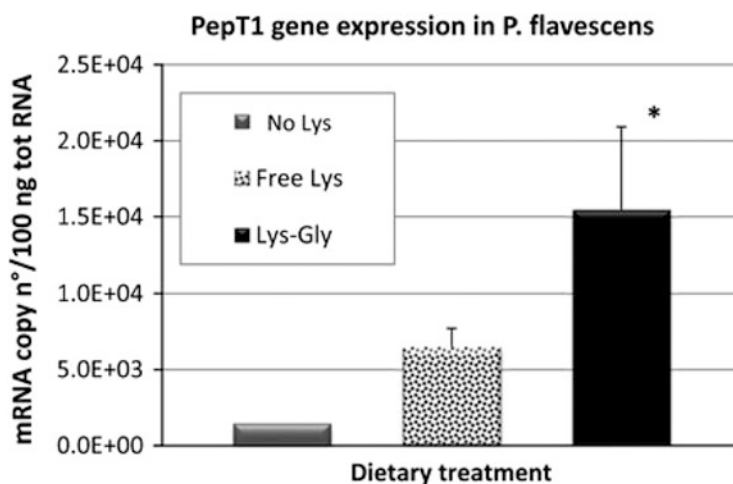


Fig. 4.3 Expression levels of the oligopeptide transporter *pept1* in yellow perch (*Perca flavescens*) whole digestive tract. The *pept1* mRNA copy number was normalized as a ratio to 100 ng Σ RNA. Bars indicate SEM. * significant difference at $P < 0.05$. (From Kwasek et al. (2012), with permission from Springer Nature)

a teleost fish, the zebrafish peptide transporter 1 (PEPT1), was cloned and functionally characterized (Verri et al. 2003).

PEPT1, PEPT2, and other peptide transporters are responsible for the preferential uptake of peptides. Whereas PEPT1 is a low-affinity/high-capacity system, with apparent affinity constants ranging between 0.2 and 10 mM depending on the substrate, PEPT2 is a high-affinity/low-capacity system, with apparent affinity constants of 0.005–0.5 mM (Romano et al. 2006). In animals with a defined tissue distribution, the separation into the intestinal PEPT1-like form and the renal PEPT2-like form can be found; in teleosts, this separation applies to *Danio rerio* (Verri et al. 2003), *Gadus morhua* (Rønnestad et al. 2007), and others (see below).

Only recently, oligopeptide transporters have been identified also in elasmobranchs, such as bonnethead shark (*Sphyrna tiburo*) (Hart et al. 2016). PEPT1 is localized in multiple components of the digestive tract (esophagus, stomach, duodenum, intestine, rectum, and pancreas), whereas the localization of PEPT2 remains unexplored so far.

The di- and tripeptide transporters display a unique substrate specificity that could be called “nonspecifically specific” or “specifically nonspecific” (Daniel et al. 2006). They essentially transport all possible di- and tripeptides composed of α -AAs as well as a large variety of derivatives. This means that a minimum of 400 different dipeptides and 8000 different tripeptides serve as substrates (Daniel et al. 2006). It is thought that water plays an important role in accommodating the broad specificity of PEPT1 by shielding the charges of the AA side chains within the substrate-binding domain of PEPT1 that allows both charged and uncharged substrates to bind at the

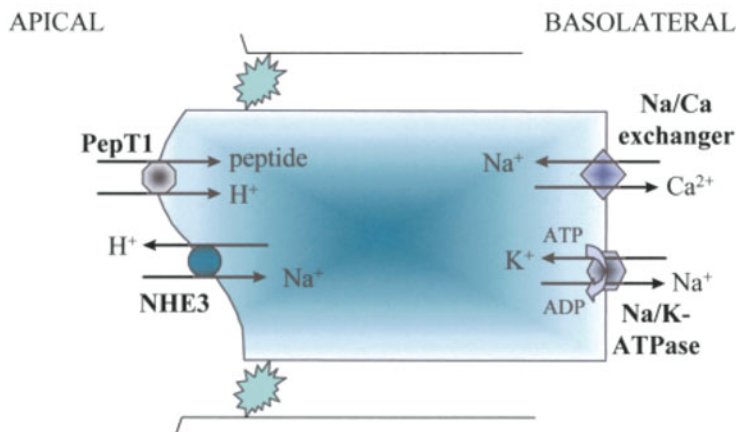


Fig. 4.4 A wide variety of di- and tripeptides are taken up across the brush border membrane by the proton-coupled symporter known as PEPT1. Anticlockwise from top left: peptides enter the cell with protons via PEPT1; the protons are exchanged for sodium via NHE3; the sodium is removed from the cell by the basolateral sodium pump (Na/K-ATPase); if the rate of sodium entry exceeds the capacity of the sodium pump to remove it, the sodium:calcium exchanger ability to keep intracellular calcium concentrations low will be compromised. The rise in calcium concentration would act to reduce the rate of peptide entry through PEPT1 and open calcium-dependent potassium channels, allowing the cell to repolarize and to restore its normal ionic balance. (From Meredith and Boyd (2000), with permission from Springer Nature)

same domain. Metal ions (Zn^{2+} , Mn^{2+} , or Cu^{2+}) also can interact with transporter proteins to enhance peptide absorption (Gilbert et al. (2008) and references therein).

The transport via PEPT1 is proton-dependent (Fig. 4.4). The processes include:

1. A Na^+/H^+ exchanger located in the brush border membrane that maintains an intracellular alkaline pH.
2. A Na^+/K^+ -adenosine phosphatase (ATPase) located in the basolateral membrane that maintains an inside negative membrane potential.
3. Several cytoplasmic peptidases that prevent intracellular accumulation of absorbed peptides. These enzymes convert most of the absorbed oligopeptides to AAs that are either used by the absorbing cells or are released into the portal circulation via the AA transporters located on the basolateral membrane of these cells (Adibi 2003).

In addition to transport by PEPT1, peptides may also be absorbed through alternate routes including paracellular movement and by cell-penetrating peptides that are capable of moving cargo across the plasma membrane (Gilbert et al. 2008). In herbivorous fishes, the basolateral intestinal dipeptide transporter appears to be distinctly different from either the high- or low-affinity brush-border transporter. It is proton dependent, electroneutral, Na^+ independent and accepts a wide variety of dipeptides (Thamotharan et al. 1996).

Meanwhile, PEPT1 has been documented in European eel (Verri et al. 2000), Mozambique tilapia, Antarctic crocodile icefish (*Chionodraco hamatus*), Atlantic salmon, rainbow trout, China rockfish (*Sebastes nebulosus*), European seabass, common carp, channel catfish (Verri et al. (2010) and references therein), zebrafish (Verri et al. 2003), Atlantic cod (Rønnestad et al. 2007), mummichog (Bucking and Schulte 2012), grass carp (Liu et al. 2013), Japanese eel (Ahn et al. 2013), yellow perch (Ostaszewska et al. 2013), crucian carp (Liu et al. 2014), and turbot (Xu et al. 2016). Therefore, the existence of PEPT1 in all teleost species appears to be very likely (Verri et al. 2012).

Vacca et al. (2019) refined the knowledge about PEPT1 by showing that it consists of two low-affinity paralogs [*pept1a* (= *slc15a1a*) and *pept1b* (= *slc15a1b*)] in zebrafish. Two paralogs of PEPT1 are also reported from mummichog (*Fundulus heteroclitus*), Nile and Mozambique tilapia, and European seabass (Vacca et al. 2019), or Atlantic salmon (Gomes et al. 2020).

In euryhaline mummichog, Bucking and Schulte (2012) discovered that the expression pattern of multiple forms of PEPT1 depends on environmental parameters, for instance, on the pH value: increased luminal pH results in decreased intestinal dipeptide transport in freshwater-acclimated individuals, but the opposite happens in seawater-acclimated fishes. The ecophysiological significance of this finding currently remains obscure. Since it has recently also been shown that the *pept1* is involved in salinity tolerance in striped catfish (*Pangasianodon hypophthalmus*) (Nguyen et al. 2016), it can be expected that not only euryhaline but also anadromous and catadromous fish species possess multiple forms of oligopeptide transporters.

In addition to PEPT1 and PEPT2, the glutamine transporter ASCT2⁴ plays a crucial role in dietary protein absorption. Hu et al. (2017) characterized ASCT2 and its dietary regulation in grass carp. Noteworthy, the protein source affects the ASCT2 expression; the ASCT2 mRNA abundance is higher in the intestine of fishes fed with soybean meal than in those fed with fishmeal (Hu et al. 2017).

Without detailed characterization of its function in nutrition, this transporter is found also in zebrafish (Tian et al. 2015), cobia (*Rachycentron canadum*) (He et al. 2019, 2020), and rainbow trout (To et al. 2019). Nevertheless, the question arises whether a biotechnical multiplication of ASCT2 can be successfully applied to increase the tolerance of fishes to high plant protein diets.

How does feed quantity affect oligopeptide transporters? Terova et al. (2009) studied this issue in seabass by analyzing fasting- and refeeding-induced changes in *pept1* transcription. *Pept1* is highly expressed in the proximal intestine with much lower levels of expression in gills, brain, heart, liver, and spleen. Nutritional status influences *pept1* mRNA copy numbers in the proximal intestine, inducing a downregulation during prolonged fasting and an upregulation during the recovery from fasting. These findings support a specific, yet to be elucidated, role of this membrane transporter protein in promoting seabass compensatory growth induced

⁴ ASCT2: AlaSerCys Transporter 2 (also called SLC1A5), a neutral amino acid transporter.

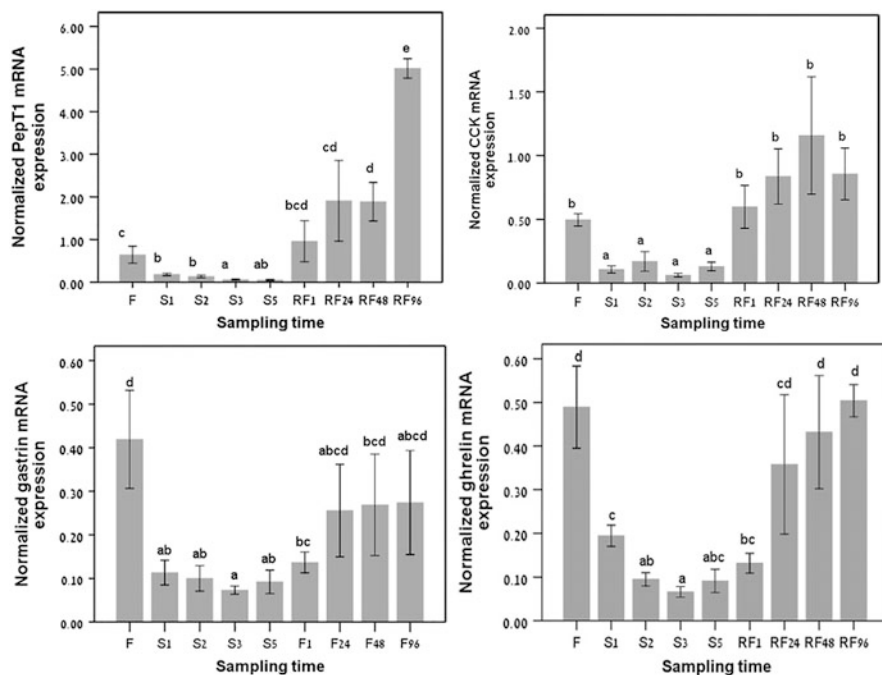


Fig. 4.5 The effect of 10-day continuous feeding (F) of zebrafish, 5-day feed deprivation (S1, S2, S3, S5) followed by immediate refeeding (RF1) and then refeeding after 24 h (RF24), 48 h (RF48), and 96 h (RF96) on normalized mRNA expressions of *pept1*, *cck*, *gastrin*, and *ghrelin*. Different letters indicate significant differences ($P < 0.05$). (From Koven and Schulte (2012), with permission from Springer Nature)

by refeeding. However, the decrease of *pept1* transcription during fasting and the increases during refeeding are the opposite of what was observed so far in mammals and birds, indicating that PEPT1 operates in direct support of fish growth.

In order to discover potential mechanisms controlling compensatory growth in adult zebrafish, Koven and Schulte (2012) studied how short-term feed deprivation and refeeding modulate not only the gastrointestinal *pept1* transcription but also how satiety hormones cholecystikinin (CCK), gastrin-releasing peptide (GRP), and ghrelin (Fig. 4.5). *Pept1* transcription increases. Successive refeedings reaches a level approximately 8 times higher than pre-fast levels, indicating a physiological mechanism for enhanced absorption efficiency to compensatory growth. *Cck*, *grp*, and *ghrelin* mRNA levels also decrease during fasting and increase with refeeding, but only to pre-fasting levels. Therefore, it is obvious that PEPT1 is central in compensatory growth via CCK secretion and, to a lesser extent, GRP and ghrelin activity.

To repeatedly demonstrate the significance of PEPT1 and its cooperation in nutrient sensing and regulation: up to 75 g kg^{-1} fishmeal can be replaced by canola meal in the diets of juvenile Wuchang bream (*Megalobrama amblycephala*) without

significant negative effects on growth and feed utilization (Zhou et al. 2018). To adapt to dietary changes, this species upregulates intestinal *pept1* and *tor* and downregulates hepatic *tor*.

4.3 Dipeptides, Health, and Stress Resistance

Not only production traits are beneficially affected by dietary dipeptides, particularly Met-Met; rather, this dipeptide gets into the focus of interests because of its effect on immunity. Two laboratories studied biomolecular traits in juvenile grass carps (Su et al. 2018) and microbiota traits in Nile tilapias (Guo et al. 2020b). Su et al. (2018) compared the efficiency of dietary DL-Met and Met-Met supplementation in terms of immunity and resistance against *Aeromonas hydrophila*. The authors provide following details:

1. Met-Met (optimal: $\sim 1.6 \text{ g kg}^{-1}$ diet) supplementation increases enteritis resistance (Fig. 4.6).
2. Met-Met supplementation increases the immune function in the intestine through a mechanism attributed to (i) Increase in innate and adaptive immune components including lysozyme and acid phosphatase activities, complement 3 (\rightarrow Box, Chap. 2), complement 4, and IgM contents and the upregulation of antimicrobial peptides genes (*hepcidin*, *leap-2a*, *leap-2b*, β -*defensin-1*, and *mucin2*); (ii) Downregulation of inflammatory cytokine genes (*tnf- α* , *ifn- γ* 2, *il-1 β* , *il-8* in the distal intestine), and the upregulation of anti-inflammatory cytokine genes (*il-4/13a*, *il-4/13b*, *il-6*, *il-10*, *il-11*, and *tgf- β 1*).

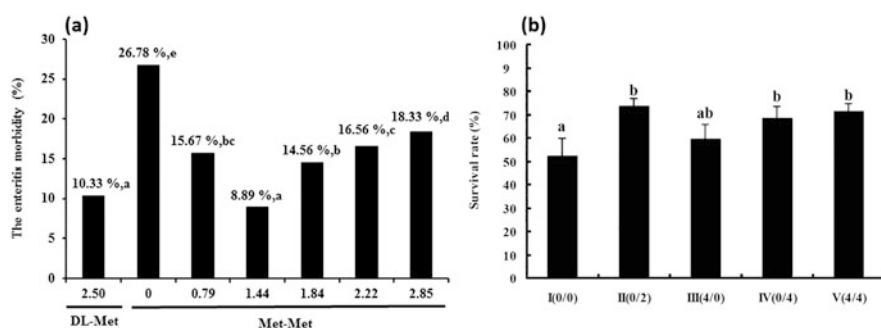


Fig. 4.6 Dietary dipeptides and fish immunity/pathogen resistance. **(a)** Effects of dietary DL-Met (g kg^{-1}) and Met-Met (g kg^{-1}) supplementation on enteritis morbidity of juvenile grass carp after infection with *Aeromonas hydrophila*. Different letters indicate significant differences ($P < 0.05$). (From Su et al. (2018), with permission from Elsevier). **(b)** Effects of Ala-Gln feeding on anti-disease ability of juvenile Jian carp. Fish were challenged by intraperitoneal injection with virulent *Aer. hydrophila*. Values are means \pm SE of three replicate groups, with five fish in each feeding group (I (0/0) = control group fed with control diet (0% Ala-Gln), II (0/2) = 2 weeks control diet fed and 2 weeks experimental diet (1% Ala-Gln) to V (4/4) = 8 weeks experimental diet fed. Different superscripts indicate significant differences ($P < 0.05$). (From Chen et al. (2016), with permission from Elsevier)

The results comply well with a study demonstrating the importance of dietary supplementation of Ala-Gln in Jian carp (Chen et al. 2016). This dipeptide improves growth (up to 16%), metabolism, crowding stress resistance, and disease resistance against *Aer. hydrophila* (Fig. 4.6). The underlying regulatory pathways remain undisclosed, but may be comparable to that in grass carps.

In the same line of evidence, Guo et al. (2020b) showed that dietary Met-Met supplementation increases growth, antioxidant capacity, content of essential AAs, and improves the diversity of intestinal microbiota in Nile tilapia. The microbial majority belongs to *Fusobacteria*, followed by *Bacteroidetes* and *Proteobacteria*. The diversity of intestinal microbiota and total antioxidant capacity in fishes is optimal at 15% dietary Met-Met.

Even abiotic environmental stresses can be mitigated by dietary dipeptides. Li et al. (2018) showed that the alanyl-glutamine dipeptide (AGD) is effective against chronic ammonia stress induced hyperammonemia in juvenile yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*).

The Ala-Gln dipeptide has even the potential to act as antidote against antinutritional factors. Soybean saponin is the major anti-nutritional factor in soybean meal, often used as protein source, and highly toxic to fishes, because of their membrane-permeabilizing activity, which causes distal intestine enteritis (Iwashita et al. 2008; Gu et al. 2014) (→AAN III “Fishmeal Replacements”). Enteritis leads to exposure of the submucosa to a variety of pathogens and microorganisms, and promotes engraftment of pathogenic bacteria in the intestinal epithelium in fishes (Iwashita et al. 2008) (from Tian et al. (2020), references added). In their zebrafish study, Tian et al. (2020) proved an antidote activity of Ala-Gln against soybean saponin. Dietary Ala-Gln at 10 g kg⁻¹ improves the adverse effects, including increasing feeding and growth performance, and improving intestinal injury. Dietary Ala-Gln supplementation promotes the breakdown of carbohydrates and fats for energy, thereby improving the growth performance of zebrafish.

4.4 Concluding Remarks

The success of dipeptide administration points out the potential for reevaluation of AA requirements in early life stages of fishes that has not been possible thus far (Dabrowski et al. 2005). Since then, the numbers of effective dipeptides are continuously increasing, and their function is more comprehensive than only nutrition and improvement of growth performance. Particularly, the improvement of the immune response on dipeptide diets deserves future attention. Therefore, it seems reasonable to apply recent concepts of bioinformatics and pharmaceutical/environmental chemistry. One novel approach could be the construction of a transcriptome atlas (Braich et al. 2019) or, to keep the approach more simple, only a reduced one (Zhang and Zhao 2018). The biomolecular techniques should be applied alone or in combination with structure-effect techniques, such as QSAR (Pudenz et al. 2002) or partial order ranking technique (Brüggemann et al. 2001a, 2001b). Structure-effect as well as

ranking techniques ease the selection of dietary dipeptides based on their effects in fishes and invertebrates. Such techniques have been successfully applied in, for instance, pharmaceutical topics in the development of novel drugs and medications.

So far, epigenetics is almost completely omitted in the studies of peptide transporters as well as peptide and AA utilization. However, recent cancer studies indicate that, for instance, miRNAs are involved in silencing peptide transporters (Dong et al. 2017; Liu et al. 2018). This indicates that the inclusion of epigenetics in AA and peptide studies is overdue.

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Chapter 5

Amino Acid Function and Requirement— *‘More than Easy Fuel’*



Abstract Conventionally, amino acids (AAs) are classified as essential AAs (EAAs) and nonessential AAs (NEAAs), although evidence is accumulating that NEAAs play important roles in regulating gene expression, cell signaling, antioxidative responses, fertility, neurotransmission, and immunity. This comprehensive function will be shown in selected wild and farmed aquatic animals. Several AAs take part in the one-carbon metabolism and, in concert with acetyl-CoA, in epigenome modulations. Furthermore, several AAs appear to regulate appetite hormone and growth factor expression. The role of microRNAs (miRs) in the biomolecular action of AAs as well as potential cross-kingdom miR transfer from plants to aquatic animals has not yet been studied in depth, but they deserve future attention.

Amino acids (AAs) are organic compounds that contain amine ($-\text{NH}_2$) and carboxyl ($-\text{COOH}$) functional groups, along with a side chain (R group) specific to each AA (Nelson and Cox 2005). AAs give rise to a variety of essential molecules in life. Based on mammalian growth studies, AAs have been traditionally classified as either nutritionally essential (indispensable) AAs (EAAs) or nonessential AAs (dispensable) (NEAAs). EAAs comprise histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val). For fishes, also proline (Pro) is essential (Wu 2013). For mammals, NEAAs are alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamate (Glu), glutamine (Gln), glycine (Gly), serine (Ser), Pro, taurine (Tau), and tyrosine (Tyr) (DelCurto et al. 2013).

A careful reevaluation of the scientific literature, however, reveals that over the past century, there has not been compelling experimental evidence to support the assumption of nonessentiality. NEAAs, such as Gln, Glu, Pro, Gly, and Arg, play important roles in regulating gene expression, cell signaling, antioxidative responses, fertility, neurotransmission, and immunity. Thus, diets for animals must contain all NEAA to optimize their survival, growth, development, reproduction, and health. In this regard, AAs should no longer be classified as nutritionally

essential or nonessential in animal nutrition (Wu 2014). However, since most papers on animal nutrition still differentiate between EAA and NEAA, this chapter follows this outdated differentiation.

5.1 Function

In mammals, the fetal synthesis of proteins depends on the balanced provision of EAAs and NEAAs (Wu et al. 2013). Emerging evidence in aquatic animals, too, shows that some AAs and their metabolites are important regulators of key metabolic pathways necessary for maintenance, growth, feed intake, nutrient utilization, immunity, behavior, larval metamorphosis, reproduction, as well as resistance to environmental stressors, including pathogens (Li et al. 2009). Furthermore, conventional definitions on essential and nonessential AAs for fishes are challenged by numerous discoveries that dietary Tau, Gln, Gly, Pro, and hydroxyproline (Hpro) promote growth, development, and health. On the basis of their crucial roles in cell metabolism and physiology, Huang et al. (2020) summarized that dietary supplementation with specific AAs is beneficial for:

1. Increasing the chemo-attractive property and nutritional value of aquafeeds with low fishmeal inclusion
2. Optimizing efficiency of metabolic transformation in juvenile and subadult fishes
3. Suppressing aggressive behaviors and cannibalism of larvae and juveniles
4. Increasing larval performance and survival
5. Mediating timing and efficiency of spawning
6. Enhancing immunity and tolerance to environmental stresses

Functional AAs hold great promise for the development of balanced aquafeeds to enhance the efficiency of global aquaculture production. More physiologically, AAs can also be classified according to the metabolic fate of the carbon skeleton after removal of ammonia from ketogenic, glucogenic, or both glucogenic and ketogenic AAs. Ketogenic AAs yield acetyl-CoA or acetoacetyl-CoA, but they do not produce metabolites that can be converted into glucose. These AAs increase blood acetoacetate (a ketone body) when consumed in high amounts. Only Lys and Leu are exclusively ketogenic AAs.

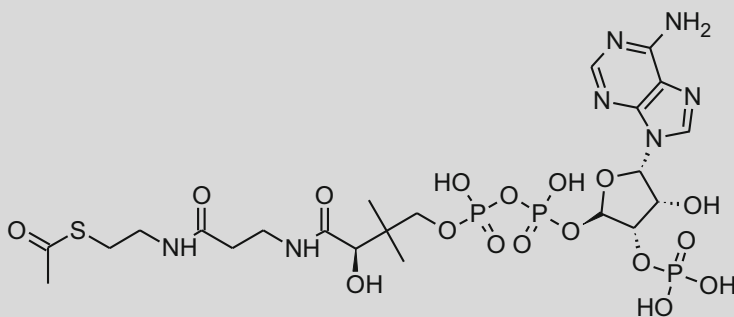
Box 5.1 Acetyl-CoA: The Key Metabolite

Acetyl-CoA (activated acetate, Box Fig. 1) is a thioester with a large, negative, standard free energy of hydrolysis. Thioesters contain a sulfur atom in the position occupied by an oxygen atom in oxygen esters. In aerobic organisms, glucose and other sugars, fatty acids (FAs), and most AAs are ultimately

(continued)

Box 5.1 (continued)

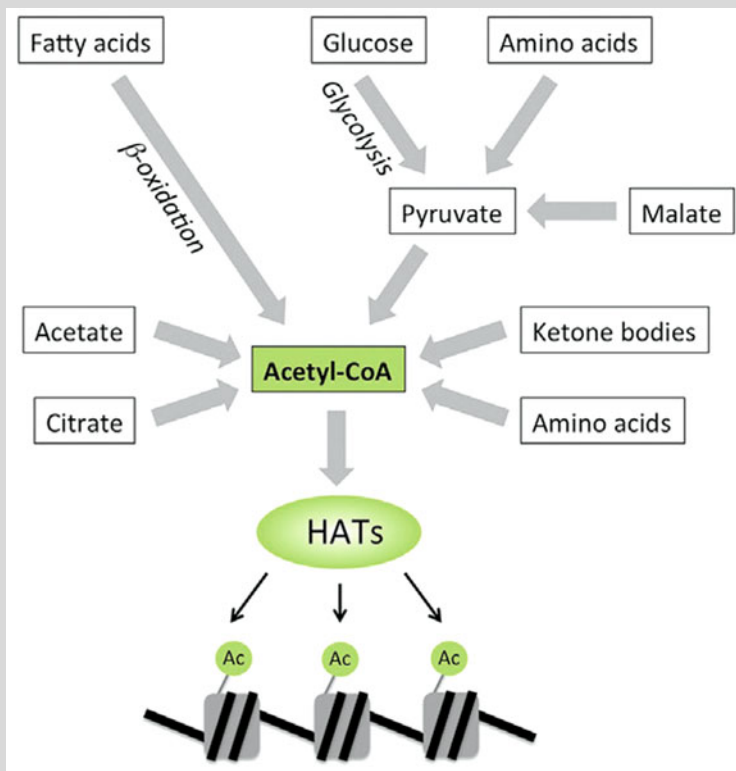
oxidized to CO_2 and H_2O via the citric acid cycle and the respiratory chain. Before entering the citric acid cycle, the carbon skeletons of sugars and FAs are degraded to the acetyl group of acetyl-CoA, the form in which the cycle accepts most of its fuel input. Many AA carbons also enter the cycle this way (Nelson and Cox 2005).



Box Fig. 1 Structure of acetyl-CoA

Acetyl-CoA is crucial in epigenetics and its position in the epigenome modulation with AAs is depicted in Box Fig. 2. It is a key intermediary metabolite produced during catabolism and anabolism. It is the universal substrate for the acetylation of histones (Etchegaray and Mostoslavsky 2016). The activity of histone acetyltransferases (HATs) relies on intracellular levels of acetyl-CoA, which stands as a prominent example of the interplay between metabolism and chromatin dynamics. Acetyl-CoA serves as a feeding supply molecule in the tricarboxylic acid (TCA) cycle within the mitochondria, and it participates as a building block in the production of macromolecules such as lipids, cholesterol, and AAs (Box Fig. 2). Acetyl-CoA is generated via activation of acetate, the thiolytic cleavage of β -ketoacyl-CoA along with β -hydroxy acids, and the oxidative decarboxylation of pyruvate. In general, the generation of acetyl-CoA is associated with the breakdown of carbohydrates and fats, via glycolysis and β -oxidation, respectively. Under conditions of limited glucose availability, such as fasting, FA β -oxidation becomes the major cellular metabolic pathway associated with the generation of acetyl-CoA promoting the TCA cycle to maintain mitochondrial-dependent ATP biosynthesis. The levels of acetyl-CoA are quite dynamic and are directly dependent on nutrient availability.

(continued)

Box 5.1 (continued)

Box Fig. 2 Interplay between intermediary metabolites and epigenetics: acetyl-CoA and histone acetylation. Various metabolic pathways lead to the formation of acetyl-CoA, which is then utilized as an acetyl group donor during histone acetyltransferase-dependent acetylation of nucleosomal histones. (From Etchegaray and Mostoslavsky (2016), with permission from Elsevier) *HAT* histone acetyltransferase

Available data strongly favor the idea that high levels of acetyl-CoA result in a more permissive chromatin configuration by increasing the acetylation of histones, which, in turn, promotes gene expression that modulates cell growth under these “nutrient-favorable” conditions. In this context, acetyl-CoA acts as a biosensor of the metabolic state that triggers the regulation of specific genes involved in growth and proliferation in response to nutrient availability through an epigenetic mechanism involving histone acetylation (Box Fig. 2) (Etchegaray and Mostoslavsky (2016) and references therein; extract taken with permission from Elsevier).

Glucogenic AAs, whose catabolism yields to the formation of pyruvate or Krebs cycle metabolites (α -ketoglutarate, oxalacetate, enol pyruvate, etc.), can be converted into glucose through gluconeogenesis (\rightarrow Chap. 18), which increase blood glucose when consumed in high amounts. Glucogenic AAs are Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Met, Pro, Ser, and Val. Some AAs can be both glucogenic and ketogenic because they can yield some precursors that can become glucose and FAs. AAs of this kind are Ile, Phe, Trp, Tyr, and Thr (Molina-Poveda (2016) and references therein).

AAs regulate gene expression at the levels of transcription, translation, and post-translational protein modification (Wu 2009). Results of cell culture studies indicate that deficiency of an AA, either an EAA or a NEAA, results in increased availability of uncharged transfer RNAs (tRNAs¹) and, eventually, in a decrease in global protein synthesis. In contrast, excess of an AA may down- or upregulate gene transcription depending on its side chains and target proteins, indicating the complexity of regulatory mechanisms for protein synthesis. For instance, either excess or deficiency of Arg modulates global gene expression in mammalian cells, whereas Met deficiency stimulates osteopontin² expression in hepatocytes through hypomethylation of DNA and protein (Wu 2009).

AAs regulating mammalian pathways for maintenance, growth, reproduction, and immunity include Arg, Cys, Gln, Leu, Pro, and Trp (Wu 2009). These AAs maximize the efficiency of feed utilization, enhance protein accretion, reduce adiposity, and improve health. It should be the focus of future studies whether these findings and statements derived from mammals can be translated to other vertebrates, such as fishes, and invertebrates alike.

Importantly, AAs, such as His, Met, Gly, and Ser, participate in one-carbon-unit metabolism that is essential for DNA synthesis as well as cell growth, development, and epigenetic regulation. These AAs regulate the availability of S-adenosylmethionine (SAM), a major methyl donor for DNA and protein methylation by methyltransferases (Jin et al. 2010). Epigenetic changes can persist through cell divisions and be transferred to subsequent generations (DeCurto et al. 2013). This notion of (indirect) transgenerational effects of AAs has important implications for animal production (Wu 2010) as well as developmental traits and health of offspring but appears to be neglected in aquatic animal nutrition so far.

In addition to the potential of epigenetic modulation, AAs have significant anti-inflammatory functions in animals (He et al. 2018) (Fig. 5.1). AAs ameliorate

¹transfer RNAs are adapter molecules that translate the information in mRNA into a specific sequence of AAs (Nelson and Cox 2005).

²Osteopontin (OPN) is central in the control of biomineralization. It is a phosphorylated glycoprotein secreted by activated macrophages, leukocytes, and activated T lymphocytes, and present in extracellular fluids, at sites of inflammation, and in the extracellular matrix of mineralized tissues. This cytokine mediates important cell matrix and cell-cell interactions. OPN is abundant in the bone, where it facilitates the attachment of osteoclasts to the bone matrix. In the immune system, OPN plays a role in chemotaxis, leading to the migration of macrophages and dendritic cells to sites of inflammation (Gravallese 2003; Kahles et al. 2014).

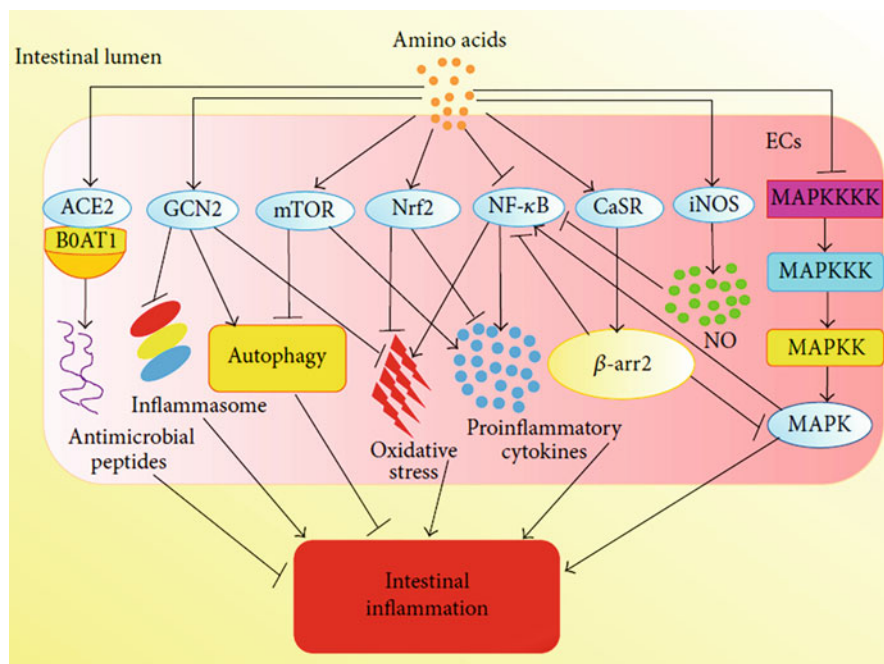


Fig. 5.1 Possible signaling mechanisms of AAs in intestinal inflammation in the epithelial cells are illustrated. AAs ameliorate intestinal inflammation by impressing the NF- κ B and MAPK pathway. AAs activate the Nrf2 pathway to regulate intestinal inflammation via inhibiting oxidative stress and the expression of pro-inflammatory cytokines. AAs activate iNOS to inhibit the NF- κ B pathway by the production of \bullet NO. ACE2 combines broad neutral (0) amino acid transporter 1 (B0AT1) to regulate the uptake of Trp in intestinal epithelial cells, which activates the expression of antimicrobial peptides to regulate intestinal microbiota. ACE2 reduces intestinal inflammation by regulating innate immune responses and intestinal microbiota (not shown). GCN2 regulates intestinal inflammation by inhibiting inflammasome activation, triggering autophagy, and preventing oxidative stress. CaSR activated by Trp exerts anti-inflammation roles via activating the complex of B-arrestin 2 (β -arr2) to inhibit NF- κ B and MAPK pathways in intestinal epithelial cells. After being activated by AAs, mTOR signaling can inhibit autophagy. (From He et al. (2018), credit Hindawi BioMed Research International)

intestinal inflammation by improving the intestinal barrier, increasing the expression of anti-inflammatory cytokines as well as tight junction proteins, and reducing oxidative stress and apoptosis of enterocytes as well as downregulation of pro-inflammatory cytokines. This function is associated with various signaling pathways with the mechanistic target of rapamycin (mTOR, \rightarrow Box TOR below), inducible nitric oxide synthase (iNOS), calcium-sensing receptor (CaSR), nuclear factor-kappa-B (NF- κ B), mitogen-activated protein kinase (MAPK), nuclear erythroid-related factor 2 (Nrf2), general controlled non-repressed kinase 2 (GCN2), and angiotensin-converting enzyme 2 (ACE2) (He et al. 2018).

5.1.1 Appetite and Growth Regulations

Food composition affects the expression of components of the growth hormone–insulin-like growth factor-I (GH-IGF) axis (Gómez-Requeni et al. 2012). Additionally, the gastric orexigenic hormone ghrelin, responsible for the release of the growth hormone (GH), is regulated by diet composition in fishes (Blanco et al. 2016): Dietary macronutrients can modulate the release of GH.

To determine whether dietary AAs regulate the hepatopancreatic expression of growth hormone receptors (*ghr-I*, *ghr-II*), and insulin-like growth factors (*igf-I*, *igf-II*) independently of circulating growth hormone (GH), Bertucci et al. (2017a) conducted organ culture experiments. Goldfish hepatopancreas sections are incubated with different doses of Trp, a precursor of serotonin (→Chap. 7). The transcription of all four genes is upregulated by Trp (Fig. 5.2). The regulatory effect depends more on the time rather than on the concentration of Trp. On the basis of their in vitro experiments, the authors hypothesize that the effects of Trp on tissue growth is mediated in vivo by serotonin and GH in the pituitary and also directly by IGFs in the liver. Future research will determine if these changes take place also with lower concentrations and if they translate into proteins.

In a companion study, Bertucci et al. (2017b) showed that single AAs directly regulate ghrelin (orexigenic) and nesfatin-1 (anorexigenic) in hepatopancreas and

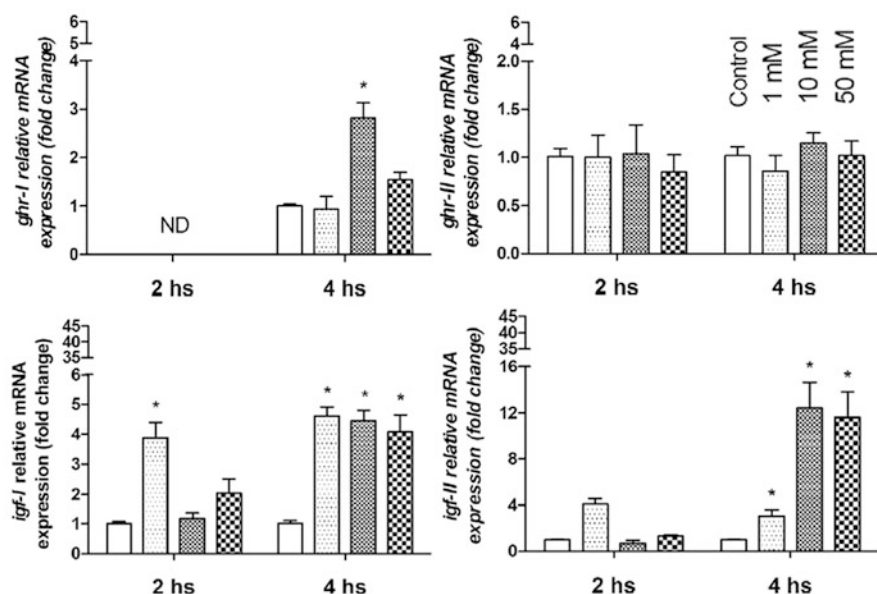


Fig. 5.2 *Ghr-I*, *ghr-II*, *igf-I*, and *igf-II* mRNA in vitro expression in goldfish hepatopancreas after 2 h and 4 h treatments with 1 mM, 10 mM, and 50 mM of L-tryptophan. Data CR are means \pm SEM ($n = 6$ fishes). * denotes significant statistical differences ($P < 0.05$). (From Bertucci et al. (2017a), with permission from Elsevier). ND not determinable

intestine cultures of goldfish. Trp downregulates the expression of both peptide hormones involved in food intake regulation. A decrease in intestinal *ghrelin* mRNA expression is in agreement with the decrease in plasma ghrelin observed after meals (Bodosi et al. 2004). Since nesfatin-1 is an anorexigenic peptide, Bertucci et al. (2017b) anticipated an increase after treatment with macronutrients. However, the opposite occurs: The decrease in *nesfatin-1* mRNA and protein expression in the intestine may indicate another, yet to be identified, function of this peptide, likely not related to food intake control.

5.1.2 Methyl-Donating Amino Acids and MicroRNAs

MicroRNAs (miRs) are an abundant class of newly identified endogenous nonprotein-coding small RNAs. They are managers of mRNA stability and translation. Furthermore, they appear to facilitate conversations between kingdoms, particularly between hosts and pathogens/parasites. MiRs play important roles in controlling DNA methylation and histone modifications, creating a highly controlled feedback mechanism. Epigenetic mechanisms such as promoter methylation or histone acetylation can also modulate miR expression. An aberrant expression of miRs is also associated with altering cell proliferation and apoptosis processes, that is, cancer development (Choi and Friso 2010).

It is much debated whether food-borne miRs have any regulating impacts on the physiology of individuals who consume feeds containing abundant miRs (Weiberg et al. 2015). Zhang et al. (2011) are the first to show that miRs from dietary plant matter (rice) survive in the consuming animals for several hours, circulate in the blood, enter multiple tissues, including the liver, and even regulate genes in the consumer. Other researchers, however, regard the reported details as artifacts (see discussion between Witwer and Zhang (2017) and reviews by Zhao et al. (2018) and Sundaram (2019)). A recent *in silico* prediction identified 942 human genes as potential targets for 277 rice miRNAs (Rakhmetullina et al. 2020) – yet, the *in vivo* proof is pending. Therefore, potential cross-kingdom effects of non-coding RNAs remain a challenging topic in nutrition.

Apart from direct regulatory potentials by feed-borne miRs, these non-coding RNAs are able to regulate methyl-deficient diets. This takes place if Met, His, Gly, or Ser is insufficiently supplied in the diets. Two mammalian examples demonstrate how severe and adverse this kind of deficient diets can become: A methyl-deficient diet that induces liver tumors in rats, also induces prominent early changes in transcription of miR genes, involved in the regulation of apoptosis, cell proliferation, cell-to-cell connection, and epithelial-mesenchymal transition in rat livers. Mice fed a methyl-deficient diet develop the nonalcoholic fatty liver disease accompanied by changes in the transcription of miRs. Changes in the expression of these miRs lead to parallel changes in protein levels of their targets. These studies indicate that alterations in the transcription of miRs are a prominent event during the development of severe diseases by dietary methyl deficiency (Choi and Friso (2010) and references

therein). These studies may serve as hypothesis generator for corresponding studies in fishes and aquatic invertebrates.

In teleost fishes, miRs have gained attention as regulating tools in ontogenetic development, physiology, and growth in general (Bizuayehu and Babiak 2014) as well as skeletal muscle growth in particular (Zhu et al. 2014, 2015). Cross-kingdom miR transfer from plants to fishes and aquatic invertebrates or inter-organismal interactions by miRs have not yet been studied but deserve future attention and can likely contribute to the improvement of rearing conditions and increasing the nutritional capacity of aquatic animals.

5.2 Requirement

5.2.1 Invertebrates

Only a few studies have been conducted on the quantitative requirements of EAA in larval crustaceans. One study is dedicated to *Macrobrachium rosenbergii* (Table 5.1) (Roustaian et al. 2000), a major freshwater prawn in aquaculture. *Macrobrachium* species are generalist predators feeding on slow-moving prey, such as minute zooplankton crustaceans in the larval stage and small worms, snails, chironomid larvae, and caddisflies in the adult stage (New 2002). In *M. rosenbergii*, the highest requirement of AAs is found for Glu and Phe [with cystine (Cys2)] ranging from 13 to 17 and 10–12%, respectively, whereas Trp (1.0–1.6%), Met (1.4–2.7%), and His (2.9–4.2%) are lower. The levels of the following EAAs do not significantly change during larval development: Arg, Leu, Phe (plus Cys2), Thr, Trp, and Val. Overall, AA the composition appears to stay relatively unchanged during larval growth and can be satisfied by a suitable protein source that resembles the larval AA profile (Roustaian et al. 2000).

In addition to the general requirement of AAs, it is worth considering the biochemical function of and biomolecular control by selected AAs. It was shown that 4 out of 20 L-AAs increase feeding behavior of the water flea *Daphnia magna*. The AAs with attractant capability are Asp, Cys, Glu, and Gln (Lari et al. 2018).

This finding complies well with earlier studies. Marine scavenging animals depend on chemoreception for detecting carrion, a scarce resource. Ide et al. (2006) found that the amphipod *Aroui* (*Scopelocheirus*) *onagawae* is efficiently attracted by Gly, Ala, and Arg, which are abundant in the tissues of (dead) marine animals.

These studies confirm that AAs are central for predators as chemical cues to find living or dead preys (McClintock and Baker 2001; Brönmark and Hansson 2012). Furthermore, the acceptance of artificial and designed feed can be improved by supplementing it with typical attracting or stimulatory AAs. However, the AA patterns appear to be species-specific or even specific for a given prey-predator system. This issue will be revisited in Chap. 6.

Table 5.1 Essential amino acid profile (% of total AAs) of *Macrobrachium rosenbergii* at different stages of development. Value for each developmental stage is the mean of two replicates. Average (\pm SD) and range of values for each AA during the larval life are presented. Means within the same row followed by different superscripts are significantly different. (From Roustaian et al. (2000), with permission from Wiley)

Amino acids	Larval stages								
	I	II	III	IV	V	VI/VII	VIII/IX	Mean \pm SD	Range
Arginine	8.9 ^a	7.9 ^a	7.2 ^a	7.1 ^a	7.3 ^a	6.9 ^a	7.0 ^a	7.5 \pm 0.71	6.9–8.9
Histidine	3.4 ^{ab}	3.9 ^{ab}	2.9 ^a	3.3 ^{ab}	4.2 ^b	3.5 ^{ab}	3.2 ^{ab}	3.0 \pm 0.44	2.9–4.2
Isoleucine	5.0 ^a	5.5 ^a	5.0 ^a	4.5 ^a	4.9 ^a	4.2 ^a	4.4 ^a	4.9 \pm 0.45	4.2–5.5
Leucine	9.2 ^a	9.8 ^a	7.6 ^a	9.0 ^a	8.3 ^a	8.5 ^a	8.4 ^a	8.7 \pm 0.71	7.6–9.8
Lysine	7.1 ^{ab}	8.9 ^b	6.4 ^a	7.0 ^{ab}	7.8 ^{ab}	8.0 ^{ab}	9.1 ^b	7.8 \pm 1.0	6.4–9.1
Methionine	2.7 ^b	1.4 ^a	2.5 ^b	2.2 ^b	2.4 ^b	2.2 ^b	2.0 ^{ab}	2.2 \pm 0.42	1.4–2.7
Phenylalanine + cystine	10.6 ^a	10.8 ^a	11.1 ^a	11.5 ^a	10.5 ^a	10.2 ^a	9.7 ^a	10.6 \pm 0.59	9.7–11.5
Threonine	4.7 ^a	4.6 ^a	4.8 ^a	4.6 ^a	4.9 ^a	4.9 ^a	4.5 ^a	4.7 \pm 0.16	4.5–4.9
Tryptophan	1.6 ^a	1.5 ^a	1.6 ^a	1.4 ^a	1.5 ^a	1.5 ^a	1.4 ^a	1.5 \pm 0.08	1.4–1.6
Valine	5.3 ^a	4.3 ^a	6.1 ^a	4.4 ^a	4.3 ^a	4.2 ^a	4.4 ^a	4.7 \pm 0.72	4.2–6.1

5.2.2 Fishes

As aquafeeds are increasingly formulated with a variety of low-cost proteins, more attention needs to be paid to ensure that the EAA requirements of fishes are met adequately; yet, little is known about these requirements. They have generally been established through dose-response studies. However, factorial modeling approach is increasingly being used to estimate EAA demands (NRC 2011). The factorial approach is based on information on maintenance requirement and AA deposition for body protein and its utilization efficiency. Reliable estimates of maintenance requirement of AAs are therefore important to develop feed formulation strategies to meet AA demands of fishes and to achieve maximum feed efficiency and minimum nitrogen (N) waste output (Hua 2013).

Hua and Bureau (2019) developed a factorial model of AA requirement for Atlantic salmon and rainbow trout by incorporating dietary effects and biological factors on AA requirement and utilization. The factorial model determines AA requirement based on species, expected body weight gain, body protein deposition, AA profile in body protein deposition, maintenance AA requirement, and EAA metabolism. Simulations with this model indicate that EAA requirement progressively decreases with increasing live weight of the fishes. Atlantic salmon appears to have higher EAA requirements than rainbow trout at the same body weight classes, and the rate of decrease in EAA requirement with the increase of body weight appears to be slower in Atlantic salmon. Model simulations also indicate that the dietary composition influences the estimates of EAA requirements. This modeling approach can be adapted to other fish species (Hua and Bureau 2019) and is a useful guideline for feed formulation, but does not yet substitute accurate estimates of AA maintenance requirement.

In her previous metastudy, Hua (2013) applied a nonlinear mixed model to estimate maintenance requirements of EAAs of fish from published studies. The maintenance requirements of Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Try, and Val are estimated to be 7.7 (± 6.6), 9.8 (± 2.4), 6.4 (± 24.8), 9.1 (± 13.7), 15.6 (± 8.2), 18.4 (± 1.9), 14.6 (± 3.2), 5.4 (± 4.4), 0.5 (± 1.3), and 9.3 (± 26.6) mg kg⁻¹ BW^{0.75} d⁻¹, respectively. The maintenance requirements of these AAs as a proportion of their total requirements for protein deposition are estimated to be 4.8%, 31.0%, 8.9%, 12.7%, 5.2%, 11.9%, 19.2%, 2.5%, 2.2%, and 10.8%, respectively. For most of the EAAs, the maintenance requirements represent a small proportion of total requirement (below 10%) with notable exceptions for His and Phe. This study indicates that maintenance requirements of fish cannot be assumed equal for all AAs.

In fasted post-larval Senegal sole (*Solea senegalensis*) and fasted herring (*Clupea harengus*) larvae, Conceição et al. (2003) showed that EAA and NEAA are differently used for metabolic purposes: high retention is found for EAA and low for NEAA in the body. Consequently, from the onset of exogenous feeding, fish larvae have high catabolic losses of AAs but use NEAA preferentially to EAA as energy

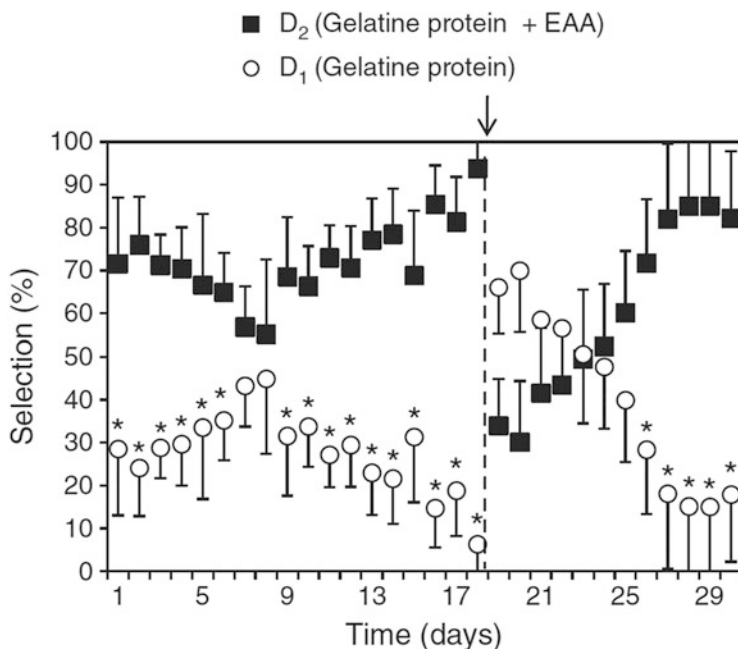


Fig. 5.3 Selection of (D₁; ○) and (D₂; black square) by Nile tilapia, considering the total of diets as 100% and the corresponding standard errors (SEM) for eight individual fishes in the 30 days of the experimental period. On day 18, the feeds were switched between feeders. Significant differences are found between days (*, $P < 0.05$). (From Fortes-Silva et al. (2012), with permission from Elsevier)

substrates, confirming previous findings in fishes general (Kim et al. 1991; Cowey and Cho 1993). This finding is also supported by a study of eggs and larvae of Asian seabass (barramundi, *Lates calcarifer*) (Dayal et al. 2003). The ratio of EAA/NEAA increases from 0.8 in the pre-feeding stage to 1.6 after 2 days of feeding. This indicates the capacity of sparing EAA at the expense of NEAA.

In an intriguing self-selection trial of Nile tilapia, Fortes-Silva et al. (2012) demonstrate that this fish is able to select its “preferred” diet (supplemented with EAA) when it is offered diets differing in EAA composition (Fig. 5.3). Tilapia not only selects the diet amended with EAA but also shows a linear behavioral relationship between protein intakes and equilibrate EAA intake.

For requirement of nine EAAs, please refer to Table 5.2 and for Met requirement to Chap. 9.

Table 5.2 Quantitative requirements for nine essential amino acids (EAA) have been established in following selected species (in percentage of dietary protein, unless otherwise indicated; *DM* dry matter)

	Arg	His	Ile	Leu	Lys	Phe	Thr	Trp	Val	References
Invertebrates										
<i>Farfantepenaeus aztecus</i>	5.2	3.2	4.5	9.8	6.1	8.4 + Trp	5.4		5.1	In Sara (2007)
<i>Haliotis rufescens</i>	6.3–8.3	1.6–2.2	3.2–4.2	7.3–8.3	5.5–6.4	3.1–4.7	4.3–4.8	0.4	4.3–5.1	Allen and Kilgore (1975)
<i>Litopenaeus vannamei</i>	5.6		3.9	5.8	3.9–4.7		3.5–3.8			In Molina-Poveda (2016)
<i>Lytechinus variegatus</i> (% diet DM)	6.1	1.6	2.7	4.7	4.8	5.4 + Trp	2.5		3.1	In Sara (2007)
	2.1	0.7	1.3	2.4	1.9	1.5			1.4	Hefin et al. (2016)
<i>Macrobrachium malcolmsonii</i> (% diet DM)	1.3–19.3	9.9–16.5	3.1–3.8 +met, Tyr, Leu +asp, Thr		1.9–2.4	1.5–1.9 +Trp			2.8–3.7 +Gln	Radhakrishnan et al. (2015)
<i>Macrobrachium rosenbergii</i>	8.1	2.3	5.0	7.5	5.3	3.9	3.4	0.9	4.4	In Molina-Poveda (2016)
<i>M. rosenbergii</i> (% diet DM)	1.7		1.0		0.9					Radhakrishnan et al. (2016)
<i>M. rosenbergii</i> (% <i>Artemia</i> DM)	1.8	0.8	1.0	1.7	1.4	1.0	1.0		1.2	Anh et al. (2009)
<i>Marsupenaeus japonicus</i>	3.2–5.3	1.2	2.6	3.8	3.8	3.0	2.6	0.8	2.8	In Molina-Poveda (2016)
	5.6	1.6	3.1	5.5	5.8	6.2 + Trp	3.0		3.0	In Sara (2007)
<i>Palaeomonetes varians</i>	5.1				5.6					Palma et al. (2015)
<i>Penaeus monodon</i>	5.5	1.7	3.1	5.3	5.2	2.9	2.7	0.8	3.6	Chen (1998)
		2.2	2.7	4.3		3.7		0.5		Millamena et al. (1999)
	5.3–7.6	1.6–2.0	3.0–4.6	5.3–9.1	4.0–6.5	4.4–8.3 + Trp	3.3–4.8		2.9–4.6	In Sara (2007)

(continued)

Table 5.2 (continued)

	Arg	His	Ile	Leu	Lys	Phe	Thr	Trp	Val	References
Fishes										
<i>Acipenser transmontanus</i>	3.9	2.6	2.6	4.7	5.3	Phe + Tyr: 4.5	2.9	0.7	3.0	In Akiyama et al. (1997)
<i>Anguilla japonica</i>	4.5	2.1	4.0	5.3	5.3	Phe + Tyr: 5.8	4.0	1.1	4.0	
<i>Aspianax fasciatus</i>	4.6	1.2	3.3	5.6	5.5	2.8	3.4	0.7	3.5	Furuya et al. (2015)
<i>Chanos chanos</i>	5.3	2.0	4.0	5.1	4.0	2.8/4.2 2.7/1.0% Tyr	4.5	0.6	3.6	Borlongan and Coloso (1993)
<i>Cirrhinus mrigala</i>	4.6	2.1	3.2	3.9	5.8	3.3; 0.1% Tyr	4.5	1.0	3.8	In Molina-Poveda (2016)
<i>Clarias gariepinus</i> × <i>C. macrocephalus</i>	4.5– 5.0									
<i>Ctenopharyngodon idella</i>	5.7			4.2– 4.3		3.4	3.6		4.8	Gao et al. (2014, 2015), Deng et al. (2014), Luo et al. (2014), Li et al. (2015)
<i>Cyprinus carpio</i>	4.3	2.1	2.5	3.3	5.3– 5.7	3.3; 2.9% Tyr	3.9	0.8	3.6	In Akiyama et al. (1997)
<i>C. Carpio</i> var. Jian			3.8				5.1		4.0	Feng et al. (2013), Zhao et al. (2012), Dong et al. (2013)
<i>Dicentrarchus labrax</i>	3.9				4.4		2.3– 2.6			In Molina-Poveda (2016)
<i>Gibelion (Catla) catla</i>	4.8	2.5	2.4	3.7	6.2	3.7; 0.1% Tyr	5.0	1.0	3.6	Ravi and Devaraj (1991)
<i>Heteropneustes fossilis</i>		0.9– 1.0						0.6– 0.7		Farhat and Khan (2013, 2014)
<i>Heterotis niloticus</i>	4.0	2.6	3.0	4.5	5.1	Phe + Tyr: 4.7	3.0	0.3	3.3	Monentcham et al. (2010)
<i>Ictalurus punctatus</i>	4.3	1.5	2.6	3.5	5.0– 5.1	2.1; 0.6% Tyr	2.2	0.5	3.0	In Molina-Poveda (2016)

<i>Labeo rohita</i>	3.1–3.5	3.8–4.0	3.8–3.9 4.5–4.8			2.9–3.1 Incl 2.5% Tyr	3.8–4.2	3.8	In Molina-Poveda (2016), Giri et al. (2015), Abidi and Khan (2009)
<i>Larimichthys crocea</i>							4.1		He et al. (2015)
<i>Lates calcarifer</i>	3.8			4.5					In Molina-Poveda (2016)
<i>Megalobrama amblycephala</i>	5.6						1.6	3.7–3.9	Habte-Tsion et al. (2015), Ren et al. (2015), Liang et al. (2016)
<i>Morone saxatilis</i>	1.4	0.6	0.9	1.9	2.2	Phe + Tyr: 1.7	1.1	1.0	Small and Soares (1998)
<i>Morone saxatilis</i> × <i>M. chrysops</i>	4.4				4.0		1.8–2.6	0.6–0.7	In Molina-Poveda (2016)
<i>Oncorhynchus keta</i>	6.5	1.6	2.4	3.8	4.8	Phe + Tyr: 6.3	3.0	0.7	In Akiyama et al. (1997)
<i>Oncorhynchus kisutch</i>	3.2–5.8	0.9–1.8	1.2	3.4–3.8	3.8	Phe + Tyr: 4.5	2.0	0.5	In Akiyama et al. (1997)
<i>Oncorhynchus tshawytscha</i>	6.0	1.8–2.2	2.6	3.0–5.0	5.0	4.4 0.4% Tyr	2.3	0.5	De Long et al. (1962); in Akiyama et al. (1997)
<i>Oncorhynchus mykiss</i>	3.5–4.2	1.0–1.6	1.5–2.8	2.3–9.2	3.0–8.4	2.0	2.6	0.3–0.9	In Akiyama et al. (1997)
<i>Oreochromis mossambicus</i>					4.1				In Akiyama et al. (1997)
<i>Oreochromis niloticus</i>	4.2	1.7	3.1	3.4	5.1–5.8	3.8 0.5% Tyr	3.8–4.7	1.0	In Molina-Poveda (2016)
<i>Pagrus major</i>	4.7								Rahimnejad and Lee (2013, 2014)
<i>Paralichthys olivaceus</i>	4.1–4.9				3.3–4.6				In Molina-Poveda (2016)

(continued)

Table 5.2 (continued)

	Arg	His	Ile	Leu	Lys	Phe	Thr	Trp	Val	References
<i>Pelteobagrus fulvidraco</i>	2.4–2.7									Zhou et al. (2015)
<i>Perca flavescens</i> In g kg ⁻¹ diet	4.9 2.0	0.9	1.4	2.3	2.6	1.4	1.4	0.3	1.6	In Molina-Poveda (2016), Hart et al. (2010)
<i>Rachycentron canadum</i>	6.2				5.3					In Molina-Poveda (2016)
<i>Rhamdia quelen</i>								2.5–3.4%		Pianesso et al. (2015)
<i>Salmo salar</i>	4.0	1.5			5.3	Phe + Tyr: 5.6	2.7	0.7	3.1	Rollin et al. (2003)
<i>Scophthalmus maximus</i>	3.0–5.4				5.0					In Molina-Poveda (2016)
<i>Sciaenops ocellatus</i>	3.7	1.7	2.9	4.7	4.4–5.7	Phe + Tyr: 4.5	2.8	0.8	3.1	In Akiyama et al. (1997)
<i>Seriola quinqueradiata</i>	3.9	2.6	2.6	4.7	5.3	Phe + Tyr: 4.5	2.9	0.7	3.0	
<i>Sparus aurata</i>	5.4	1.8	2.5	4.6	5.0	Phe + Tyr: 5.6	2.9	0.7	3.1	Peres and Oliva-Teles (2009)
<i>Sparus macrocephalus</i>	7.7–8.1				8.6					In Molina-Poveda (2016)
<i>Symphysodon aequifasciata</i>	6.9	2.6	5.0	6.7	8.1	4.7	4.4	0.7	4.8	Chong et al. (2004)

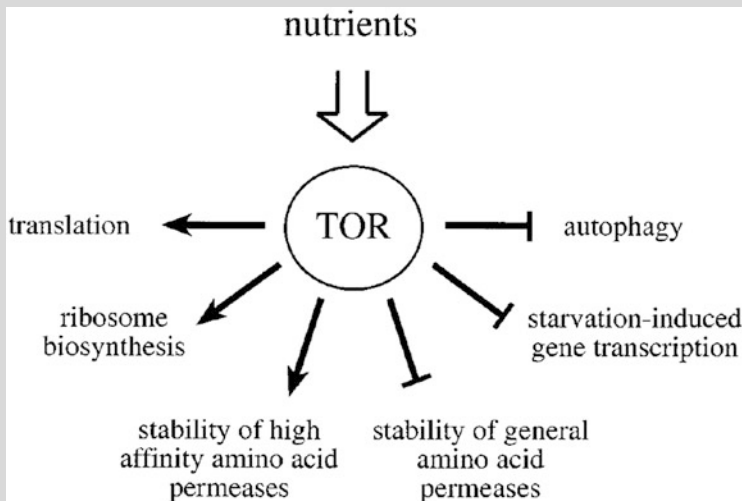
Box 5.2 *Yin and Yang of Energy Regulation*

AMP-activated protein kinase (AMPK) and target of rapamycin³ (TOR) pathways are interlinked, opposing signaling pathways involved in sensing availability of nutrients and energy and regulation of cell growth. AMPK (*Yin* or the “dark side”) is switched on by lack of energy or nutrients and inhibits cell growth, while TOR (*Yang* or the “bright side”) is activated by nutrient availability and promotes cell growth. Genes encoding the AMPK and TOR complexes are found in almost all eukaryotes, indicating that these pathways arose very early during eukaryotic evolution (González et al. 2020).

The TOR proteins regulate.

1. The initiation and elongation phases of translation
2. Ribosome biosynthesis
3. Amino acid import
4. Transcription of numerous enzymes involved in multiple metabolic pathways
5. Autophagy

Studies indicate that TOR signaling plays a critical role also in brain development, learning, and memory formation. Inactivation of the TOR proteins, or rapamycin treatment, mimics nutrient deprivation in yeast, *Drosophila*, and mammalian cells (Raught et al. 2001). Thus, a current working model for TOR signaling proposes that these kinases relay a permissive signal to downstream targets only in the presence of sufficient nutrients to fuel protein synthesis (Box Fig. 3).



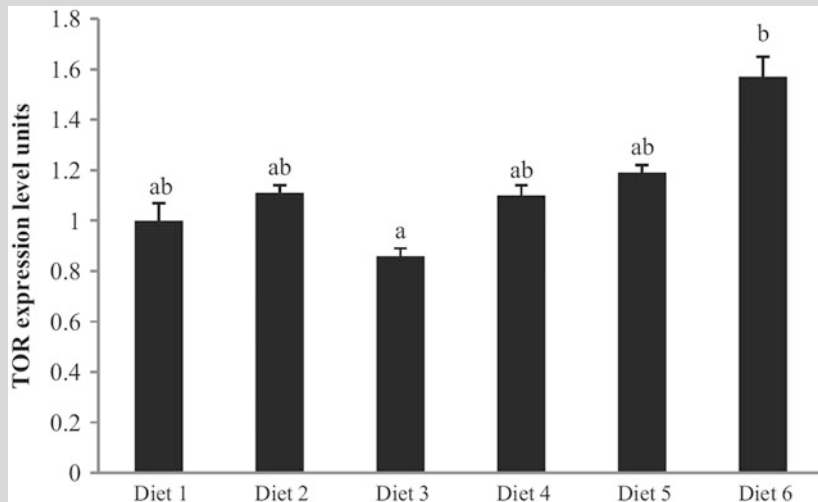
(continued)

³Rapamycin is a macrolide (large macrocyclic lactone ring to which one or more deoxy sugars are attached) compound produced by the bacterium *Streptomyces hygroscopicus*.

Box 5.2 (continued)

Box Fig. 3 TOR proteins regulate the balance between protein synthesis and protein degradation. TOR signaling is active in the presence of sufficient nutrients to fuel protein synthesis. The TOR signal allows for the translation of mRNAs coding for components of the translation machinery, ribosome biosynthesis, and the stabilization of high-affinity AA permeases. At the same time, TOR signaling destabilizes general AA permeases, inhibits autophagy, and represses the transcription of a subset of genes required for AA biosynthesis (from Raught et al. (2001), with permission from The National Academy of Sciences)

The response of TOR on modulated AA content can be demonstrated in Wuchang bream (*Megalobrama amblycephala*): Mokrani et al. (2019) replaced fishmeal (FM) by plant proteins plus EAAs. Supplementation with Lys, Met, and Thr upregulates *tor* in the liver as compared with low-FM diet without EAA supplementation (Diet 3, Box Fig. 4). During obvious nutrient sufficiency, this intracellular pathway is activated via the TOR pathway, eventually resulting in upregulated protein synthesis.

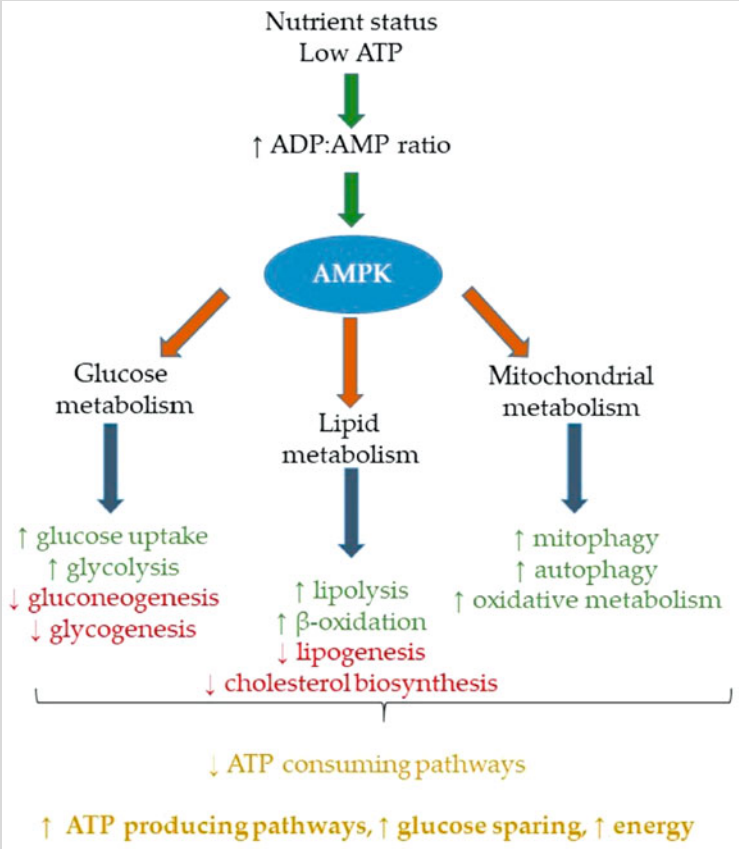


Box Fig. 4 Relative expression of Target of Rapamycin (*tor*) in the liver of blunt snout bream fed pelleted diets with different fishmeal levels supplemented or not with EAA. Different letters indicate significant differences ($P < 0.05$). (From Mokrani et al. (2019), with permission from Wiley). Diets 5 and 6 are devoid of fishmeal and contain soybean and canola meal. Diet 5 is without and Diet 6 is supplemented with Lys, Met, and Thr

AMPK activation, in response to low energy status, re-configures glucose, lipid, and mitochondrial metabolism toward adenosine triphosphate (ATP) production and decreases anabolic pathways that would otherwise further deplete ATP levels (Box Fig. 5) (Lyons and Roche 2018). Notably, AMPK

(continued)

Box 5.2 (continued)
activation reduces hepatic lipid content by increasing fat oxidation in vivo (Foretz et al. 2018).



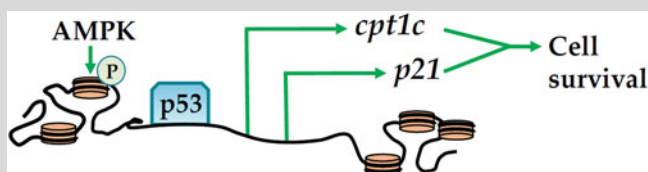
Box Fig. 5 The role of AMP-Activated Protein Kinase (AMPK) on whole-body metabolism. AMPK is a nutrient sensor, which is activated in response to low adenosine triphosphate (ATP) levels and an increased adenosine diphosphate: adenosine monophosphate (ADP:AMP) ratio. As a result, it activates pathways that produce ATP through glucose, lipid, and mitochondrial metabolism pathways, thus increasing ATP levels. Conversely, pathways that deplete ATP are inhibited by AMPK. An ↑ arrow represents an upregulation of the process and ↓ represents a downregulation of the process. *AMPK* AMP-Activated Protein Kinase, *ATP* Adenosine triphosphate, *ADP* Adenosine diphosphate, *AMP* Adenosine monophosphate. (From Lyons and Roche (2018), credit MDPI, Basel)

Moreover, AMPK regulates a diverse set of signaling networks that converge to mediate transcriptional events epigenetically. Reversible histone and DNA modifications result in structural chromatin alterations that influence transcriptional machinery access to genomic regulatory elements. AMPK

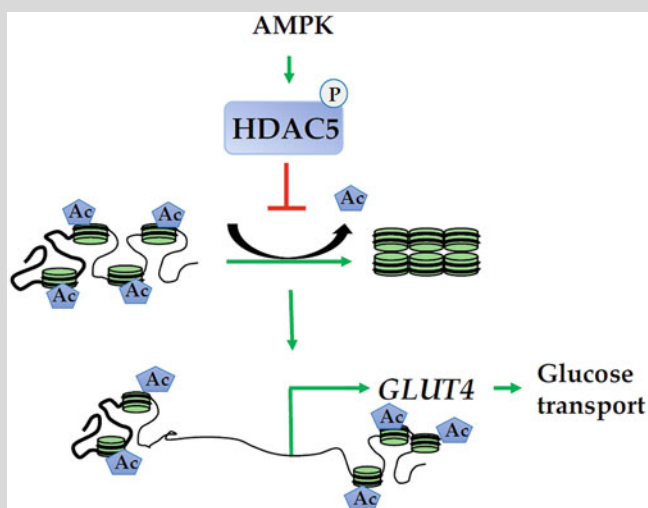
(continued)

Box 5.2 (continued)

phosphorylation of histones, DNA methyltransferases, and histone post-translational modifiers establish AMPK as a key player in epigenetic regulation (Gongol et al. 2018). Two examples from mammalian studies: AMPK is crucial for cell survival and adaptation as evident by its activation upon nutrient depletion and stressors resulting in histone phosphorylation (Box Fig. 6). AMPK phosphorylates histone deacetylase 5 (HDAC5), promoting its dissociation from the promoters releasing its suppressive effects to increase expression of an array of metabolic genes, including *glucose transporter type 4* (GLUT4) (Box Fig. 7).



Box Fig. 6 AMPK promotes cell survival through histone phosphorylation. AMPK phosphorylates H2B to promote chromatin relaxation at tumor protein p53 (p53) recognized promoters and transcription of *carnitine palmitoyltransferase 1C* (*cpt1c*) and *cyclin-dependent kinase inhibitor* (*p21*) to enhance cell survival. (From Gongol et al. (2018), credit MDPI, Basel)



Box Fig. 7 AMPK inhibits histone deacetylase activity to promote histone acetylation. AMPK phosphorylates and inhibits histone deacetylase 5 (HDAC5) enhancing acetylation at the glucose transporter type 4 (GLUT4) promoter increasing its transcription and subsequent transport of glucose to the corresponding tissues. (From Gongol et al. (2018), credit MDPI, Basel)

5.3 Concluding Remarks

During the period of rapid growth, fishes rely on proteinaceous substrates as source of structural units for proteins, for energy, and for synthesis of other macromolecules such as nucleic acids. Less well documented, this statement seems to apply also to invertebrates; but it deserves future studies.

Some short remarks remain to be made:

- Surplus of dietary NEAAs appears to induce beneficial effects in growth, health, etc. Therefore, these AAs deserve future attention with studies on the phenotypic as well as biomolecular level.
- Individual AAs are key compounds of growth, development, and metamorphosis, immunity, and health (e.g., anti-inflammatory capacity) as well as reproduction.
- Energy regulation, AAs, and epigenetic pathways appear to be closely connected; the state of knowledge does not yet transgress the inventory state. Thus, the picture remains fragmentary.
- Proteins in deficiency always and in excess often induce adverse symptoms in aquatic animals. Some mechanisms of how hyperproteic diets may act adversely are limited trypsin capacity, oxidative stress, and further, yet to be elucidated, mechanisms.
- To date, it is well understood that a central function in protein synthesis is played by the target of rapamycin (TOR) proteins (→Box *Yin* and *Yang* of Energy Regulation). Another central pathway involves the *insulin-like growth factor 1* (*igf-1*) gene.
- Besides these two central pathways triggered by AAs, several essential AAs appear to induce specific pathways. This can best be seen with Trp, Phe, and Met.
- If supplied in dietary excess, many AAs have an adverse potential. Obviously, an adverse action of AAs does not apply to all aquatic animals. Furthermore, the mechanisms behind the adverse actions remain mostly obscure and need disclosure.
- Two emerging fields of academic and economic interests come into the focus of attention: interaction of various AAs sparing of essential AAs. Some corresponding results are promising; however, these issues are still premature and deserve future attention.

Evidence is emerging that excess supply of certain AAs can lead to inflammation of the intestine, as found particularly with branched-chain AAs (→Chap. 8). It deserves further studies to verify the generalizability of this finding and underlying modes of action and pathways.

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Chapter 6

Simple Amino Acids: Gly, Ala, Asp, Gln— *‘Pure Easy Fuel?’*



Abstract Evidence is growing that even “simple” amino acids have important controlling functions. Gly is critical in the osmoregulation of fishes and shellfishes and improves growth in many farmed animals. Whether immunity can also be strengthened deserves future studies. There is rare indication that also Ala can improve growth of aquatic animals. Recently it was shown that Asp injection improves pathogen resistance. If dietary Asp is similarly successful, is open to studies. Dietary Glu and Gln improve growth, intestinal development, innate and adaptive immune responses, skeletal muscle development, ammonia removal, and the endocrine status. Evidence is increasing that Glu has, in concert with probiotics, a biomolecular regulatory potential.

Asparagine was first isolated in 1806 in a crystalline form by French chemists from asparagus juice (Aders Plimmer 1908), in which it is abundant—hence the chosen name. It is the first amino acid (AA) to be isolated. Glycine (Gly), alanine (Ala), glutamine (Gln), and asparagine (Asn) are simply structured AAs (Fig. 6.1) with, in the traditional view, more or less only fueling functions. However, the body of evidence is growing that even these “simple” AAs have significant controlling functions; therefore, the following chapters focus mainly on these functions in aquatic animals.

6.1 Glycine and Alanine

Glycine is the smallest proteinaceous AA (Fig. 6.1) and essential for animals under stress conditions (Wu 2013). Gly is an inhibitory neurotransmitter in nervous systems and can function as regulator of gene expression in fishes. This has been shown in rainbow trout by dietary supplementation with Gly, which increased hepatic thyroxine 5′monodeiodinase activity; thereby, enhancing the efficiency of nutrient absorption and anabolic processes. Furthermore, Gly plays a critical role in the osmoregulation of fishes and shellfishes, such as oysters, in response to environmental stress: Oysters rapidly take up free Gly from the surrounding water.

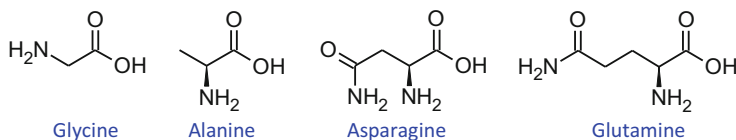


Fig. 6.1 Glycine, L-alanine, L-asparagine, and L-glutamine

Furthermore, they synthesize Gly in the gills in response to rapid changes in salinity or anoxia. Gly enrichment enhances the survival of oysters after transfer from seawater to freshwater (Li et al. 2009). Further recent studies indicate that endogenous synthesis of Gly from serine, choline, threonine, and 4-hydroxyproline is inadequate for maximal growth, collagen production, or feed efficiency in fishes; therefore, it has to be supplemented via diets (Li and Wu 2018).

Early studies in shrimps, such as *Marsupenaeus japonicus* (Deshimaru and Yone 1978) and *Penaeus monodon* (Murai et al. 1981), identified that feed intake is stimulated by Gly. Moreover, Gly and Ala supplementation can significantly enhance weight gain in *Litopenaeus vannamei* (Xie et al. 2014) and rainbow trout (Hunt et al. 2014). In *L. vannamei*, best growth occurs at 2.6% dry dietary Gly. Growth decreases, if dietary Gly supplementation increases further. Importantly, dietary Gly improves response to environmental stress: shrimps fed appropriate Gly show improved survival and antioxidative capacity upon osmotic stress (Xie et al. 2014). In subsequent papers, Xie et al. (2016, 2017) are able to extend this statement to Nile tilapia, but not to grass carp which does not profit from dietary Gly supplementation. In Nile tilapia, dietary Gly significantly improves growth performance and antioxidative capacity, but not survival after a *Streptococcus iniae* challenge.

Alanine (Fig. 6.1) is, in the traditional classification, a non-essential α -AA (Wu 2013) and plays a key role in glucose–alanine cycle between tissues and liver in gluconeogenesis and recycling of glucose carbon (Molina-Poveda 2016). Ala has rarely been in the focus of nutritional studies in aquatic animals. Nevertheless, there are reports on beneficial effects of Ala: Jin et al. (2016) supplemented a soybean meal-based diet for juvenile grass carp and find that the addition of Ala (besides Gly) improves growth, liver function, and protein utilization, but does not enable the performance of a full fishmeal diet.

6.2 Asparagine

Asparagine (Fig. 6.1) is, in the traditional classification, also a non-essential α -AA (Wu 2013). Besides Ala, Asn is the major glucogenic precursors and important energy substrates for fishes. Asn is essential to purine nucleotide synthesis in all cell types. In contrast to most aquatic animals and if experimental flaws can be excluded,

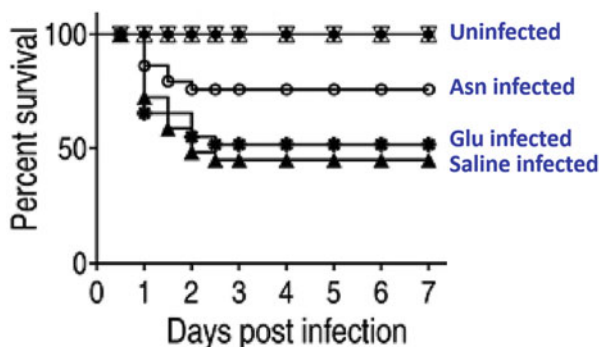


Fig. 6.2 Exogenous aspartic acid promotes zebrafish survival to *Vibrio alginolyticus*: Percentage of survival of zebrafish pretreated with L-aspartic acid. Zebrafish were injected with saline, L-aspartic acid, or glutamate for 3 days followed with bacterial infection. Mortality was monitored for 7 days and analyzed by Log-rank (Mantel-Cox) test, $**P < 0.01$. (From Gong et al. (2020), with permission from Elsevier)

Macrobrychium rosenbergii apparently lacks the ability to biosynthesize Asn and has to satisfy its demand via dietary uptake (Smith et al. 1987).

In meagre (*Argyrosomus regius*), dietary supplementation with Asn modulates immune response (Gonzalez-Silvera et al. 2018); however, the results are inconsistent. Very recently, Gong et al. (2020) showed that injected Asn, but not Glu, promotes survival of zebrafish against *Vibrio alginolyticus* infection through nitric oxide-induced phagocytosis (Fig. 6.2).

6.3 Glutamine and Glutamate

Glutamine (Gln, Fig. 6.1) and its anion, *glutamate* (Glu; abbreviation for both forms together is Glx), are conditionally essential proteinaceous α -AAs (Wu 2013). Gln is related to the immune system via modulation of cytokine and $\cdot\text{NO}$ production (Molina-Poveda 2016). Contrary to mammalian leukocytes, in fish leukocyte, the metabolic role of Gln is complex and appears to be species-specific. Conflicting reports document Gln-dependent and -independent responses of proliferating cultured lymphocytes (Pohlenz and Gatlin 2014).

In mammals, Gln is involved in the immune response via effects on lymphocytes and monocytes. Furthermore, Gln stimulates heat shock protein (HSP) synthesis and regulates AMP kinase (AMPK) activity in an energy-saving manner (Roth 2013). The central position of Gln in human immune cell function is depicted in Fig. 6.3.

Comparable, not yet fully consistent effects are being detected also in fishes. In ongoing studies in juvenile Jian carp, comprehensive insight into the mode of action of Gln and other AAs (see below) is presented with respect to growth promotion, innate (nonspecific) immunity, and stress response. Hu et al. (2015) reported that in

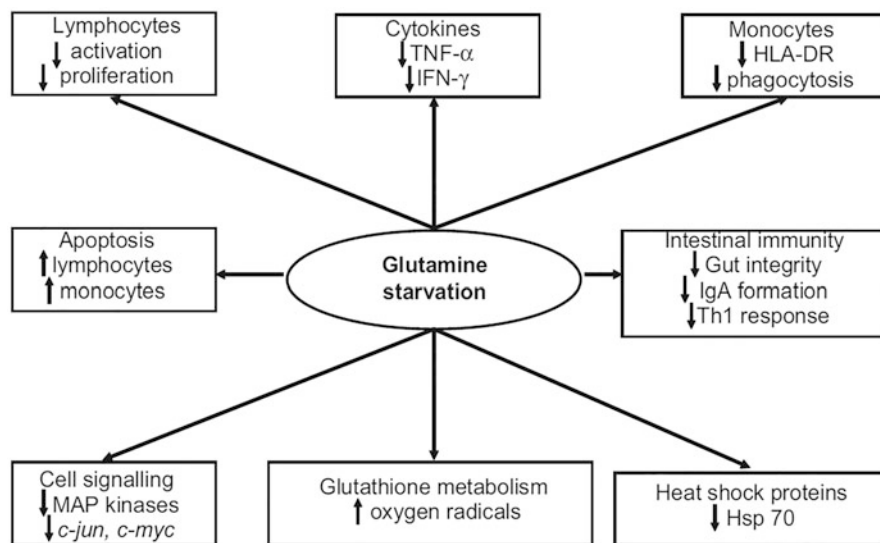


Fig. 6.3 Influence of glutamine starvation on aspects of immune cell function. Glutamine starvation influences several cell systems and pathways leading to a state of immunosuppression. (From Roth (2013), with permission from Woodhead Publishing)

the spleen, Gln downregulates the transcription of the pro-inflammatory cytokine *il-1β* gene, and increases the level of phosphorylation of TOR protein. Furthermore, it inhibits protein oxidation through enhanced $\cdot\text{OH}$ -scavenging via elevating GSH content in the spleen. In head kidney, Gln upregulates *tgf-β2* (anti-inflammatory cytokine) and downregulates *tnf-α* (pro-inflammatory). However, also the anti-inflammatory cytokine *il-10* gene transcription as well as p-TOR and total TOR protein levels are downregulated. Moreover, the antioxidant-related parameters are not affected by Gln in the head kidney of fish. This indicates that further research is still necessary to elucidate the Glu metabolism as well as the role of its metabolites particularly in the head kidney of fish. Nevertheless, it is obvious that Glu has a biomolecular regulatory potential. In Pacific white shrimp, Chien et al. (2020) detected an indirect role of Gln in controlling the immune response: probiotics, such as *Bacillus subtilis*, upregulate the immune response via Gln metabolism.

As functional AAs, Glu and Gln are major metabolic fuels for tissues and play important roles not only in protein synthesis but also in glutathione synthesis and antioxidative reactions. The universality of Glu and Gln as abundant intracellular AAs depends on their enormous versatility in metabolism. Dietary supplementation with Glu and Gln to farmed aquatic animals improves growth, intestinal development, innate and adaptive immune responses, skeletal muscle development and fillet quality, ammonia removal, and the endocrine status. This indicates the shift of the nutritional paradigm of non-essentiality of AAs with respect to Glu and Gln (Li et al. 2020).

Table 6.1 Typical effect of dietary glutamine on life history traits in farmed fishes

Species	Doses g kg ⁻¹	Affected traits	References
<i>Ctenopharyngodon idella</i> , grass carp	0→12	Growth↑, LPO↓, intestine function↑, <i>ppar-γ</i> ^a ↓	Qu et al. (2019)
<i>Cyprinus carpio</i> var. Jian, Jian carp	12	Pro-inflammatory cytokines↓, protein oxidation↓	Hu et al. (2015)
	12.3	Erythrocyte function↑, LPO↓, apoptosis↓	Li et al. (2017)
	0→32	AOC↑, myogenesis↑ (Fig. 6.5)	Zhao et al. (2019)
<i>Oreochromis niloticus</i> , Nile tilapia	0, 10, 20	WG↑, spleen lysozyme activity↑	Pereira et al. (2017)
<i>Scophthalmus maximus</i> , turbot	0, 10, 20	WG↔, innate immunity↑, pathogen resistance↔	Zhang et al. (2017)

↑ support/increase, ↓ reduction/downregulation, ↔ no obvious effect, WG weight gain, LPO lipid peroxidation, AOC antioxidant capacity, IgM, IgT immunoglobulins, *ppar-γ* peroxisome proliferator-activated receptor γ

^aEncodes peroxisome proliferator-activated receptor γ , which regulates fatty acid storage and glucose metabolism (Jones et al. 2005)

Besides taurine, Gln is one of the most abundant free AAs in plasma and muscle and essential for the synthesis of purine and pyrimidine nucleotides in all cells. Through renal ammoniagenesis, Gln plays an important role also in regulating the acid-base balance in the body. Most likely, Gln stimulates muscle protein synthesis in aquatic animals (Li et al. (2009) and references therein).

Gln has the potential to mitigate dietary intoxications. Jiang et al. (2015) reported how dietary glycinin (an anti-nutritional protein in soybeans (→AAN III “Fishmeal Replacement”) exposure causes intestinal oxidative damage and disruption of intestinal physical barriers and functions and reduces growth of juvenile Jian carps. However, appropriate dietary Gln can reverse these adverse effects in carps (Jiang et al. 2015) or turbot (Gu et al. 2017).

A short survey of dietary Gln effects on life history traits in farmed fishes is presented in Table 6.1. A more comprehensive review on effects as well as metabolism of dietary Glu and Gln is reviewed by Li et al. (2020).

Also the sea cucumber (*Apostichopus japonicus*) responds positively to Gln supply (Yu et al. 2016). Appropriate dietary Gln (0.4–0.8%) increases growth and intestinal function by improving certain digestive enzymes and villus height and density of the intestine, as well as increasing antioxidant enzyme activities.

As Gln, also Glu has beneficial controlling capacities as documented in Jian carps. Jiang et al. (2017) identified preventive effects on lipopolysaccharide (LPS)-induced oxidative damage, mRNA expression changes of tight junction and defensin proteins (Fig. 6.4), and inflammatory and apoptosis response signaling molecules in the intestine. 71–82 g Glu per kg diet is optimal (Zhao et al. 2020). Furthermore, in Jian carp muscles, the transcription of genes coding muscle growth (*myogenic*

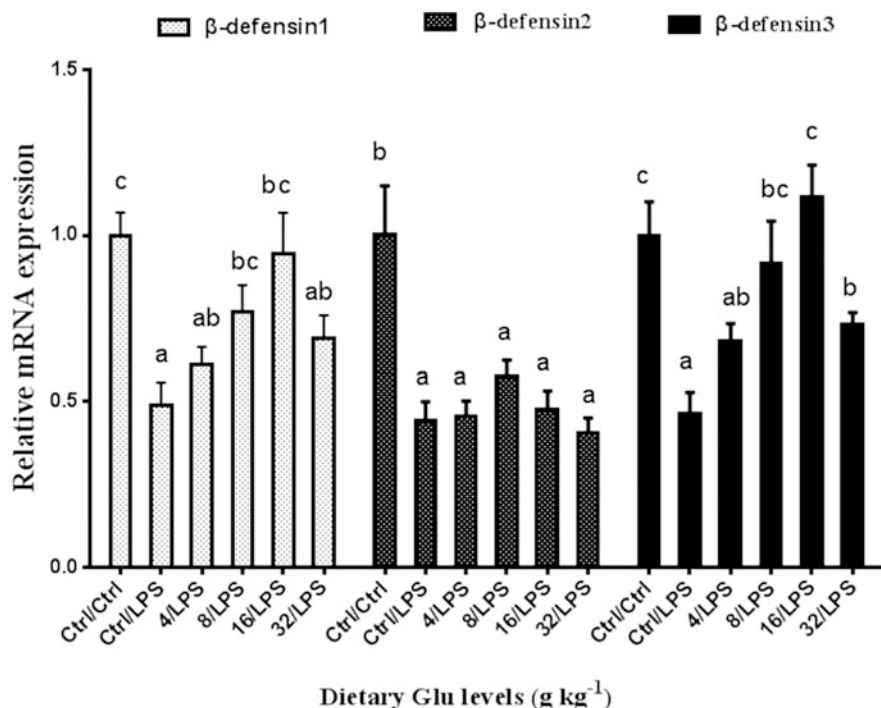


Fig. 6.4 The β -defensin1, β -defensin2, and β -defensin3 mRNA expression in the intestine of Jian carp fed graded dietary levels of Glu for 9 weeks, followed by LPS challenge (injected intraperitoneally with LPS at 4 mg of LPS kg⁻¹ of fish). Values are means \pm SEM ($n = 6$), and different letters denote significant differences ($P < 0.05$). (From Jiang et al. (2017), with permission from Elsevier)

regulatory factor 4, *myogenic factor 5*, *myoblast determination protein*, and *myogenin*) increases with dietary supplementation of Glu levels up to 8 g kg⁻¹ diet, whereas no effect is observed in *myostatin* (Fig. 6.5). Myostatin acts as inhibitor of satellite cell proliferation (Seiliez et al. 2012).

6.4 Concluding Remark

In addition to the general remarks in Chap. 5, it is obvious, but understudied, that even “simple” AAs have controlling functions in animal development and health. This issue is just starting to be understood—with fishes even better than with aquatic invertebrates. Besides the genetic regulatory pathway, epigenetics as well as indirect pathways via gut microbiota deserve future attention. Simple production-oriented dose-response studies should be approaches of the past. Furthermore, although in

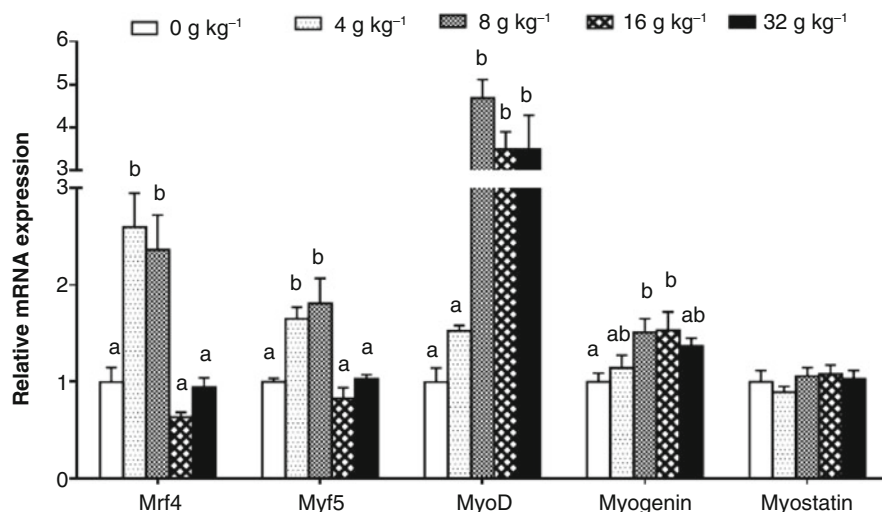


Fig. 6.5 Effects of dietary Glu supplementation (g kg^{-1} diet) on *mrf4* (myogenic regulatory factor 4), *myf5* (myogenic factor 5), *myod* (myoblast determination protein), *myogenin*, and *myostatin* gene expressions in muscle of Jian carp. Values are means \pm SEM, of three replicates with six fishes in each replicate, and different letters denote significant difference ($P < 0.05$). (From Zhao et al. (2019), with permission from Elsevier)

human toxicity of, for instance, Gln up to 14 g d^{-1} has not been observed (Shao and Hathcock 2008), adverse effects of imbalanced dietary AA composition are only sparsely studied and should be in the focus of studies to come. Biomolecular tools for such studies are well developed and must be applied.

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Chapter 7

The Versatile Amino Acid: Tryptophan— *‘More Controlling than Fueling’*



Abstract Tryptophan is a major constituent of proteins and precursor of the neurotransmitter serotonin and the neurohormone melatonin. It serves as substrate for niacin and nicotinamide adenine dinucleotide (NAD) biosynthesis. Adequate dietary Trp supply increases stress resistance and innate immunity in fishes and aquatic invertebrates. Deficient as well as excess dietary Trp supplies reduce fitness and health of fishes by deteriorating immune competence, increasing programmed cell death, and disrupting the physical barrier function in gills. Adequate Trp supply reduces aggressive behavior. Dietary Trp can reduce plasma cortisol levels in stress situations. Kynurenic acid, a degradation product of Trp, has been identified as stressor to young fishes. As immunomodulatory metabolite, this acid is Janus-faced. From studies in mammals, there are indications that Trp or its metabolites take part in epigenetic pathways; however, this issue remains to be identified in aquatic animals. The same applies to the role of Trp metabolism along the gut microbiota-brain axis.

Aromatic amino acids possess aromatic rings: tryptophan (Trp), phenylalanine, tyrosine, and histidine. Besides Met, aromatic AAs are highly important, since they take part as educts or as their metabolites in various central pathways. Based on current knowledge and because of its versatile function, the most interesting aromatic AA appears to be Trp.

L-Tryptophan (Fig. 7.3) is an essential AA with a huge functional versatility, in addition to its participation in protein synthesis. Because of the complexity of its metabolism, and the functional relevance of several of its metabolites, it participates in a wide array of physiological pathways (review by Hoseini et al. (2019)). This review lists Trp requirement for fishes ranging from 0.30 to 1.30% of dietary protein level in *Cyprinus carpio* and *Ctenopharyngodon idella*, respectively (Fig. 7.4). Requirement in shrimps lies in the same range: *Marsupenaeus japonicus* 0.4% of the diet and 0.8% of dietary protein; *Penaeus monodon* 0.2% and 0.5%, respectively (NRC 2011); and 0.4% and 0.9–1.0% for *Litopenaeus vannamei*, even under hypo-osmotic stress (Jin et al. 2017).

Dietary Trp beneficially modulates growth, digestive and absorptive enzyme activities, intestinal antioxidant capacity, appetite, and GH-IGF axis-related gene expression (Fig. 7.1), as recently shown in hybrid catfish (*Pseudobagrus*

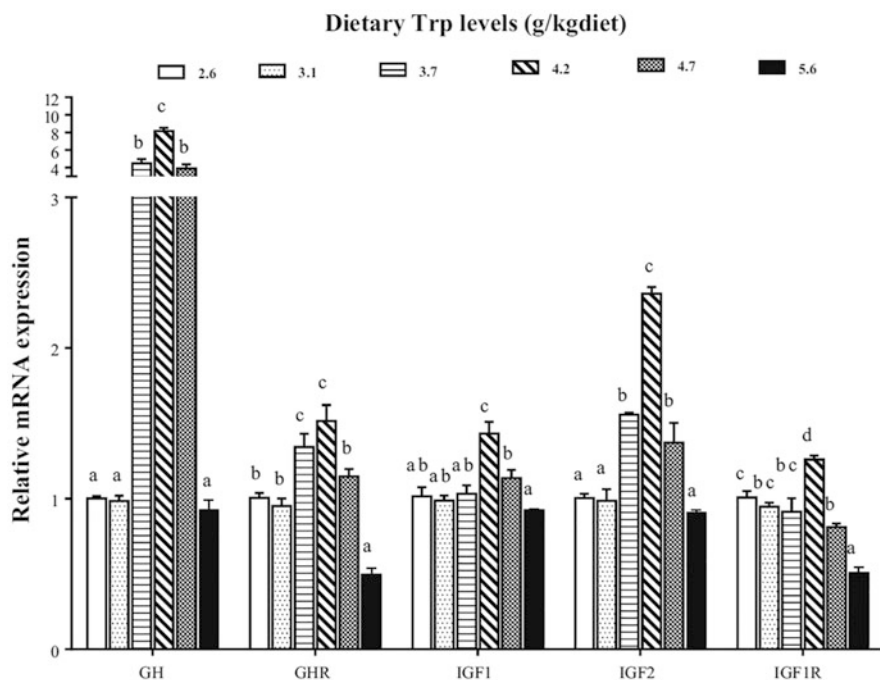


Fig. 7.1 Relative mRNA expression of GH gene in the pituitary, GHR, IGF1, and IGF2 genes in the liver and IGF1R gene in the intestine of hybrid catfish fed diets containing graded levels of Trp. Data are means \pm SEM, of three replicates. Different letters indicate significant differences ($P < 0.05$). (From Zhao et al. (2019), with permission from Springer Nature). GH growth hormone, GHR growth hormone receptor, IGF1, IGF2 insulin-like growth factors, IGF1R insulin-like growth factor 1 receptor

(*Pelteobagrus*) *vachelli*♀ \times *Tachysurus dumerili*♂ (*Leiocassis longirostris*) (Zhao et al. 2019).

Trp is indispensable in all fish species studied (NRC, 2011). Trp deficiency results in depressed growth, low feed efficiency, or poor protein retention (Tang et al. 2013). There is increased Trp requirement under stressful conditions (Fig. 7.2). In *Cirrhinus mrigala* and *Labeo rohita*, for instance, the 45–90 days Trp administration under constant stressful conditions suppresses cortisol and glucose responses and improves growth (Tejpal et al. 2009; Kumar et al. 2014) (Fig. 7.9). Under such conditions, which usually result in oxidative stress and lipid peroxidation (Steinberg 2012), the activity of antioxidant enzymes decreases, when *L. rohita* is fed 0.75 and 1.5% Trp diets (Ciji et al. 2015) indicating relief from the stress. All these studies have used Trp levels higher than needed for regular growth confirming increased Trp requirement under oxidative stress conditions (Hoseini et al. 2019). Such conditions emerge by abiotic as well as biotic environmental stressors (Table 7.1). Even more, dietary Trp supply increases the resistance against forthcoming stress, such as handling and transport stress (see below).

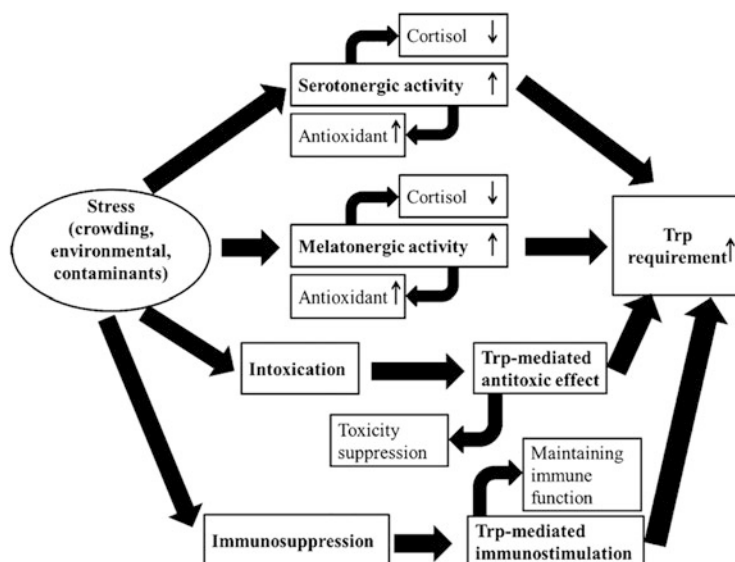


Fig. 7.2 Factors affecting dietary tryptophan requirement of teleosts. (From Hoseini et al. (2019), with permission from Wiley)

One severe stress to farmed animals is the replacement of fishmeal and fish oil by plant-based diets, which usually leads to enteritis (→AAN III “Fishmeal Replacement”). Dietary supplementation with Trp, however, stimulates the immune system and alleviates enteritis (Cerqueira et al. 2020) with gut microbiota being central (Gao et al. 2018).

Trp functions as biochemical precursor for the following compounds (Fig. 7.3):

- *Serotonin*, a neurotransmitter that also inhibits the production of inflammatory cytokines and superoxide and feed intake in teleost fishes and invertebrates (e.g., *C. elegans* (Sawin et al. 2000), mosquito larvae (Kinney et al. 2014)).
- *Melatonin* (MT), a neurohormone with similar inhibitory function as serotonin. MT is central in the biological clock, MT synthesis is coupled to the expression of clock genes (Ben-Moshe et al. 2014; Schwarzenberger and Wacker 2014). Dietary MT can enhance immunity, antioxidant capacity (Jaworek 2006), and antibacterial activity of *Eriocheir sinensis* (Yang et al. 2020).
- Trp serves as substrate for *niacin* (vitamin B₃ or nicotinic acid) biosynthesis (→Chap. 33).
- In addition, nicotinamide adenine dinucleotide (NAD) can be synthesized from Trp as found in mammals. NAD, in turn, is required by enzymes involved in DNA repair, recombination, apoptosis, and maintenance of genomic stability (Oommen et al. 2005). It is subject to future studies, whether this mechanism applies also to fishes and aquatic invertebrates.

Table 7.1 Recent studies on stress attenuation by dietary tryptophan in fishes

Species, common name	Level, %	Stress response	References
<i>Argyrosomus regius</i> , meagre	1.0	Resistance against handling stress↑	Asencio-Alcudia et al. (2019)
	0.1→0.16	Chronic stress resistance↑	Herrera et al. (2020)
<i>Dicentrarchus labrax</i> , European seabass	0.5	Pathogen resistance↑	Azeredo et al. (2017a)
<i>Labeo rohita</i> , Rohu	0→1.5	Resistance against thermal stress↑	Kumar et al. (2018)
<i>Ompok bimaculatus</i> , butter catfish	0→3	Cannibalism↓	Biswas et al. (2019)
<i>Oncorhynchus mykiss</i> , rainbow trout	0→1.0	Crowding stress↓, growth @ high densities↑: Optim. @ 0.5	Hoseini et al. (2020a, b, c)
<i>Salmo salar</i> , Atlantic salmon	1×, 2×, 3× regular dietary dose	Long-term crowding stress resistance↑	Höglund et al. (2017)
<i>Solea senegalensis</i> , Senegalese sole	0.86, 1.72	Pathogen resistance↑	Azeredo et al. (2019)
	1.0	Stress by permanent light↓	Salamanca et al. (2020)
<i>Totoaba macdonaldi</i> , Totoaba	1.0→2.2	Homeostasis under crowding conditions↑	Cabanillas-Gómez et al. (2020)

↑ support/increase; ↓ reduction/decrease

Several observations from animals other than fishes demonstrate that Trp increases the activity of digestive and brush border enzymes. Trp activates amylase, lipase, as well as trypsin. The Trp metabolite MT exerts stimulatory effects on the secretion of pancreatic enzymes (Jaworek 2006). Studies on protein structures indicated that Trp residues play important roles in catalytic activity, targeting, stabilization, and conformation of several enzymes such as alkaline phosphatase in green crab (*Scylla serrata*) (Zheng et al. 1997), creatine kinase in common carp (Sun et al. 1998), and ATPase in zebrafish (Rajaroo et al. 2001).

Increased health and stress resistance after dietary Trp supply applies also to farmed invertebrates. In pond cultures of *E. sinensis*, fighting, defense, and foraging, can cause a high rate of limb autotomy that reduces the immune defense. Appropriate doses of dietary Trp improve the immune status and enhances survival and disease resistance against *Aer. hydrophila* under chelipeds autotomy stress (Zhang et al. 2018a). Even more impressive, Zhang et al. (2019) identified that dietary supplementation of Trp promotes limb regeneration by regulating corresponding gene expressions and hepatopancreatic digestion, which may be achieved by regulating MT levels and binding of 5-hydroxytryptophan (5-HTP) and dopamine (DA) to receptors. The regulation of limb regeneration by Trp is mainly achieved

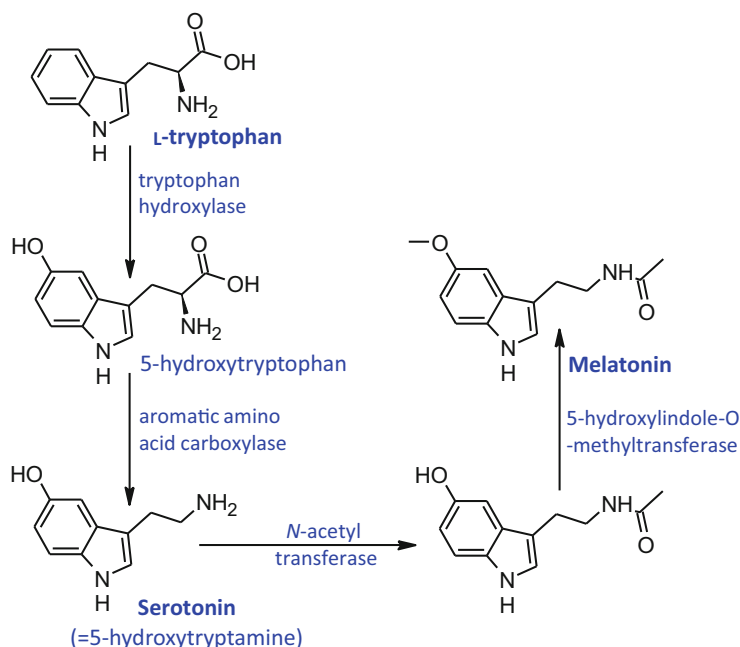


Fig. 7.3 Transformation of L-tryptophan into serotonin and melatonin

by 5-HT (serotonin), DA, and MT. The digestion and absorption capacities of digestive organs and the repair of physiological functions of regenerative tissues occur by altering the expression of 5-HT- and DA-related receptor genes in digestive organs and nervous tissues (Zhang et al. 2019).

7.1 Requirement

As illustration of Trp requirement in fishes and aquatic invertebrates, we refer only to a few studies. In a dose-response study, Rodehutsord et al. (1997) evaluated weight gain (WG), dry matter intake, and macronutrient composition in young rainbow trouts. These variables increase with increasing dietary Trp contents. However, lipid concentrations in WG decrease with increasing dietary Trp concentrations. To achieve 95% of the maximum protein deposition, dietary Trp concentration of 2.0 g kg^{-1} dry matter is required.

Feeding juvenile Jian carps, Tang et al. (2013) showed that WG increases up to $3.8 \text{ g Trp kg}^{-1}$ diet (Fig. 7.4) and then decreases, indicating that both Trp deficiency and excess lead to adverse growth effects. A similar growth curve with a similar optimum dietary Trp content is obtained in young grass carps (Fig. 7.4). As can be expected, also in black tiger shrimp (*Penaeus monodon*), Trp requirement follows an

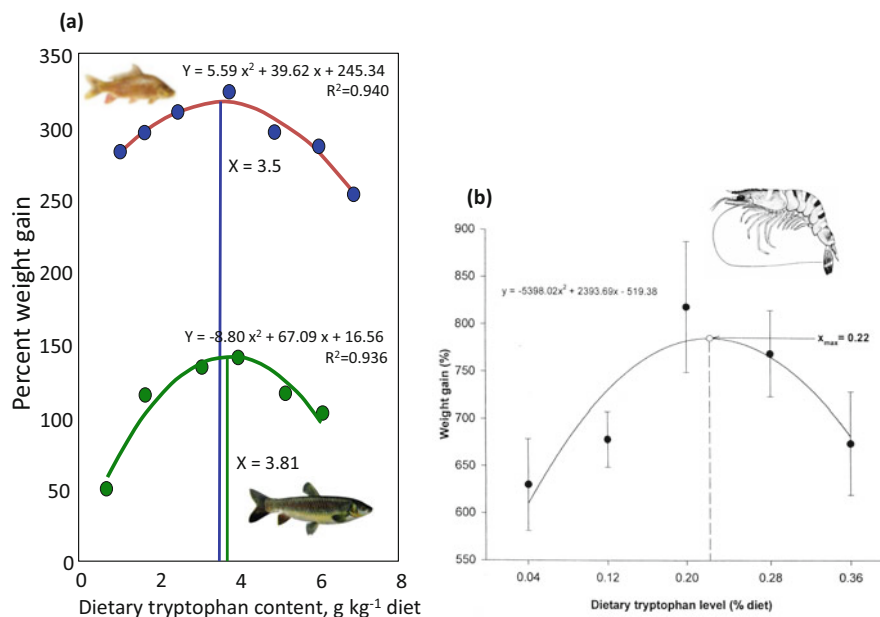


Fig. 7.4 (a) Dependence of weight gain on dietary tryptophan in juveniles of *Cyprinus carpio* var. Jian (above) and *Ctenopharyngodon idella* (below) (redrawn from Tang et al. (2013) and Wen et al. (2014), with permission from Elsevier). (b) Growth response of black tiger prawn (*Penaeus monodon*) fed graded levels of tryptophan for 8 weeks. (From Millamena et al. (1999), with permission from Elsevier; image credit FAO)

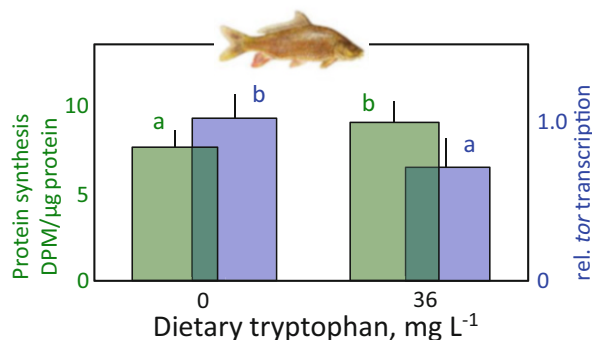
optimum curve (Fig. 7.4) and reaches a similar optimum as the fishes (Millamena et al. 1999). For optimal growth, the Pacific white shrimp requires 0.4% dietary Trp (Sun et al. 2014).

Trp supplementation is beneficial not only for growth but also for survival: it affects the 5-HT, 5-HT₁ receptor mRNA transcription, as well as γ -aminobutyric acid (GABA) levels in different tissues, indicating that 5-HT as well as the GABA pathway is central in growth and survival of shrimps. GABA is a non-proteinogenic amino acid and inhibitory neurotransmitter (→Chap. 12).

How do phenotypic findings translate into biomolecular regulatory mechanisms? Trp supplementation increases protein synthesis and decreases the *target of rapamycin (tor)* mRNA level (Fig. 7.5). TOR is a highly conserved, nutrient-sensitive protein kinase and central controller of cell growth and aging (Loewith and Hall 2011) leading to induced mRNA translation (→Chap. 5). Trp promotes fish protein synthesis through the TOR pathway via alterations of *tor* gene transcription (Tang et al. 2013).

Optimal dietary Trp contents also improve the intestinal mucosal immunity and barrier function of the mucous cells by changing the expression of tight junction proteins as exemplified in young grass carps. Furthermore, the antioxidant capacity (Fig. 7.6a) increases by upregulating anti-oxidant enzymes, such as copper/zinc

Fig. 7.5 Effect of tryptophan supplementation on protein synthesis rate and relative *target of rapamycin* (*tor*) gene expression of carp enterocytes. DPM disintegrations per minute. Different letters indicate significant differences ($P < 0.05$). (Redrawn from Tang et al. (2013), with permission from Elsevier)



superoxide dismutase (SOD1) (Fig. 7.6b) and glutathione peroxidase (GPx) (Fig. 7.6c), and reduction of lipid peroxidation (LPO) as well as protein carbonyl (PC) formation in the gills (Wen et al. 2014).

In fishes, the innate immune response induces an inflammatory response primarily mediated by cytokines. Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF- α) and interleukin 8 (IL-8), increase the synthesis of small inflammatory mediators and initiate inflammatory processes. Conversely, the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor- β (TGF- β) are produced to inhibit the excessive activation of the inflammatory response and initiate tissue recovery processes. In fact, optimal Trp supply reduces the transcript of pro-inflammatory cytokines and increases the transcript of anti-inflammatory cytokines in gills of young grass carps (Fig. 7.6d, e) (Jiang et al. 2015). Although several details of the biomolecular regulation remain to be elucidated, it is obvious that the intestinal immune response significantly and comprehensively can be optimized by appropriate dietary Trp contents. To stress the term “appropriate,” Jiang et al. (2016) reported that in young grass carps, Trp-deficient as well as Trp-excess diets induce oxidative stress. Only appropriate dietary Trp doses enhance the anti-oxidant ability through the master regulator, Nrf2, and through TOR (\rightarrow Box: Yin and Yang of Energy Regulation in Chap. 5).

Effects of deficient supply can be summarized as follows (Jiang et al. 2015):

1. Increase of ROS content and severe oxidative damage to the gills with LPO and protein carbonyl formation.
2. Changes of the tight junction proteins and the transcription of cytokines. The tight junctions are comprised of transmembrane proteins (such as occludin and claudins) and cytoplasmic proteins (such as ZO-1) encircling the fish gill epithelial cells and form a semipermeable seal that limits the movement of water and solutes across the paracellular pathway. Trp deficiency causes decreases in the mRNA levels of *zo-1*, *occludin*, *claudin-c*, and *claudin-3* in the gill, indicating that Trp deficiency leads to disruption of the physical barrier function.
3. Increase of pro-inflammatory cytokine transcription.

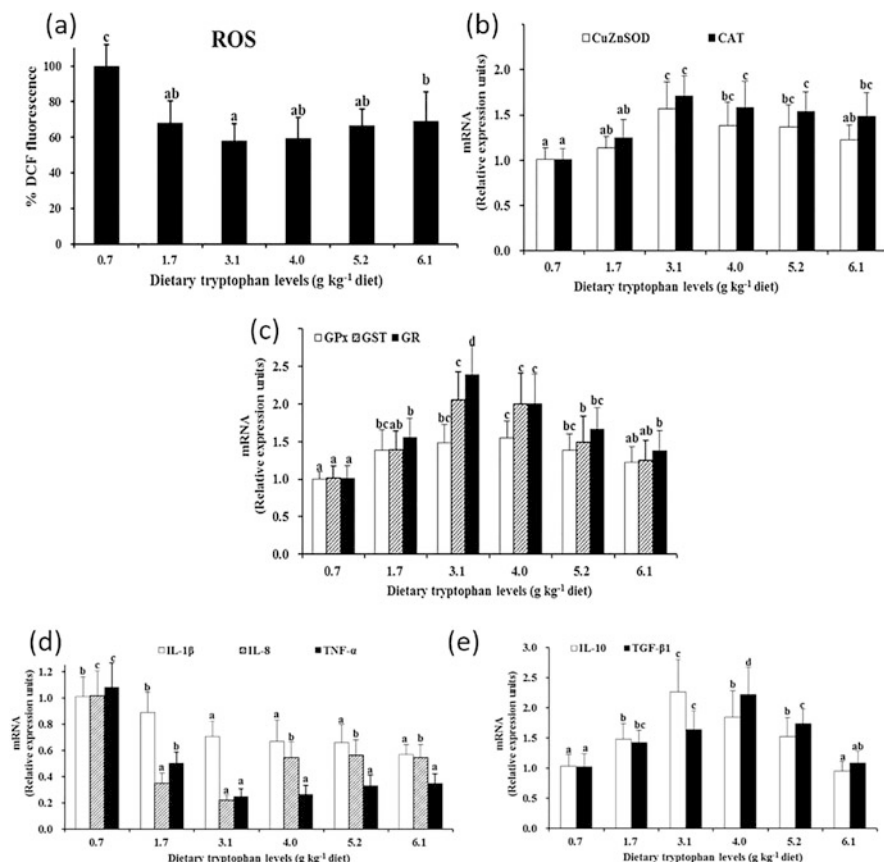


Fig. 7.6 The reactive oxygen species (ROS) content (a) and relative expression of (b) *copper zinc superoxide dismutase* (*CuZnSOD*) and *catalase* (*cat*), and (c) *glutathione-transferase* (*gst*), *glutathione peroxidase* (*gpx*), and *glutathione reductase* (*gr*); relative expression of (d) *interleukin 8* (*il-8*), *tumor necrosis factor α* (*tnf-α*), and *interleukin 1β* (*il-1β*), and (e) *interleukin 10* (*il-10*) and *transforming growth factor β1* (*tgf-β1*) in the gill of young grass carp fed diets with graded tryptophan contents (g kg⁻¹ diet) for 8 weeks. In gills of young grass carp fed diets containing increasing contents of tryptophan for 8 weeks. The 0.7 g kg⁻¹ variant served as control. Different letters denote significant differences between treatments ($P < 0.05$). (From Jiang et al. (2015), with permission from Elsevier)

4. Upregulation of apoptosis signaling via *caspase-3*, *caspase-8*, and *caspase-9* transcription. Conversely, appropriate Trp supply protects fish gill structural integrity by preventing the cell apoptosis via downregulation of *caspase-3*, *caspase-8*, and *caspase-9* transcription.
5. Oxidative damage, accompanied by decreases in the antioxidant enzyme activities and their mRNA levels associated with the Keap1-Nrf2 signaling pathway. The Keap1-Nrf2 pathway is the major regulator of cytoprotective responses to oxidative stress (Kansanen et al. 2013).

Excessive Trp causes similar adverse effects on the structural integrity of fish gills and immune system as Trp deficiency. This means that deficient or excess dietary Trp supply of fishes is much more than only retarded growth; rather, it reduces fitness and health of the fishes by deteriorating the immune competence, by increasing the programmed cell death, and by disruption of the physical barrier function in fish gills.

7.2 Stress Tolerance and Behavior

7.2.1 Fishes

Since Trp is converted into 5-hydroxytryptophan (5-HTP) and subsequently into the neurotransmitter serotonin (5-HT) (Fig. 7.3), it can be hypothesized that dietary Trp or 5-HTP supplementation has the potential to improve stress tolerance and change behavioral traits. Several reports prove this assumption (Table 7.1). For instance, dietary supplementation of Trp mitigates crowding stress and improves thermal tolerance in mrigal crap (*Cirrhinus mrigala*) fingerlings (Tejpal et al. 2009, 2014). Furthermore, Ciji et al. (2015) reported an increased tolerance of *Labeo rohita* juveniles to nitrite exposure. The normal requirement of Trp (0.4% of the diet) is unable to combat the nitrite-induced stress; dietary fortification up to 0.75%, in addition to the normal dose, is effective in compensating nitrite-induced stress and triggering immunity. Moreover, dietary Trp is shown to advance maturation in male and female ayu sweetfish (*Plecoglossus altivelis*) (Akiyama et al. 1996). More recently, Azeredo et al. (2017b) denoted a beneficial Trp effect in European seabass challenged with *Photobacterium*. Several immune-related genes are upregulated in the gut indicating a neuroendocrine-immune response. In contrast, skin mucosal immunity is inhibited pointing out the tissue specificity of Trp immunomodulation. Usually, immunosuppressive conditions increase dietary Trp requirements (Hoseini et al. 2019). However, the stress response appears to be species-specific, since Diógenes et al. (2019) found that dietary Trp supplementation does not counteract adverse stocking effects on growth in gilthead seabream.

A characteristic behavioral response to stress is reduced feed intake. Consequently, Höglund et al. (2007) investigated whether pretreatment with Trp-enriched feed affects subsequent stress-induced changes in feeding behavior in brown trout (*Salmo trutta*) (Fig. 7.7): isolated fishes were fed control or Trp-supplemented feed for 7 d, where upon they were transferred to a novel environment, in which all fishes received control feed. This transfer results in decreased feeding in both groups. However, this decrease is more pronounced in the control group. This study shows that even pretreatment with dietary Trp attenuates stress-induced anorexia. Recently, Fernández-Alacid et al. (2019) described that dietary Trp has been successfully applied mitigating also hypoxia and netting stress in meagre (*Argyrosomus regius*).

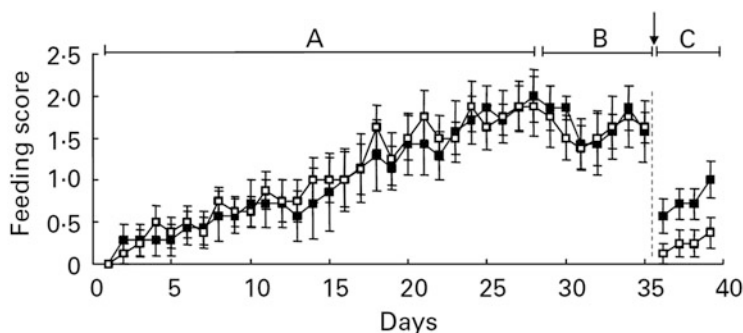


Fig. 7.7 Mean feeding score during the acclimatization (a), L-tryptophan (Trp) pretreatment (b) and stress phase (c) in juvenile brown trout. One group was fed wet feed supplemented with 3 g kg^{-1} Trp 7 d before transfer and unsupplemented control feed during the acclimatization and stress phase, while the control-treated group was fed unsupplemented feed throughout the experiment. An arrow indicates transferring the fish to a new environment. Values are means and SEM. (From Höglund et al. (2007), with permission from the Cambridge University Press) (black square) Trp; (white square) control

In an educational cartoon, Höglund et al. (2019) summarize the fate of Trp in the brain of fishes (Fig. 7.8). Trp enters the brain in competition with other large neutral AAs (LNAAs, such as Val, Ile, Leu, Tyr, Phe, and Met) through a common transporter protein. Therefore, the amount of internalized Trp depends on the plasma concentrations of Trp in relation to the other LNAAs. Hence, ingestion of a normal protein source, usually containing 0.5–1% Trp, results in a relatively small increase in Trp, but a larger elevation of plasma concentrations of other LNAAs. This results in a decrease in the plasma Trp/LNAA ratio and thus reduced Trp influx to the brain (Fig. 7.8). Dietary carbohydrates, on the contrary, increase the brain Trp levels. This is due to elevated insulin, which, in turn, promotes uptake of LNAAs except Trp to the skeletal muscles, thereby increasing plasma Trp/LNAA ratio and Trp influx to the brain (Fig. 7.8). This differential AA uptake to skeletal muscles is caused by the fact that Trp in blood plasma is bound to albumin whereas other LNAAs are not. Trp influx to the brain is then promoted by the common LNAA transporter protein in the blood-brain barrier having a much higher affinity for Trp compared to albumin (Höglund et al. (2019) and references therein).

Since the intriguing study by Höglund et al. (2007), the number of reports on stress attenuation in fishes by dietary Trp has increased and major results are briefly summarized in Table 7.1.

Stress is indicated by the universal stress biomarker, plasma cortisol. Lepage et al. (2002) showed that in non-stressed rainbow trouts, supplementary dietary Trp results in slightly elevated cortisol levels. In response to stress, fishes fed the control diet show elevated plasma cortisol levels, but fishes fed the Trp-supplemented diet have a significantly reduced cortisol levels. The authors assume that Trp-supplemented diets stimulate the brain 5-HT system, which stimulates the hypothalamic–pituitary–interrenal axis.

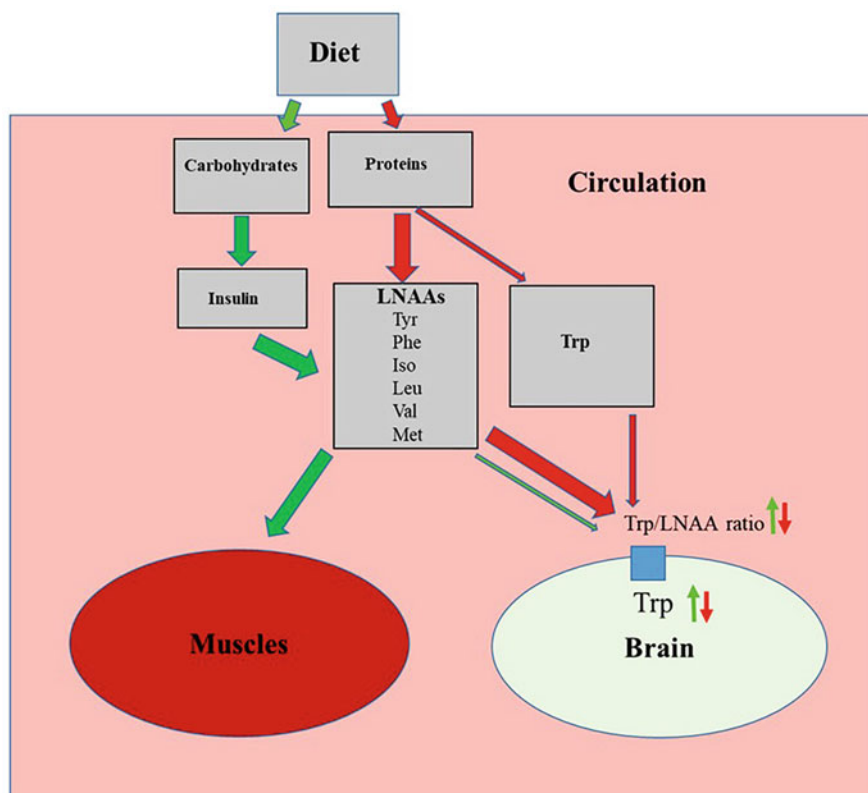


Fig. 7.8 Effects of the proteins and carbohydrates on influx of tryptophan (Trp) to the brain. Green arrows indicate activation of carbohydrate induced pathway, resulting increased muscle uptake of large neutral amino acids (LNAAs; *Tyr* tyrosine, *Phe* phenylalanine, *Iso* isoleucine, *Leu* leucine, *Val* valine, and *Met* methionine) which in turn increases plasma Trp/LNAA ratio and brain Trp levels. Red arrows indicate how a normal dietary protein source, with relatively low Trp content, decreases the plasma Trp/LNAA ratio and brain Trp levels. (From Höglund et al. (2019), credit Frontiers Media)

Seawater-reared Atlantic salmon responds unlike rainbow trout (Basic et al. 2013a). Fishes are given feed containing 1, 2, 3, or 4 times the Trp content in a regular feed for 1 week. Subsequently, fishes are reintroduced to feed containing the lowest Trp level, corresponding to standard feed for a number of days prior to exposure to an acute confinement stressor. Treatment with Trp-enriched feed results in suppressed basal levels of cortisol at 1 and 10 days after the feed regime is terminated. Ten days post-Trp treatment, this pattern is reversed and inhibitory effects of dietary Trp are observed. This demonstrates that the effects of Trp on stress response are time-specific even after termination of dietary pretreatment.

The clearest short-term dose-response after Trp treatment is found in Atlantic cod. A dose-dependent decline in plasma cortisol levels occurs in stressed fishes already

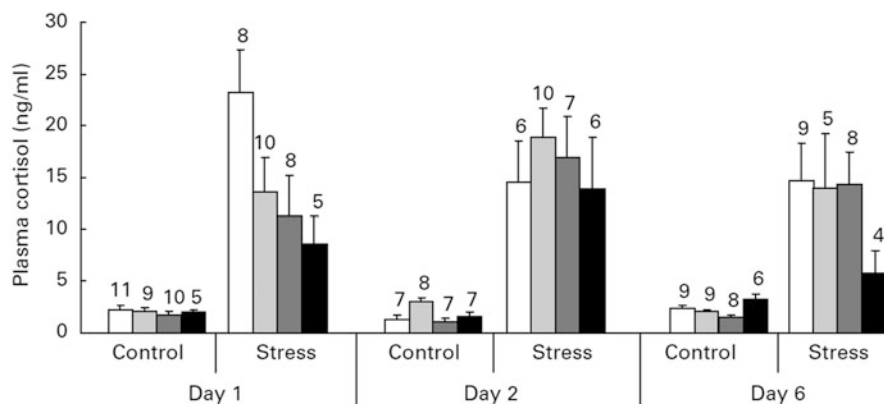


Fig. 7.9 Plasma cortisol levels in juvenile cod (*Gadus morhua*) following the reintroduction of control diet after 7 d of Trp supplementation: control feed containing 0.47% Trp (1 × Trp; light grey), experimental feed containing 0.82% Trp (2 × Trp; grey), experimental feed containing 1.14% Trp (3 × Trp; dark grey), experimental feed containing 1.65% Trp (4 × Trp; black). The values above the bars indicate the number of individual fish. (From Basic et al. (2013b), with permission from the Cambridge University Press)

1 day after the termination of the dietary Trp treatment (Fig. 7.9). The effect is no longer evident on days 2 and 6. Unstressed fish show low cortisol levels all the time. This study confirms that pretreatment with Trp-enriched feed results in a dose-dependent reduction in post-stress plasma cortisol levels. Conversely in Nile tilapia, no clear differences can be detected in cortisol levels after an acute stress between control and Trp-enriched diets (Martins et al. 2013).

Under farming conditions, intra-cohort cannibalism is one of the main factors that adversely affect growth and survival of carnivorous fishes, especially during early life stages, and causes substantial losses under conditions of intensive rearing. The complete elimination of cannibalism in larviculture is virtually impossible, but it can be mitigated. Juvenile rainbow trout fed for 7 days significantly suppressed the aggressive behavior (Winberg et al. 2001). Highly, aggressive interactions and cannibalism occur also in juvenile Atlantic cods and orange-spotted groupers: the latter reduce their cannibalism, if supplied with complimentary Trp (Fig. 7.10a) (Hseu et al. 2003). Two more successful examples: 2% dietary Trp significantly diminishes cannibalism in butter catfish (*Ompok bimaculatus*, Fig. 7.10b) (Biswas et al. 2019) and $\leq 1.0\%$ in juvenile mud crab (*Scylla serrata*) (Fig. 7.10c), respectively (Laranja Jr et al. 2010).

Conversely, Król et al. (2014) did not find any significant reduction of cannibalism in aquacultured and Trp-fed larvae of European catfish (*Silurus glanis*) indicating that control of aggressive behavior is more complex than only controlled by Trp and its metabolites, at least in this species and in the fighting fish (*Betta splendens*) (Clotfelter et al. 2007). In pikeperch (*Sander lucioperca*) postlarvae, Trp

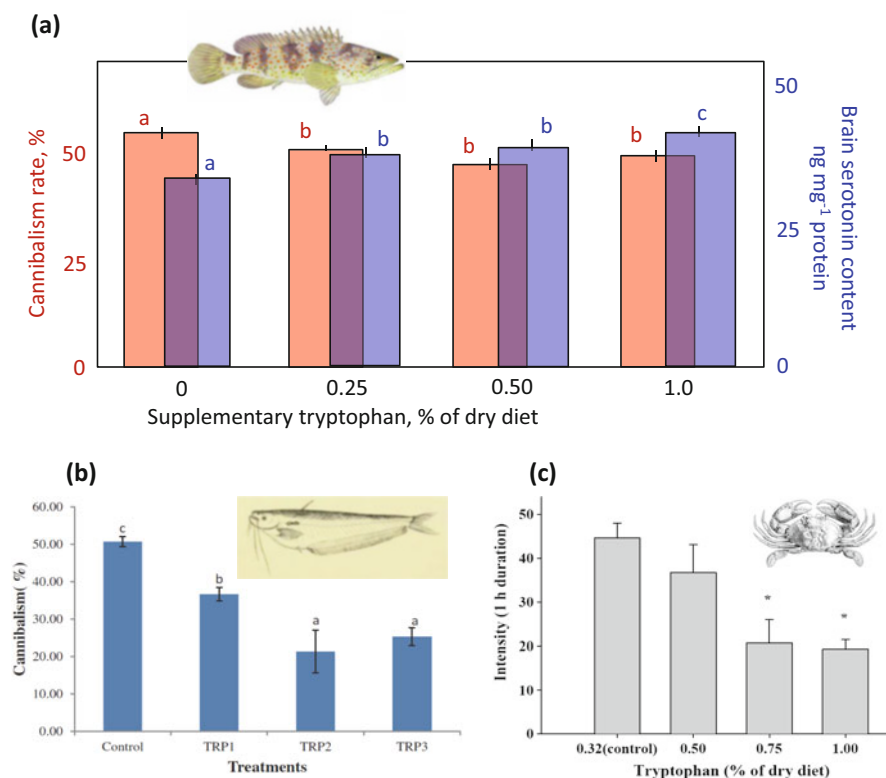


Fig. 7.10 (a) Reduction of the cannibalism rate of juvenile orange-spotted grouper (*Epinephelus coioides*) and increases of brain serotonin content by providing graded tryptophan diets. Different letters indicate significant differences. (Redrawn from Hseu et al. (2003), with permission from Elsevier). (b) Effects of dietary tryptophan on cannibalism (%) of *Ompok bimaculatus* fry. Mean values are data of three replicates. Different letters indicate significant differences ($P < 0.05$). Control, TRP1, TRP2, and TRP3 show 0%, 1%, 2%, and 3% tryptophan-supplemented experimental diets, respectively. (From Biswas et al. (2019), with permission from Taylor and Francis; image credit Beavan (1877)). (c) Intensity of attacks performed by juvenile mud crabs fed graded levels of tryptophan. Values are means \pm SEM for $N = 3$. * indicates significant difference ($P < 0.05$) from the control by 2-tailed Mann–Whitney U test. (From Laranja Jr et al. (2010), with permission from Elsevier; image credit FAO)

supplementation results only in slight decrease of cannibalism; and Trp treatment has no clear influence on the final survival of postlarvae (Król and Zakęs 2016).

One likely solution to this obvious inconsistency of Trp effects on behavior appears to be the change over time of the anti-aggressive effect of Trp and metabolites. Wolkers et al. (2014) demonstrated that Trp alters the aggressive behavior of the commercially interesting *Brycon amazonicus*; however, this effect is limited to the beginning of the fight indicated by body bites (Fig. 7.11), pointing out an only transient effect of Trp on aggressive behavior.

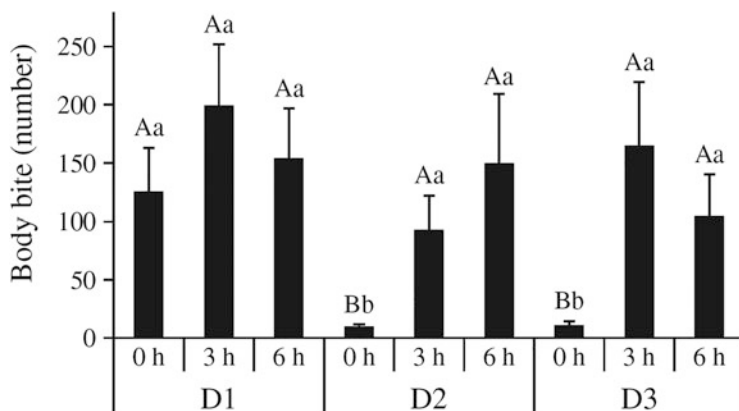
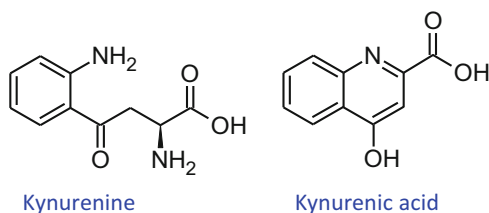


Fig. 7.11 Body bites of *Brycon amazonicus* fed with an L-tryptophan supplemented diet and fighting with an intruder up to 6 h. Treatments: **D1** 4.6 g kg⁻¹; **D2** 9.4 g kg⁻¹; **D3** 18.8 g kg⁻¹. Upper-case letters indicate significant differences among the treatments at the same time ($P < 0.05$), lower-case letters indicate significant differences among the times for the same treatment. (From Wolkers et al. (2014), with permission from Wiley)

Fig. 7.12 Structures of kynurenine and kynurenic acid



7.2.1.1 Kynurenic Acid

Recently, the integrative metabolism of Trp has been unraveled for the first time in fishes, namely, in Senegal sole. The findings provide new insights in Trp and derived metabolite accumulation. In mammals, the intestine is the main serotonin store; and the formation of the AA kynurenine (Kyn, Fig. 7.12) seems to be a key under stress conditions (Salamanca et al. 2020). Usually, the Kyn pathway eliminates excess Trp. However, based on histopathological examinations, Kaczorek et al. (2017) proved that dietary kynurenic acid (Kyna, Fig. 7.12), a metabolite of either kynurenine or Trp, is a stressor to young fishes.

In addition to adverse effects, the metabolite Kyn is identified as sex attractant in female masu salmon (*Oncorhynchus masou*) (Yambe et al. 2006). Moreover, Kyn has also an epigenetic potential. It can modulate histone methylation and acetylation (Hayakawa et al. 2019) and enhance the expression of certain miRs (Beischlag et al. 2016; Elmansi et al. 2020). Furthermore, it is considered as one means of diet-mediated disorders via epigenetics, namely, of the Central Nervous System (Huang

et al. 2020). As immunomodulatory Trp metabolite, also Kyna is Janus-faced (Wirthgen et al. 2018).

7.2.2 Invertebrates

The few available reports of Trp-supplemented diet affecting behavior of invertebrates appear to be rather consistent. As shown above, high mortality of the mud crab (*Scylla serrata*) can be attributed to aggressive encounters and cannibalism. The survival of juvenile crabs can be improved by increasing the level of Trp to max. 1% (Fig. 7.10). However, higher Trp levels adversely affect growth. Trp supplementation results in an increase of 5-HT in the hemolymph which is clearly seen after a fight, indicating that 5-HT plays an important role in suppressing the agonistic behavior of mud crabs during aggressive encounters (Laranja Jr et al. 2010); the same applies to Chinese mitten crabs (Pang et al. 2019).

In addition, the Galician crayfish (*Astacus leptodactylus*) can be calmed by feeding Trp-enriched diet. Harlioğlu et al. (2014) showed that the aggressive behavior of female, but not male, crayfish can be suppressed (Fig. 7.13), and the rearing efficiency can be improved by increasing dietary Trp levels up to 1.0%.

Increased stress resistance on Trp diets applies not only to fishes (Table 7.1) but also to invertebrates. In sea cucumbers, Zhang et al. (2018b) showed that dietary supplementation of Trp increases growth rate, intestinal digestive enzyme activity, and energy allocation for growth, enhances immune response, and augments growth of farmed animals under crowding stress.

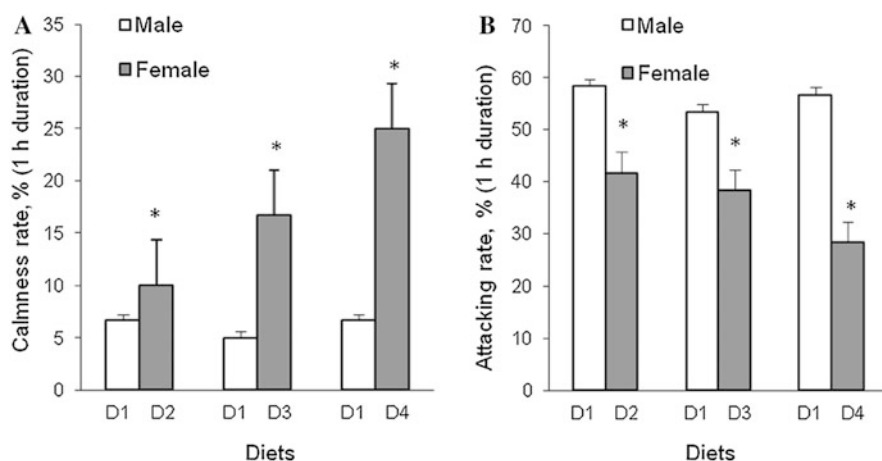


Fig. 7.13 (a) Rates of calmness and (b) of attacks of *Astacus leptodactylus* males and females during 1 h fed increasing dosages of tryptophan. D1 0.33; D2 0.50; D3 0.75; D4 1.0% Trp in diet dry matter. * Significant difference ($P < 0.05$) from the control. (From Harlioğlu et al. (2014), with permission from Springer Nature)

Overall, appropriate dietary Trp formulations may assist fishes and aquatic invertebrates coping with predictable stressful events, particularly crowding stress.

7.3 Concluding Remarks

Tryptophan appears to be one of the most versatile and most comprehensively studied individual AAs in aquatic animals. Nevertheless, some remarks remain to be made.

1. The “microbiota-gut-brain axis” plays a fundamental role in maintaining host homeostasis, and different immune, hormonal, and neuronal signals participate to this interkingdom communication system between eukaryota and prokaryota. In mammals, the essential Trp, as a precursor of several molecules acting at the interface between the host and the microbiota, is fundamental in the modulation of this bidirectional communication axis. In the gut, Trp undergoes three major metabolic pathways, the 5-HT, kynurenine, and aryl hydrocarbon receptor (AhR) ligand pathways, which may be directly or indirectly controlled by the saprophytic microflora (Bosi et al. 2020). Furthermore, first indications disclose that gut microbiota-derived Trp metabolites modulate inflammatory responses (Krishnan et al. 2018). Moreover, the inhibition of the kynurenine pathway prevents behavioral disturbances and oxidative stress in the brain of adult rats (Réus et al. 2018). Although many details in the mammalian studies are currently still obscure, these few examples may serve as role models for corresponding studies in aquatic animals.
2. Also from mammalian studies and as sketched above, it emerges that Trp and its metabolites (particularly Kyn) play roles in epigenetics. Histone modification (Etchegaray and Mostoslavsky 2016; Hayakawa et al. 2019), interaction in the expression of miRs (Beischlag et al. 2016; Elmansi et al. 2020), or DNA methylation (Fertan et al. 2019) have been identified so far. In aquatic animals, the participation of Trp and its metabolites in epigenetic processes waits to be discovered.

It can easily be predicted that the physiological and biomolecular role of Trp in aquatic animal nutrition will become even more comprehensive.

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Chapter 8

A Bunch of Amino Acids: Phe, Tyr, Branched-Chain AAs, Ser and Thr—‘*Much More than Easy Fuel*’



Abstract This chapter shows the nutritional requirement of selected farmed fishes and a few invertebrates for a variety of amino acids (AAs). The requirement of the essential AAs follows optimum curves with adverse effects if the AAs are in deficiency or excess. The mechanisms behind adverse effects of dietary excess deserve future attention; as one underlying mode of action antagonism or even toxicity of branched-chain AAs are discussed, but it needs substantiation. Based on functional metabolomics, evidence is increasing that AAs can run into deficit during pathogen challenge; exogenous supply of these AAs can mitigate infection effects. These AAs have the potential to act as metabolic modulators and strengthen the immune response. So far, the involvement of the intestinal microbiome or epigenetic mechanisms of dietary AAs are treated only cursorily.

This chapter comprises some so-called essential and some non-essential amino acids (AAs), namely phenylalanine (Phe), tyrosine (Tyr), leucine (Leu), isoleucine (Ile), valine (Val), serine (Ser), and threonine (Thr) and focuses, as the previous chapters, too, not only on corresponding requirements but also particularly on controlling functions.

8.1 Phenylalanine and Tyrosine

8.1.1 Phenylalanine

Phenylalanine (Phe, Fig. 8.1) is an essential proteinogenic AA (Wu 2013). Gisbert et al. (2012) assumed that Phe plays an important role in skeletogenesis, and Kim et al. (2012) proved that fishes, exemplified in red seabream (*Pagrus major*), can utilize Phe in both the free and the dipeptide form equally well.

Phe uses the same active transporter as Trp to cross the blood–brain barrier and can enter the serotonin production pathway. Phe can biologically be hydroxylated into L-tyrosine (Tyr). Tyr, in turn, is converted into L-DOPA that is further transformed into the neurotransmitters dopamine, noradrenaline, and adrenaline

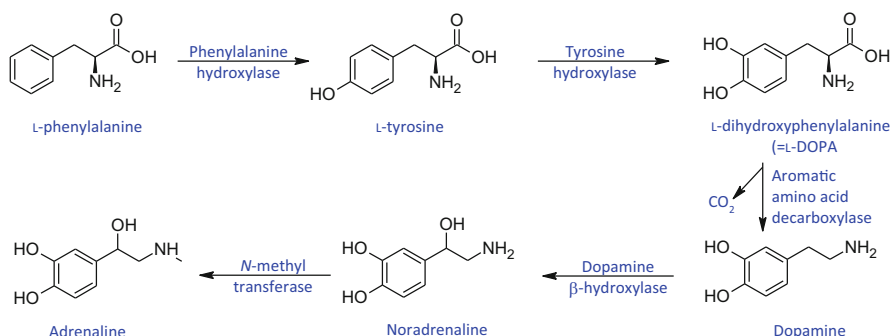


Fig. 8.1 Transformation of L-phenylalanine into L-tyrosine, neurotransmitters (dopamine, noradrenaline), and the hormone adrenaline

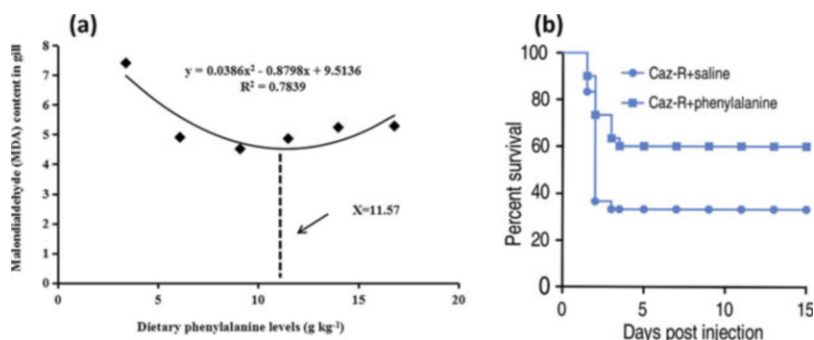


Fig. 8.2 (a) Phe requirement in young grass carps. (From Feng et al. (2017b), with permission from Elsevier). (b) Phe enhances innate immune response to clear ceftazidime-resistant *Vibrio alginolyticus* (Caz-R) in zebrafish. (From Jiang et al. (2019b), with permission from Elsevier)

(Fig. 8.1). Phe is the sole precursor of Tyr, but Phe cannot be synthesized back from Tyr. In recent trials, 37% of the Phe requirement can be replaced by Tyr in *Gibelion (Catla) catla* fingerlings (Zehra and Khan 2014), 40% in fingerlings of another Indian major carp (*Cirrhinus mrigala*) (Ahmed 2009), and 50% in juvenile Wuchang bream (*Megalobrama amblycephala*) (Ren et al. 2015b). In the diet for juvenile red drum (*Sciaenops ocellatus*), Tyr can account for up to 60% of the total aromatic AA requirement (Castillo et al. 2015). In rainbow trout, the replacement value of Tyr for Phe is ~53% on a weight basis (Kim 1993), 50% in channel catfish (Robinson et al. 1980), and 60% in common carp (Nose 1979). In Atlantic cod under stress, Phe can be used as dietary additive for stress attenuation (Herrera et al. 2017).

In their studies in grass carp, Feng et al. (2015a, 2017b) showed that Phe improves growth (Fig. 8.2a), digestive ability, and absorptive function as well as antioxidant capacity in the intestine. Phe controls the intestinal immune response and the gene expression of cytokines, tight junction proteins, antioxidant enzymes, and signaling molecules, including NF-κB, an inhibitor of nuclear factor κBα

(IkB α), TOR, Nrf2, and Keap (\rightarrow Chap. 7). Applying functional metabolomics in zebrafish, Jiang et al. (2019b) identified that exogenous Phe enhances the survival after infection with antibiotic-resistant *Vibrio alginolyticus* (Fig. 8.2b).

Except of a few previous studies noticing that Phe is essential to shrimps (Cowey and Forster 1971; Miyajima et al. 1976; Fair and Sick 1982), current papers on Phe metabolism in aquatic invertebrates are almost completely lacking. Only recently, Jin et al. (2019) determined Phe requirement of *Litopenaeus vannamei* in low-salinity water at 15.8 g kg⁻¹ of dry diet. This value lies slightly above the demand of *Marsupenaeus japonicus* (Teshima et al. 2002) and complies well with that in developing *Macrobrachium rosenbergii* larvae (Roustaian et al. 2000). More interestingly, deficiency as well as excess of dietary Phe adversely affects energy metabolism in *L. vannamei* indicated by the metabolite profiles in the hemolymph (Jin et al. 2019).

8.1.2 Tyrosine

Tyrosine (Tyr) is a non-essential proteinogenic α -AA. Via phenylalanine hydroxylase, Phe can easily be converted into Tyr (Fig. 8.1): Tyr can be spared by sufficient dietary Phe supplementation (Wilson and Halver 1986), whereby the sparing amount appears to be species-specific. Therefore, studies on dietary Tyr alone are sparse.

Tyr occurs in proteins that take part in signal transduction and metamorphosis. Pinto et al. (2010) showed that dietary Tyr supplementation results in an increase of its availability in Senegalese sole body fluids at metamorphosis, for coping with metamorphosis-related processes, such as the production of thyroid hormones. On the other hand, gilthead seabream larvae seem to be able to biosynthesize Tyr at a sufficient rate at pre-metamorphosis and do obviously not require an additional supply of aromatic AAs during metamorphosis. The different results obtained for sole and seabream are related to the complexity of metamorphosis that each species undergoes and to the needs for thyroid hormone production, which affects the aromatic AA demands throughout this physiological process. In addition, appropriate Tyr supply has the potential of reducing skeletal deformities and enhancing stress resistance, as demonstrated in white seabream (*Diplodus sargus*) challenged by a short-term temperature drop from 21 to 10 °C (Saavedra et al. 2010).

8.2 Branched-Chain Amino Acids

Branched-chain AAs (BCAAs) possess aliphatic side chains. Among the proteinogenic AAs, the three BCAAs, Leu, Ile, and Val (Fig. 8.3), are the most hydrophobic ones and are central in determining the structures of globular proteins as well as the interaction of the transmembrane domains of membranous proteins with phospholipid bilayers. Therefore, the occurrence of BCAA in nature is

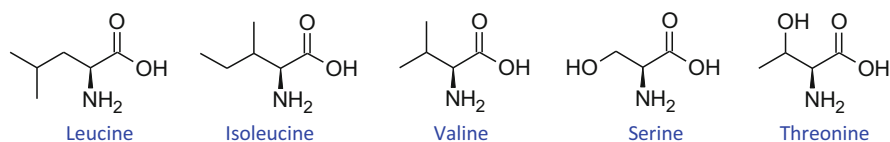


Fig. 8.3 L-Leucine, L-isoleucine, L-valine, L-serine, and L-threonine

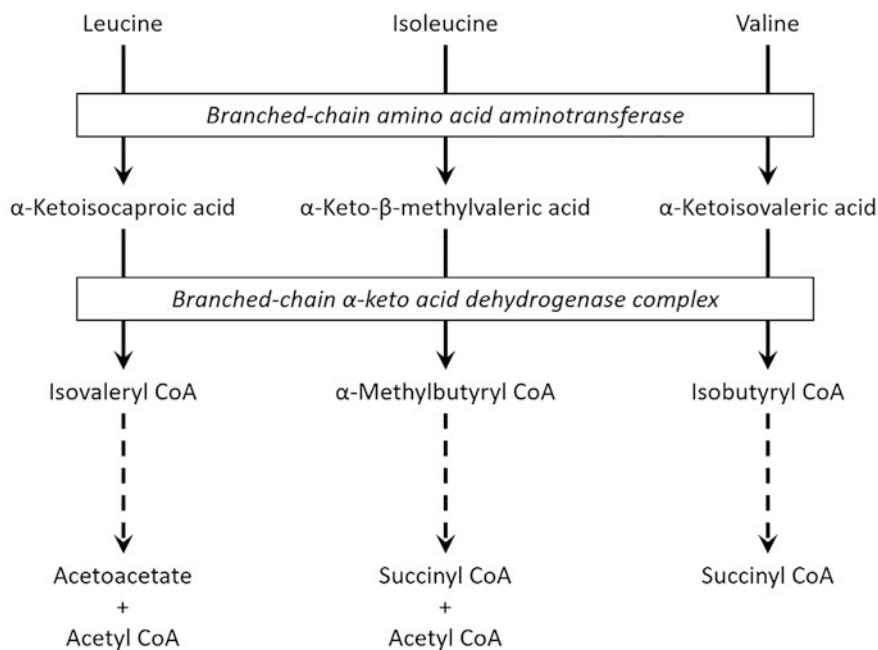


Fig. 8.4 Branched-chain amino acid catabolism. Step 1, transamination (removal of the amino group): branched-chain amino acid aminotransferase catalyzes the conversion of leucine, isoleucine, and valine into α -ketoisocaproic acid, α -keto- β -methylvaleric acid, and α -ketoisovaleric acid, respectively. Step 2, oxidative decarboxylation (removal of the carboxyl group): the α -keto acids are further metabolized by the branched-chain α -keto acid dehydrogenase complex to form isovaleryl CoA, α -methylbutyryl CoA, and isobutyryl CoA, respectively. Catabolism of leucine ultimately yields acetoacetate and acetyl CoA; isoleucine is metabolized to succinyl CoA and acetyl CoA, and valine yields succinyl CoA. CoA coenzyme A. (From Gorissen and Phillips (2019), with permission from Elsevier)

attributable to their primary role in protein structure, not to their secondary metabolic roles (Brosnan and Brosnan 2006).

Some four decades ago, Hughes et al. (1983a) identified the enzyme BCAA aminotransferase in five tissues of fingerling lake trout (*Salvelinus namaycush*). The activity of this aminotransferase is a first proof that this catabolism is common to all three BCAAs. In contrast to other AAs, BCAAs are metabolized stronger by skeletal muscles than by liver (Gorissen and Phillips 2019). The BCAA catabolism is sketched in Fig. 8.4.

In fishes, requirements lie between 2.2 and 4.0% protein for Val, 2.6 and 5.3 for Leu, and 1.2 and 4.0 kg⁻¹ for Ile, respectively (Ahmed and Khan 2006). For penaeid shrimps, Sara (2007) reported the corresponding demands collectively as 4.0, 8.26, and 4.2, respectively. Of the BCAAs, Leu has the highest anabolic potential (Gorissen and Phillips 2019), indicated by its high requirement.

8.2.1 Leucine and Isoleucine

8.2.1.1 Leucine

Leucine (Leu, Fig. 8.3) is an essential proteinaceous α -AA (Wu 2013) and mTOR activator and, thereby, regulates cell growth and stimulates muscle protein synthesis (Ren et al. 2015a). In addition, Leu stimulates the immune response (Jiang et al. 2015). Also, Leu—but not Met or Lys—possesses signaling properties in rainbow trout hepatocytes by regulating gluconeogenesis and lipogenesis, but not glycolysis (Lansard et al. 2011).

Uptake and Transport

In *Litopenaeus setiferus*, Leu, Met, and Phe share a Na⁺/K⁺-dependent AA transporter in membrane vesicles of the hepatopancreatic brush border (Duka and Ahearn 2013) as found in Atlantic lobster (Balon and Ahearn 1991; Duerr and Ahearn 1996). However, contrary to their marine relatives, freshwater crustaceans appear not to apply protons as driving force for AA uptake as exemplified in *Echinogammarus stammeri* (Berra et al. 2006). It seems that this finding can be expanded to other freshwater shrimps, such as the economically important *Macrobrachium* spp., since Brick and Ahearn (1978) described that the uptake of Lys in *M. rosenbergii* is not influenced by pH. Overall, an improved understanding of which dietary components modulate AA transport across gastrointestinal organs will lead to the development of an artificial diet that increases growth rate and decreases grow-out time in these animals.

Controlling Function

Dietary Leu can successfully be used as metabolic modulator to elevate survival ability of tilapias infected by *Streptococcus iniae* (Ma et al. 2015). In this fish, Du et al. (2017) detected that dietary Leu potentiates the phagocytosis of macrophages and increases the resistance against *Str. iniae* (Fig. 8.5). This finding is supported by Wu et al. (2017), who found that adequate dietary Leu enhances the nonspecific immunity in black carp (*Mylopharyngodon piceus*) juveniles, stimulates appetite,

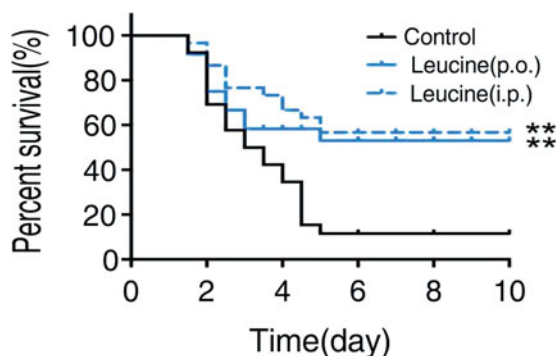


Fig. 8.5 L-leucine potentiates phagocytosis of tilapia macrophages to bacterial pathogens. Percent survival of tilapias challenged by *Str. iniae* in the presence or absence of exogenous leucine by injection (i. p.) or oral (o.p.) administration. Control: sterile saline. Significantly higher protection was observed in the two groups compared to the control ($P < 0.01$, Chi-squared test). (From Du et al. (2017), with permission from the American Chemical Society)

improves feed utilization, and promotes growth; the dietary Leu demand is estimated to be $\sim 24 \text{ g kg}^{-1}$, corresponding to 6.0% of dietary protein.

Also Giri et al. (2016) noticed an immunity strengthening by appropriate Leu nutrition. Exposing *Labeo rohita* fingerlings to lipid polysaccharides (LPS) induces inflammatory response and upregulation of pro-inflammatory cytokine and interleukin genes (*tnf- α* , *il-8*, *il-1 β*) in fish hepatocytes. Dietary pre-supplementation of an optimal amount of Leu (4.7 g kg^{-1} diet), however, prevents LPS-induced inflammatory responses. The protective effect of Leu is associated with reduced transcription of pro-inflammatory cytokines. Furthermore, Leu pretreatment enhances *il-10* mRNA expression that independently functions as an anti-inflammatory agent.

Excessive dietary Leu increases feed efficiency but depresses feed intake, growth, and protein deposition in rainbow trout likely due to antagonism among AAs, particularly BCAAs (Choo et al. 1991). Notably, in recent years, there has been growing interest in the stimulatory effect of supplemental β -hydroxyl- β -methylbutyrate (a Leu metabolite) on growth and immunity in several cold-water fishes. However, such a benefit has not been observed in warm-water species (tilapia, hybrid striped bass). This discrepancy may be explained by species differences in Leu metabolism. In addition, studies with rainbow trout, Arctic charr, and shrimps have revealed that BCAAs can be rapidly mobilized from white and red muscles during exhaustive exercise or seawater acclimation, but the rates of Leu release vary greatly among species (Li et al. (2009) and references therein).

As with the majority of nutrients, also the supply of Leu follows an optimum curve. Giri et al. (2015) showed that appropriate Leu supplementation enhances weight gain (WG), feed intake, and protein efficiency ratio and decreases the plasma ammonia content. Similarly, optimal Leu supplementation increases head kidney glutathione (GSH) content and stimulates superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. The increased antioxidant capacity reduces LPO.

The reduced oxidative stress is also reflected by modulated transcription of corresponding genes: increased transcription of *sod*, *gpx*, *nuclear factor erythroid 2-related factor 2 (nrf2)*, *natural killer-cell enhancing factor β (nkef- β)*, and *toll-like receptor-22 (tlr22)* in the head kidney and decreased transcription of *tumor necrosis factor- α (tnf- α)*, *kelch-like-ECH-associated protein 1 (keap1)*, and *interleukin 1 β (il-1 β)*. Inappropriate Leu supply, however, be it below or even above the tested optimum of 4.6 g kg⁻¹ diet, increases internal oxidative stress and, therefore, reduces the growth of rohita fingerlings. Growth retardation by excess dietary Leu is also found in Japanese flounder (Han et al. 2014).

In Japanese Amberjack (*Seriola quinqueradiata*), Kawanago et al. (2014) demonstrated that dietary Leu supplementation increases WG and influences the hepatic growth factor system: mean WG increases on a 1% Leu-supplemented diet, so does also the expression of hepatic *IGF-binding protein-1 (igfbp-1)* and of hepatic *insulin-like growth factor-1 (igf1)*, whereas that of *igf2* decreases on the 3% Leu-supplemented diet.

Almost identical results as in rohita occur in Leu-fed young grass carps (Fig. 8.6) (Deng et al. 2014). Appropriate Leu improves growth, enhances the anti-oxidant status through accelerating non-enzymatic and enzymatic anti-oxidant capacity. This is upregulated and controlled by the signaling molecules Nrf2 and Keap1. Adverse effects, such as increased LPO or decreased transcription of stress-protective genes, take place, if dietary Leu levels fall below or exceed 4.3% Leu in feed proteins. Supporting evidence is found also in juvenile Wuchang bream (Liang et al. 2018).

In their favorite farmed fish, namely, gibel carp, Zou et al. (2018) found that dietary Leu improves growth, tissue protein synthesis via TOR signaling pathway (\rightarrow Box: *Yin and Yang of Energy Regulation*, Chap. 5) and protein content.

Leu demand as optimum curve with excess supply causing adverse effects applies also to Wuchang bream juveniles. Ren et al. (2015a) provided insight into potential mechanisms: the lowest *tnf- α* mRNA expression is observed on 1.7% Leu, while the highest value is found on excess Leu (3.0%). TNF- α is a pro-inflammatory cytokine, which is involved in systemic inflammation, able to induce fever, apoptotic cell death, or cachexia¹ (Schwabe and Brenner 2006). This indicates that optimum dietary Leu level attenuates the inflammation, while excess dietary Leu aggravates the inflammatory response in juvenile Wuchang bream, which is in agreement with the report in Jian carp fed graded Ile (another BCAA) levels (Fig. 8.8) (Zhao et al. 2013). Excess dietary Leu might induce insulin resistance and, thereby, affect glucose metabolism, which is confirmed by high plasma glucose concentration in fish on excess Leu (Ren et al. 2015a).

With particular reference to immunity of young grass carps, proposed mechanisms behind adverse actions of non-appropriate dosages of feed ingredients have recently been and are going to be evaluated by a Chengdu (China) consortium. The biomolecular pathways of adverse effects caused by imbalanced different nutrients

¹Complex syndrome associated with an underlying illness causing ongoing muscle loss that is not entirely reversed with appropriate nutritional supplementation (Abrigo et al. 2018)

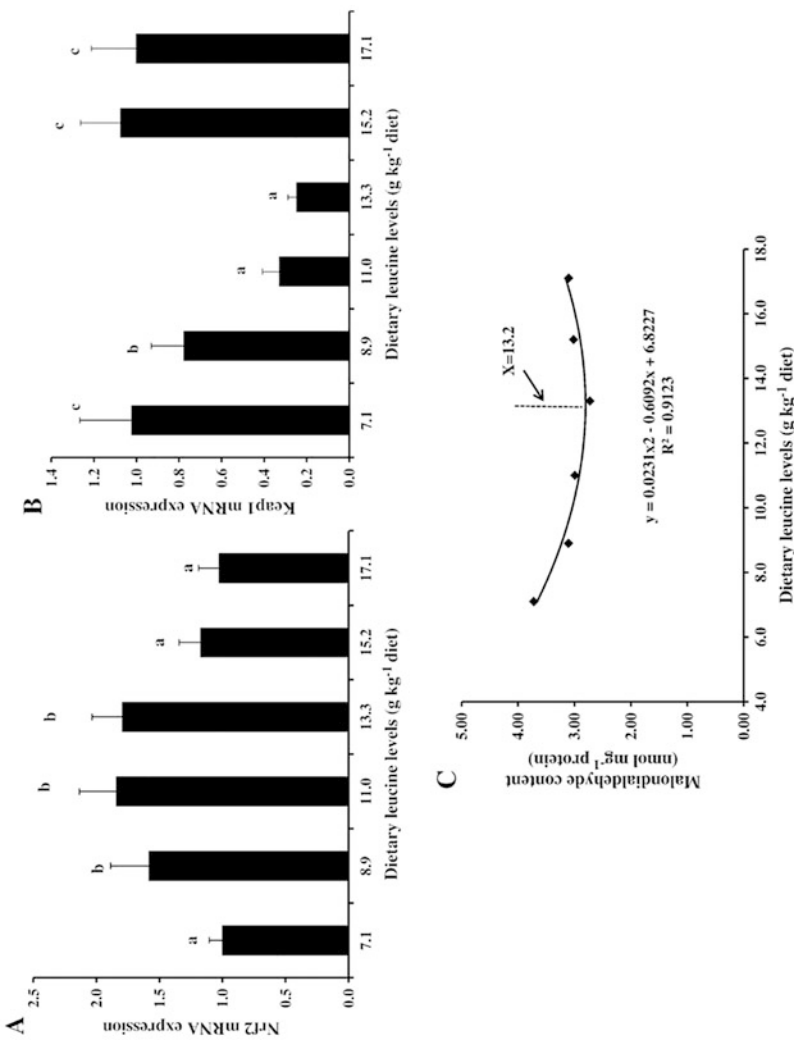


Fig. 8.6 Transcription of nuclear factor erythroid 2-related factor 2 (*nrf2*) (a) and kelch-like-ECH-associated protein 1 (*keap1*) (b), and intestinal oxidative stress (malondialdehyde content) (c) in young grass carp fed diets with graded levels of Leu (g kg⁻¹) for 8 weeks. (From Deng et al. (2014), with permission from Elsevier)

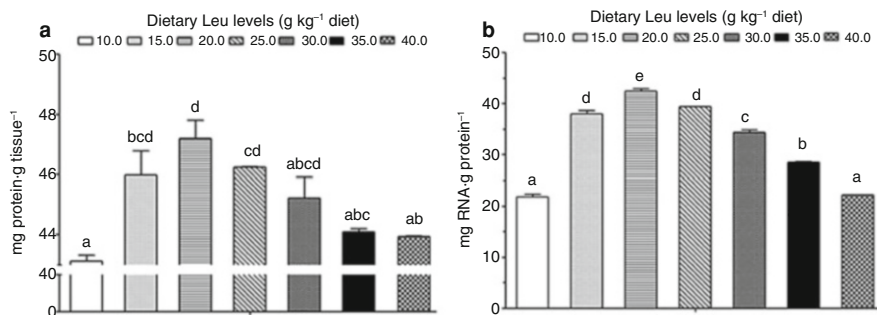


Fig. 8.7 Effect of dietary Leu on protein content (mg protein × g tissue⁻¹ (a)) and RNA/protein ratio (mg RNA × g protein⁻¹ (b)) in hybrid catfish muscle. Data represent means ± SEM of three replicates, with six fish in each replicate. Values having different letters are significantly different ($P < 0.05$). (From Zhao et al. (2020b), credit MDPI, Basel)

strongly resemble one another. With respect to Leu, Jiang et al. (2017) describe that deficiency induces oxidative damage and apoptosis which disrupts the cellular structural gill integrity by impairing the tight junction protein complex (through regulation of several *claudins*). Optimal dietary Leu level for gill protection is ~13 g kg⁻¹ diet.

Recently, Zhao et al. (2020b) figured out that dietary Leu improves muscle protein synthesis (Fig. 8.7) in hybrid catfish (*Pseudobagrus (Pelteobagrus) vachelli*) × *Tachysurus dumerili* (*Leiocassis longirostris*) by activating the PI3K/Akt/TOR signaling pathway and downregulating muscle protein degradation via the Akt/FoxO3a signaling pathway. The optimal dietary Leu level for growth is ~20 g kg⁻¹ diet.

8.2.1.2 Isoleucine

Compared to Leu, isoleucine (Ile, Fig. 8.3) is less well studied. It is an essential proteinaceous α-AA (Wu 2013) and part of the innate immune responses (Rahimnejad and Lee 2014); it enhances disease resistance and survival of juveniles after pathogen challenges (Fig. 8.8a). The improved survival coincides inversely with increased transcription of immune-related genes and directly with the *tor* gene in the intestine (Fig. 8.9) (Zhao et al. 2013). TOR is involved in protein production, cell survival, autophagy, and transcription (→Chap. 5). Appropriate Ile supply improves intestinal immune function (Zhao et al. 2014). Vice versa, non-optimal Ile supply increases the risk of inflammation: deficient supply elicits *tnf-α* and excess stimulates *tnf-α* and *il-1β* transcription (Fig. 8.8b). IL-1β is another pro-inflammatory cytokine, involved in cell proliferation, differentiation, and apoptosis.

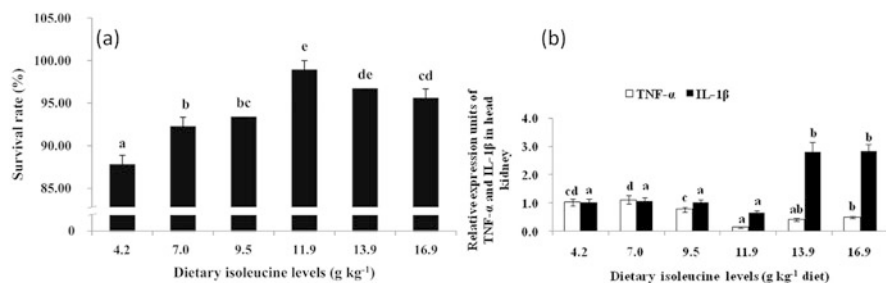


Fig. 8.8 (a) Survival of juvenile Jian carps challenged with *Aeromonas hydrophila* and fed diets with graded isoleucine levels (g kg⁻¹). (b) Survival rate, relative expression of *tumor necrosis factor α* (*tnf-α*, white columns), and *interleukin 1β* (*il-1β*, black columns) genes in the head kidney. Different letters denote significant differences ($P < 0.05$). (From Zhao et al. (2013), with permission from Elsevier)

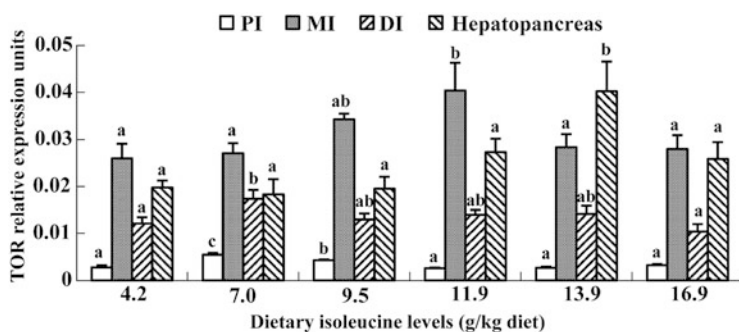


Fig. 8.9 Relative transcription of target of rapamycin (*tor*) genes in proximal intestine (PI), mid-intestine (MI), distal intestine (DI), and hepatopancreas of juvenile Jian carp fed diets containing graded levels of isoleucine (g kg⁻¹). Different letters above the columns denote significant difference ($P < 0.05$). (From Zhao et al. (2012), with permission from Elsevier)

In Jian carp, Zhao et al. (2012) showed that appropriate Ile supplementation increases:

1. Specific growth rate and feed intake
2. Body protein content and protein retention
3. Intestine fold height
4. Activities of trypsin, chymotrypsin, lipase, and amylase in hepatopancreas and intestine
5. Activities of alkaline phosphatase in distal intestine, Na⁺/K⁺-ATPase and γ-glutamyl transpeptidase in intestine
6. Glutamate–oxaloacetate transaminase activity in hepatopancreas
7. mRNA expression of chymotrypsin, lipase and amylase in hepatopancreas, γ-glutamyl transpeptidase in mid-intestine, Na⁺/K⁺-ATPase in intestine, and TOR in hepatopancreas and mid-intestine

It decreases:

1. Feed conversion ratio
2. Glutamate–pyruvate transaminase activity in the hepatopancreas
3. mRNA expression of trypsin in the hepatopancreas, alkaline phosphatase in the intestine, γ -glutamyl transpeptidase in distal intestine, and eIF4E-binding protein (4E-BP, a translation repressor protein) in the hepatopancreas, proximal, and mid-intestine

The repression of these genes results in increased growth, so that this paper collectively indicates that dietary Ile improves fish growth, promotes digestive and absorptive abilities, and coordinately regulates gene expression of the digestive and brush border enzymes, TOR, and 4E-BP.

Ile supplementation, too, follows an optimal curve as can be observed with *tor* transcription (Fig. 8.9). In the mid-intestine and the hepatopancreas, *tor* transcription is maximal, if the fish are fed 11.9 or 13.9 g kg⁻¹ Ile.

More comprehensively, Feng et al. (2017a) continued the above study of Zhao et al. (2012) and proposed biomolecular pathways for adverse effects of non-appropriate Ile diets on gill structure and immunity of young grass carps. The authors show—as with several other imbalanced diets—that

- (a) Ile deficiency downregulates several *claudins*, *occludin*, and *zo-1* to disrupt the intercellular structural integrity of fish gills.
- (b) Furthermore, pro-inflammatory cytokines (*il-1 β* , *il-8*, *tnf- α*) are upregulated and anti-inflammatory cytokines (*il-10*, *tgf-1 β*) downregulated.
- (c) Ile deficiency induces apoptosis and oxidative damage to disrupt the intercellular structural integrity of fish gills by increased transcription of several *caspase* genes and impairs antioxidant defense via Nrf2 signaling.
- (d) Ile excess results in similar adverse effects as Ile deficiency.
- (e) All adverse effects caused by Ile deficiency and excess can be reversed by appropriate Ile supplementation.

8.2.2 Valine

Valine (Val, Fig. 8.3) is an essential protein BCAA (Wu 2013) and involved in tissue repair and maintenance of body nitrogen balance (Molina-Poveda 2016). Appropriate Val supply improves the innate immune responses of fishes (Rahimnejad and Lee 2013) and enhances hepatopancreatic and intestinal enzyme activities as well as influences the balance of intestinal microflora of juvenile Jian carp (Dong et al. 2013). In addition to these beneficial effects of appropriate supply, Luo et al. (2014) reported adverse effects of Val deficiencies in young grass carps. As can be expected, deficiency is associated with decreased immune status and tight junction protein transcription. Furthermore, also Val demand supply follows an optimum curve with excess Val reducing feed intake and WG of the individuals (Fig. 8.10a). Furthermore, excess Val increases the risk of inflammation in the proximal intestine

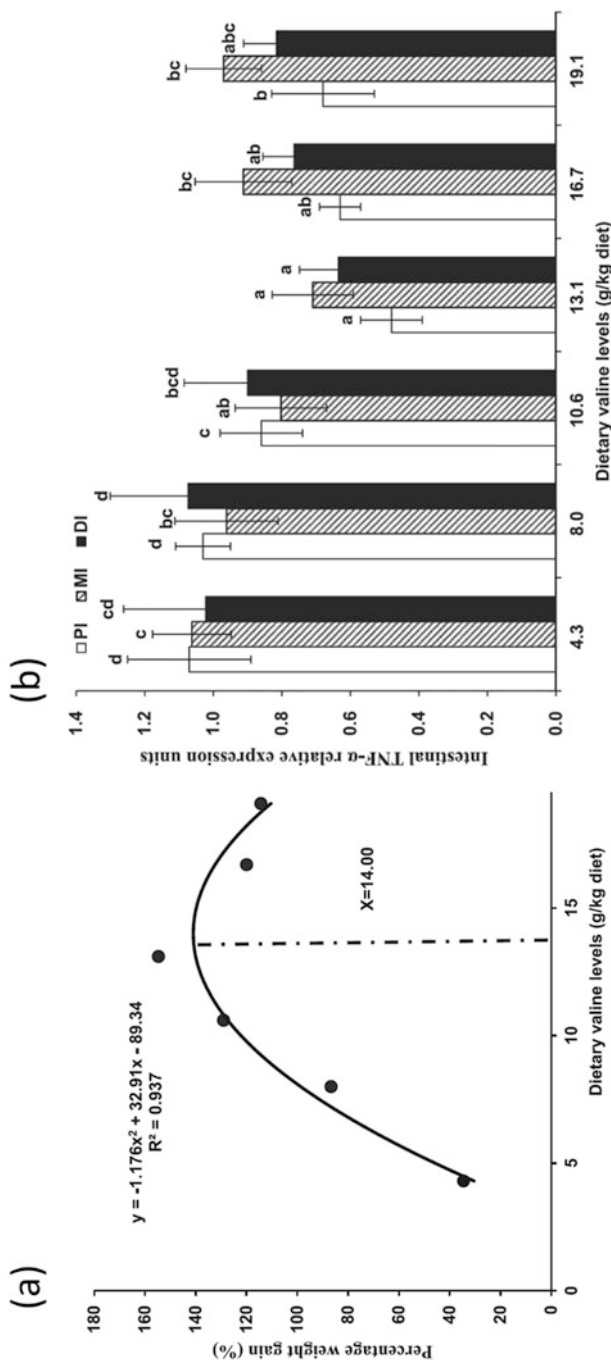


Fig. 8.10 Quadratic regression analysis of percentage weight gain (a) and *tnf-α* expression in the proximal intestine (PI), mid-intestine (MI), and distal intestine (DI) of juvenile grass carp fed diets containing graded levels of valine. (From Luo et al. (2014), with permission from Elsevier)

and mid-intestine by upregulating pro-inflammatory cytokines, such as *tnf- α* (Fig. 8.10b), *il-8* (comparable to the other two BCAAs), and downregulating anti-inflammatory cytokines, such as *tgf- β 1*.

In the intestine of young grass carps, Val deficiency elicits the following symptoms (Feng et al. 2015b):

1. Dietary Val deficiency disrupts immune barrier in the intestine by regulating the antibacterial compounds, increasing pro-inflammatory cytokines (IL-8 and TNF- α), and decreasing anti-inflammatory cytokines (IL-10 and TGF- β 1).
2. Val deficiency disrupts intestinal physical barrier by decreasing transcript levels of transmembrane tight junction proteins (occludin and claudins) and cytosolic tight junction proteins in the intestine.
3. However, optimum dietary Val supplementation restores intestinal barrier function by regulating the abovementioned antibacterial compounds, cytokines, signaling molecule expression, and the tight junction protein transcription.

8.2.3 Antagonism

Since the late 1940s and early 1950s, imbalanced dietary levels of BCAAs are known to produce antagonistic effects in pigs, rats, chicks, and humans, affecting the concentration of BCAAs in plasma and ultimately depressing growth (Grau and Kamei 1950; Anderson et al. 1951; Russell et al. 1952; Henderson et al. 1953).

Later, Hughes et al. (1984) identified this phenomenon also in lake trout and reported that Leu and Ile excesses seem to follow the pattern of AA antagonism described in other animals. The Ile antagonism appears to be less dramatic than that of Leu. The Leu antagonism causes an increase in the requirement of lake trout for Val, but the Ile requirement appears to be unaffected. Excess dietary Ile alone does not increase the dietary requirements of this fish for Leu or Val. However, when supplemental Leu is added to this diet, the Val requirements increase. The lacking effect of Leu and Ile additions to ameliorate growth reductions of excess Val, along with the persistent elevation of BCAS aminotransferase, is quite different from the patterns presented by the Ile and Leu antagonisms, since dietary Val excess does not increase the dietary requirement of lake trout for Leu or Ile. For these reasons, the effect of dietary Val excesses on lake trout is interpreted as AA toxicity rather than antagonism (Hughes et al. 1984).

Beside lake trout, antagonisms among BCAAs are proven in Chinook salmon (Chance et al. 1964), rainbow trout (Choo et al. 1991; Yamamoto et al. 2004), channel catfish (Wilson et al. 1980; Robinson et al. 1984), and olive flounder (Han et al. 2014). The latter authors found not only depression of growth at high levels of Leu but also stimulating effects of increased Val levels, at low Leu supplies. In rainbow trout, BCAA antagonism is induced by excess protein-bound Leu, and

enrichment with Ile and Val to a Leu excess diet relieves adverse effects on the growth performance and AA metabolism (Yamamoto et al. 2004). These authors conclude that there are antagonist effects owing to excess Leu derived from intact proteins in rainbow trout.

Robinson et al. (1984) reported an interrelationship between the BCAA in channel catfish. This antagonism between BCAA does not occur when the dietary levels of the three BCAA meet the requirements. Ahmed and Khan (2006) observed growth reductions of *Cirrhinus mrigala* fed excess Val, Ile, and Leu and attribute this to an antagonism between BCAA.

Experimental results on antagonisms among BCAA in fishes, however, are inconsistent between species and between major BCAA. Chance et al. (1964) reported that the demand of Ile increases with increasing dietary Leu in Chinook salmon. The authors also mentioned that Val demand may be affected by relatively high levels of Ile and Leu. Nose et al. (1974) noted that fish growth is suboptimal when Leu is in excess. Hughes et al. (1983b) found that antagonistic effects clearly occur in lake trout on Leu excess. Val supplementation of these excess Leu diets relieves the depression of growth and feed utilization.

Recently, Wang et al. (2017) described interactive effects of Leu and Ile on growth of Japanese flounder. Not only antagonistic effects are observed on high dietary Leu, but stimulatory effects also occur on growth at low dietary Leu with increasing dietary Ile. This indicates that dietary Leu and Ile function interactively, and the best combinations of these two BCAAs are low Leu-high Ile and high Leu-low Ile. Castillo and Gatlin (2018) determined the effects of imbalanced dietary levels of BCAAs on growth and AA utilization of juvenile red drum. Growth performance of juveniles is significantly depressed by dietary Leu excess, but not by excesses of Ile or Val.

Excess dietary Leu in mammals leads to imbalanced concentrations of plasma BCAAs with reduced levels of plasma Ile and Val. This is attributed to competitive inhibition during intestinal absorption and to the role of Leu as a promoter of BCAA catabolism. Corresponding results are found in rainbow trout with low concentrations of Ile and Val in plasma when fed diets with Leu excess (Castillo and Gatlin (2018) and references therein). Furthermore, excess Leu reduces the postprandial concentration of α -ketoglutarate in plasma in red drum, possibly indicating an increased ratio of transamination due to the imbalanced postprandial concentration of BCAAs. Ultimately, these results are a step forward in understanding the nature of antagonistic effects among BCAAs in fishes and can potentially prevent impaired performance due to the imbalanced profile of BCAAs in some alternative protein feedstuffs commonly used in aquafeeds (Castillo and Gatlin 2018).

Overall, interaction and antagonism of BCAAs are still challenging research issues in aquatic animal nutrition and more biomolecular studies are needed to identify the underlying modes of action.

8.3 Serine

Serine (Ser, Fig. 8.3) is a non-essential, proteinaceous α -AA (Wu 2013), important in the biosynthesis of purines and pyrimidines. Furthermore, it plays a key role in providing methyl donors for DNA and histone methylation (Ji et al. 2016). Together with Gly, Ser is interconverted primarily in liver and kidneys by tetrahydrofolate-dependent hydroxymethyl-transferase. The two AAs participate in gluconeogenesis, sulfur AA metabolism, and fat digestion. They stimulate feed intake in many fishes. Similarly, tissue levels of Ser in fish and shrimp increase by abrupt changes in salinity or hardness indicating a potential osmolytic activity of Ser. At present, little is known about whether dietary Ser supplementation may also optimize fish survival and growth under suboptimal culture conditions (Li et al. (2009) with references therein).

8.4 Threonine

Threonine (Thr, Fig. 8.3) is an essential 4-carbon proteinaceous α -AA (Wu 2013) with a hydroxyl group; it is 3-methyl serine. Optimum dietary Thr content ($\sim 12 \text{ g kg}^{-1} \text{ DM}$) improves growth, protein depositions, and gut micromorphology as shown in juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ \times *Epinephelus lanceolatus* ♂).

Furthermore, Thr modulates the immune function: Habte-Tsion et al. (2015c) reported that Thr influences the absorption capacity and brush border enzyme gene transcription and activity in juvenile Wuchang bream: alkaline phosphatase, Na^+/K^+ -ATPase transporter, and γ -glutamyl transpeptidase in the proximal intestine, mid-intestine, and distal intestine. The transpeptidase is involved in the transfer of AAs across cellular membranes.

Furthermore, optimum dietary Thr level reduces hepatopancreatic alanine transaminase activities and plasma ammonia content. However, excess dietary Thr triggers hepatic damage by stimulating hepatopancreatic aspartate aminotransferase activities as well as *tnf- α* transcription in the intestine, thereby eliciting inflammatory responses (Habte-Tsion et al. 2015a). Thr deficiency leads to severe dysplasia. For instance, grass carps fed Thr-deficient diets exhibits intestinal *villus* exfoliation, twist, severely fusion, lower anterior intestinal *villus* height, and serosa thickness (Gao et al. 2014).

In a detailed study in juvenile Jian carps, Feng et al. (2013) showed that Thr improves growth, digestive and absorptive capacity, enterocyte proliferation and differentiation, protein synthesis, downregulates *tor*, and upregulates *4e-bp2* transcription in juvenile Jian carp (Fig. 8.11). eIF4E-binding protein2 binds to mRNA, allowing the recruitment of ribosomes and translation initiation. The exact mechanism, however, whereby Thr controls protein synthesis through *tor* and *4e-bp2* gene transcription in Jian carps warrants further studies.

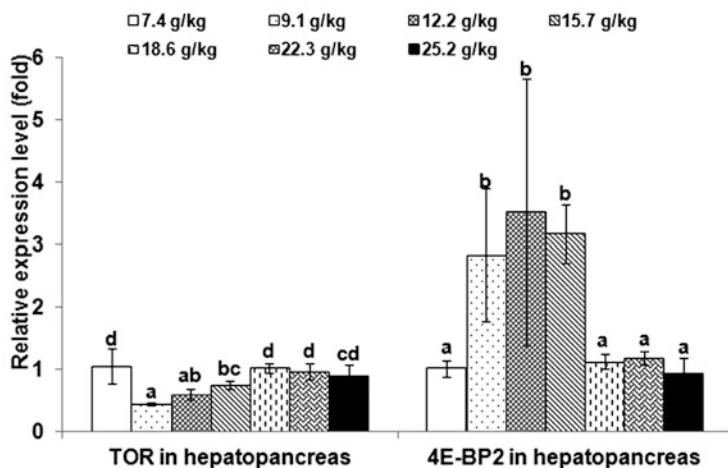


Fig. 8.11 Effects of dietary Thr on gene expression in hepatopancreas of juvenile Jian carp. Values are means with standard deviations. Different letters indicate significant differences among treatments ($P < 0.05$). Thr levels are 7.4, 9.1, 12.2, 15.7, 18.6, 22.3, and 25.2 g kg⁻¹ diet, respectively. (From Feng et al. (2013), credit Public Library of Science). *tor* target of rapamycin; *4e-bp2* eIF4E-binding protein2

To add some more symptoms to the catalog above, Habte-Tsion et al. (2015a) found that deficiency as well as excess of dietary Thr level affects WG, enzyme activity, immune response, and immune-related gene transcription in Wuchang bream; or vice versa: only the optimum dietary Thr (1.6% Thr per feed protein (Habte-Tsion et al. 2015b)) improves growth, enhances immune function, and maintains fish health. Non-appropriate Thr supply decreases the transcription of *tor* and *igf-1* but increases that of *4e-bp2*. In addition, dietary Thr regulates also the transcription of digestive enzymes (chymotrypsin, trypsin, amylase, and lipase), most enzymes with maximal transcription at 1.6% Thr in the diet. Furthermore, Habte-Tsion et al. (2016) found also transcription of stress- and immune-related genes modulated. Again, most genes transcribe best at 1.6% dietary Thr. This value complies well with further recent determinations: dietary 1.4% is optimal to hybrid catfishes (Zhao et al. 2020a) and ~1.5% to grass carps (Dong et al. 2017), respectively.

In their model fish (*Ctenopharyngodon idella*), Dong et al. (2017, 2018) disclosed effects of imbalanced dietary Thr in juveniles. Thr deficiency decreases intestinal immunity and aggravates inflammation associated with NF- κ B and TOR signaling pathways after infection with *Aer. hydrophila* by reducing innate and adaptive immune components (Fig. 8.12). Furthermore, Thr deficiency downregulates hepatic antimicrobial peptides (*leap-2a*, *leap-2b*), *hepcidin*, *igz*, *igm*, and *β -defensin1*; upregulates intestinal pro-inflammatory cytokines *tnf- α* , *il-1 β* , *il-6*, *il-8*, and *il-17d*. Correspondingly, anti-inflammatory cytokines are downregulated, namely, *tgf- β 1*, *tgf- β 2*, *il-4/13a*, and *il-10*. In their companion paper, Dong et al. (2018) showed that Thr deficiency also impairs gill structure by

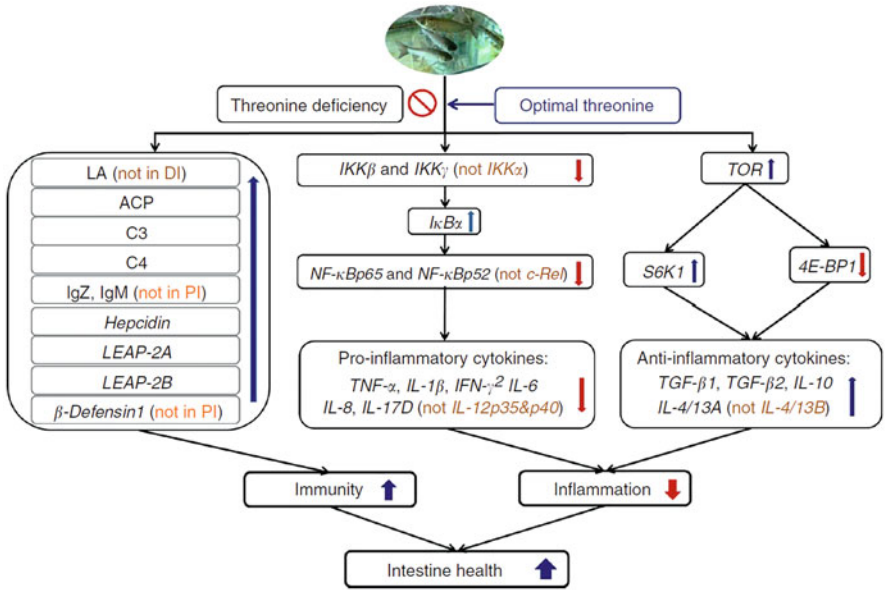


Fig. 8.12 Potential action pathways of dietary threonine regulating intestinal immunity and inflammation response in young grass carps. *LA* lysozyme activities, *DI* distal intestine, *IKK* IκB kinase, *TOR* target of rapamycin, *ACP* acid phosphatase, *C3* and *C4* complements, *PI* proximal intestine, *S6K1* ribosomal protein S6 kinases 1, *4E-BP1* eIF4E-binding protein 1, *LEAP* liver-expressed antimicrobial peptide, *IFN-γ* interferon γ, *TGF* transforming growth factor. (From Dong et al. (2017), with permission from the Cambridge University Press)

reducing transcription of *occludins* and, consequently, disrupts the intercellular structural integrity of fish gills and reduces resistance against further pathogens, such as *Flavobacterium columnare*.

Metabolomics in crucian carps, reared at elevated temperatures, reveal deficiencies in Thr after challenge with *Edwardsiella tarda*. The metabolome can be reprogrammed by adding exogenous Thr (Jiang et al. 2019a) (→Chap. 26).

8.5 Concluding Remarks

By comparing studies of fishes and invertebrates, it becomes obvious that the level of sophistication in the latter lags behind those of finfishes. Simple dose-response studies combined with the expression of a few monitoring genes, at best, are dominating the journals. There is no reason why this imbalance should be perpetuated.

It is beginning to be understood that, besides the “classical” functions of AAs in protein forming and energy metabolism, all AAs have a controlling function on the

biomolecular level in growth, innate immunity, or pathogen resistance. Metabolomic studies show that, during pathogen infection, several AAs become deficient; consequently, these AAs, supplemented exogenously, increase pathogen resistance even against antibiotic-resistant strains. Worthwhile, this approach should be developed as alternative of antibiotic application in aquaculture.

Less well understood are currently the role of intestinal microbiota, epigenetics, and the epigenetics-mediated transgenerational effect of certain nutrients, particularly those that take part in the one-carbon unit metabolism. Moreover, it is not well understood whether metabolic programming of succeeding generations occurs and, if so, how this phenomenon can be optimized.

The demand of all AAs follows optimum curves with detrimental effects if the AAs are in deficiency or in excess. The same applies to nutritionally imbalanced diets. In the case of BCAAs, antagonistic mechanisms are discussed. However, these mechanisms need genetic and epigenetic confirmation. Only based on robust mechanistic understanding, sustainable future developments of farmed aquatic animals (as outlined in the following chapters) can get be facilitated.

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Chapter 9

Sulfur Amino Acids—‘*Much More than Easy Fuel*’



Abstract Methionine and cysteine are essential proteinaceous amino acids (AA) with Met as one of the most versatile and important AAs. Met is central in the one-carbon metabolism and appears to be toxic in elevated doses, whereby the underlying modes of action remain to be identified in aquatic animals. Moreover, it plays a role in the synthesis of creatine, polyamines, and choline. In many feedstuffs, Met is one of the most limiting AAs, particularly in plant-based aquafeeds. Met has a pronounced beneficial influence on the innate immune response. The demand for appropriate immune response appears to be elevated in animals on plant-based diets. A few attempts show that, at least in several fishes, Met can successfully be replaced with Met hydroxyl analog. Due to mutual sparing effects of total sulfur AAs, growth requirements should be expressed in terms of total SAA rather than Met or Cys alone – a classical request in aquatic animal nutrition. Concluding, this chapter points out gaps in the understanding of controlling by sulfur AAs. Although Met is one major methyl donor, epigenetics in the metabolism of and controlling by sulfur AAs is sparsely studied. The same applies to the microbiota-host axis of interactions.

The major sulfur amino acids (SAAs), methionine (Met) and cysteine (Cys), are essential proteinaceous α -AAs (Wu 2013). Fishes have a total sulfur AA requirement rather than a specific Met or Cys demand (Wilson and Halver 1986). Besides these two AAs, the oxidized dimer of Cys, cystine (Cys₂), and ergothioneine play also roles in aquatic animal nutrition and metabolism. Appropriate Met supplies improve intestinal microbial diversity; this aspect, however, deserves more attention.

9.1 Methionine

Doubtless, methionine (Met, Fig. 9.1) is the most intriguing S-containing AA, not least because it takes part in the one-carbon metabolism. However, it is also the most toxic AA when fed in excess (Benevenga and Steele 1984). As one major toxicity mechanism, Dever and Elfarra (2008) show that Met transamination is central in Met

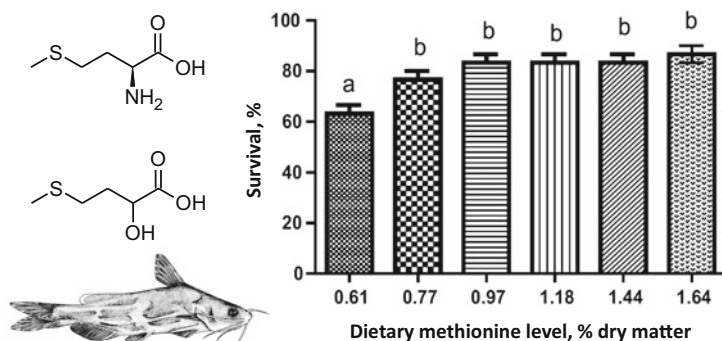


Fig. 9.1 Left: L-Methionine and methionine hydroxy analog. Right: Increases of post challenge survival of yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*) after infection with *Aeromonas hydrophila* by increasing the Met dose in the diet. Different superscripts indicate significant differences ($P < 0.05$). (From Elmada et al. (2016), with permission from Wiley; image credit FAO)

toxicity as found in male mouse hepatocytes. This mechanism, however, could not be confirmed in humans (Garlick 2006). Whatever mechanism, corresponding reports in aquatic animals are not (yet) available, although dietary Met requirements often follow optimum curves (Figs. 9.3 and 9.8) showing clear adverse effects on dietary Met excess.

The NRC (2011) recommendation mentions Met requirement for proper growth to be below or equal 1.0% of dietary dry matter for fishes and shrimps. This figure complies well with individual evaluations in, for instance, common carp (Schwarz et al. 1998), European seabass (*Dicentrarchus labrax*) (Machado et al. 2018), Nile tilapia (Guo et al. 2020b), or Pacific white shrimp (Façanha et al. 2016).

Met has an important and specific function in mRNA translation (Métayer et al. 2008), plays a role in the synthesis of creatine and polyamines, and is a precursor of choline (Molina-Poveda 2016). Usually, appropriate Met supplies also improve intestinal microbial diversity (Guo et al. 2020b); this aspect, however, deserves more attention. Met and derivatives display pronounced antioxidative activities (Feng et al. 2011; Coutinho et al. 2017).

Within the one-carbon metabolism, Met is vital for DNA synthesis, cell growth and development, and methylation of DNA and histones (Wang et al. 2012). It is methyl donor to more than 60 products (Hollenbeck 2012).

Box 9.1 Methionine Restriction: General Aspects

In many feedstuffs, Met is usually one of the most limiting AAs, particularly in plant-based aquafeeds (→AAN III Chapters on “Fishmeal Replacement”) (Bulbul et al. 2016). Mammalian studies report that individuals subjected to Met restriction (MR) display changes in metabolic flexibility indicated by increases in energy expenditure, glucose tolerance, and life span (Latimer et al.

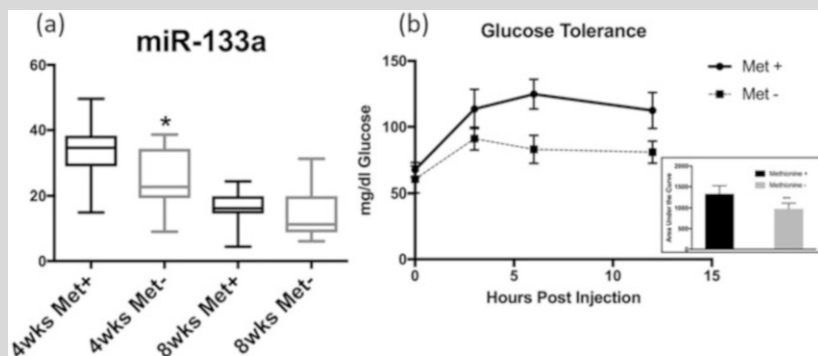
(continued)

Box 9.1 (continued)

2018a). MR prevents steatosis hepatis, delays the onset of age-related diseases, and enhances fat oxidation in obese subjects with metabolic syndromes (Mladenović et al. 2019). Further beneficial effects of MR are improvement of epithelial tight junctions, modulation of cancer progression, and improvement of stress tolerance (Ables and Johnson 2017).

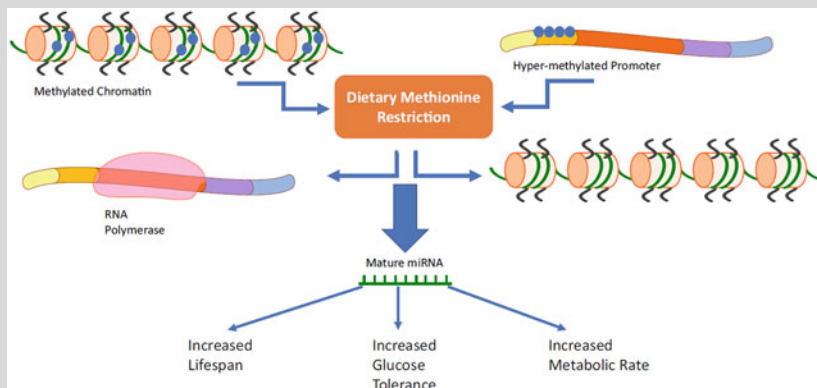
Some of the protective effects of MR are due to increased trans-sulfuration flux to increase the expression of the *cystathionine gamma lyase* (catalyzes the last step in the trans-sulfuration pathway from Met to Cys) and the production of hydrogen sulfide (Canfield and Bradshaw 2019). Furthermore, dietary MR inhibits mTOR (\rightarrow Box: Yin and Yang of Energy Regulation, Chap. 5) activity to initiate autophagy and increases plasma and liver levels of *miR-31* in mice which, in turn, alter bone structure (Latimer et al. 2018b).

Comparable studies in aquatic animals are still in their infancy: several miRNAs involved in myogenesis are elevated in rainbow trout muscle stem cells exposed to Met-deficient cell media (Latimer et al. 2017). MR blocks cell differentiation by decreasing *myoD1* and *myogenin* expression. Moreover, these cells regulate miRNAs that reduce their capacity for differentiation (*miR-133a*, *miR-206*, and *miR-210*). Rainbow trouts fed an MR diet for 4 weeks have lower levels of *miR-133a* in the skeletal muscle at 4 weeks (Box Fig. 1) and increased glucose tolerance (lower plasma glucose level, Box Fig. 1) following a glucose challenge at 8 weeks (Latimer et al. 2018a). Finally, Latimer et al. (2018b) provide an educational sketch of the potential epigenetic pathway of MR (Box Fig. 2).



Box Fig. 1 (a) Expression of *miR-133a* during 8 weeks of Met- or Met+ feeding qPCR analysis of miR abundance. * indicates a significant difference ($P < 0.0163$, $N = 15$) as indicated by Two-Way ANOVA. Error bars indicate standard deviation. (b) Analysis of Glucose Area under the curve following a glucose challenge at 8 weeks in fishes fed Met- or Met+ diets. ***indicate significant differences in area under the curve ($P < 0.0005$, $N = 9$) as indicated by unpaired t-test. (From Latimer et al. (2018a), with permission from Elsevier)

(continued)

Box 9.1 (continued)

Box Fig. 2 Proposed epigenetic pathway of methionine restriction. Changes in chromatin methylation or methylation status of DNA promoters due to dietary MR has the ability to activate or repress microRNAs (miRs) involved in the cell response to MR. These distinct changes could then be related to MR phenotype characterized by increased life span, glucose tolerance, and metabolic rate. (From Latimer et al. (2018b), credit Frontiers Media)

Although the current studies in aquatic animals did not investigate the MR-mediated aging response, they do indicate that miRs are regulated by Met availability and potentially play a role in the metabolic phenotype (Latimer et al. 2018b). Interesting details can be expected in aquatic animals in future studies.

A portion of dietary Met is converted to Cys2, if Cys2 is not adequately supplied in the diet. And vice versa, the presence of Cys2 or Cys reduces the demand of Met for maximum growth. Many studies show that dietary Cys2 suppresses trans-sulfuration, thus providing a metabolic basis for the sparing effects of Cys2 on Met requirements. For instance, Zehra and Khan (2016) reported that up to 40% of the Met requirement can be spared by Cys in the diet for *Gibelion (Catla) catla* fingerlings.

Met has a pronounced beneficial influence on the innate immune response to inflammation, as shown in, e.g., European seabass or yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*). In the latter species, even the lowest supplementary Met dose (0.6%) increases the post challenge survival after infection with *Aeromonas hydrophila* (Fig. 9.1) (Elmada et al. 2016). In seabass, the immune strengthening effect of Met is even more pronounced than that of Trp (→Chap. 7) and points out its potential to be incorporated in functional feeds and prophylactic strategies against predictable unfavorable events (Machado et al. 2015, 2018).

No information is available on Met-modulated immune response in aquatic invertebrates.

A study in rainbow trouts fed Met-deficient diets shows that this species preferentially utilizes AAs for lipid synthesis (Rolland et al. 2016). The transcription of hepatic genes involved in AA catabolism (*alt1*, *gdh*, *gls01*, and *gls02*) is differently affected by dietary levels of crystalline Met. *Alt1* and *gdh* transcription is upregulated with increasing Met level, whereas the transcript of *got2* is not affected. The expression of *gls01* and *gls02* shows similar patterns, being the highest in fishes fed 1.0% Met. GLS01 and GLS02 catalyze the conversion of glutamate to glutamine by fixing free ammonia; alanine aminotransferase (ALT1) catalyzes the transfer of an amino group from L-alanine to α -ketoglutarate. Conversely, high dietary Met level upregulates the transcription of *alt1* and *gdh* involved in the synthesis of intermediates of the Krebs cycle (pyruvate and glutamate), and excess dietary Met can be oxidized as an energy substrate directly through the Krebs cycle or via gluconeogenesis (Rolland et al. 2016).

Harmful effects are found in turbot on a Met diet with increasing ratios of Met +Cys (Fig. 9.2a) (Klatt et al. 2016). The breakpoints reflect the lower critical

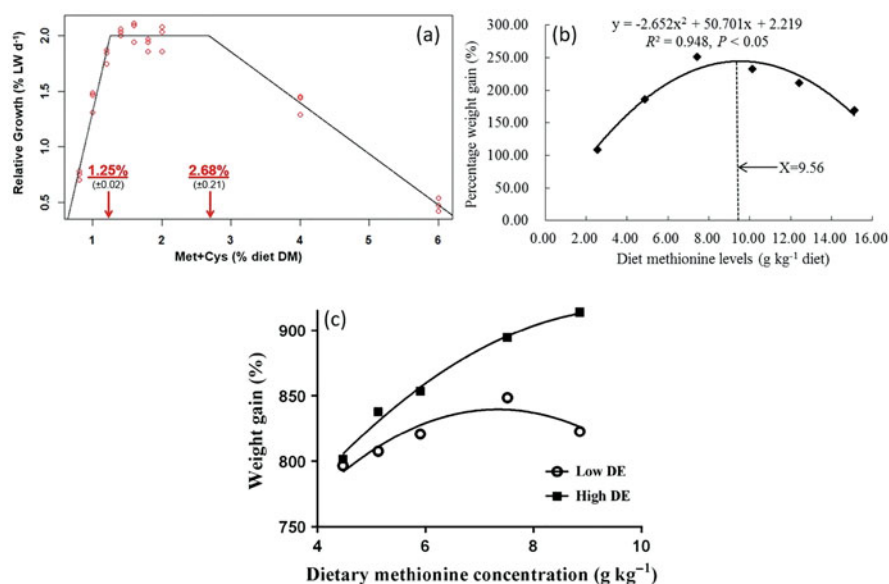


Fig. 9.2 (a) The relationship between the Met+Cys concentration (% of diet dry matter, DM) in the diets and the relative growth (% LW d⁻¹) of juvenile turbot (*Scophthalmus maximus*) fed with 9 different diets for 56 days. (From Klatt et al. (2016), with permission from Elsevier). (b) Quadratic regression analysis of percentage weight gain (PWG) for on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of Met (g kg⁻¹) for 60 days. (From Fang et al. (2020), with permission from the Cambridge University Press). (c) Weight gain responses of juvenile Nile tilapia to dietary Met concentrations (g kg⁻¹) at low (10.9 MJ kg⁻¹ digestible energy, DE) and high (12.4 MJ kg⁻¹ DE) energy levels for 8 weeks using quadratic regression analysis. Estimates of Met requirements (g kg⁻¹ diet, low versus high diet DE diets) were 7.34 versus 9.90, respectively. (From He et al. (2017), with permission from Wiley)

concentration with 1.25% Met+Cys and the upper critical concentration at 2.7% Met+Cys. At a strong oversupply of Met+Cys, the concentration of hepatic *S*-adenosyl-L-homocysteine (SAH) is negatively correlated to growth indicating Met+Cys toxicity. The Met requirement of the model fish of the Chengdu (China) laboratory, *Ctenopharyngodon idella*, is in the same range as the turbot value (Fig. 9.2b). Reasons for Met toxicity are diverse: Oversupplied Met has to be catabolized leading to an excess of sulfur that cannot be completely eliminated, increasing the concentration of sulfuric acid with metabolic acidosis (Klatt et al. 2016).

Furthermore, high hepatic levels of SAH are toxic as found in rats. SAH is an intermediate product in the Met metabolism, a metabolite of *S*-adenosylmethionine (SAM) and a precursor of L-homocysteine, HCy. SAH accumulation in hepatic tissues can partly be explained by a dysfunction of the liver due to non-enzymatic methylation of macromolecules by SAM, the most important donator of methyl groups. Furthermore, SAH is known as a strong oxidant related to several disorders like vascular diseases in humans (Klatt et al. (2016) and references therein). This function of SAH, however, has still to be checked in fishes and aquatic invertebrates.

A compilation of Met-requirement is presented in Table 9.1.

Met requirement for growth depends on available energy (digestible energy, DE) in the feed as described in juvenile Nile tilapia (He et al. 2017). Fishes fed high-DE diets require higher Met for maximum growth than fishes fed low-DE diets (Fig. 9.2c). This is mainly because tilapias on high-DE diets retain more AAs and protein in the whole body, but less lipids. Furthermore, Met acts as methyl donor and is the precursor of carnitine that plays an important role in fatty acid (FA) β -oxidation. Therefore, increased Met is needed for synthesizing carnitine; thus, transferred FAs to participate β -oxidation can also explain the elevated Met requirement (He et al. 2017).

Despite its dependency on total available energy, Met requirement is rather uniform (Table 9.1): It appears to be independent of whether it is a finfish or a crayfish, be it herbivorous, omnivorous, or carnivorous, not depending of the zoogeographic origin of the species and even independent of the methods applied. Met requirement oscillates around 1.0 (0.6–1.7)% diet (DM) with only two exceptions: the first one is the swimming crab with only 0.3 and the second one is the Plata pompano with 2.3% diet. The uniform requirement indicates that Met is needed, beside protein accretion and carnitine synthesis, for primordial and basal pathways that are conserved during evolution from invertebrates to fishes. This basic function of Met is its role as one major methyl donor for synthesis of other substrates as well as epigenetic pathways.

9.1.1 Invertebrates

Penaeus monodon juveniles serve as model invertebrate in Met-triggered transcriptomic studies. Fed an optimal crude protein level to this shrimp, Richard et al. (2010a, 2010b) noticed that a 30% Met deficiency diminishes protein accretion

Table 9.1 Methionine requirement of selected aquatic animal juveniles

	Requirement,% of		References
Species	Dietary dry matter	Dietary protein	
Invertebrates			
<i>Apostichopus japonicus</i> , sea cucumber	0.6–0.7	2.8–3.4	Li et al. (2020a)
<i>Artemia franciscana</i> , brine shrimp	1.8		Vikas et al. (2014)
<i>Haliotis discus hannai</i> , Japanese abalone		2.1	Mai et al. (1994)
<i>H. rufescens</i> , red abalone		2.3–2.8	Allen and Kilgore (1975)
		2.0	Chen (1998)
<i>H. tuberculata</i> , abalone		2.07	Mai et al. (1994)
<i>Litopenaeus vannamei</i> , Pacific white shrimp	0.7–0.9		Lin et al. (2015)
	0.5		Forster and Dominy (2006)
	0.8		Gu et al. (2013)
	0.6		Fox et al. (1995)
		3.5	Zhou et al. (2013)
<i>Lytechinus variegatus</i> , Green Sea urchin	0.6		Heflin et al. (2016)
<i>Macrobrachium rosenbergii</i> , Giant river prawn		3.4–3.5	D'Abramo and Sheen (1994)
		3.0	Mukhopadhyay et al. (2003)
<i>Marsupenaeus japonicus</i> , Kuruma shrimp	0.6–0.8		Teshima et al. (2002)
<i>Palaemonetes varians</i> , Atlantic ditch shrimp	~1.0		Palma et al. (2015)
<i>Penaeus monodon</i> , black tiger shrimp	0.9 (+0.4 Cys)	2.4	In Nunes et al. (2014)
	0.8		Richard et al. (2010a)
<i>Portunus trituberculatus</i> , swimming crab	0.3		Jin et al. (2015)
Fishes			
<i>Acipenser transmontanus</i> , White sturgeon		2.2 Σ SAA ^a	In Akiyama et al. (1997)
<i>Anguilla japonica</i> , Japanese eel		3.2 Σ SAA	In Wilson and Halver (1986)
<i>Argyrosomus regius</i> , meagre	0.8		de Moura et al. (2018)
<i>Astyanax altiparanae</i> , Lambari	1.4 Σ SAA	4.4 Σ SAA	Campelo et al. (2020)
<i>Astyanax fasciatus</i> , Colliroja		2.01	Furuya et al. (2015)
<i>Atractoscion nobilis</i> , White weakfish	0.9 (+0.5 Cys)		In Xu et al. (2019)
<i>Carassius gibelio</i> , Gibel carp	0.8–1.0	1.7–2.3	Wang et al. (2016a)
<i>Gibelion (Catla) catla</i> , Indian major carp		3.6	Ravi and Devaraj (1991)
		3.9 Σ SAA	Zehra and Khan (2016)
		3.4 Σ SAA	In Akiyama et al. (1997)

(continued)

Table 9.1 (continued)

Species	Requirement,% of		References
	Dietary dry matter	Dietary protein	
<i>Chanos chanos</i> , milkfish		3.2 Σ SAA	In Akiyama et al. (1997)
		2.5 (0.8 Cys2)	Borlongan and Coloso (1993)
<i>Cirrhinus mrigala</i> , Indian major carp	1.6–1.7		Khan and Abidi (2013)
<i>Ctenopharyngodon idella</i> , grass carp	0.6		Pan et al. (2017)
	0.6 (+0.2 Cys)	2.2	In Xu et al. (2019)
	0.96 (Fig. 9.2)		Fang et al. (2020)
<i>Cyprinus carpio</i> , common carp	0.8 (+0.2 Cys)		In Nunes et al. (2014)
		2.7 Σ SAA	In Abimorad et al. (2010)
		3.1 Σ SAA	In Wilson and Halver (1986)
<i>Dicentrarchus labrax</i> , European seabass	~0.9 (+0.4 Cys)		In Nunes et al. (2014)
	0.8	2.0	Tulli et al. (2010)
		4.0 Σ SAA	In Akiyama et al. (1997)
	1.3		Coutinho et al. (2017)
<i>Epinephelus coioides</i> , grouper	1.3	3.3 Σ SAA	In Klatt et al. (2016)
<i>E. fuscoguttatus</i> ♀ \times <i>E. lanceolatus</i> ♂, hybrid grouper	1.5	2.9	Li et al. (2020b)
<i>Heteropneustes fossilis</i> , stinging catfish	0.7 (+0.8 Cys)		Farhat and Khan (2014)
	1.1 (+0.4 Cys)	2.73	Ahmed (2014)
<i>Heterotis niloticus</i> , African bonytongue		2.5 Σ SAA	Monentcham et al. (2010)
<i>Ictalurus punctatus</i> , channel catfish	0.6		In Nunes et al. (2014)
		2.3 Σ SAA	In Wilson and Halver (1986)
<i>Labeo rohita</i> , Rohu	0.6 (+0.3 Cys)		Abidi and Khan (2011)
		2.7	In Klatt et al. (2016)
<i>Larimichthys crocea</i> , large yellow croaker	1.4	3.3	Mai et al. (2006)
		2.8	Li et al. (2013)
<i>Lates calcarifer</i> , barramundi		1.8–2.3 2.9–3.4 Σ SAA	Poppi et al. (2017)

(continued)

Table 9.1 (continued)

Species	Requirement,% of		References
	Dietary dry matter	Dietary protein	
<i>Lepomis macrochirus</i> , bluegill	1.3		Masagounder et al. (2011)
<i>Megalobrama amblycephala</i> , Wuchang (blunt snout) bream	0.9 (+0.2 Cys)	2.5	Liao et al. (2014)
	0.8	2.3	In Xu et al. (2019)
<i>Morone saxatilis</i> , striped bass	1.0 Σ SAA		Small and Soares Jr (1998)
<i>Morone chrysops</i> \times <i>M. saxatilis</i> , hybrid striped bass	$\sim 0.7 \Sigma$ SAA		Griffin et al. (1994)
		2.9 Σ SAA	Keembiyehetty and Gatlin (1993)
	1.0 Σ SAA		Whiteman and Gatlin (2005)
<i>Myxocyprinus asiaticus</i> , Chinese sucker	1.4 (+0.4 Cys)	3.2	Chu et al. (2014)
<i>Oncorhynchus keta</i> , chum salmon	1.2	3.0 Σ SAA	In Gurure et al. (2007) In Akiyama et al. (1997)
<i>O. kisutch</i> , Coho salmon		2.7 Σ SAA	In Akiyama et al. (1997)
<i>O. mykiss</i> , rainbow trout	0.7	2.7 Σ SAA	Boonyoung et al. (2013)
		1.5	In Akiyama et al. (1997)
		2.3 Σ SAA	In Klatt et al. (2016)
	0.8		Skiba-Cassy et al. (2016)
		2.2	In Wilson and Halver (1986)
<i>O. tshawytscha</i> , Chinook salmon	1.6		In Gurure et al. (2007)
		4.0 Σ SAA	In Wilson and Halver (1986)
<i>Oreochromis aureus</i> , blue tilapia		2.8 Σ SAA	In Akiyama et al. (1997)
<i>O. mossambicus</i> , Mozambique tilapia		1.0–3.2 Σ SAA	In Akiyama et al. (1997)
<i>O. niloticus</i> , Nile tilapia	0.8 (+0.2 Cys)		In Nunes et al. (2014)
	1.1 Σ SAA		In Nunes et al. (2014)
		3.0 Σ SAA	Nguyen and Davis (2009)
	0.9 Σ SAA	3.2 Σ SAA	In Abimorad et al. (2010)
	Up to 47% Σ SAA by Cys2		He et al. (2016)
	0.7–1.0 (+0.5 Cys)		He et al. (2017)
	0.9 (+0.1 Cys)		In Xu et al. (2019)

(continued)

Table 9.1 (continued)

Species	Requirement,% of		References
	Dietary dry matter	Dietary protein	
<i>O. niloticus</i> × <i>O. mossambicus</i> , hybrid tilapia	1.3–1.6		Figueiredo-Silva et al. (2015)
<i>Pampus argenteus</i> , silver pomfret		1.9 Σ SAA	Hossain et al. (2011)
<i>Paralichthys olivaceus</i> , olive flounder	1.4–1.7	3.0	Alam et al. (2000, 2001)
	1.9		Forster and Ogata (1998)
<i>Pagrus major</i> , Japanese seabream		2.2	Forster and Ogata (1998)
<i>Perca flavescens</i> , yellow perch	0.8		Hart et al. (2010)
<i>Piaractus mesopotamicus</i> , Pacu		2.2	Abimorad et al. (2010)
	0.45		Khan et al. (2020)
<i>Pseudosciaena crocea</i> , large yellow croaker	1.4	3.3	Mai et al. (2006)
<i>Pseudobagrus ussuriensis</i> , Ussuri catfish	1.4 (+0.25 Cys)	3.5	Wang et al. (2016b)
<i>Rachycentron canadum</i> , cobia	1.2 (+0.7 Cys)	2.6	Zhou et al. (2006)
	1.0–1.2	2.2–2.5	Wang et al. (2016c)
<i>Rhamdia quelen</i> , Jundiá catfish		2.1	Meyer and Fracalossi (2005)
<i>Salmo salar</i> , Atlantic salmon	0.7 (+0.6 Cys)	3.4	In Nunes et al. (2014), Rollin et al. (2003)
<i>Salvelinus alpinus</i> , Arctic charr	1.1		Gurure et al. (2007)
		1.8–2.6	Simmons et al. (1999)
<i>Sciaenops ocellatus</i> , red drum	1.1 Σ SAA ^b		Goff and Gatlin (2004)
		~3.0 Σ SAA	In Akiyama et al. (1997)
		2.6	In Klatt et al. (2016)
<i>Scophthalmus maximus</i> , turbot	~1.3 Σ SAA		Klatt et al. (2016)
	1.6	3.3	Ma et al. (2013)
	1.0 as MHA ^c		Hu et al. (2015)
<i>Sebastes schlegelii</i> , Korean rockfish	1.4 (+0.1 Cys)	2.8	Yan et al. (2007)
<i>Seriola lalandi</i> , yellowtail kingfish	1.0; 1.7 Σ SAA		Jirsa et al. (2014)
	1.0–1.3 ^d		Aguillón et al. (2020)
	1.9 (+0.56 Cys) 2.45 Σ SAA		Candebat et al. (2021)
<i>S. Quinqueradiata</i> , yellowtail		2.4 Σ SAA	In Akiyama et al. (1997)
	1.1 (+0.3 Cys)	2.6	In Klatt et al. (2016)

(continued)

Table 9.1 (continued)

Species	Requirement,% of		References
	Dietary dry matter	Dietary protein	
<i>S. aurata</i> , gilthead bream		4.0 Σ SAA	In Wilson and Halver (1986)
		2.5	Peres and Oliva-Teles (2009)
<i>Sparus macrocephalus</i> , black seabream	1.7 (+3.1 Cys)	4.5	Zhou et al. (2011)
<i>Symphysodon aequifasciata</i> , discus		3.0 ^e	Chong et al. (2004)
<i>Tachysurus fulvidraco</i> , yellow catfish	1.1		Chen et al. (2014)
	1.2 (+0.4 Cys)	2.35	Elmada et al. (2016)
<i>Trachinotus blochii</i> , silver pompano	1.7	~2.8	Ebenezar et al. (2020)
<i>T. marginatus</i> , Plata pompano	1.7–2.3 Σ SAA		Tesser et al. (2014)
<i>T. ovatus</i> , Golden pompano	1.1–1.3	2.5–3.0	Niu et al. (2013)

^a Σ SAA = sum of sulfur-containing amino acids

^bCys2 was able to spare approximately 50% of the dietary Met requirement of red drum

^cMHA methionine hydroxy analog (Fig. 9.1)

^dOn low cholesterol diet (0.23 g kg⁻¹ diet)

^eBased on the content of whole-body tissues of adult fish

and increases deamination, showing a change in AA catabolism in individuals receiving an imbalanced dietary Met supply. Subsequently, Richard et al. (2011) studied Met as C1 donor for methylation reactions and as a precursor of other sulfur-containing compounds, such as Cys or taurine (Tau). Emphasis was put on the activity of two key enzymes of remethylation (betaine-homocysteine methyltransferase, BHMT) and trans-sulfuration (cystathionine β -synthase, CBS). As can be expected, N accretion is lower with the SAA-limiting diets but increases back to control levels by extra choline or Cys2 supplies, demonstrating their sparing effect on Met utilization for protein accretion. Consequently, the authors recommend expressing growth requirements in terms of total SAA rather than Met alone. This is in good compliance with the classical statement by Wilson and Halver (1986) for fishes.

Furthermore, Richard et al. (2011) demonstrated the activity of BHMT and CBS in the hepatopancreas in response to SAA supplementation. Only BHMT, catalyzing the remethylation of L-homocysteine (HCys) into Met, responds to SAA deficiency by reduced activity. This indicates that MR affects remethylation in *P. monodon*, but not trans-sulfuration. Furthermore, the addition of choline and Cys2 to the SAA-limiting diets neither stimulates remethylation by BHMT nor downregulates the CBS trans-sulfuration activity. However, the constant Met and HCys concentrations in the hemolymph, independent of dietary treatments, point out the capability of the shrimps to maintain homeostasis in terms of remethylation/trans-sulfuration.

In the related Atlantic white shrimp (*Litopenaeus setiferus*), Duka and Ahearn (2013) showed that three essential AAs (Leu, Met, and Phe) are transported across

hepatopancreatic brush border membrane vesicles by a single shared carrier. This energy-dependent carrier can drive the uptake of AAs into the vesicles against a transient concentration gradient using either Na^+ or K^+ as co-transport substrates, whereby the identity of the shared carrier protein is presently unclear (Duka and Ahearn (2013)).

9.1.2 Fishes

Espe et al. (2014) communicated an important finding of Met requirement and metabolism in juvenile Atlantic salmon: Met limitation reduces growth and protein accretion, but does not deplete hepatic SAM. Gene expression of SAM decarboxylase and the rate-limiting enzyme of polyamine synthesis ornithine decarboxylase are not affected. Again, this clearly indicates that one, probably the, major function of Met is acting as methyl donor in DNA methylations.

In addition to this function, Met can directly affect the expression of genes involved in its hepatic metabolism, particularly involved in Met re-synthesis and net Met loss, such as Tau synthesis or cystathionine β -synthase, as found in Atlantic salmon liver (Kwasek et al. 2014). Furthermore, transcriptional responses to MR in rainbow trout demonstrate a reduction in the de novo synthesis of FAs and cholesterol, and a potential decrease in hepatic fat oxidative capacity. Muscle adenylate charge is depressed under MR, and increased expression of *AMP-activated protein kinase $\alpha 1$* is detected, indicative of reduced energy availability (Craig and Moon 2013).

Feeding cobia (*Rachycentron canadum*) graded Met diets, including deficient and excess doses, Wang et al. (2016c) identified that the optimum Met dose covers rather a small plateau (Fig. 9.3a): 1.04–1.15% of dry matter. Met deficiency suppresses the transcription of the *insulin-like growth factor-1* (IGF-1) (He et al. 2019). Furthermore, Wang et al. (2016c) confirmed that the lipid metabolism is significantly affected by both Met deficiency and excess. The expression of genes involved in the hepatic lipid synthesis (*srebp-1*, *ppary*, *fas*, *scd-1*) is markedly higher in fish on optimal diet with 1.02% Met (Fig. 9.3a, b), whereas the transcriptional levels of lipolytic genes (*ppara*, *cpt-1*, *lpl*) are elevated in fishes on Met-deficient diet (Fig. 9.3c): The lipid content decreases on non-optimal Met diets.

Recently, Gao et al. (2019) added some more symptoms of Met deficiency to the existing catalog. In turbot, transcription of *S*-metabolizing enzymes (cystathionine β -synthase, SAM decarboxylase) is upregulated, while that of Met synthase is depressed. Furthermore, Met deficiency decreases the height of intestinal villus and microvilli, as well as the number of goblet cells. Transcription of *proliferating cell nuclear antigen* (*pcna*, a cofactor of DNA polymerase delta) and *mucin-2*¹

¹The protein encoded by this gene is secreted onto mucosal surfaces. Mucin 2 is particularly prominent in the gut where it is secreted from goblet cells in the epithelial lining into the lumen of the large intestine. There, mucin 2, along with small amounts of related mucin proteins, polymerizes into a gel (Johansson and Hansson 2016).

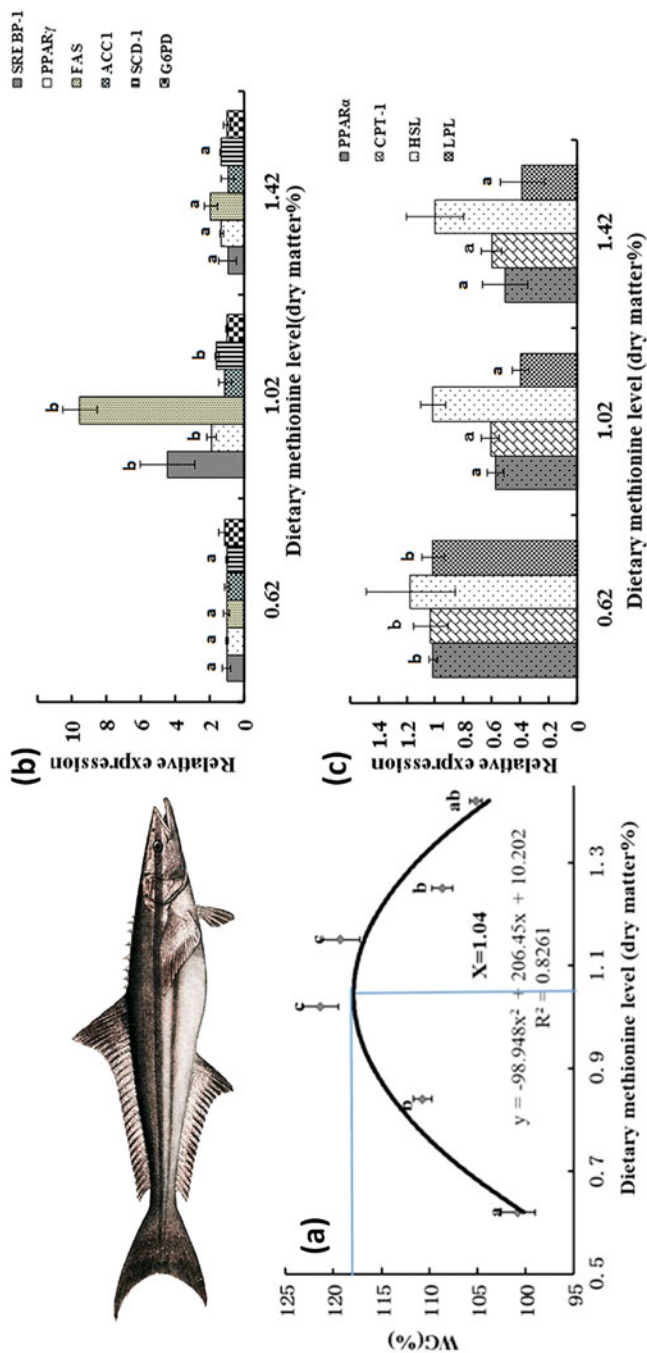


Fig. 9.3 (a) Relationship between dietary methionine level and weight gain of cobia (*Rachycentron canadum*) fed experimental diets for 10 weeks. Response of cobia fed diets containing deficient (0.62%), optimal (1.02%), and excess (1.42%) levels of methionine on: (b) expression of hepatic lipogenesis-related genes: *srebp-1* sterol regulatory element binding protein-1; *ppar γ* peroxisome proliferator activated receptor γ ; *fas* fatty acid synthetase; *acc-1* acetyl-coenzyme A carboxylase-1; *scd-1* stearoyl-CoA desaturase-1; *g6pd* glucose-6-phosphate dehydrogenase; (c) hepatic fatty acid oxidation-related genes: *ppara* peroxisome proliferator activated receptor α ; *cpt-1* carnitine acyl transferase-1; *hsl* hormone sensitive lipase; *lpl* lipoprotein lipase. Values are means \pm SEM; different letters indicate significant differences. (From Wang et al. (2016c), with permission from Elsevier, image credit U.S. National Oceanic and Atmospheric Administration)

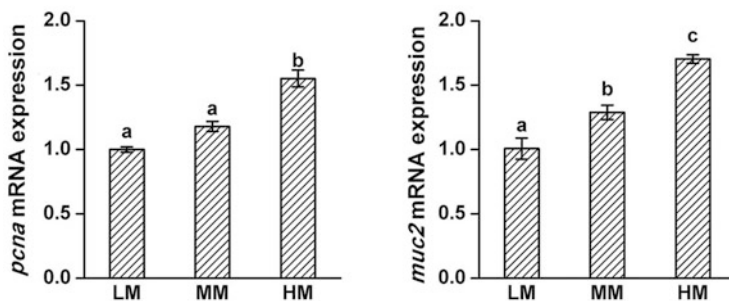


Fig. 9.4 Relative *pcna* and *muc-2* mRNA levels in the intestine of turbot fed with differential methionine levels. *Pcna* and *muc-2* mRNA levels were measured by quantitative real-time PCR (qRT-PCR) and normalized by *rpsd*. The value of LM (Met deficient) group was normalized to 1.0 and the rest of the groups were expressed as relative expression values to the LM group. The data are means \pm SEM ($n = 9$); different letters indicate significant differences ($P < 0.05$). (From Gao et al. (2019), with permission from Elsevier)

(Fig. 9.4), as well as the content of glutathione, are also reduced on Met-deficient diets.

In rainbow trout, MR leads to lower body weight and feed efficiency – not surprising. This growth reduction is accompanied by an increase of autophagy in the white muscles. Similarly, the mRNA levels of several proteasome-related genes are upregulated by MR (Belghit et al. 2014). Subsequently, the same laboratory detected that dietary Met imbalance alters the transcriptional regulation of genes involved in hepatic glucose, lipid, and AA metabolism (Skiba-Cassy et al. 2016). MR activates the GCN2/eIF2 α pathway, part of the general AA control pathway, when one or more AAs become growth limiting (Zhang et al. 2002). In contrast, dietary Met excess produces broader changes in hepatic gene expression by increasing the levels of transcripts related to FA synthesis and oxidation, gluconeogenesis, and AA catabolism.

Feeding five plant-based diets with increasing contents of crystalline Met to rainbow trouts, Rolland et al. (2015) showed that Met availability affects the expression of genes involved in the GH–IGF axis response and protein turnover. The transcript levels of *growth hormone receptor 1* and *insulin-like growth hormone binding protein-1* rise linearly with the increase of dietary Met content, indicating growth. The apparent capacity for hepatic protein degradation decreases with increasing dietary Met levels: protein is allocated to growth.

Espe et al. (2016) extended this list of effects by studying Atlantic salmon. Individuals fed low Met diet gain reduced protein, but growth is not reduced. Deficient fishes maintain the concentrations of hepatic sulfur AAs and reduce free Met in muscles. Correspondingly, methylation capacity is maintained in the liver, but reduced in muscles. Gene transcription of muscle *igf-1* and synthesis of protein in the white trunk muscle are reduced. Thus, the dietary protein needs to be balanced in AAs to support metabolism in all compartments of the body and secure maximal protein gain.

Further details of the essentiality of Met come from a study in European seabass. Machado et al. (2020) found that dietary Met supplementation improves the immune status following long-term feeding on fishmeal (FM)-free diets. Moreover, the authors point out that the requirement of Met needed for immune support is higher in individuals on plant protein-based diet than on FM-based diet.

9.1.2.1 Transgenerational Effect

Recently, Fontagné-Dicharry et al. (2017) and Seiliez et al. (2017) showed that the dietary Met content of the broodstock influences the metabolism of the progeny in rainbow trouts. Lowering the Met levels in broodstock diets below the requirement for rainbow trout (NRC 2011) alters the levels of Met and Cys as well as SAM and SAH in the produced eggs (Fontagné-Dicharry et al. 2017). Furthermore, the size of the eggs as well as survival of the resulting swim-up fry is reduced. This study also reveals that parental Met nutritional history affects the growth response of fry subjected to different dietary Met levels (Fig. 9.5). Lowest offspring growth is observed from Met-deficient broodstock (BD, Fig. 9.5). Best survival from the swim-up stage onward is noticed in offspring from Met-excess broodstock (BE), whereas best growth is recorded in offspring from Met-adequate broodstock (BA). Fry growth, but not survival, is affected by Met levels of the fry diet, being the lowest in fry fed the lowest dietary Met level (Met-deficient fry, FD). Fry fed Met-excess diet (FE) exhibits the highest growth (Fig. 9.5). Furthermore, dietary Met affects the expression of certain genes involved in Met metabolism, the control of feed intake and of muscle growth in offspring.

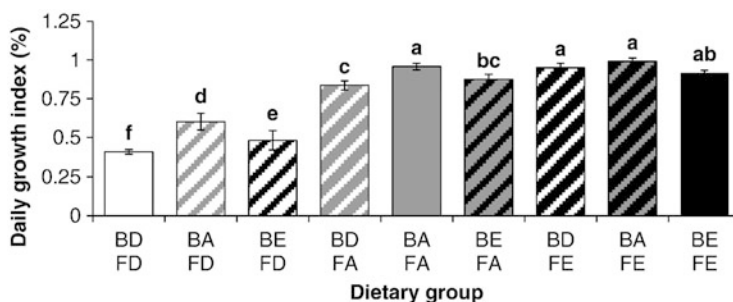


Fig. 9.5 Interacting effects of rainbow trout broodstock and fry nutrition on daily growth of offspring fed different Met levels at a constant water temperature of 17 ± 1 °C for 3 weeks. Columns represent means \pm SD. Means not sharing a common superscript letter are significantly different ($P < 0.05$). (From Fontagné-Dicharry et al. (2017), with permission from Elsevier). *BD* Met-deficient broodstock, *BA* Met-adequate broodstock, *BE* Met-excess broodstock, *FD* Met-deficient fry, *FA* Met-adequate fry, *FE* Met-excess fry

Veron et al. (2018) demonstrated that Met can be of critical importance in metabolic programming of rainbow trout offspring. This stimulus affects DNA methylation of specific *loci*, among them, an interesting CpG site in the gene *bnip3a* has been identified and may be positively related with mRNA levels. (→AAN III “Nutritional Programming”).

Also Seiliez et al. (2017) described the possibility of nutritional programming in rainbow trouts by means of parental nutrition: gluconeogenesis and autophagy in the offspring are only affected by parental Met-deficient diets. In studies to come, it is interesting to figure out whether these effects are accompanied by epigenetic phenomena, such as DNA and histone methylation at the promotor or silencer of the corresponding genes. Another important issue will be to determine the persistence of the observed broodstock effects on offspring metabolism.

9.2 Methionine Hydroxy Analog

Due to substitution of the amino by a hydroxyl group, Met hydroxyl analog (MHA, Fig. 9.1) is not a real AA, but widely used as dietary Met substitute because of its lower price (Robinson et al. 1978). MHA is slightly more efficient than L-Met in promoting growth (Goff and Gatlin 2004; Savolainen and Gatlin 2010). Juvenile turbot and Jian carps fed MHA also show elevated serum ascorbic acid concentrations pointing out improved anti-oxidative activity and immune function (Xiao et al. 2012; Ma et al. 2013). In young grass carps, dietary MHA improves the innate immunity and enhances the capacity against enteritis emergence. Furthermore, it increases the inflammation inhibition in the intestine via regulating cytokine expression, the repression of oxidative damage and apoptosis in the intestine, and the upregulation of tight junction proteins genes in the intestine (Pan et al. 2016, 2017).

In a meta-analysis, Guo et al. (2020a) evaluated the effect of MHA supplementation on final weight (FW), weight gain (WG), protein efficiency ratio (PER), feed conversion ratio (FCR), and feed efficiency (FE) in diverse common aquaculture taxa. Twenty-three published studies are included that accounted for 249 effect sizes estimated across eight fish (*Cyprinus carpio*, *Ictalurus punctatus*, *Sciaenops ocellatus*, *Lateolabrax japonicus*, *Oncorhynchus mykiss*, *Oreochromis niloticus* (GIFT), *Larimichthys crocea*, *Scophthalmus maximus*) and one shrimp (*Litopenaeus vannamei*) species. Adding MHA to the diet can improve FW, WG, PER, and FE, and decrease FCR for fishes, but not for the shrimp. Obviously, the shrimp has more difficulties utilizing the applied crystalline AA additive than fishes. Moreover, a classical study in channel catfish, not included in the meta-analysis, revealed a contrasting result: MHA is only about 26% as effective in promoting growth as L-Met (Robinson et al. 1978).

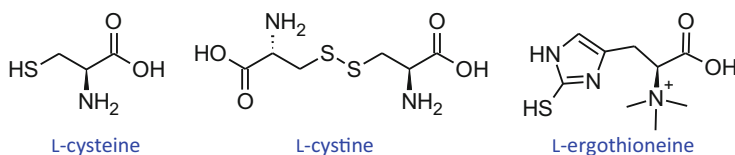
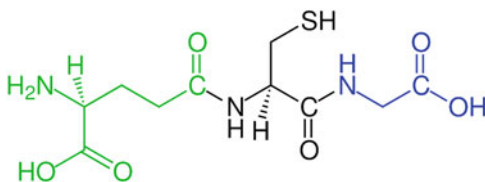


Fig. 9.6 L-Cysteine and L-cystine (L-Cys2), the oxidized dimer of cysteine, and L-ergothioneine

Fig. 9.7 Tripeptide glutathione: Glu (green), Cys (black), and Gly (blue) (credit Jü, Wikimedia)



9.3 Cysteine

Cysteine (Cys, Fig. 9.6) is a constituent of glutathione (GSH, Fig. 9.7) and can be converted into Tau. Cys is important in disulfide linkages (Molina-Poveda 2016) and can be converted from Met via Hcy and Ser (Toennies and Callan 1939). Due to its redox sensitivity, cysteine has antioxidant properties as best expressed in the tripeptide GSH.

Although not methyl donor itself, Cys takes part in the folic acid metabolic pathway resulting in DNA methylation (Vanhees et al. 2014) (\rightarrow AAN I “Transgenerational Effects,” Steinberg (2018)). When Cys or Tau is deficient in the diet, their synthesis from Met will increase in vivo, thus decreasing total SAM availability for DNA or protein methylation. Therefore, AA deficiency can alter the epigenetic code through changes in DNA methylation or histone modifications (Wang et al. 2012).

Due to mutual sparing effects of total SAA, growth requirements should be expressed in terms of total SAA rather than Met or Cys alone (see above). This statement receives support by a previous study in red drum (Goff and Gatlin 2004), indicating that this fish is able to use various SAA compounds equally as effective as Met to meet its SAA requirement. Cys2 also is able to spare approximately 50% of the dietary Met required in red drum. SAA requirements are presented in Table 9.1.

9.4 Cystine

Cystine (Cys2, Fig. 9.6) is the oxidized dimer of cysteine and a non-essential AA. If deficient, Cys2 is synthesized from Met; and vice versa, Cys2 has the potential of sparing Met (Abidi and Khan 2011). Cys2 can replace approximately 60% of Met in

channel catfish, 50% in Chinook salmon, 40–50% in red drum, 49% in Nile tilapia, 44% in blue tilapia, 42% in rainbow trout, 40% in hybrid striped bass, rainbow trout, yellow perch, and Indian major carp (Zehra and Khan 2016), and 33–39% in rohu (Abidi and Khan 2011).

The only available study on Met sparing in invertebrates was carried in juvenile *P. monodon* and indicates that about 50% of the Met requirement can be spared by cyst(e)ine (Richard et al. 2011).

9.5 Ergothioneine

Ergothioneine (Fig. 9.6) is a naturally occurring AA and a thiourea derivative of histidine. It can be found in *Actinobacteria*, *Cyanobacteria*, and certain fungi. Animals have to take up ergothioneine via feed. It has high antioxidant properties, higher than, e.g., glutathione or histidine (Nguyen et al. 2013). The antioxidative property protects from lipid peroxidation (LPO) in fishes, crabs, and shrimps (Bao et al. 2009; Encarnacion et al. 2010). It inhibits *prophenoloxidase* transcription in shrimps, thereby avoiding melanosis, but increasing risk of infection, since a major innate defense system in invertebrates comprises melanization of pathogens and damaged tissues via phenoloxidase (Cerenius and Söderhäll 2004).

9.6 Glutathione

The tripeptide glutathione (GSH) is composed of γ -glutamic acid, Cys, and Gly (Fig. 9.7). GSH is a common antioxidant in plants, animals, fungi, some bacteria, and archaea (Pompella et al. 2003). It increases the antioxidant capacity (AOC) and is a major redox buffer.

The few available dietary studies in aquatic animals are summarized in Table 9.2 and show that some more beneficial effects than only antioxidant functions can be identified. Liu et al. (2019) found that also genes encoding anti-lipopolysaccharide factors or antimicrobial peptides are transcribed by GSH action (Table 9.2, Fig. 9.8). This indicates that even the innate immunity increases after feeding GSH-supplemented diets. Nevertheless, the optimal dietary dose has to be figured out for each individual species, since Han et al. (2020) reported that beneficial and adverse doses are in close proximity. *L. vannamei* supplemented with 75 mg kg⁻¹ GSH completely eliminates ROS overproduction and reduces LPO. Moreover, it increases bacterial diversity and the relative abundance of beneficial bacteria community such as *Rhodobacteraceae* preventing pathogen (e.g., *Vibrio*) invasion in the intestine under specific hypoxia conditions and enhancing survival and growth. However, doubling the dose leads to serious impairment of survival and growth.

Table 9.2 Effect studies of dietary glutathione in aquatic animals

Species	Dietary level g kg ⁻¹	Affected trait	References
<i>Eriocheir sinensis</i> , Chinese mitten crab	0→1.2	@ 0.6–0.9: Immunity↑, <i>alfs</i> ↑, <i>crus</i> ↑ (Fig. 9.8), AOC↑, apoptosis↓	Liu et al. (2019, 2020a, 2020b)
<i>Ictalurus punctatus</i> , channel catfish	0, 0.1, 1.0	↔	Gatlin and Bai (1993)
<i>Ctenopharyngodon idella</i> , grass carp	0.4	WG↑, beneficial microbial community↑	Yuan et al. (2015)
	0.01→0.8	@ 0.4: WG↑, FCR↓, mortality↓, <i>igf-I</i> ↑; innate immunity↑, AOC↑	Ming et al. (2015, 2019)
<i>Litopenaeus vannamei</i> , Pacific white shrimp	0→0.3	AOC↑, innate immunity↑, ammonia stress resistance↑	Xu et al. (2012)
	0→0.2 @ reduced FM	AOC↑, SGR↓, mortality(↓)	Wang et al. (2018)
	0.1→0.3	WG↑, innate immunity↑	Xia and Wu (2018)
	0, 0.08, 0.15	@ 0.08: ROS↓, LPO↓, intestinal microbial diversity↑	Han et al. (2020)
<i>Salmo salar</i> , Atlantic salmon	0→0.4	WG↑, FCR↓, AOC(↑)	Ma et al. (2019)

↑ enhancement; ↔ no apparent effect; ↓ decrease/reduction; *alf* anti-lipopolysaccharide factor, *crus* crustin (important antimicrobial peptides in crustaceans (Vargas-Albores et al. 2004)), AOC anti-oxidant capacity, FCR food conversion rate, SGR specific growth rate, WG weight gain, FM fishmeal, ROS reactive oxygen species, LPO lipid peroxidation

9.7 Concluding Remarks

Several times, this chapter pointed out gaps in the knowledge about SAAs and their metabolic as well as controlling functions; therefore a few concluding remarks can be kept briefly.

Relatively unsolved is the issue of dietary AA imbalance as well as excess (Benevenga and Steele 1984) and toxicity of individual sulfur AAs (Olney et al. 1990). These authors identified a neurodegenerative capacity of L-Cys.

From Figs. 9.2, 9.3, and 9.8 it becomes obvious that beneficial and adverse dietary doses are in close proximity; excess doses are easily reached and adverse results are to be anticipated. To demonstrate this gap of knowledge, the reader is referred to Dilger et al. (2007) and Dilger and Baker (2008), who found that excess dietary Cys, but not Cys2, is lethal for chicks, but not for rats or pigs. In contrast, in intestinal porcine epithelial cells, excessive Cys induces vacuole-like cell death by activating endoplasmic reticulum stress (→Chap. 23) and mitogen-activated protein kinase signaling (Ji et al. 2016). And in fishes? Only one very brief paper is available studying this toxicity in fishes. In rainbow trouts, the injection of Cys, rather than

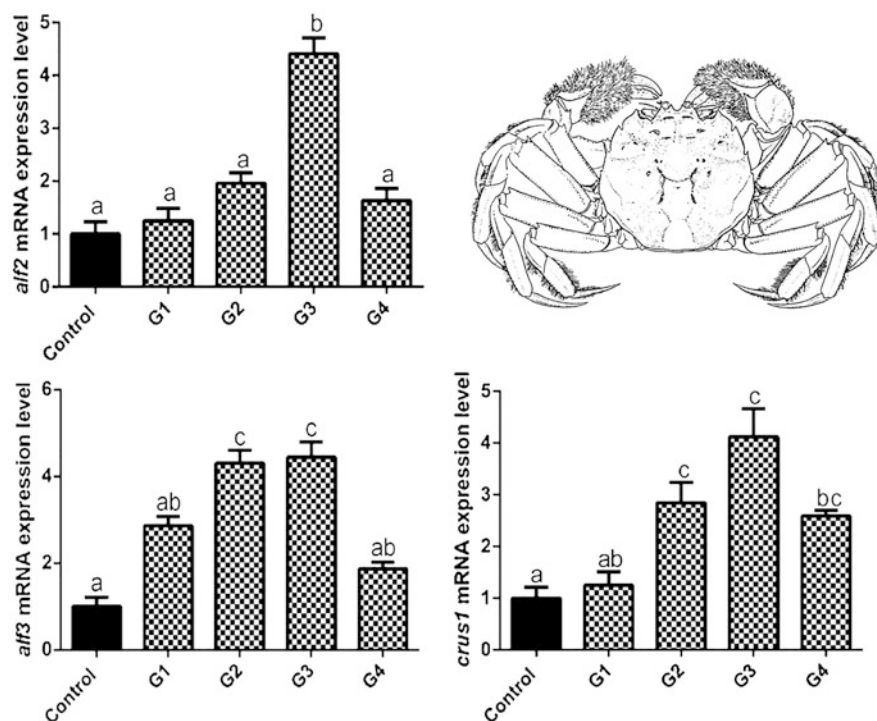


Fig. 9.8 Relative expression of immune-related genes in the hepatopancreases of *Eriocheir sinensis* fed different concentrations of dietary glutathione (GSH): *anti-lipopolysaccharide factor* here 2 and 3 (*alf2*, *alf3*) and *crustin 1* (*crus1*, an antimicrobial peptide). Each datum (mean \pm SEM) is the mean of four replicates. Different superscripts indicate significant differences ($P < 0.05$). Control 0 mg kg⁻¹ GSH; G1 300; G2 600; G3 900; G4 1200 mg kg⁻¹ GSH. (From Liu et al. (2019), credit Elsevier; image credit FAO)

dietary administration, resulted in markedly swollen epithelia of secondary lamellae of the gills (Yokoyama and Sakaguchi 1996).

Met restriction (MR) and the underlying mechanism are poorly understood in fishes and even less in aquatic invertebrates. Some reports on miR-mediated mechanisms in fishes seem to contradict those in mammals. Does MR lead to life span extension also in aquatic animals? If so, can it be passed to succeeding generations? Can the MR-mediated increased glucose tolerance be used to increase the share of plant-derived feed in carnivorous farmed animals? Again, if so, can this increased tolerance be passed to succeeding generations?

Last point, but not less important: What about the role of intestinal microbiota? This symbiotic metabolic pathway has received only sparse attention; however, the GSH diet in crustaceans allows the expectation of promising results in fishes.

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Chapter 10

Basic Amino Acids and Prolines—‘Again: Much More than Easy Fuel’



Abstract Crucial as building blocks and immunostimulants, basic amino acids and prolines are limited nutrients in aquafeeds. Excess of basic AAs leads often to diminished growth and impaired immunity, whereby the underlying mechanism remains obscure. Arg can elicit immune-associated gene expression and improve humoral and cellular immunity. In the cladoceran *Daphnia* spp., His and Arg contribute to the switch between asexual and sexual reproduction and trigger longevity. Arg can stimulate the release of various hormones (insulin, growth hormone, glucagon) and is a more potent activator of insulin release in fishes than glucose. Dietary Arg deficiency impairs intestinal immune function and optimum supplementation can restore these changes. His participates in the one-carbon metabolism. Pro is considered a conditionally essential AA, whereas Hyp is a common nonproteinogenic AA and a major component of collagen. In euryhaline fishes, Pro and Hyp function as osmolytes. Moreover, Hyp appears as a limiting factor when high levels of plant protein are used in the diets. Studies of dietary Pro and Hyp in invertebrates are sparse and reveal mixed results. It is beginning to be understood that basic AAs have a controlling function in metabolism and immune response in fishes. These aspects deserve future attention.

Among the essential amino acids (AAs), basic AAs as well as prolines stand out by their structural features and have gained attention as limited nutrients in aquafeeds, since, besides serving as a building block for proteins, they play central roles in the functioning of the immune system (Zhao et al. 2015).

10.1 Basic Amino Acids

Basic AAs comprise Lys, Arg, His, and derivatives (Fig. 10.1). Their side chains contain nitrogen and resemble ammonia. Their dissociation constant is low enough that they tend to bind protons, gaining a positive charge. Recently, Yaghoubi et al. (2017) reconfirmed the essentiality of Arg and Lys by showing that silvery-black porgy (*Sparidentex hasta*) juveniles fed Arg- and Lys-deficient diets have a

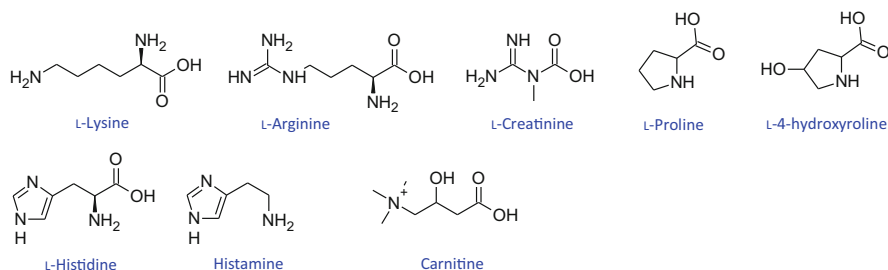


Fig. 10.1 Structures of L-lysine, L-arginine, L-creatine, L-proline, L-4-hydroxyproline, L-histidine, histamine, and carnitine

compromised innate immune system; they have lowered plasma complement components 3 and 4, lysozyme, total immunoglobulin contents, and total superoxide dismutase activity.

10.1.1 Lysine

Lysine (Lys, Fig. 10.1) is an essential proteinaceous α -AA (Wu 2013) and plays a major role in calcium absorption, building muscle protein, recovery from injuries, and production of hormones, enzymes, and antibodies. Furthermore, it acts as precursor of carnitine (Car, Fig. 10.1), which is required for the transport of long-chain FAs into mitochondria (\rightarrow Chap. 12) (Molina-Poveda 2016).

Lys is often one of the most limiting AA in ingredients for commercial fish feeds, especially when fishmeal (FM) is replaced by plant proteins (Mai et al. 2006) (\rightarrow AAN III Chapters on Fishmeal Replacement); dietary Lys levels critically affect growth and health. Dietary Lys supplementation also enhances the immune responses and gastrointestinal development of agastric fishes, such as cyprinids (Li et al. 2009).

Interestingly, free Lys is utilized for growth less efficiently than protein-bound Lys, as shown in young channel catfish (Zarate and Lovell 1997). One reason appears to be that free Lys passes out of the stomach faster than protein-bound Lys (Zarate et al. 1999). Furthermore, free Lys supports lipid deposition more effectively than bound Lys (Zarate and Lovell 1997).

Lys limitation does not deplete the muscle Car depot during the on-growing seawater phase of Atlantic salmon, but affects the deposition pattern of nutrients; it reduces hepatic Car (Rathore et al. 2010). Thus, low-Lys diets do not likely affect the fatty acid (FA) oxidation capacity. This fact is supported by unaffected FA profiles and lipid classes between treatments during the 3-month feeding study.

Applying whole-body proteomics, Gómez-Requeni et al. (2011) identified effects of dietary Lys imbalance in *Danio rerio*. Fishes on Lys-deficient diets show that a decrease for proteins is involved in the growth decrease and even apoptosis.

Conversely, the higher growth rates exhibited by fishes on Lys excess are accompanied by an increase of glycolysis-related proteins and additional proteins preceding steroidogenesis and sexual maturation. Surprisingly, Lys excess seems to have no adverse effect in zebrafish – contrary to several other AAs, if in excess, or other aquatic animals. For instance, *Penaeus monodon* decreases growth, if the Lys content exceeds ~2% of diet (Millamena et al. 1998).

Some dietary Lys requirement data are collected in Table 10.1. Based on different response parameters, the data vary between 10 and some 29 g Lys kg⁻¹ diet. The NRC (2011) lists values between 16 (channel catfish, tilapia, hybrid striped bass) and 24 g kg⁻¹ diet (salmonids). Often, the authors did not distinguish between minimal and optimal dietary requirement. Furthermore, parallel studies with supposedly identical species reveal differences of up to 100% (e.g., grass carp, red drum) indicating not only that the experimental approaches were not identical but also that the tested animals differed in genetic, developmental, metabolic, or biomolecular properties. Lee et al. (2020) showed differences in response in two rainbow trout strains. Optimum dietary Lys levels for a fast-growing strain and a slowly growing strain of rainbow trout are 2.8% and 2.2% of diet, respectively. More examples of intraspecific variability will follow in Chap. 40.

Hauler and Carter (2001) reported a Lys requirement of rainbow trout between 16 and 21 g Lys kg⁻¹ based on the model of constant Lys requirement during ontogenetic development. Also methodologically traditional, Campelo et al. (2018) identified 19 g kg⁻¹ in adult lambari (*Astyanax altiparanae*) and Madrid et al. (2019) the same value in juvenile *Totoaba macdonaldi* (Table 10.1), thus confirming the figures for rainbow trout and assuming a constant requirement during development. This issue will be revisited in the conclusions below.

Excess dietary Lys can decrease growth as found in Japanese seabass (Mai et al. 2006), *Cirrhinus mrigala* (Ahmed and Khan 2004b), *Micropterus salmoides* (Dairiki et al. 2007), *Salminus brasiliensis* (Dairiki et al. 2013), *Rachycentron canadum* (Zhou et al. 2007), *Piaractus mesopotamicus* (Bicudo et al. 2009), and *Polydactylus sexfilis* (Deng et al. 2010). Toxicity of excess Lys is assumed. However, so far no study has clearly elucidated the mechanism of Lys “toxicity” in fishes. Previous studies in rainbow trout (Kaushik and Fauconneau 1984) and Atlantic salmon (Berge et al. 1997, 1998) hypothesized a Lys-Arg antagonism, which was documented in livestock before (Jones 1964). Further studies are needed to obtain a better understanding of adverse interaction of AAs in aquatic animals on dietary AA excess.

Interesting novel aspects of dietary Lys have very recently been discovered, namely, controlling biomolecular functions. Appropriate dietary Lys levels (2.44%) elicit glycolysis by regulating *glucokinase* and *pyruvate kinase* mRNA levels without affecting gluconeogenesis in grass carp fry. In addition, the appropriate Lys level promotes lipolysis by regulating the PPAR- α ¹ signaling pathway

¹The peroxisome proliferator-activated receptor (PPAR) signaling pathway is vital in promoting adipocyte differentiation and maintaining lipid metabolism balance (Blaschke et al. 2006). PPAR- α and PPAR- γ are two important transcription factors in the PPAR signaling pathway that regulate lipolysis, lipid synthesis, and lipid metabolism (Hashimoto et al. 2000).

Table 10.1 Dietary lysine requirement in selected fish species

Species	Dietary level, g kg ⁻¹ diet	Response parameter	References
<i>Astyanax altiparanae</i>	18.7	WG	Campelo et al. (2018)
<i>Bidyanus bidyanus</i>	20.6		Yang et al. (2011)
<i>Cirrhinus mrigala</i>	23.0		Ahmed and Khan (2004b)
<i>Ctenopharyngodon idella</i>	~10	Lipid peroxidation↓	Li et al. (2014)
	20.7	WG, FER	Wang et al. (2005)
<i>C. idella</i> fry	24.4	WG	Huang et al. (2021a)
<i>Cyprinus carpio</i>	14.5		D'Mello et al. (1989)
	18.9		Signor et al. (2017)
<i>Cyprinus carpio</i> var. Jian	23.6		Zhou et al. (2008)
<i>Dicentrarchus labrax</i>	22.2	PG	Tibaldi and Lanari (1991)
<i>Gibelion catla</i>	17–18	WG, PG, LysG	Zehra and Khan (2013b)
<i>Heteropneustes fossilis</i>	13–14	WG, PG	Khan and Abidi (2011b)
	20–23	WG, PG, FCR, LysG	Farhat and Khan (2013a)
<i>Labeo rohita</i>	22.4	WG, SGR, FER	Murthy and Varghese (1997)
<i>Larimichthys crocea</i>	~33.5	WG, survival	Xie et al. (2012)
<i>Lateolabrax japonicus</i>	~25.0	SGR, FER, PER	Mai et al. (2006)
<i>Morone saxatilis</i>	21	WG, SGR, FCR	Small and Soares (2000)
<i>Oncorhynchus keta</i>	19	WG	Akiyama et al. (1985)
<i>Oncorhynchus mykiss</i>	16–21		Hauler and Carter (2001)
	13–23		Van Larebeke et al. (2018)
<i>O. mykiss</i> FGS	28		Lee et al. (2020)
SGS	22		
<i>Oreochromis niloticus</i>	14.6	Fillet yield	Michelato et al. (2016)
<i>Pagrus major</i>	14.4–17.6	SGR, FER	Forster and Ogata (1998)
<i>Paralichthys olivaceus</i>	13.2–18.4		
<i>Piaractus mesopotamicus</i>	14–15	WG, FER	Bicudo et al. (2009)
<i>Polydactylus sexfilis</i>	~23	SGR, FER, PER, PG	Deng et al. (2010)
<i>Rachycentron canadum</i>	23.3	SGR	Zhou et al. (2007)
<i>Salmo salar</i> , medium-sized	16–18	WG, SGR, FCR, PER	Berge et al. (1998)
<i>S. salar</i> , smolt	28.8	WG	Grisdale-Helland et al. (2011)
<i>Sciaenops ocellatus</i>	~23	WG, FER	Brown et al. (1988)
	15.5		Craig and Gatlin (1992)

(continued)

Table 10.1 (continued)

Species	Dietary level, g kg ⁻¹ diet	Response parameter	References
<i>Totoaba macdonaldi</i>	19.3	WG	Madrid et al. (2019)
<i>Trachinotus blochii</i>	~24	WG, SGR, PER, FER	Ebenezzar et al. (2019)

SGR specific growth rate, FCR feed conversion ratio, FER feed efficiency ratio, PER protein efficiency ratio, PG protein gain, WG weight gain, ↓ reduction, FGS fast growing strain, SGS slowly growing strain, LysG lysine gain

resulting in decreased whole-body lipid content; the PPAR- γ pathway is not remarkably influenced by dietary Lys levels (Huang et al. 2021a). Even more intriguing, Huang et al. (2021b) showed in a companion paper that optimal dietary Lys improves immunity and antioxidant status via TOR, p38 MAPK, Nrf2, and NF- κ B pathways. Noteworthy, as one of the mitogen-activated protein kinase (MAPK) family, p38 mitogen-activated protein kinase (p38 MAPK) participates in the regulation of cellular processes, including inflammation and apoptosis.

10.1.2 Arginine

Arginine (Arg, Fig. 10.1) is an essential proteinogenic α -AA and has great potential as free AA (Wu 2013). It is involved in numerous metabolic processes including protein deposition, synthesis of ornithine (precursor of polyamines, →Chap. 12), immune responses (via nitric oxide production), and removal of nitrogenous waste as urea (Fig. 10.2). Arg also stimulates the release of growth-promoting hormones such as insulin, glucagon, and growth hormone in fishes. In rainbow trout, there is generally a high Arg demand (1.5–2% of the diet), reflecting the lack of a de novo synthesis due to an inefficient urea cycle (Clark et al. 2020).

Arg is central in the innate immune response. Han et al. (2018) reported that Arg elicits immune-associated gene expression and improves humoral and cellular immunity. In *Epinephelus coioides*, different tissues have different responses to dietary Arg: the intestinal gene expression is presented in Fig. 10.3. In the same line of evidence, Wu et al. (2018) showed in hybrid grouper that graded dietary Arg leads to transient upregulation of pro-inflammatory genes. This is the early phase of anti-inflammatory response in order to get the anti-inflammatory machinery started.

Pointing out the later phase of immune responses, Liang et al. (2018) showed that the optimal dietary Arg level lowers pro-inflammatory gene mRNA levels and increases anti-inflammatory gene mRNA levels in juvenile Wuchang breams. Furthermore, optimal Arg diet (~3.6% dry matter) increases the transcription of growth factor genes, such as *igf-1*, *tor*, and *s6k1*, and suppresses the transcription of *4e-bp2* in hybrid grouper juveniles. 4E-BP2 (eukaryotic translation initiation factor 4E-binding protein 2) is a translation repressor protein and agonist of growth factors. We shall revisit this issue.

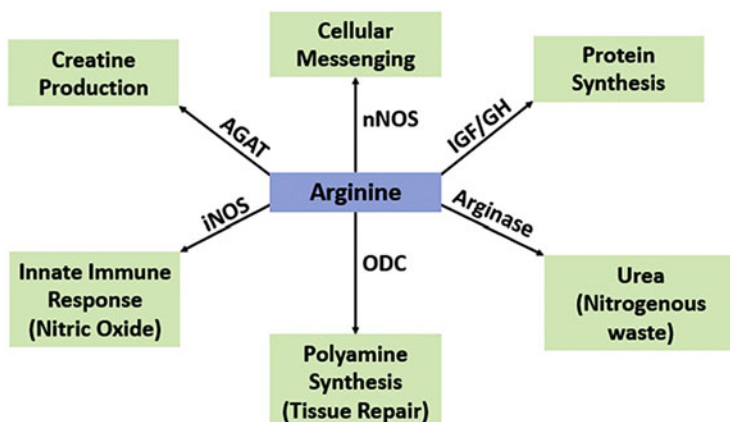


Fig. 10.2 Arginine's metabolic pathways and associated enzymes: *nNOS* neural nitric oxide synthase, *IGF/GH* insulin like growth factor/growth hormone, *ODC* ornithine decarboxylase, *iNOS* inducible nitric oxide synthase, *AGAT* arginine:glycine amidinotransferase. (From Clark et al. (2020), with permission from Elsevier)

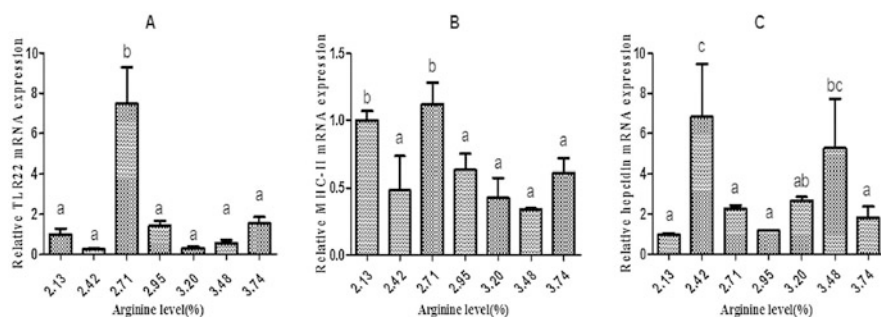


Fig. 10.3 The *toll-like receptor tlr22* (a), *major histocompatibility complex-II mhc-II* (b), and *hepcidin* (c) mRNA expression in the intestines of juvenile orange-spotted grouper (*Epinephelus coioides*) fed diets containing graded arginine levels for 8 weeks. Data are means \pm SD ($n = 3$). Columns of the same gene bearing the same letters are not significantly different (Tukey's test, $P > 0.05$). (From Han et al. (2018), credit Elsevier)

To sum up the immunity issue, Huang et al. (2020) proposed a pathway of how AAs can enhance the immunity in aquatic animals with Arg being central (Fig. 10.4).

10.1.2.1 Invertebrates

The planktonic crustacean genus *Daphnia* is extremely successful in freshwater ecosystems—at least partly due to their cyclical parthenogenetic life cycle, in which asexual and sexual reproduction alternates periodically. This temporal change

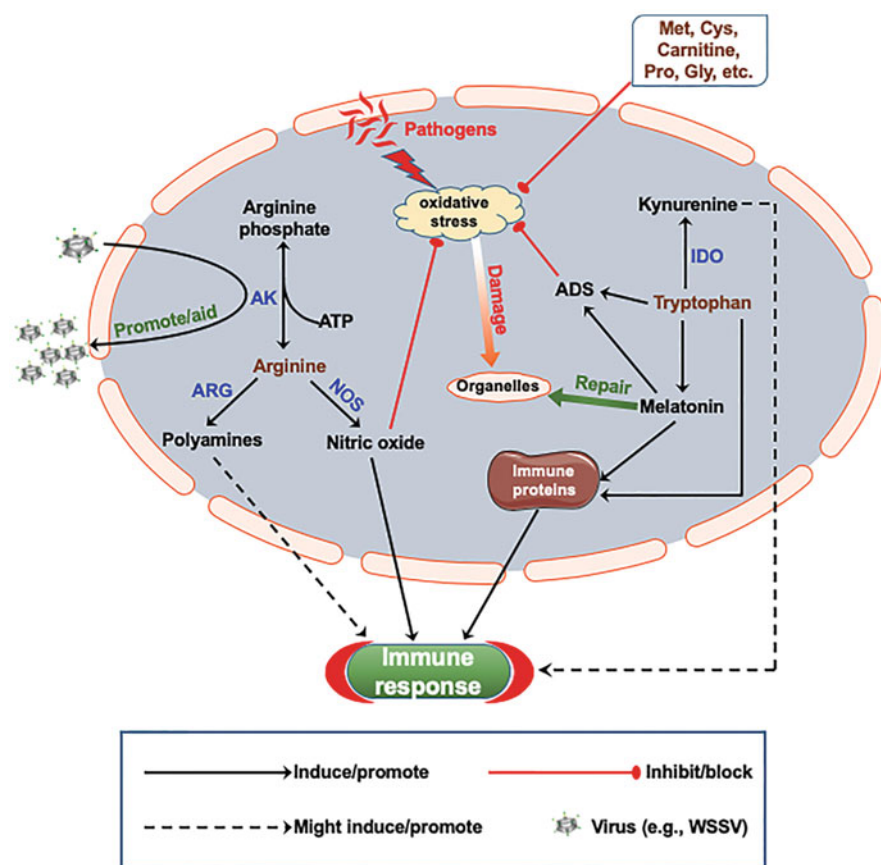


Fig. 10.4 Proposed schematic representation of how amino acid metabolism and immune modulation occur in crustaceans. Nitric oxide ($\bullet\text{NO}$) is generated from arginine (Arg) by the action of nitric oxide synthase (NOS), which counteracts pathogen-induced oxidative stress and promotes immune response. Polyamines, as downstream products of Arg metabolism, promote immune response while arginine kinase (AK) can catalyze the conversion of Arg to arginine phosphate, which can be coopted to promote replication of white spot syndrome virus (WSSV). Tryptophan (Trp) and its metabolite melatonin can activate the antioxidant system (ADS) and promote the expression of immune proteins as well as counteract oxidative stress. Melatonin also promotes the repair of oxidative stress-induced organelle damage. Trp could also suppress inflammatory response through the kynurenine pathway. Some amino acids such as Met, Cys, Pro, Gly, etc., develop direct antioxidant activity due to their chemical properties. *IDO* Indoleamine-2,3-dioxygenase, *ARG* arginase, *Met* methionine, *Cys* cysteine, *Pro* proline, *Gly* glycine. (From Huang et al. (2020), credit Frontiers Media)

between reproductive strategies allows for (1) rapid population growth via subitaneously developing eggs when environmental conditions are appropriate and stable and (2) the maintenance of genetic diversity via sexual reproduction and the production of resting eggs when environmental conditions deteriorate.

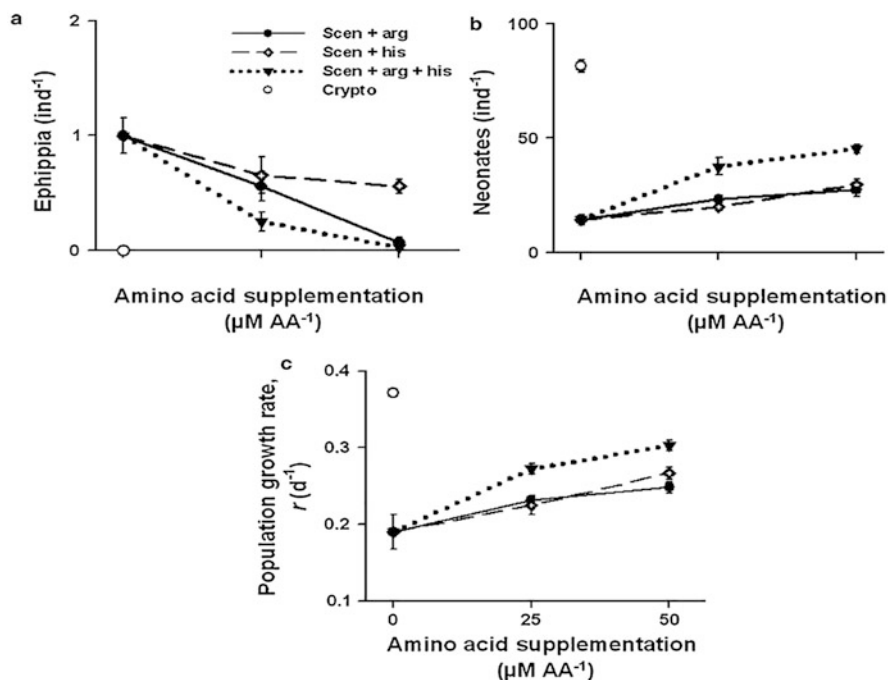


Fig. 10.5 Cumulative numbers (means \pm SE) of ephippia (a), neonate production produced per individual (b) and population growth rates (r) (c) within 16 days of *Daphnia pulex* fed *Cryptomonas* sp. (Crypto), or *Scenedesmus obliquus* (Scen) supplemented with 1, 25, and 50 $\mu\text{M AA}^{-1}$, respectively, of arginine (Arg) and/or histidine (His) separately or combined. (From Koch et al. (2011), with permission from Springer Nature)

Dietary AAs are involved in triggering the switch between the reproductive modes in *Daphnia pulex* (Koch et al. 2011). Supplementation with graded Arg and His supply (either separately or in concert) averts crowding-induced resting egg (ephippia) production and accordingly increases neonate production and thereby population growth rate (Fig. 10.5).

In a series of feeding experiments, Bouchnak and Steinberg (2013, 2014) evaluated how feed stoichiometry triggers life history traits of clones of two cladocerans without and with additional environmental stress. Fed low phosphorus algae, Arg and His trigger longevity in *D. magna*; reproduction appears to be triggered by C:P and N:P ratios. Exposure to dissolved humic substances, natural xenobiotics, and a major matrix of any ecosystem (Steinberg 2003) reduces offspring numbers. Under these conditions, the total AA content seems to trigger reproduction (Bouchnak and Steinberg 2013). Conversely in the second cladoceran, *Moina macrocopa*, α -linolenic acid (ALA) controls life span modulation even under stress by humic substances, whereas modulation of offspring numbers appears to be controlled by C and total AA content (Bouchnak and Steinberg 2014). This indicates that, upon

exposure to humic substances, the investment of energy in body maintenance or reproduction depends, at least partly, on the biochemical composition of the feed available. Environmental stress by dissolved humic substances is a realistic scenario, since the concentration of this biogeochemical matrix will increase due to global warming (Shurin et al. 2010).

10.1.2.2 Fishes

Fishes have high requirements for dietary Arg. Elasmobranchs and ureogenic teleosts may be able to convert citrulline (Cit, →Chap. 12) into Arg via hepatic argininosuccinate synthase and lyase. However, it is unknown whether there is a net synthesis of Cit or Arg by this organ. There is circumstantial evidence for an Arg sparing effect of dietary Glu in channel catfish, but this effect may result from an inhibition of Arg degradation, rather than de novo synthesis. At present, pathways for Arg synthesis from Gln, Glu, Pro, or AAs other than Cit have not been established for fishes (Li et al. 2009).

As depicted in Figs. 10.2 and 10.4, Arg is one of the most versatile AAs. In addition, Arg stimulates the release of various hormones such as insulin, growth hormone, and glucagon. Arg is a more potent activator of insulin release in fishes than glucose itself. Furthermore, Arg radicals are central for gonadotropin secondary structure and function (as shown in *Acipenser gueldenstaedtii*, Zenkevičs et al. (2010)).

Growth- and health-promoting effects of dietary Arg beyond the requirement for protein synthesis have been occasionally reported. The survival of channel catfish in response to challenge with *Edwardsiella ictaluri* depends upon the dietary Arg levels (Pohlenz et al. 2012). Enhanced immunity and resistance against pathogens are also found in juvenile yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*) (Zhou et al. 2015) or Nile tilapia (Yue et al. 2015). In terms of FM replacement and comparable to dietary Gln, Arg supplementation can successfully be used to improve immunity and intestinal morphology in fishes on soybean meals (Gu et al. 2017).

Dietary excess Arg reveals diverse effects on fish performance. Many reports demonstrate beneficial effects in terms of increasing titers of insulin, IGF-1, and GH. This remarkable endocrine modulatory capacity of Arg has been discussed as main cause for enhanced growth performance and protein efficiency observed in several fishes (channel catfish, juvenile Atlantic salmon, hybrid striped bass). However, in black sea bream (*Acanthopagrus schlegelii*), European seabass, Japanese flounder, red drum, grouper (*Epinephelus coioides*), and adult Atlantic salmon (Andersen et al. 2014), dietary Arg surplus does not change growth performance and feed utilization. In rainbow trout and Wuchang bream, dietary excess Arg results even in growth depression likely associated with an Arg-Lys antagonism (Coutinho et al. (2016) and references therein). Similarly, *Penaeus monodon* responds with decreased growth to dietary Arg excess (Millamena et al. 1998).

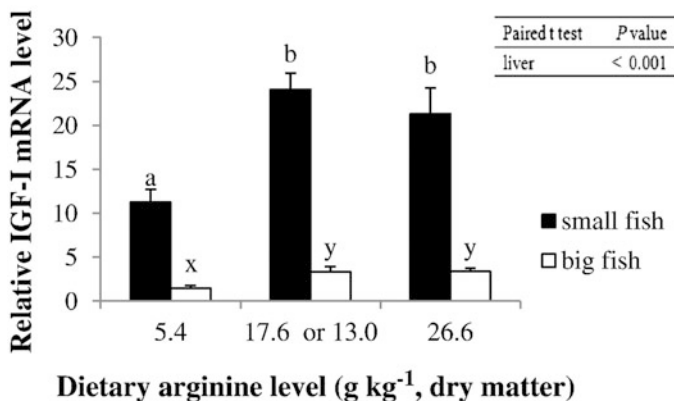


Fig. 10.6 Relative expression of *igf-1* genes of gibel carp fed diets with different arginine levels in small and big gibel carps, respectively. Different superscripts indicate significant differences ($P < 0.05$). (From Tu et al. (2015), with permission from Elsevier)

In juvenile Wuchang bream, Liang et al. (2016) showed that life history traits and protein efficiency ratio increase with increasing dietary Arg level up to 4.9% Arg in protein, and thereafter follows a decrease. This biphasic response is facilitated via gene transcription of the TOR signaling pathway. In addition to this study, Tu et al. (2015) demonstrated that young gibel carps require higher dosages of Arg than older ones, coinciding with reduced transcription of genes of the TOR signaling pathway, again shown with *igf-1* (Fig. 10.6).

Moreover, Arg supplementation is effective in enhancing non-enzymatic and enzymatic antioxidative capacities, as identified in young grass carp. This enhancement can be attributed to the upregulation of antioxidant enzyme gene expression mediated by several signaling molecules, such as *nrf2*, *keap1*, *ck2*, *tor*, and *s6k1* (Wang et al. 2015). The dietary Arg requirement for optimal growth of young grass carp is determined to be 13.5 g kg⁻¹ diet.

One alarming report with unexpected results, however, was published for European seabass. Aiming at finding out how Arg affects the innate immune status or improves resistance against vibriosis, fish were fed two Arg-supplemented diets (1% and 2%) (Azeredo et al. 2015). Surprisingly, a general inhibitory effect of Arg on the immune defenses and disease resistance is discovered. This may be due to the direct role of Arg on cell activation and differentiation leading to a restrained humoral response or to the action of Arg on cell communications by inhibiting the production of pro-inflammatory mediators. Therefore, it is important to have full and robust knowledge of each specific situation (e.g., developmental stage, fish species, etc.) and conditions (e.g., water temperature, densities, water quality), when supplementing aquafeeds with Arg aiming at health improvement.

Coutinho et al. (2016) and Oliva-Teles et al. (2017) contributed another critical example: Dietary excess Arg does not improve intestinal nutrient absorption capacity, AA metabolism, and oxidative status of gilthead seabream juveniles.

Contrastingly, the 1% Arg supplementation facilitates skin wound healing and prevents a systemic inflammation reaction by alleviating the inflammatory response and enhancing the re-epithelialization and extracellular matrix biosynthesis in skin wound sites (Chen et al. 2020).

Applying biochemical and biomolecular techniques rather than only phenotypic ones, Andersen et al. (2015) showed that even no changes in growth can be connected with adverse biochemical indicators. In adult Atlantic salmon on almost twice the actual Arg requirement, no significant effect on growth is detected. However, there are significant differences in the metabolite profile in plasma and liver: increased concentrations of biliverdin, prostaglandin $F_{2\alpha}$ (→Chap. 29), oxidized glutathione, selenocysteine, two monoacylglycerols, and a tripeptide in the liver as well as decreased concentrations of Val and a vitamin D₃ metabolite in the plasma of Arg supplemented fishes. The most alarming finding is the decreased concentration of the vitamin D₃ metabolite, since this deficiency has been reported to impair Arg-induced insulin secretion in rats. Vitamin D is linked to the $\cdot\text{NO}$ pathway, as supplementation of vitamin D₃ induces $\cdot\text{NO}$ production in cultured endothelial cells. Moreover, vitamin D is involved in calcium homeostasis, a cofactor used by $\cdot\text{NO}$ synthase when converting Arg into $\cdot\text{NO}$. If vitamin D₃ is involved in Arg-induced $\cdot\text{NO}$ synthesis or insulin secretion in the Arg-supplemented fishes, this can explain the decreased plasma concentrations of the vitamin D₃ metabolite observed after Arg supplementation (Andersen et al. (2015) and references therein). Currently, it is open to future long-term studies whether such vitamin D₃ deficiency adversely affects also other life history traits (→Chap. 35 for effects of dietary VD deficiency) and even succeeding generations.

Which responses does Arg deficiency elicit in fishes? Wang et al. (2016) provided evidence that Arg deficiency upregulates pro-inflammatory cytokines *il-1 β* , *il-8*, and anti-inflammatory cytokine *tgf- β* as well as downregulates another anti-inflammatory cytokine *tnf- α* – overall, an induction of intestinal inflammation. Furthermore, dietary Arg deficiency disrupts the intestinal physical barrier by regulating tight junction proteins (*occludin* and *claudin 7*), increased lipid peroxidation, protein oxidation, as well as downregulation of antioxidant enzymes. In sum, dietary Arg deficiency impairs intestinal immune function; however, optimum supplementation can restore these changes.

In the same line of evidence, Hoseini et al. (2019) showed in common carps that optimal dietary Arg inhibits stress-induced cortisol and lysozyme elevation and mitigates stress-induced *il-1 β* and *il-8* downregulation; however, dietary Arg excess impairs growth and health. Similarly in rainbow trout, dietary Arg excess increases the expression of non-hepatic *arginase* and *hsp70* in the intestine (Fauzi et al. 2019). Arginase removes excess Arg by catalyzing the hydrolysis of Arg to ornithine and urea.

Some dietary Arg requirement data are collected in Table 10.2, varying between 10 and $\sim 30 \text{ g kg}^{-1}$ with an extreme value found in Pacific salmon (49 g kg^{-1} , Luzzana et al. (1998)). The NRC (2011) lists values between 10 (hybrid striped bass) and 22 g kg^{-1} diet (*Oncorhynchus* spp.). Again, the authors do often not differentiate

Table 10.2 Dietary arginine requirement in selected fishes

Species	Dietary level, g kg ⁻¹	Response parameter	References
<i>Bidyanus bidyanus</i>	27	WG	Ngamsnae et al. (1999)
<i>Carassius gibelio</i>	Small 16.4 Big 12.9	SGR	Tu et al. (2015)
<i>Cirrhinus mrigala</i>	18.4	FCR, PER	Ahmed and Khan (2004a)
	7.0	Immunostimulation	Varghese et al. (2020)
<i>Ctenopharyngodon idella</i>	21.7	SGR	Gao et al. (2015)
<i>Cyprinus carpio</i>	18.5	Intestinal inflammation	Jiang et al. (2015)
<i>Dicentrarchus labrax</i>	18.1	WG	Tibaldi et al. (1994)
	17.8	Protein gain	Fournier et al. (2002)
<i>Epinephelus awoara</i>	28.0	WG, SGR	Zhou et al. (2012b)
<i>Gibelion catla</i>	16.7	WG	Zehra and Khan (2013a)
<i>Hemibagrus wyckioides</i>	9.0 in plant-rich diets	WG, SGR	Gu et al. (2020)
<i>Heteropneustes fossilis</i>	20.4–22.6	WG, FCR, PER	Khan and Abidi (2011a)
	16.3	FCR, PER	Ahmed (2013)
<i>Ictalurus punctatus</i>	10.0	WG	Robinson et al. (1981)
<i>Labeo rohita</i>	12.2–13.9	SGR, FCR, PER	Abidi and Khan (2009)
	23.0	SGR, FCR	Murthy and Varghese (1995)
<i>Megalobrama amblycephala</i>	24.6	SGR, PER	Ren et al. (2013)
<i>Micropterus salmoides</i>	19.1	WG	Zhou et al. (2012a)
<i>Oncorhynchus kisutch</i>	49.0	WG	Luzzana et al. (1998)
<i>O. mykiss</i>	13.8	PG	Fournier et al. (2002)
<i>Pagrus major</i>	23.7	WG	Rahimnejad and Lee (2014)
<i>Paralichthys olivaceus</i>	10–~21.0	WG, FCR	Alam et al. (2002a, 2002b)
	27	WG, SGR	Han et al. (2013)
<i>Rachycentron canadum</i>	28.5	SGR, FER	Ren et al. (2014)
<i>Salminus brasiliensis</i>	~14.5	WG, FCR	Dairiki et al. (2013)
<i>Salmo salar</i> in sea water	16	WG, FER	Lall et al. (1994)
<i>Sciaenops ocellatus</i>	14.4–17.5	WG, PER	Barziza et al. (2000)
<i>Scophthalmus maximus</i>	16.7	Protein gain	Fournier et al. (2002)
<i>Seriola quinqueradiata</i>	14.3–16.3	WG	Ruchimat et al. (1998)
<i>Sparus aurata</i>	16.6	Protein gain	Fournier et al. (2002)
<i>S. macrocephalus</i>	27.9	SGR	Zhou et al. (2010)
	30.9	PER	

SGR specific growth rate, FCR feed conversion ratio, FER feed efficiency ratio, PER protein efficiency ratio, PG protein gain, WG weight gain

between minimal and optimal dietary requirement. The concerns of the methodological approaches, raised in the Lys section above, apply here, too.

Creatine

Creatine (Fig. 10.1) is found in vertebrates where it facilitates an almost complete (98.3%) recycling of ATP, the energy currency of the cell (Wu 2020). It plays also a role as neuroprotector (Marques and Wyse 2019). Creatine is found in white muscle both in its free and phosphorylated forms (Danulat and Hochachka 1989). It contributes to muscle relaxation and contraction.

Based on the hypothesis that creatine loading improves muscle performance, a few studies addressed this potential. In fact, creatine supplementation leads to enhanced sprint endurance and elevated white muscle glycogen stores in juvenile rainbow trout (McFarlane et al. 2001). In contrast, Schrama et al. (2018) fed gilthead seabream graded dietary creatine supplementation and do not find improved productivity or muscle quality. However, differences at the proteome level are detected: in muscles, tropomyosin, β -enolase, and creatine kinase are differently expressed; and in the liver, several proteins involved in immunity, cellular processes, stress, and inflammation response are modulated.

Recently, Burns and Gatlin (2019) confirmed the body building potential of creatine by reporting that the euryhaline red drum (*Sciaenops ocellatus*) benefits from dietary creatine supplementation by improved growth. At a salinity of 5‰, optimal creatine supplementation is found at 2.0‰, and dietary creatine up to 3.8‰ does not result in any deleterious effects. Besides WG, also feed efficiency is improved by supplementation with creatine, whereas its demethylated form, guanidinoacetic acid, proves ineffective (Stites et al. 2020).

10.1.3 Histidine

Histidine (His, Fig. 10.1) is an imidazole-containing semi-essential, proteinaceous AA. It is a precursor to histamine, a vital inflammatory agent in immune responses (Molina-Poveda 2016). Furthermore, His-related (imidazole) compounds, His itself, anserine (β -alanyl- π -methyl-L-histidine), and carnosine (β -alanyl- τ -methyl-L-histidine) function as proton buffers in white muscles (Davey 1960). As antioxidants, carnosine and related compounds protect macromolecules, such as lipids, proteins, or DNA, from oxidative damages (Leinsoo et al. 2006; Feng et al. 2013) and prevent eye cataracts (Waagbø et al. 2010). Very high levels of His-related compounds are typical for migratory pelagic marine fishes (Suzuki et al. 1987).

His participates in the one-carbon metabolism, therefore affecting DNA and protein synthesis as well as epigenetic pathways (Li et al. 2009). Dry matter intake and WG rise with increasing dietary His concentrations in rainbow trouts (Rodehutscord et al. 1997). Dietary demands reach from 1.0 to 1.7 (stinging catfish,

silver pomfret, common carp, Nile tilapia, and channel catfish) over 2.6 (African bonytongue) to 4.5% of dietary protein (barramundi) (Monentcham et al. (2010); Hossain et al. (2011); Farhat and Khan (2013).

Dietary His requirements show clear optima for many species with adverse effects at deficient as well as excess concentrations. This can be illustrated by His requirement in fingerling stinging catfish (Farhat and Khan 2013b). Absolute WG and feed conversion ratio, protein gain, and His retention against graded dietary His levels exhibit optima. For deficient or excess supply of His, symptoms other than suboptimal growth are rarely reported; therefore, underlying mechanisms deserve evaluation with “omics” techniques.

Data of dietary His demand in fishes are collected in Table 10.3. The demand for growth lies between ~3 and ~16 g kg⁻¹ diet (DW). The NRC (2011) lists values

Table 10.3 Dietary histidine requirement in selected fish species

Species	Dietary level, g kg ⁻¹	Response parameter	References
<i>Ctenopharyngodon idella</i>	12.1	WG	Gao et al. (2016)
	~8	LPO↓	Wu et al. (2020)
<i>Cyprinus carpio</i> var. Jian	7.8	SGR	Zhao et al. (2012)
	8.6	LPO↓	Feng et al. (2013)
<i>Gibelion catla</i>	~6.5	WG	Zehra and Khan (2016)
<i>Heteropneustes fossilis</i>	9.4	WG, FCR	Farhat and Khan (2013b)
<i>Ictalurus punctatus</i>	3.7	WG	Wilson et al. (1980)
<i>Labeo rohita</i>	9	SGR, FCR	Murthy and Varghese (1995)
<i>Megalobrama amblycephala</i>	8	Intestinal health	Yang et al. (2019)
	11.2	WG, SGR	Wilson-Arop et al. (2018)
<i>Oreochromis niloticus</i>	8.2	WG	Michelato et al. (2017)
<i>Oncorhynchus keta</i>	7		Akiyama et al. (1985)
<i>O. kisutch</i> , <i>O. tshawytscha</i>	2.8		Klein and Halver (1970)
<i>Oreochromis niloticus</i>	8	WG, myogenin↑	Zaminhan-Hassemer et al. (2020)
<i>Paralichthys olivaceus</i>	15.6	WG, SGR	Han et al. (2013)
<i>Salmo salar</i> , adult in seawater	≥13	Eye cataract↓	Waagbø et al. (2010)
<i>Sciaenops ocellatus</i>	5.9	WG	Peachey et al. (2018)
<i>Seriola dumerili</i>	15	Fecundity↑, fertilization↑, egg & larval quality↑	Sarih et al. (2019)

SGR specific growth rate, FCR feed conversion ratio, FER feed efficiency ratio, LPO lipid peroxidation, PER protein efficiency ratio, WG weight gain, ↑ support/increase, ↓ reduction

between 5 (common carp) and 10 g kg⁻¹ diet (tilapias). Again, various authors do often not differentiate between minimal and optimal dietary requirement, and the methodological concerns continue to apply as well as the use of diverse genetic materials.

Low His requirements for optimal growth apply to crustaceans as shown for postlarval tiger shrimp (Millamena et al. 1999) or kuruma prawn (*Marsupenaeus japonicus*) (Teshima et al. 2002). As in many fish and invertebrate species, His demand follows optimum curves.

More sophisticated, non-appropriate His supply induces changes in integrity of the gill of young grass carps due to depression of cellular antioxidative ability, induction of apoptosis, inflammation and impair of cell-cell tight junctions related to Nrf2, TOR, and NF- κ B signaling (Jiang et al. 2016). Conversely, optimal dietary His level improves immunocompetence in juvenile Wuchang bream by enhancing intestinal anti-inflammatory and downregulating pro-inflammatory genes (Fig. 10.7) (Yang et al. 2019).

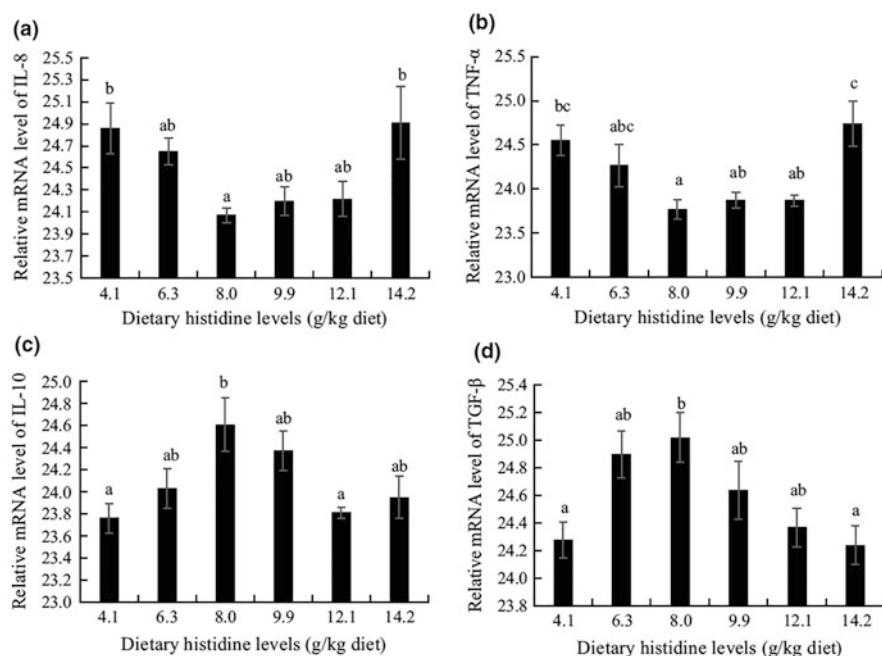


Fig. 10.7 Relative gene expression of inflammatory genes in the intestine of Wuchang (blunt snout) bream on graded histidine diet: (a) *interleukin 8* (*il-8*, pro-inflammatory); (b) *tumor necrosis factor- α* (*tnf- α* , pro); (c) *interleukin 10* (*il-10*, anti-inflammatory); (d) *transforming growth factor- β* (*tgf- β* , anti). Data are means \pm SEM. Different letters indicate significant differences ($P < 0.05$). (From Yang et al. (2019), with permission from Wiley)

10.1.4 Basic Amino Acid Antagonism

Contradictory reports exist whether a basic AA antagonism does exist, whereby excess of one AA reduces the utilization of another one. Clarification and knowledge of potential underlying modes of action are needed. Green and Hardy (2008) reviewed that excess Arg in plant protein-based diets causes a reduced protein efficiency in *Oncorhynchus mykiss*, but not in turbot. Berge et al. (2002) found that neither Arg nor Lys in excess has detrimental effects on growth and protein retention in *Salmo salar*.

In studies with documented antagonism, both quality of tested AAs and the degree to which an AA is in excess seem to influence the outcome. Adverse effects appear more likely when the dietary level is high, namely 2–3 times the dietary requirement or even more (Green and Hardy 2008). Again, more detailed information and identification of potential modes of action are central, but unavailable.

In channel catfish and gilthead seabream, however, growth and feed efficiency indicate the lack of an antagonism when excess Arg is added to diets marginal in Lys (Robinson et al. 1981; Coutinho et al. 2016). In rainbow trout, Chiu et al. (1987) found that excess Lys causes depressed growth and efficiency of feed utilization. The authors attribute these effects to Lys toxicity rather than Lys-Arg antagonism, as the effects cannot be mitigated by supplemental dietary Arg.

An interesting interaction between His and Arg occurs in juvenile Japanese flounders (Han et al. 2013). Growth is best on high Arg/medium His diet. Significantly better nutrient utilization is also observed in the higher Arg-supplemented groups. Japanese flounders on low dietary Arg together with low dietary His show high oxidative stress. Low dietary Arg together with low dietary His increases the plasma glutamyl oxaloacetic transaminase and glutamic-pyruvate transaminase, but decreases lysozyme activity. This symptom can be mitigated by increasing either the Arg or His level in the diet. The best combination is 2.7% Arg and 1.7% His.

An antagonism between basic AAs appears to exist also in a few invertebrates. Growth rate, feed conversion rate, body protein, body Lys and Arg contents in Pacific white shrimp are significantly affected by dietary Lys and Arg. An optimal ratio of these two AAs lies around 1:1 (Molina-Poveda 2016).

10.2 Proline and Hydroxyproline

10.2.1 Proline

Proline (Pro, Fig. 10.1) is the only proteinogenic AA with a secondary amine. In fishes, Pro is essential: While most mammals can synthesize Pro from Arg and Glx, rates of endogenous synthesis are inadequate in many fishes (Wu et al. 2011). Pro is now considered conditionally essential for fishes at least in early life stages (Li et al.

2009). Dabrowski et al. (2010) reviewed that high growth rates go with high Hyp:Pro ratios (5–10:1) in rainbow trout muscles.

In invertebrates, at least in Pacific white shrimp under stress, Pro appears to be essential, too (Xie et al. 2015). Pro is a major nitrogenous substrate for the synthesis of polyamines (Molina-Poveda 2016). 4-hydroxyproline (Hyp, Fig. 10.1) is a common nonproteinogenic AA and a major component of collagen (Szpak 2011). Approximately 99.8% of the Hyp in the body stores of mammals are found in collagen (Barbul 2008). Also Pro supports collagen biosynthesis (Zhang et al. 2015; Lin et al. 2020).

Both Pro and Hyp can function as osmolytes in euryhaline fishes, such as Arctic charr or mangrove killifish (*Kryptolebias marmoratus*) (Frick and Wright 2002; Bystriansky et al. 2007). Pro has the potential to increase pathogen resistances. For instance, *Streptococcus agalactiae* causes massive tilapia kills (streptococcosis) under elevated water temperatures. Applying functional metabolomics, Zhao et al. (2015) identified that, among other AAs, particularly Pro decreases at high temperatures and this decrease, in turn, can be responsible for the increased susceptibility to streptococcosis. This assumption is validated by injection or oral addition of Pro, which leads to elevated survivals.

Wei et al. (2020) studied the interaction of Pro and vitamin C on growth, muscle quality, and antioxidative capacity in large yellow croaker. Dietary Pro (2.4% and 3.0%) rather than vitamin C increases growth when dietary FM level is 30%. Dietary vitamin C and Pro interact on muscle quality and antioxidative capacity: Pro increases the activities of glutathione peroxidase and copper/zinc superoxide dismutase in muscle at higher level of dietary vitamin C; these nutrients act synergistically.

It is well understood that AAs function as signaling molecules in protein synthesis and animal growth by regulating several genes in the TOR pathway. Recently, Rong et al. (2020b) found in juvenile *Protonibea diacanthus* that (1) dietary Pro promotes protein synthesis and (2) the increased protein synthesis takes place via transcription of *tor*.

Studies on dietary Pro in invertebrates are scarce. One of the few studies is in juvenile Pacific white shrimps (Xie et al. 2015) shows that 2.3% Pro supplementation improves antioxidative capacity, immune response, and ammonia stress tolerance, but has no effect on growth. In slow-growing *Halotis midae* individuals, the addition of Pro to a standard diet serves as a substrate for AA catabolism and growth. This happens by means of Pro breakdown and energy production via the tricarboxylic acid cycle (Venter et al. 2019).

10.2.2 Hydroxyproline

As a post-translational modification, Hyp (Fig. 10.1) is produced by hydroxylation of Pro by prolyl hydroxylase. This reaction takes place in the lumen of the

endoplasmic reticulum. Although it is not directly incorporated into proteins, Hyp comprises roughly 4% of all AAs found in animal tissues (Gorres and Raines 2010).

Dietary supplementation of Hyp, but not Pro, increases growth, promotes formation of collagen, and modifies bone composition of Atlantic salmon and large yellow croaker fed diets rich in plant protein (Aksnes et al. 2008; Wei et al. 2016). Although the underlying mechanisms are not yet completely understood, these findings support the notion that AA derivatives are potent growth promoters in many fishes.

Opposing effects are observed in turbot, a species with high dietary protein requirement. Growth performance is reduced in turbot fed diets with high levels of plant material. One assumption is that, among other, Hyp is not sufficiently supplied in these diets (Zhang et al. 2013). Therefore, the authors evaluated the effects of graded Hyp diets on survival, growth, feed utilization, body composition, Hyp and collagen concentrations in tissues, and *prolyl 4-hydroxylase $\alpha(I)$ (*p4h $\alpha(I)$)* expression of juvenile turbot. P4H $\alpha(I)$ catalyzes the formation of 4-Hyp in collagens and similar proteins. Supplementation of Hyp in high plant protein diets does not induce beneficial effects on growth performance of juvenile turbot. Free Hyp in plasma and total Hyp contents in liver and muscle increase as dietary Hyp increased. Total collagen concentration in muscle increases with increasing dietary Hyp. The expression of *p4h $\alpha(I)$* transcription remains constant in the liver, indicating that hepatic P4H(I) activity is independent of dietary Hyp levels. However, *p4h $\alpha(I)$* in the muscle decreases as dietary Hyp increases. This may eventually result in lower collagen synthesis in the muscle of fishes fed diets with high Hyp levels. All this indicates that the improved collagen concentration in the muscle of fishes fed high dietary Hyp levels cannot be attributed to the increased ability of collagen synthesis, but may be due to suppression of collagen degradation of fishes fed the diet with high Hyp levels. The final result is identical.*

The assumption that Hyp in plant proteins can be limiting in FM replacements gets strong support by a study of Liu et al. (2014) also in turbot. The authors added graded levels Hyp to decreasing FM, but increasing plant-derived protein levels. The results are convincing: without Hyp inclusion, the plant protein mixture can only substitute 40% of FM without affecting growth. However, Hyp supplementation improves the replacement ratio up to 60%. Therefore, Hyp is a limiting factor when high levels of plant protein are used in the diets – at least in turbot that makes dietary supplementation of this AA necessary.

Feeding graded levels of Hyp to juvenile spotted drum (*Nibea diacanthus*), Rong et al. (2020a) demonstrated that Hyp improves growth and the ability to synthesize proteins through the mTOR pathway. The optimum amount of dietary Hyp is estimated as $\sim 17 \text{ g kg}^{-1}$. The expression levels of *mechanistic target of rapamycin (tor)* and *ribosomal protein S6 kinase1 (s6k1)* genes in the liver, muscle, and swim bladder increase with increasing Hyp content of diets, while the mRNA expression level of *eukaryotic translation initiation factor 4E-binding protein (4e-bp)* gene decreases in these tissues.

As with Pro, only a few studies are conducted with aquatic invertebrates on Hyp-supplemented diets. Vosloo et al. (2013) showed that *Haliotis midae* juveniles increase their body mass when their feed is supplemented with Pro. However,

Hyp-amended abalones from fluctuating, stressful environments do not benefit from increased antioxidant capacity. Therefore, the underlying mechanism deserves further attention.

10.3 Concluding Remarks

Somewhat repetitive, this chapter frequently pointed out optimum curves of response traits on graded AA feeds. Several figures above show that excess of a given AA elicits reduction of growth and other traits. Some authors offer an obscure AA toxicity as potential explanation; others discuss antagonism between basic AAs as underlying mechanism. In the light of “omics” techniques and epigenetic pathways, these explanations alone do no longer convince. Consequently, the adverse effects of dietary AAs excess deserve mechanistic biomolecular studies. The same applies to the antagonism between basic and other AAs, since, in some fish species, a replacement or antagonism can be shown, in others not. Abstracting from individual cases by applying biomolecular and *in silico* techniques will support solving this problem.

Finally, in a some 10-year-old paper, Hua (2012) raised her concern about the currently applied methods of requirement determinations. She questioned the commonly applied reference parameter, since she found that dietary protein content has a significant effect on Lys requirement, while the more often applied digestible energy does not. To express AA requirement as % diet, % protein or g MJ⁻¹ digestible energy is simple, straightforward and will probably continue to be used in feed formulation, as one can find in the nutrient requirement tables published by the NRC (2011). The current practice adopted by most fish nutritionists to express requirement as a fixed concentration of diet provides less goodness of fit than to express requirement as a percentage of dietary protein. (For more details, →Chap. 5.)

In addition to this fuzziness, the AA utilization is not a constant as recently exemplified with Nile tilapia and Lys. Hua et al. (2019) demonstrated that marginal Lys utilization efficiency is influenced by fish sizes and life stages in Nile tilapia. Marginal utilization efficiency of Lys stays relatively high for fish below 100 g, but decreases significantly when fish grow larger. This non-constant marginal utilization efficiency of Lys across life stages indicates that a novel approach that embodies the effects of biological and dietary factors has to be adopted to describe the utilization and requirement of EAAs.

It is beginning to be understood that basic AAs elicit controlling functions in metabolism and immune response. These aspects deserve future attention.

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Chapter 11

Taurine—‘*Controlling Rather than Fueling*’



Abstract Taurine is an amino acid that differs from the more familiar AAs in possessing a sulfonic rather than a carboxylic group. It is central in membrane stabilization, antioxidation, detoxification, immune response, calcium transport, myocardial contractility, retina development, bile acid metabolism, and endocrine functions. It comprises up to 50% of the free AA pool and is central in osmoregulation. Tau is an essential nutrient in many aquatic animals. Appropriate Tau supplementation sustains many health and metabolism aspects and endurance of farmed fish, while excess Tau seems to be “toxic” to shrimps and fishes. Dietary Tau increases phagocytic activity as well as innate immunity by upregulation of immunity gene transcription. Many details of the metabolism of dietary normal and excess Tau have been identified only phenotypically; details remain to be proven by innovative biomolecular approaches. Furthermore, the role of the intestine microbiota in Tau metabolism has not yet been considered appropriately.

In addition to the proteinaceous amino acids (AAs) described in previous chapters, another major AA, taurine (Tau, Fig. 11.1a), plays an overwhelming controlling role in animals (Laidlaw et al. 1990). Tau is an AA that differs from the more familiar ones in being a sulfonic rather than a carboxylic AA and in being a β -AA rather than an α -AA. Compared with carboxylate groups, the sulfonate group is a strong acid (Huxtable 1992).

The replacement of the carboxyl group by the sulfonate group disables the molecule to form peptide bonds; hence, Tau can usually not be part of peptide chains. Consequently, Tau is the most abundant free AA in animal tissues, accounting for up to 25% of the free AAs pool in the liver, 50% in the kidney, 53% in the muscle, and 19% in the brain (Salze and Davis 2015). Tau plays significant roles in membrane stabilization, antioxidation, detoxification, immune response, calcium transport, myocardial contractility, retina development, bile acid metabolism, and endocrine functions (El-Sayed 2014)—at least in mammals.

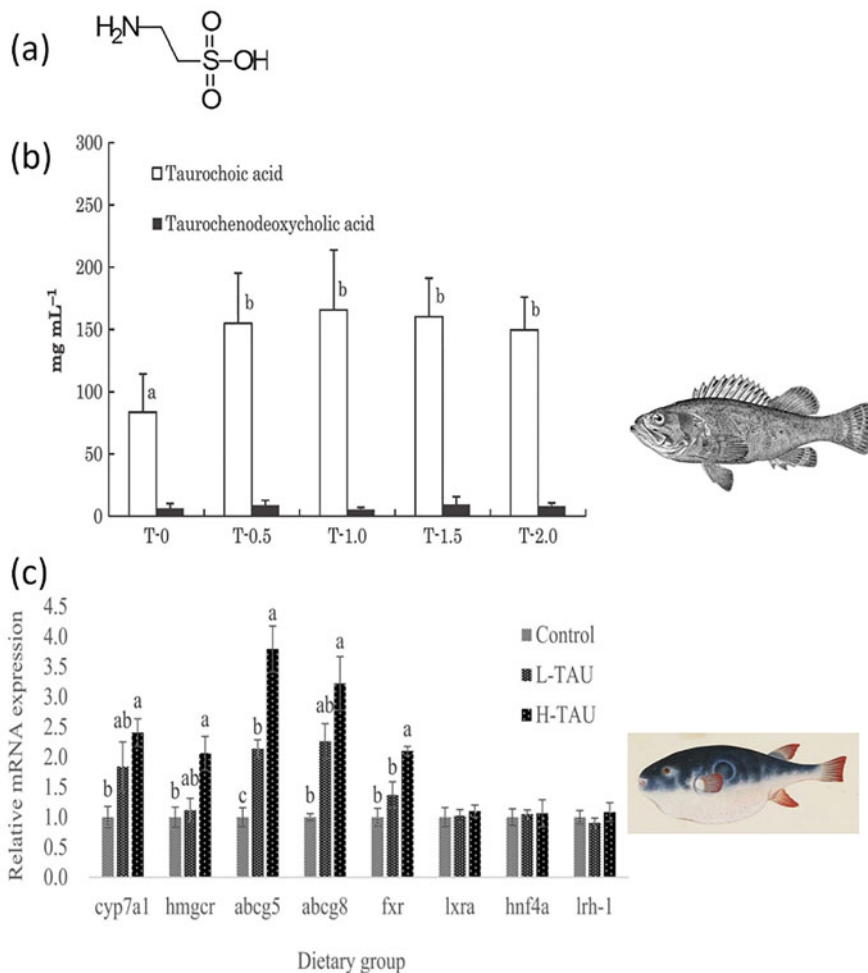


Fig. 11.1 (a) Taurine (=2-aminoethanesulfonic acid). (b) Conjugated bile acids detected in gallbladder bile of juvenile Korean rockfish (*Sebastes schlegelii*) at the end of the 4-month feeding study. **T-0** to **T-2.0**: diets with increasing Tau content (0.7 to 17 mg g⁻¹ dry matter). Different superscripts indicate significant differences ($P < 0.05$). (From Kim et al. (2015), with permission from Wiley). (c) Relative mRNA expression of genes related to bile acid and cholesterol metabolism in the liver of tiger puffer (*Takifugu rubripes*). Different superscripts indicate significant differences ($P < 0.05$). (From Xu et al. (2020), with permission from the Cambridge University Press; images credit Naturalis Biodiversity Center). L-TAU 8 g kg⁻¹ diet; H-TAU 14 g kg⁻¹ diet; *cyp7a1* cholesterol 7 α -hydroxylase; *hmgcr* 3-hydroxy-3-methylglutaryl-CoA reductase; *abcg* ATP binding cassette subfamily G; *fxr* farnesoid X receptor; *lxra* liver X receptor α ; *hnf4a* hepatocyte nuclear factor 4 α ; *lrh-1* liver receptor homolog-1

Since Tau comprises up to 50% of the free AA pool, it plays a role in osmoregulation as shown in:

- Marine fishes: European flounder (*Platichthys flesus*), winter flounder (*Pseudopleuronectes americanus*), and little skate (*Leucoraja erinacea*).
- Brackish fishes: mangrove killifish.
- Freshwater fishes: walking catfish, tilapia, and common carp (Fugelli and Zachariassen 1976; Frick and Wright 2002; Chara et al. 2011).

The osmoregulatory function of Tau applies also to euryhaline invertebrates in intertidal ecosystems, such as penaeid shrimps *Marsupenaeus japonicus*, *Penaeus monodon* (Richard et al. 2011), and *Litopenaeus vannamei* (Li et al. 2017), prawns *Palaemon elegans*, bivalves *Mytilus galloprovincialis* (Ji et al. 2013) and *Ruditapes philippinarum* (Liu et al. 2013), and the freshwater giant shrimp *Macrobrachium rosenbergii* (El-Sayed 2014). Another proof of the osmoregulatory activity of Tau is the increase in Tau transporter (*tauT*) mRNA in carp and tilapia tissues during salinity-induced stress (Takeuchi et al. 2000a, b). Consequently, in maintaining the osmotic homeostasis, the sufficient dietary supply with Tau or its precursors is central (Chara et al. 2011).

Except of spontaneous basic physiological functions, such as antioxidation or osmoregulation, the functions of Tau in fishes and aquatic invertebrates are much less well documented than in mammals. In teleost species where Tau has been identified as an essential nutrient, poor growth and reduced survival are consistently observed during Tau deficiency. However, such phenotypic symptoms are uninformative to the roles of Tau (Salze and Davis 2015) and still subject to extensive studies. As effective antioxidant in aquatic animals (Zhao et al. 2017), Tau counteracts oxidative stresses by scavenging reactive oxygen species (ROS) that are produced as multipurpose tools after the impact of different internal as well as external stressors (Steinberg 2012).

Tau accelerates growth and improves immunity and survival of fish larvae (Kim et al. 2016). Furthermore, it is involved with several important biological functions including fat digestion as a conjugator with bile acids, such as cholic acid or chenodeoxycholic acid in the liver (Fig. 11.1b), and stimulates the hepatic biosynthesis of both bile acid and cholesterol (Fig. 11.1c).

11.1 Taurine Biosynthesis and Physiology

Over the past decade, it has become obvious that Tau is an essential nutrient in many aquatic animals, as shown in octopus (Lopes et al. 2016), sea cucumber (Liu et al. 2016), shrimps (Yue et al. 2013; El-Sayed 2014), and fishes (El-Sayed 2014; Salze and Davis 2015; Magalhães et al. 2019). Meanwhile, several endogenous biosynthesis pathways have been discussed (see below); however, numerous fish species benefit from dietary Tau supplementation, thus showing that the two endogenous pathways are insufficient to provide the necessary amounts of Tau for maximal

growth (Salze and Davis 2015). In this review, the authors summarize the recommended Tau supplement for various fish species from dietary 0.2% (common dentex, *Dentex dentex*; European seabass; rainbow trout) to 1.7% (Japanese flounder).

In larval cobia, Salze et al. (2012) found that Tau supplementation increases specific amylase and trypsin activities in early stages, later also lipase and pepsin. These increased enzymatic activities lead to enhanced nutrient availability, thus providing some explanation to the improved development, growth, and post-weaning survival observed in Tau-supplemented larvae.

Moreover, Tau is an important neurochemical factor in the animal visual system. Abundant Tau is localized in the retinal photoreceptor and neural layers of, for instance, the anadromous ayu (*Plecoglossus altivelis*), juvenile Japanese flounders, glass eel, and young goldfish (Omura et al. 1997; Omura and Yoshimura 1999; Nusetti et al. 2006, 2010), demonstrating that Tau is involved in the protection of the photoreceptor outer segment, the regulation of neural transmission, and photoreceptor differentiation.

The Tau biosynthetic pathways have been the subject of research for several decades, in spite of which some parts remain poorly characterized. The cysteinesulfinatase-decarboxylase (CSD) pathway is the main pathway in mammals. CSD activity occurs in freshwater fishes, such as rainbow trout, common carp, tilapia, bluegill (*Lepomis macrochirus*) as well as Atlantic salmon (El-Sayed 2014); however, the activity remains an order of magnitude lower than in small mammals. Furthermore, studies with common carp indicate that this species relies on a different pathway than the CSD pathway for Tau production, possibly the pathway using cysteic acid decarboxylase; the precise pathway, however, remains to be ascertained (Salze and Davis 2015). Consequently, one cannot systematically assume that all teleost rely on the CSD pathway for the biosynthesis of Tau. Recently, Ma et al. (2021) found that golden pompano (*Trachinotus ovatus*) possesses the key enzyme for Tau biosynthesis through two main pathways, and exogenous Tau intake directly affects the expression of synthesis-related genes in the liver. Tau biosynthesis alone, however, is insufficient to satisfy the demands of *T. ovatus*; the results indicate that approximately 10 g kg⁻¹ of Tau still needs to be provided via diet.

Tau is mainly synthesized in the fish liver. Consequently, the liver is the most-impacted organ in the event of deficiencies. Green liver syndrome is arguably the most specific symptom of Tau deficiency. The green liver originates from the accumulation of biliverdin and reduced excretion of bile pigment from the liver to the bile (Salze and Davis 2015). Tau supplementation effectively reduces the severity of green liver disease in yellowtail fed a diet devoid of fishmeal and containing less than 0.1% Tau (Takagi et al. 2005). This indicates that Tau deficiency can be at the origin of this nutritional disease.

As mentioned, Tau is a major compound of bile and central in lipid metabolism via bile acids. Bile acids are steroids derived from cholesterol, synthesized in the liver, stored in the gallbladder, and released into the intestinal lumen to emulsify fats and help in the absorption of lipids and fat-soluble vitamins. Bile acids are

conjugated mainly with Tau and—to a lesser extent—with Gly. Therefore, Tau enhances lipid metabolism in fishes, through increase in the bile salt-activated hepatic lipase activity. Therefore, Tau as well as bile salts can be used as olfactory stimuli to increase feed uptake as documented in European glass eel, European seabass fry, gilthead seabream fry as well as rainbow trout, and channel catfish (El-Sayed 2014; Jirsa et al. 2014).

Less well understood is the Tau biosynthesis in invertebrates. The enhanced growth of *P. monodon* after dietary addition of Tau indicates limited Tau endogenous synthesis in this shrimp (Shiau and Chou 1994). More recently, Richard et al. (2011) showed that *P. monodon* has a capacity to regulate Tau synthesis in relation to dietary Cys2 levels. In contrast to *P. monodon*, the giant river shrimp (*M. rosenbergii*) appears to cover its Tau requirement by biosynthesis (Smith et al. 1987). However, in both shrimps, details of the biosynthesis pathway remain to be discovered.

Tau is absorbed by the intestinal epithelium through a specific, Na^+/Cl^- -dependent Tau transporter (TauT). In mammals, compounds are identified to inhibit specifically the uptake of Tau in the intestine, particularly β -alanine. These substances can be classified as anti-nutritional factors (Salze and Davis (2015). TauT is found, for instance, in common carp and tilapia, Senegal sole, Mediterranean blue mussel, and Pacific oyster (Hosoi et al. 2005, 2007). However, information of anti-nutritional substances is lacking; but a deeper understanding of the interactions between these antinutrients (alone or in combination) with Tau—and other nutrients—in respect to bioavailability and metabolism would be invaluable to refine requirement estimates and necessary supplementation levels (Salze and Davis 2015). Furthermore, it has to be evaluated which other factors affect the Tau transport. In addition, it is also unexplored whether the transcription of transporter genes underlies a diurnal rhythmicity itself as it is well understood from genes encoding detoxification enzymes, such as cytochrome P-450 monooxygenases or glutathione transferases (Hooven et al. 2009; Brager et al. 2011) (for more detail, →AAN I “Chrononutrition” (Steinberg 2018)).

In Nile tilapia muscle, Shen et al. (2018) visualized the metabolic trajectory and revealed the metabolic mechanisms of dietary Tau supplementation on growth. Nineteen Tau-induced metabolic changes are involved in carbohydrate, AA, lipid, and nucleotide metabolism; Tau has a central metabolic position (Fig. 11.2). This paper presents pathways and supports the notion that Tau supplementation significantly regulates growth and development.

In a series of papers, Pinto and coworkers demonstrated the role of Tau in gilthead seabream larvae. The essentiality for Tau appears not to exist; this species can grow on Tau-free diets, since it biosynthesizes Tau from Met. However, Tau dietary supplementation may beneficially affect larval metabolism by increasing Met availability for several other important physiological purposes in the one-carbon metabolism (Pinto et al. 2013a).

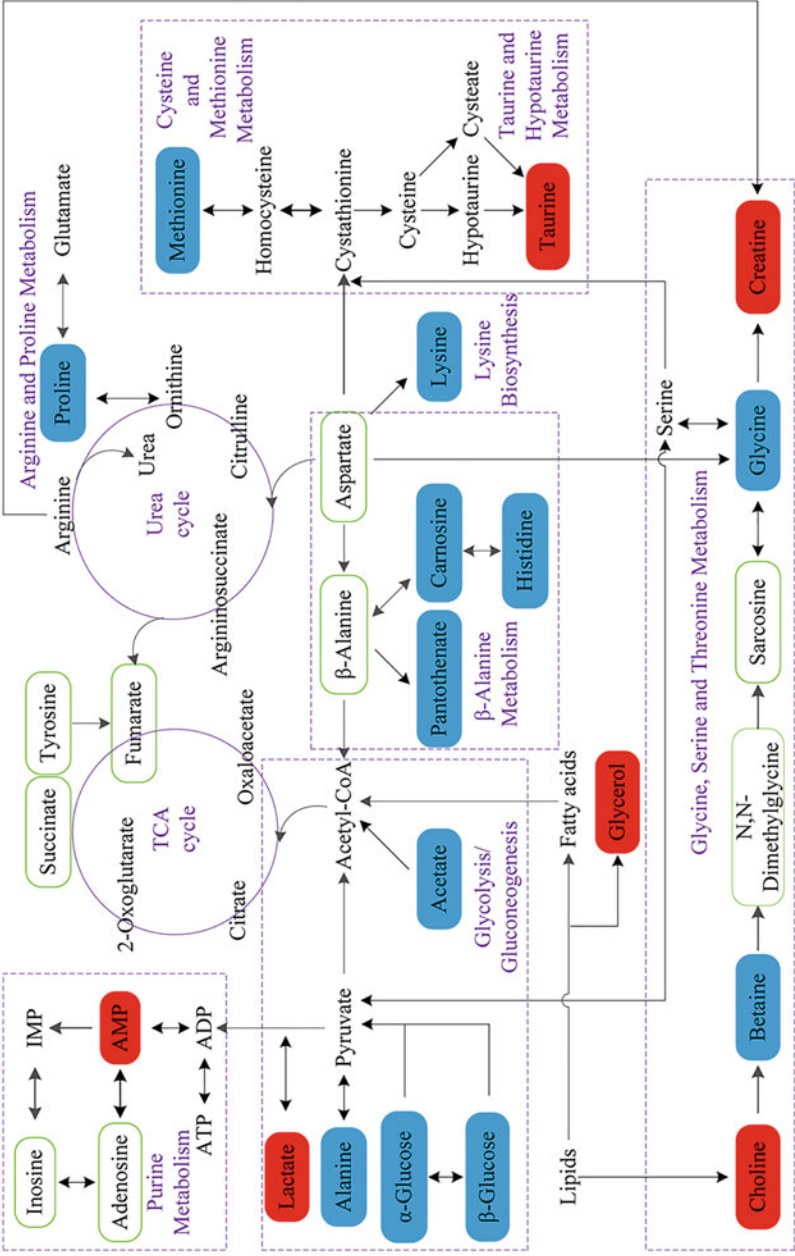


Fig. 11.2 Metabolic pathways affected by dietary Tau in tilapia muscle extracts. Metabolites in red and blue represent higher or lower levels in tilapia muscle extracts of the Tau-supplemented groups, when compared with the control group. Metabolites in green frames represent nonsignificant change and were detected by ¹H NMR, and metabolites with no color marking were not detected. (From Shen et al. (2018), with permission from the American Chemical Society)

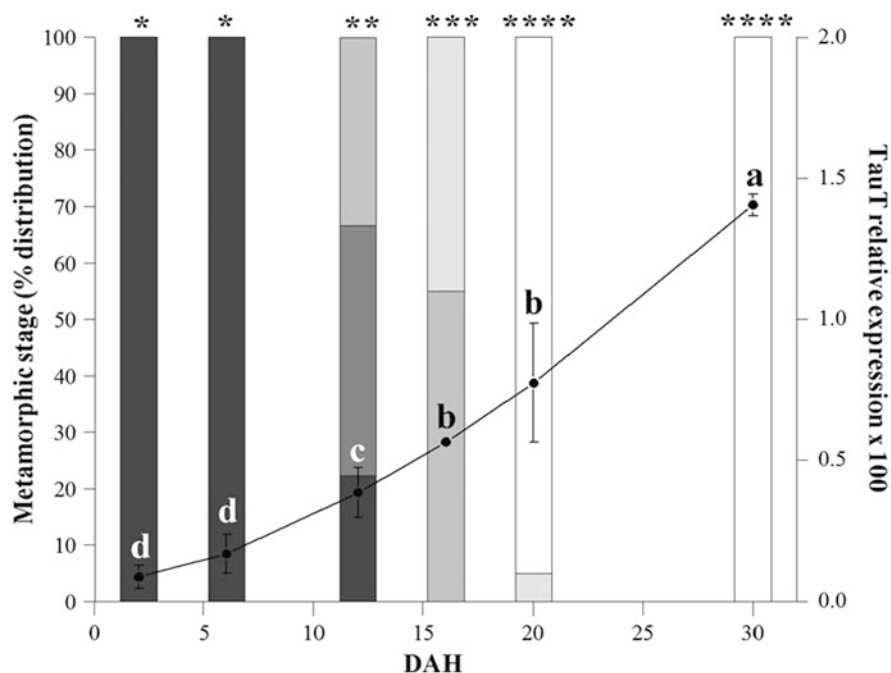


Fig. 11.3 Metamorphosis pattern and *tauT* expression in *Solea senegalensis* larvae. **DAH** days after hatching (=dph). Results for metamorphosis pattern are expressed as percentage of each metamorphic stage (Pre-■; Early■, Middle□, and Late□). Results for *tauT* expression (—●—) are given as means \pm SD. Asterisks represent significant differences for the mean metamorphic stage at a certain age. Different letters represent significant differences for the expression of the *tauT* throughout larval development. (From Pinto et al. (2013b), with permission from Springer Nature)

In contrast to gilthead seabream, flatfishes, such as the Senegalese sole, require dietary Tau. Flatfishes undergo a visibly marked metamorphosis. Pinto et al. (2013b) assessed the effect of Tau supplementation in sole larvae and juveniles via growth, metamorphosis success, AA metabolism, and transcription of *tauT*. The transcription increases during larval development, increasing at the onset of metamorphosis (12 days after hatching, DAH) and achieving the highest values at post-metamorphosis (30 DAH; Fig. 11.3). In Senegalese sole juveniles, *tauT* mRNA is ubiquitously expressed in all analyzed tissues, with high expression in the brain, heart, and eye. In the digestive tract, high *tauT* expression occurs in hindgut and stomach tissues, indicating that dietary Tau is readily absorbed when the digestive process begins. Furthermore, Tau endogenously used for bile salt conjugation can be reabsorbed at the posterior end of the digestive tract pointing out the presence of an enterohepatic recycling pathway for Tau in Senegalese sole, at least in the juvenile stage. This process appears to be important for the maintenance of the Tau pool in the body—likely not only in this but also in other flatfish species.

Furthermore, Tau induces important protective biochemical mechanisms. In the liver and anterior intestine of European seabass, stress protein levels and the MAPK¹ pathway as well as lactate dehydrogenase are upregulated (Feidantsis et al. 2014). Furthermore, dietary Tau increases immunity and resistance against hyperammonemia in juvenile yellow catfish (Li et al. 2016) pointing out that appropriate Tau supplementation sustains health and endurance of farmed fish.

In many fishes, replacement of marine proteins with plant proteins leads to reduced growth (Espe et al. 2012) and development of enteritis. Plant ingredients are low in Tau (Liaset and Espe 2008); and thus Tau is conditionally indispensable if the plant ingredients become the main protein source in aquafeeds. Several studies exist in which either voluntary feed intake or growth performance improves following exogenous Tau addition to diets based on non-marine protein ingredients. However, in juvenile Atlantic salmon, the addition of 0.1% Tau to high plant protein diets has an adverse effect on growth (Espe et al. 2012). It decreases the lipid content in the entire fish, without affecting protein accretion, and thus reduces the body lipid-to-protein ratio and increases the liver pool of free AAs. A decreased lipid deposition is also observed in Japanese flounder after Tau supplementation (Han et al. 2014). Taken together, the partitioning of growth and nutrient deposition pattern following the availability of sulfur AAs, including Tau, has to be studied in more detail because such knowledge can facilitate the production of slimmer and healthier fish during the growth period, in which the majority of fat deposition occurs.

Some more studies of dietary Tau effects in farmed animals are listed in Table 11.1. The results span from inconspicuous to beneficial effects. Many studies replace fishmeal by plant-based proteins and supplement Tau.

Supplementing 3.5% Tau in an all-plant protein diet, Hu et al. (2018) showed that this AA is a phagostimulant in large yellow croaker (*Larimichthys crocea*) and upregulates a variety of olfactory receptor genes in the olfactory epithelium (Fig. 11.4). The phagostimulation aids the development of an all-plant protein feed for this fish. Moreover, Tau-enriched rotifers and *Artemia nauplii* fed to gilthead seabream larvae have beneficial effects on retinal opsin abundance and gene expression of five main opsins. The Tau effect on the pattern of gene expression of selected opsins in the larvae is age-dependent and potentially improved vision leading to increased hunting success and weight gain (Gaon et al. 2020).

Dietary Tau mitigates oxidative stress and apoptosis at low temperatures as shown in obscure pufferfish (*Takifugu obscurus*) (Fig. 11.5). Apoptosis is a form of programmed cell death that occurs in multicellular organisms (Elmore 2007), and caspases are its executioners. Caspase-3 is the major player responsible for the proteolytic cleavage of many critical cellular proteins (Elmore 2007). Dietary Tau

¹Mitogen-activated protein kinases are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock, and pro-inflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis (Pearson et al. 2001).

Table 11.1 Recent effect studies of dietary taurine in aquatic animals

Species	Diet	Dietary Tau level, %	Effects	References
Invertebrates				
<i>Eriocheir sinensis</i> , Chinese mitten crab	Regular diet	0→1.6	WG↑, immunity↑, AOC↑ Optim @ 0.4–0.8	Dong et al. (2018)
<i>Penaeus monodon</i> , black tiger shrimp	41% P	1.5	Feeding attractant→SGR↑	Hartati and Briggs (1993)
Fishes				
<i>Acanthopagrus latus</i> , yellowfin seabream	Low FM diet	0→1.6	SGR↑, FCR↑ optim ~1.1	Dehghani et al. (2020)
<i>A. schlegelii</i> , black seabream	All-plant diet	0→2.0	WG↑, cholesterol→bile acid ^a <i>cyp7a1</i> ↑	Tong et al. (2019)
			Serum protein↑, serum cholesterol↓, hepatic bile acid↓	Tong et al. (2020)
<i>Argyrosomus regius</i> , meagre	High plant diet	0, 1.0	WG↔, FCR↔	de Moura et al. (2018)
<i>Clarias gariepinus</i> , African catfish		0→4.0	WG↑, FCR↔	Adeshina and Abdel-Tawwab (2020)
<i>Ctenopharyngodon idella</i> , grass carp		0→0.2	WG↑, intestinal health↑	Yan et al. (2019)
<i>Cyprinus carpio</i> , common carp	Regular diet	0→2.0	Salinity stress resistance↑, optim @ 1.5	Abdel-Tawwab and Monier (2018)
<i>Danio rerio</i> , zebrafish	High plant diet	0→1.5	WG↔, reproduction↔	Guimarães et al. (2018)
<i>Dicentrarchus labrax</i> , European seabass	Low FM diet	0, 0.26	AOC(↑)	Coutinho et al. (2017)
		1.5	WG↑, ROS↓, liver <i>cat</i> ↓, muscle <i>gpx</i> ↓	Ceccotti et al. (2019)
		0, 1.5, 5.0	Spectral sensitivity↓	Brill et al. (2019)
		0, 1.0	WG↑, AOC↑, <i>casp3</i> ↓, <i>casp9</i> ↓, inflammation↓, lipogenesis↓, β-oxidation↓, <i>cyp7a1</i> ↔	Martins et al. (2019, 2021)
	Low FM diet; 32% soy	0→1.0	Growth↔	Kotzamanis et al. (2020)
	FM-based	1.0	Growth↑, immunity↑	Saleh et al. (2020)

(continued)

Table 11.1 (continued)

Species	Diet	Dietary Tau level, %	Effects	References
<i>Larimichthys crocea</i> , large yellow croaker	All-plant protein diet	3.5	Phagostimulation↑, <i>olfactory receptors</i> ↑ (Fig. 11.4)	Hu et al. (2018)
<i>Lates calcarifer</i> , barramundi	Plant-based diet	0.1→1.5	Growth↑, if S-AAs deficient	Poppi et al. (2018)
<i>Oreochromis niloticus</i> , Nile tilapia	Soy-based diet	0→1.0	Met sparing	Michelato et al. (2018)
<i>Rachycentron canadum</i> , cobia	Plant-based diet	0.5	WG↑	Raggi et al. (2019)
<i>Scophthalmus maximus</i> , turbot		0.8	WG↔	Wei et al. (2019)
		0→2.0	Muscle growth↑, collagen↑	Wijerath Wiriduge et al. (2020)
			WG↑, hepatic AOC↑, ER stress↓, inflammation↓	Zhang et al. (2021)
<i>Seriola dumerili</i> , greater amberjack	Regular diet	0.3→1.1	Broodstock fecundity↑	Sarih et al. (2019)
<i>S. (dorsalis) lalandi</i> , California yellowtail, broodstock		0.3→2.7	Yolk sac volume↑, larval survival↑	Salze et al. (2019)
<i>Sparus aurata</i> , gilthead seabream	Via <i>Brachionus rotundiformis</i> and <i>Artemia nauplii</i>	0.17→6.2	WG↑, vision↑, hunting success↑	Gaon et al. (2020)
<i>Takifugu rubripes</i> , tiger puffer	Regular diet @ low temperature	0→0.17	WG↑, immunity↑, apoptosis↓ (Fig. 11.5), oxid. Stress↓	Cheng et al. (2018)
	Low FM diet	0.6, 1.2	Bile acid biosynthesis↑, cholesterol biosynthesis↑, lipogenic genes↑	Xu et al. (2020)
	FM + plant-based diet	0→2.0	Met sparing	Wei et al. (2020)
<i>Thunnus thynnus</i> , bluefin tuna	Via <i>Brachionus rotundiformis</i>	0→2.0 g (10 ⁶ rotifers) ⁻¹	<i>tauT</i> ↓ ≥1.0 g: WG↑	Betancor et al. (2019)

↑ support/increase; ↓ reduction/decrease; ↔ no apparent (adverse) effect; AOC antioxidant capacity; P protein; FM fishmeal; WG weight gain; SGR specific growth rate; FCR food conversion ratio; ER stress endoplasmic reticulum stress (→Chap. 23); cat catalase; gpx glutathione peroxidase; *cyp7a1* cholesterol 7α-hydroxylase; *tauT* taurine transporter

^a*cyp7a1* encodes cholesterol 7α-hydroxylase, the key enzyme in the process of converting hepatic cholesterol to bile acid (Yokogoshi et al. 1999)

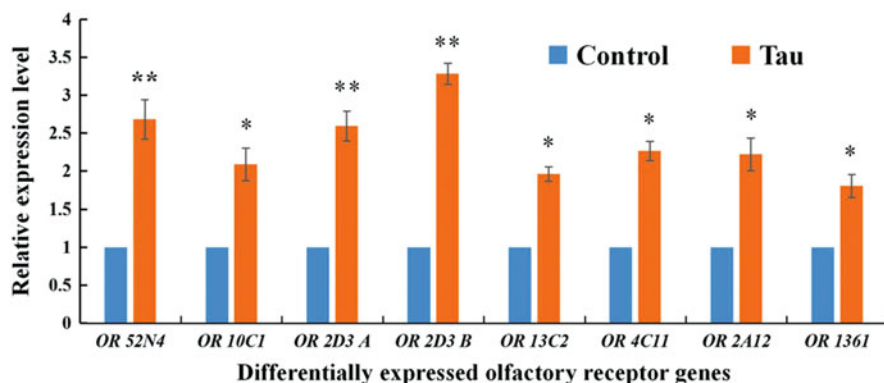


Fig. 11.4 Upregulation of eight olfactory receptor genes of large yellow croaker fed 3.5% dietary Tau. Relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ using β -actin as an internal reference gene. * $P < 0.05$ and ** $P < 0.01$. (From Hu et al. (2018), with permission from Wiley)

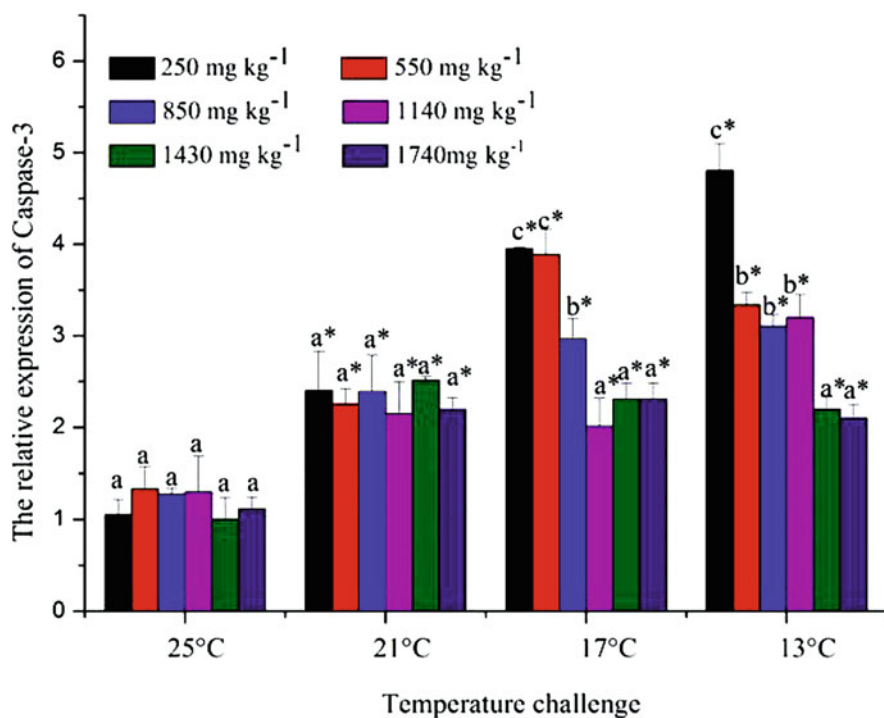


Fig. 11.5 Effects of Tau on relative expression levels of *caspase-3* under low-temperature stress in obscure pufferfish (*Takifugu obscurus*). Note: Data are expressed as mean + SD ($n = 6$). Diverse small letters show significant differences ($P < 0.05$) in different groups at the same time point in Duncan's test. (From Cheng et al. (2018), with permission from Elsevier)

exerts anti-apoptotic action by reduction of *caspase-3* transcription at low temperatures (Fig. 11.5).

The effect of dietary Tau supplementation to aquatic broodstock animals and their offspring is scarcely studied. One of the few papers shows that parental 2.7% dietary Tau increases the yolk sac volume and, thus, survival of newly hatched larvae of the California yellowtail (Salze et al. 2019).

Because Tau is that central in many physiological processes, the question arises whether animals can decide to feed on Tau-rich diets if they have the choice. Angell et al. (2012) offered this choice to ass’s-ear abalone (*Haliotis asinina*) in the form of eight species of brown, red, and green macroalgae varying in nutrient composition. The abalone consistently prefers *Hypnea pannosa* (red alga) and *Ulva flexuosa* (green alga). *H. pannosa* is rich in Tau, but *U. flexuosa* lacks this AA. Furthermore, the richest alga in Tau is *Jania crassa* (red alga) which is less preferred by *H. asinina* than the Tau-free *U. flexuosa*. This shows that no direct link exists between algal Tau content and the preference of *H. asinina*.

Similarly in planktonic freshwater cladocerans: in feeding experiments with three different coccal green freshwater algae, differing in Tau contents, it occurs that the Tau content does not steer life history traits in *Daphnia magna*, *Moina macrocopa*, and *M. micrura* (Bouchnak and Steinberg 2013, 2014). Instead, the contents of the AAs, Arg and His, influence the life span of *Daphnia*, whereas the traits of the *Moina* sp. appear to be independent of individual AAs.

Only a few trials are dedicated to the effects of dietary Tau on physiology and growth in shrimps. These studies indicate that penaeids (*P. monodon*, *L. vannamei*) require this AA for optimal performance during larval and grow-out stages, accelerated larval molting rates, and improved survival rates. However, excess Tau appears to be toxic to *L. vannamei* larvae (El-Sayed 2014). This statement is in good compliance with several recent fish studies showing that excess Tau has adverse effects on the development of fish larvae. Studying the survival rates of larval yellowtail kingfish (*Seriola lalandi*), Partridge and Woolley (2016) assume toxic action of Tau excess.

Also in Persian sturgeon (*Acipenser persicus*), adverse effects of dietary Tau are reported. Increased dietary Tau results in decreased feed intake and reduced growth (Hoseini et al. 2017). The authors assume that the tested Tau levels (0.25–1.6%) exceed the actual demand (Hoseini et al. 2018). Studies with lower dietary Tau levels are pending.

11.2 Taurine and Immunity

Table 11.1 includes several hints that dietary Tau increases innate immunity of aquatic animals. Comprehensive surveys of underlying mechanisms, however, are scarce. In one of their biomolecular studies in young grass carps, Yan et al. (2019) identified the multifaceted role of dietary Tau in the immune function—besides improvement of growth. Dietary Tau

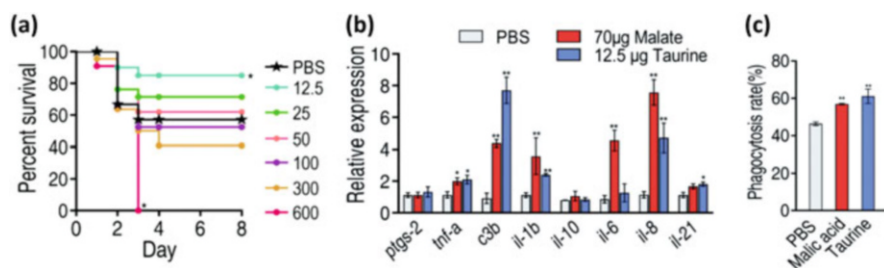


Fig. 11.6 (a) Survival of zebrafish in the presence of graded doses of Tau (in μg), 21 zebrafish each group. (b, c) Expression of innate immunity genes in zebrafish in the presence of 70 μg malate or 12.5 μg Tau (b). Twenty-five zebrafish spleens were collected in each group. Five were pooled as a sample, yielding five biological repeats for analysis of gene expression. Samples were collected in 24 h after the injection of malate or Tau for 3 days. (c) Phagocytosis in the absence or presence of 70 μg malate or 12.5 μg Tau. Macrophages were separated from the head kidney of Nile tilapia and incubated with 20 mM malate or Tau. Then, 1:100 bacterial cells were added. Three biological repeats were performed. Results are displayed as mean \pm SEM, and significant differences are identified (* $P < 0.05$, ** $P < 0.01$) as determined by two-tailed Student's t-test. (From Yang et al. (2020), credit Taylor and Francis). *ptgs-2* prostaglandin-endoperoxide synthase 2 involved in the conversion of arachidonic acid to prostaglandin H₂, an important precursor of prostacyclin, which is expressed in inflammation; *tnf- α* , tumor necrosis factor α , a pro-inflammatory cytokine; *c3b*, part of complement component 3 (\rightarrow box in Chap. 2); *il-1 β* pro-inflammatory interleukin 1 β ; *il-10* anti-inflammatory interleukin 10; *il-6* interleukin 6 pro-inflammatory cytokine and anti-inflammatory myokine; *il-8* pro-inflammatory interleukin 8; *il-21* interleukin 21 induces cell division/proliferation; *PBS* phosphate-buffered saline. Inflammation follows a biphasic stage starting with initiation, where cells are attracted toward the affected side and release pro-inflammatory signal molecules and ROS (Kany et al. 2019). In the resolving phase, anti-inflammatory molecules are released to end the inflammation (Opal and DePalò 2000).

- (a) Strengthens the ability against enteritis.
- (b) Increases antimicrobial compounds.
- (c) Downregulates pro-inflammatory cytokines and upregulates anti-inflammatory cytokines.

In zebrafish, dietary malate triggered a metabolic shift via activating the TCA cycle, leading to elevated Tau production (Yang et al. 2020). To prove the efficiency of Tau, this AA is supplemented. In fact, exogenous Tau increases the survival of zebrafish after *Vibrio alginolyticus* infection (Fig. 11.6a) as malate did as well. Moreover, exogenous Tau and malate regulate the transcription of innate immunity genes (Fig. 11.6b), boost phagocytosis (Fig. 11.6c), and promote the generation of ROS and activated nitrogen oxide. The two metabolites alleviate excessive immune responses to bacterial challenge and protect from bacterial infection. Reprogramming the metabolome with exogenous Tau is also beneficially effective in crucian carp, reared at elevated temperatures (Jiang et al. 2019) (\rightarrow Chap. 26).

11.3 Interactions with Nutrients

An intensive interplay exists between Tau and other AAs, not only with the S-containing AAs. Recently, Candebat et al. (2020) showed that adequate dietary Met spares dietary Tau; and vice versa, insufficient dietary Met induces increased Tau demand in juvenile yellowtail kingfish (*Seriola lalandi*).

In larval Nile tilapia, a number of AAs (Trp, Arg, His, Leu, Ile, Val, Ala, Gly, Thr, and Tau) increase with increasing supplemental Tau up to 10 g kg⁻¹. However, with further Tau increase, body AAs decrease (Al-Feky et al. 2015). This indicates that excessive Tau is excreted to keep body Tau at optimal concentrations. This process is energy demanding and therefore reduced growth occurs. This phenomenon is documented in juvenile Pacific white shrimp (Yue et al. 2013), rainbow trout, and gilthead seabream (Al-Feky et al. 2015). At the first glance, the above explanation seems plausible; however, it is unlikely that the pure excretion without any transformation is that energy demanding that growth retardation results (Steinberg 2012). Therefore, an additional or even alternate mechanistic background for the adverse effect must apply. Excessive dietary Tau can also lead to cessation of growth rates through reducing feed intake, as shown in Japanese flounder (Park et al. 2002) or rainbow trout (Gaylord et al. 2006). Furthermore, Glover and Hogstrand (2002) showed that Tau increases subepithelial zinc accumulation in rainbow trout but decreases the passage of zinc to post-intestinal compartments. Since zinc is both a nutrient and a toxicant of importance and since Tau is the most abundant free AA in intestinal mucosa of fishes (Auerswald et al. 1997), this may be an alternate toxicity mode of action of excess Tau.

11.4 Concluding Remark

Tau can be considered essential to most aquatic animals. However, many details of its metabolism of dietary normal and excess doses have been identified only phenotypically. Dietary excess Tau deserves special attention. Details remain to be proven and abstracted from individual levels by innovative biomolecular approaches. Here, innovative approaches means application of omics techniques not only as sophisticated monitoring tools, for instance, some transcription of genes as weak hint of the underlying pathway; rather, it means to apply these techniques so comprehensive that signaling pathways can be proposed. Furthermore, the role of the intestine microbiota in Tau metabolism has not yet been considered. Combining next-generation sequencing with proteomics or metabolomics will possibly identify comprehensive signaling pathways for growth or reproduction. Moreover, beneficial effects have to be observed if, or how, they translate into succeeding generations.

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Chapter 12

Nonprotein Amino Acids—‘*Fuel at All?*’



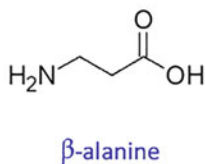
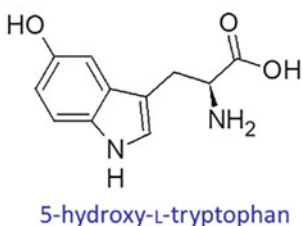
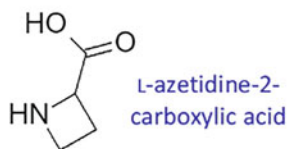
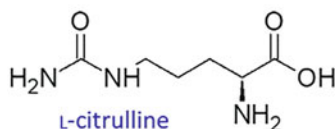
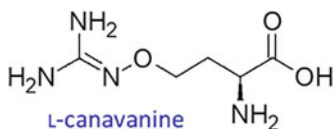
Abstract Several of the numerous nonprotein amino acids (NPAAs) act as repellents, antinutrients, or antimetabolites for herbivores or omnivores. There are first, yet still inconsistent, indications, however, that some NPAAs are metabolites in the immune response. Many dietary trials with NPAAs suffer from the lack of biomolecular fundamentals and remain on the phenotypic level; consequently, inconsistent outcomes are not rare. The microbiota can play a role in reducing adverse effects of the so-called anti-nutritional factors. It can be supported, or even structured, by dietary prebiotics and probiotics, respectively. There are first reports that improving the gut microbiota appears to be a means to overcome toxicity of dietary compounds. This aspect of aquatic animal nutrition has not yet been considered in depth, but it deserves future attention.

In addition to the common L-amino acids (AAs) used for protein biosynthesis and Tau, plants produce numerous nonprotein AAs (NPAAs). More than 600 of such NPAAs have been identified (Rosenthal 1991), e.g., in seeds of various legumes, such as soybean *Glycine max* (Martínez-Villaluenga et al. 2006), a common proteinaceous replacement of fishmeal. NPAAs often function as antinutrients or antimetabolites (Yamane et al. 2010), i.e., they may interfere with the metabolism of herbivores or omnivores (Rosenthal 1991; Wink 2007), but are not toxic to the producers themselves (Fürstenberg-Hägg et al. 2013). Many NPAAs are structural analogs to protein AAs (Fig. 12.1).

12.1 L-Canavanine

L-Canavanine (Can, Fig. 12.1) is found as repellent in certain legumes, such as soybean or lupin. It is structurally related to Arg and, therefore, an agonist of this protein AA. The arginyl-tRNA synthase of most animals cannot distinguish between Arg and Can, resulting in incorporation of Can into proteins leading to deleterious effects (Fürstenberg-Hägg et al. 2013). A transfer RNA is an adaptor molecule, necessary for protein translation.

Nonprotein Amino Acids



Protein Amino Acids

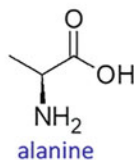
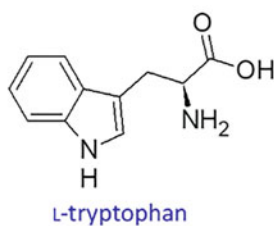
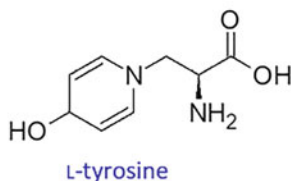
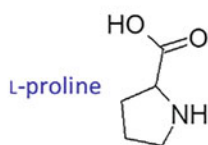
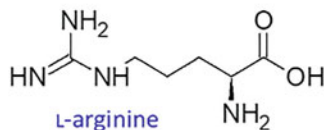


Fig. 12.1 Structures of selected nonprotein amino acids and their protein analogs

Mozambique tilapia fed diets containing Can exhibit depressed appetite, lethargic movements, and high mortality (Martinez-Palacios et al. 1988). Cracked legume seeds, such as jack beans (*Canavalia ensiformis*), containing Can can be detoxified using wet thermal methods and then serve as protein source for Nile tilapia

(Fagbenro et al. 2007). North African catfish (*Clarias gariepinus*) appears to be less sensitive to raw jack bean meal than tilapia; its fingerlings tolerate up to 20% replacement of soybean meal by jack bean meal without adverse effects on growth and nutrient utilization (Solomon et al. 2018).

12.2 L-Citrulline

L-Citrulline (Cit, Fig. 12.1) originates from the urea cycle and is a byproduct of the enzymatic production of nitric oxide from Arg (Buentello and Gatlin III 2001). It does not appear to have direct dietary significance; nevertheless, Fauzi et al. (2020) recently found that dietary Cit improves survival of rainbow trout juveniles challenged with *Vibrio anguillarum*—even better than dietary Arg or Orn (Fig. 12.1). Obviously, dietary Cit strengthens innate immunity by increasing *inducible nitric oxide synthase (inos)* expression in the kidneys (Fig. 12.2). iNOS binds calmodulin¹ at physiologically relevant concentrations and produces free ·NO as immune defense mechanism. In contrast, Clark et al. (2020) showed also in rainbow trout that, although dietary Cit supplementation increased circulating Arg levels, it has only little effects on improving the immune response or survival to pathogen challenge. This contradiction remains unresolved.

As immunostimulant, citrulline reveals mixed results. Supplementation of dietary Cit induces immunostimulation in gilthead seabream (*Sparus aurata*), particularly at the 1% inclusion level (Ramos-Pinto et al. 2020). However, European seabass (*Dicentrarchus labrax*) does not respond to dietary Cit different from the control if infected with *Photobacterium damsela* (Fig. 12.3) (Azeredo et al. 2020). The response appears to be host- and/or pathogen-specific.

12.3 Mimosine

As alkaloid, mimosine (β -3-hydroxy-4 pyridone, Mim, Fig. 12.1) is toxic. It is chemically similar to Tyr and displays deleterious activities, such as disruption of reproduction and teratogenicity. Mim is present in *Leucaena leucocephala* (Mimosaceae, used as livestock fodder) leaves and seeds. Dietary *Leucaena* leaf meal is not well tolerated by fishes. Without any pretreatment (cooking, drying) of *Leucaena* leaves, growth reduction is common as shown in Nile tilapia on 0.5–1.6% Mim (Wee and Wang 1987), common carp on up to 6% Mim (Ter Meulen and El-Harith 1983), or Mozambique tilapia (no data on Mim content available) (Jackson et al. 1982). Nile tilapia studies indicate that *Leucaena* leaf proteins are poorly

¹Calmodulin acts within the calcium signal transduction pathway by modifying its interactions with various target proteins such as kinases or phosphatases (Nelson and Cox 2005).

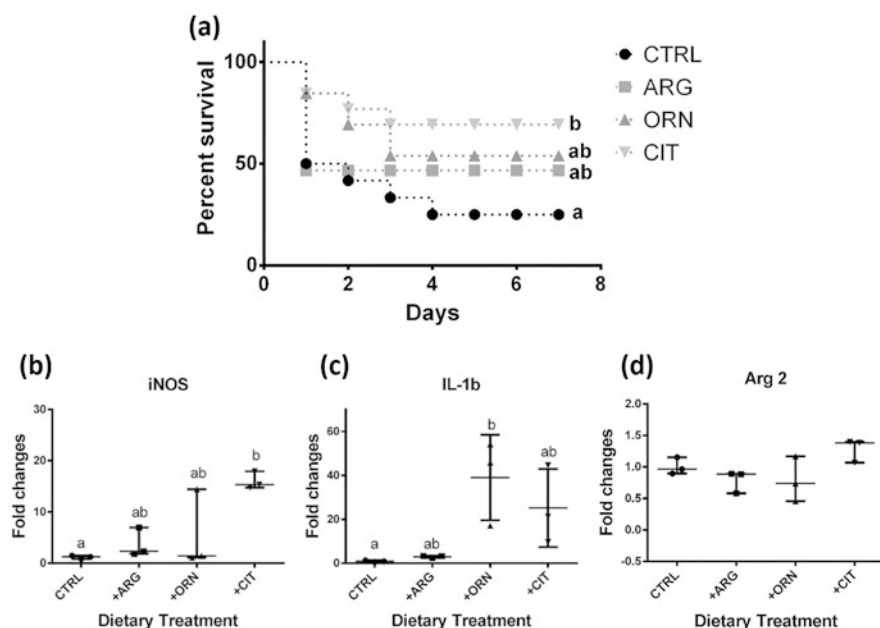


Fig. 12.2 (a) Survival of rainbow trout pre-fed with supplemental level of arginine, ornithine, and citrulline upon *Vibrio anguillarum* challenge through peritoneal injection. Disease challenge was performed for 7 days. Analysis was conducted using log-rank test ($P = 0.0211$). Relative expression of inducible nitric oxide (*inos*) (b), interleukin-1-beta (*il-1b*) (c), and arginase 2 (*arg 2*) (d) in the kidney at 24 h after disease challenge with *Vibrio anguillarum* after 30 days fed with dietary supplementation of arginine ($P = 0.0165$). (From Fauzi et al. (2020), with permission from Elsevier). Arginase catalyzes the hydrolysis of arginine to ornithine and urea. In the intestine of rainbow trouts, *arginase II* is upregulated when excessive dietary arginine is given (Fauzi et al. 2019). Arginine is one important compound in activation of macrophage. The described immune response in rainbow trout does obviously not use this pathway, since *arg2* transcription is not influenced by dietary Arg, ornithine, or citrulline

digested. Furthermore, tilapia males seem to tolerate Mim better than females; the underlying mechanism remains unclear. Production of fry is reduced beyond the 40% inclusion level (Santiago et al. 1988). Cataracts, as observed in livestock (Holmes et al. 1981), are not yet found in fishes on Mim diets (Wee and Wang 1987).

Again, the North African catfish appears to be less sensitive than tilapia: Verma et al. (2018) reported that, although growth is reduced, humoral immunity and resistance to pathogens are improved in individuals fed diets with 33% *L. leucocephala* seed powder. This indicates that more than one life history trait is necessary for a robust effect evaluation.

Shrimps, such as *Macrobrachium rosenbergii* and *Penaeus monodon*, are not adversely impacted by dietary pretreated *Leucaena* leaves (Vogt et al. 1986). The authors fed postlarvae with formulated diets containing either soaked or unsoaked

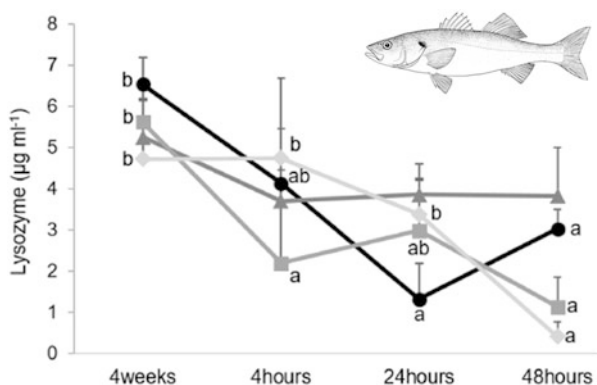


Fig. 12.3 Plasma lysozyme content in European seabass fed CTRL (●), ARG1 (0.5%■), ARG2 (1.0%▲), and CIT (0.5%◆) after i.p. infection with *Photobacterium damsela* subsp. *piscicida* (means \pm SD, $n = 6$). Different letters indicate significant differences attributed to dietary treatment. (Nested ANOVA; Tukey post hoc test; $P \leq 0.05$). (From Azeredo et al. (2020), with permission from Elsevier; image credit FAO)

Leucaena leaves. Weight gain and survival of shrimps fed with both *Leucaena* diets slightly increase but do not differ from the soybean controls.

Bairagi et al. (2004) applied a promising approach to feed *Leucaena* material to rohu (*Labeo rohita*) fingerlings, in order to overcome its toxic effects: the authors added specific strains of fish intestinal bacteria. *Bacillus subtilis* (isolated from *Cyprinus carpio*) and *B. circulans* (isolated from *Oreochromis mossambicus*), having extracellular cellulolytic and amylolytic activities, are inoculated with *Leucaena* leaf meal for 15 days at 37 °C. The Mim content decreases, whereas free AAs and fatty acids (FA) increase in the treated leaf meal. Now, the inclusion of *Leucaena* leaf meal improves growth. Diet formulated with 30% *Leucaena* leaf meal inoculated with *B. circulans* results in best growth.

12.4 γ -Aminobutyric Acid (GABA)

GABA (Fig. 12.4) is the decarboxylation product of glutamate and acts as neurotransmitter in brains. Upon graded dietary GABA, life history traits of juvenile grass carps react in an optimum response curve (Wu et al. 2016). The specific growth rate increases with dietary GABA levels up to 100 mg kg⁻¹ diet and then declines on 200 mg kg⁻¹ dietary GABA. Diets supplemented with 50 to 100 mg kg⁻¹ GABA enhance also antioxidant status and the transcription of neuropeptide Y and ghrelin in the brain. Similarly, GABA improves growth, serum total protein, and superoxide dismutase activity in juvenile Nile tilapia with an optimum dietary GABA level of ~160 mg kg⁻¹ (Temu et al. 2019). With ~100 mg kg⁻¹, a slightly lower optimum dietary GABA level is determined in Jian carps (Chen et al. 2021), which is indicated

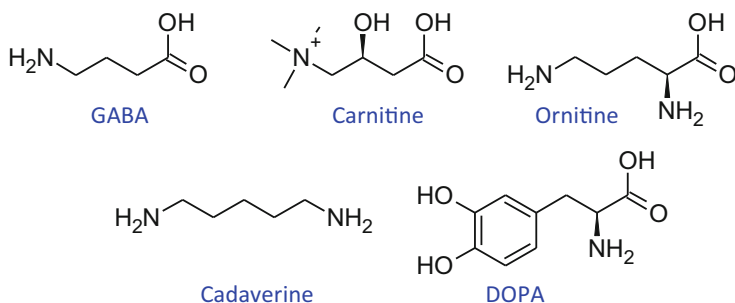


Fig. 12.4 γ-Aminobutyric acid (GABA), L-carnitine, ornithine, cadaverine, and dihydroxyphenylalanine (DOPA)

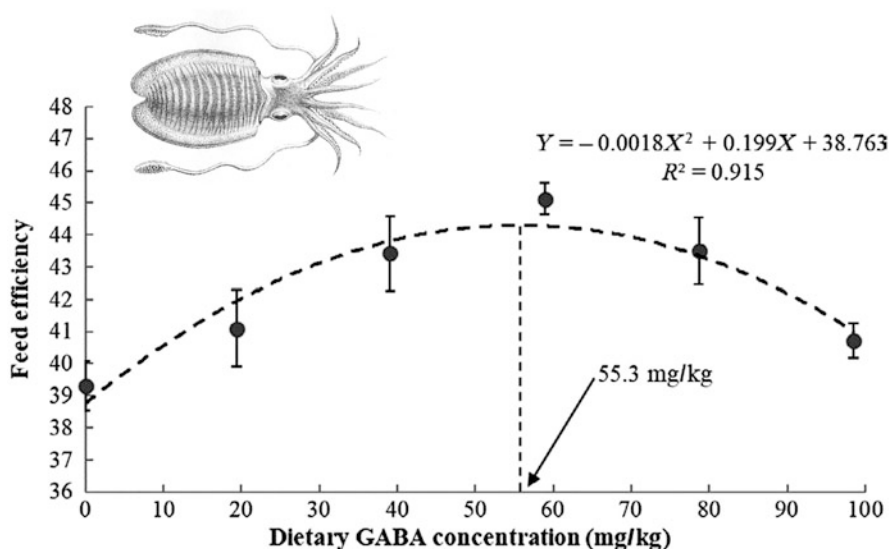


Fig. 12.5 Quadratic regression analysis of feed efficiency to dietary GABA concentration for *Sepia pharaonis*. (From Li et al. (2020a), with permission from Wiley; cuttlefish dorsal view credit FAO)

by reduced transcription of pro-inflammatory and increased transcription of anti-inflammatory genes as well as optimal lysozyme and alkaline phosphatase activities and nitric oxide content.

GABA is functional also in invertebrate nutrition. Xie et al. (2017) found that GABA supplementation improves growth and ammonia stress tolerance of juvenile *Litopenaeus vannamei* on low fishmeal diet. This is due to enhanced antioxidant and immune response. Shrimps on 150 mg kg⁻¹ GABA diet show best growth, antioxidative capacity, and survival after ammonia stress.

Supplementary GABA improves survival rate and growth performance also in pharaoh cuttlefish (*Sepia pharaonis*) (Fig. 12.5) to a certain range that coincides with

the improvement in serum nonspecific immunity and antioxidative capacity. However, GABA excess adversely affects traits. Optimal dietary GABA is estimated to be 55 mg kg⁻¹ (Li et al. 2020a). Dietary excess induces adverse effects also in other farmed animals, such as inhibition of feed intake in Japanese flounder (Kim et al. 2003) or growth reduction in *M. rosenbergii* postlarvae (Chettri et al. 2007).

12.5 L-3,4-Dihydroxyphenylalanine (L-DOPA)

L-DOPA (Fig. 12.4) is a neurotransmitter and transformation product of Tyr (→Chap. 8). DOPA has been studied as chemical cue for planktonic invertebrate larvae to settle and metamorphose, rather than as organic nutrient. These studies seem to lack field relevance.

12.6 L-Carnitine

L-Carnitine (Car, Fig. 12.4) is a Lys derivative and involved in lipid metabolism. It is required for the transport of long-chain FAs into mitochondria; hence, L-Car is essential in normal oxidative metabolism as well as in the formation of ketone bodies (Brody 1999). In contrast, D-Car is toxic because it inhibits the activity of L-Car (Bremer 1983). Furthermore, dietary D-Car induces lipotoxicity, hepatic inflammation, oxidative stress, and apoptosis in Nile tilapia (Li et al. 2019a).

Benefits of appropriate dietary Car include growth promotion. However, excess Car results in growth reduction with oxidative stress as one underlying mechanism, indicated by lipid peroxidation (Ma et al. 2008); further mechanisms may be inhibition of enzyme systems and/or loss of energy while excreting excess Car (Jayaprakas and Sambhu 1998). Regulating pathways, however, deserve future studies on the omics level.

To figure out the systemic regulation by dietary L-Car, Li et al. (2017) used zebrafish and found that L-Car decreases the lipid contents in the liver and muscle, accompanied by increased concentrations of total and free Car in the tissues. In addition, L-Car enhances mitochondrial β -oxidation and the transcription of *carnitine palmitoyltransferase 1*,² whereas it depresses the transcription of adipogenesis-related genes. In Nile tilapia, dietary L-Car supplementation efficiently stimulates lipid catabolism and increases glycogen and protein deposition in muscles (Li et al. 2020b).

Car has often been fed as growth promoter and antidote (Table 12.1). Nunes et al. (2014) found histopathological traits in Car-fed rainbow trouts. This study is in

²Carnitine palmitoyltransferase 1 is a mitochondrial enzyme responsible for the transfer of acyl groups of a long-chain fatty acyl-CoA from coenzyme A to L-carnitine (Bonfont et al. 2004).

Table 12.1 Dietary L-carnitine impacts life history traits in selected aquatic animals

Species, common name	Dietary level, %	Life history trait	References
<i>Astacus leptodactylus leptodactylus</i> , narrow-clawed crayfish	0.001→0.125	Optim SGR, LP, PER @ 0.078–0.093	Safari et al. (2015)
<i>Litopenaeus vannamei</i> , white Pacific shrimp	0.01	Apoptosis↓	Zhou et al. (2016)
<i>Macrobrachium rosenbergii</i> , giant river prawn	0→0.1	Optim WG, SGR, FCE @ 0.05	Singh et al. (2008b)
<i>Acanthopagrus schlegelii</i> , blackhead seabream	0.01→0.11	Optim WG @ 0.028, LPO↓	Ma et al. (2008)
	0.03	WG↔, FCE↔, inflammatory response↓	Jin et al. (2019)
<i>Bidyanus bidyanus</i> , silver perch	0.04	WG↑, body lipids↓	Yang et al. (2012)
<i>Cirrhinus mrigala</i> , mrigal carp	0→1	Max WG @ 0.25	Singh et al. (2008a)
<i>Clarias batrachus</i> , Philippine catfish	0→0.1	Optim WG, SGR, FCE, PER @ 0.05	Desai et al. (2010)
<i>C. gariepinus</i> , African catfish	0.013→0.39	WG↑, protein sparing?	Torrelee and Verreth (1993)
	0, 0.2	WG↔, FCE↔, PER↔	Yilmaz et al. (2004)
	0.02, 0.1	WG↔, FA oxidation↑, AA oxidation↓	Ozório et al. (2001, 2002)
	0.0015, 0.1	Crowding stress resistance↑	Ozório et al. (2005)
<i>Cyprinus carpio</i> , common carp	0.02→0.06	Max FCR, SGR, PER @ 0.04 and 0.06	Focken et al. (1997)
	0.05→0.2	Resistance against microcystin↑	Chen et al. (2017)
	0→0.1	WG↔, LPO↓	Sabzi et al. (2017)
<i>Danio rerio</i> , zebrafish	0.05	Hepatic <i>mTOR</i> ↑, glycolysis↓	Li et al. (2017)
<i>Dicentrarchus labrax</i> , European seabass	100→250 mg kg ⁻¹ d ⁻¹	SGR↑, body lipids↓, FA oxidation↑	Santulli and D’Amelio (1986),
	1.5 g L ⁻¹	Leu incorporation↑	Santulli et al. (1990)
	0.03→0.3	WG↔, FCE↔, PER↔, body lipids↔	Dias et al. (2001)
<i>Etroplus suratensis</i> , pearlspot	0.075	WG↑, body lipids↓, lipase↑, protease↑	Jayaprakas and Sambhu (1998)
<i>Huso huso</i> , beluga	0→0.12	Optim WG @ 0.03, protein sparing	Mohseni et al. (2008)
	0.005→0.125	Optim WG @ 0.035–<0.065	Mohseni and Ozório (2014)
	0, 0.03	WG↑, FCE↑	Mohseni et al. (2014)

(continued)

Table 12.1 (continued)

Species, common name	Dietary level, %	Life history trait	References
<i>Ictalurus punctatus</i> , channel catfish	0, 0.1	WG↔, body lipids↓, body protein↑	Burtle and Liu (1994)
<i>Ictalurus punctatus</i> ♀ × <i>I. furcatus</i> ♂, hybrid catfish	0.05	Body lipids↓	Li et al. (2007)
<i>Labeo rohita</i> , rohu carp	0→0.1	WG↑, body lipids↓, lipase↑, protease↑	Keshavanath and Renuka (1998)
<i>Morone saxatilis</i> ♂ × <i>M. chrysops</i> ♀, hybrid striped bass	0.002→0.037	WG↑, body lipids↔	Twibell and Brown (2000)
	0, 0.3	WG↔	Gaylord and Gatlin III (2000)
<i>Mugil cephalus</i> , flathead grey mullet	0→0.12	IGF-1↑, optim WG, SGR, FCE@0.08	Akbary (2019)
<i>Oncorhynchus mykiss</i> , rainbow trout	0→0.053	Histopathological disorder↑	Nunes et al. (2014)
	0→0.045	WG↔	Schuhmacher and Gropp (1998)
	0, 0.05	WG↔, FCE↔	Selcuk et al. (2010)
	0, 0.03, 0.06	WG↑, FCE↑	Dikel et al. (2010)
	0→0.053	Plasma osmolality↔	Ozório et al. (2012)
	0→0.053	Nutritional metabolism↔	Gonçalves et al. (2018)
<i>Oreochromis niloticus</i> , Nile tilapia	1.25	Pathogen resistance↑	Lu et al. (2019)
	0, 0.04, 0.088	Cyanotoxin resistance↑	Guzmán-Guillén et al. (2017)
<i>O. niloticus</i> , GIFT, genetically improved farmed Nile tilapia	0→0.08	Body protein↑, body lipid↓	Chen et al. (2010)
<i>O. niloticus</i> × <i>O. aureus</i> , hybrid tilapia	0, 0.015, 0.03	Optim WG, FCE @ 0.015	Becker et al. (1999)
	0, 0.015, 0.045	Optim MXR @ 0.015	Schlechtriem et al. (2004)
	0, 0.025	WG↔, FCE↔	Yang et al. (2009)
<i>Pagrus major</i> , red seabream	0.0075→0.42	Optim WG, FCE @ 0.021, free hepatic FA↓	Chatzifotis et al. (1995)
<i>P. pagrus</i> , red porgy	0.0046, 0.063	WG↔, lysis of the spleen melanomacrophages↑	Nogueira et al. (2010)
<i>Pelvicachromis pulcher</i> , rainbow krib	0→0.2	Optim cold tolerance @ 0.1	Harpaz et al. (1999)
<i>Poecilia reticulata</i> , guppy	0, 0.11	Anionic xenobiotic resistance↑	Schreiber et al. (1997)
	0, 0.11	Fry production↔	Dzikowski et al. (2001)

(continued)

Table 12.1 (continued)

Species, common name	Dietary level, %	Life history trait	References
<i>Rhynchocypris lagowskii</i> , Amur minnow	0.005→0.11	Optim antioxidant capacity 0.04–0.075	Wang et al. (2019)
	0, 0.05, 0.1	Inflammation↓	Zhang et al. (2019)
<i>Scophthalmus maximus</i> , turbot	0.004, 0.024	WG↔, ammonia excretion↓, pathogen resistance↑	Gonçalves et al. (2010); Dias et al. (2011)
<i>Tachysurus (Pelteobagrus) fulvidraco</i> , yellow catfish	0.03, 0.35	Lipogenic enzymes↑, body lipids↑	Zheng et al. (2014)

↑ increase/support; ↔ no apparent effect; ↓ decrease/reduction; *LP* lipid production; *LPO* lipid peroxidation; *WG* weight gain; *SGR* specific growth rate; *FCE* feed conversion efficiency; *PER* protein efficiency ratio; *FA* fatty acid; *AA* amino acid; *MXR* multixenobiotic resistance (transporter)

contrast to other ones in the same species (Table 12.1) and discusses the abovementioned oxidative stress as explanation. Furthermore, increased lysis of melanomacrophages and necrosis of acinar cells³ are observed in the spleen of red porgy on Car diet indicating accelerated catabolic tissue breakdown (Nogueira et al. 2010). Recently, Li et al. (2019b) elucidated the reasons for such conflicting results and summarized that the nutritional physiology studies on dietary L-Car in aquatic animals focus on using growth and body composition as primary response traits. The regulatory pathways of L-Car in energy metabolism, however, remain undisclosed, and the effective application of dietary L-Car is impeded. The same concern applies to studies in crustaceans. Despite the available studies, from which a few are listed in Table 12.1, it is obvious that the effect of dietary L-Car in crustacean metabolism and energy utilization reveals contradicting results (Li et al. 2019b) that wait to be unveiled.

Furthermore, Table 12.1 indicates a protective effect of dietary Car from biotic and abiotic stressors. Although the mechanisms of L-Car metabolism in immune functions have not been studied in detail, the anti-inflammatory effects of L-Car and its ability to improve FAs β-oxidation may be a promising tool to increase stress resistance in fishes (Li et al. 2019b).

12.7 L-Ornithine

L-Ornithine (Orn, Fig. 12.4) is a basic nonprotein AA in the urea cycle. Orn has an antifatigue effect in increasing the efficiency of energy consumption. Therefore, it is not surprising that a substantial decrease in plasma Orn concentrations can be

³ Acinar cells arise from a population of multipotent progenitor cells that also produce ductal cells, which channel the acinar secretions to the intestine, and endocrine cells, which populate the islets of Langerhans (MacDonald et al. 2010).

observed as in Senegalese sole subjected to acute stress (Aragão et al. 2008). Moreover, Orn induces the transcription of selected immunity genes as found in rainbow trouts (Fig. 12.2) (Fauzi et al. 2020).

12.8 Cadaverine

Cadaverine (1,5-pentanediamine, Cad, Fig. 12.4) is a diamine and toxic to many organisms. It is a decarboxylation product of Lys during putrefaction of animal tissue, such as fishmeal staling. Contrary to expectations, Tapia-Salazar et al. (2001) reported that combined supplementation of Cad and His can enhance feed consumption and growth of blue shrimp (*Litopenaeus stylirostris*). Tapia-Salazar et al. (2004) argued that this effect is not due to increased contents of biogenic amines in the fishmeal made from stale fish but caused by hitherto unidentified compounds.

12.9 β -Alanine

β -Alanine (Fig. 12.1, 3-aminopropanoic acid) is a product of aspartate decarboxylation. Besides its anti-nutritional action—it inhibits the taurine transporter, it is a constituent of several oligopeptides, such as carnosine (β -alanyl-L-histidine), anserine (β -alanyl-1-methyl-L-histidine), carbinine (β -alanyl-histamine), and balenine (β -alanyl-3-methyl-L-histidine). Carnosine and anserine are antioxidants and important proton buffers in skeletal muscle of aquatic animals, especially migratory pelagic marine fishes including salmon. Elevated levels of β -alanine in plasma or tissue in response to acute handling stress or an environmental change occur in fishes, clams, and oysters; however, the physiological significance of this finding has not been well elucidated (Li et al. 2009; Koyama et al. 2015).

To improve the proton buffering capacity of yellowtail, Ogata (2002) provided diets supplemented with β -alanine; as a consequence, the intramuscular concentrations of carnosine, but not of anserine, increase. Kim et al. (2003) did not find improved growth of Japanese flounders on β -alanine (1% supplementation) diet. In common carps, however, β -alanine tends to increase body weight and feed intake, whereas carnosine and anserine contents in the blood do not change confirming that carp belongs to a species that does not store His-containing dipeptides (Geda et al. 2014).

12.10 Concluding Remarks

Several times, this chapter has pointed out missing or not fully convincing mechanistic explanations for described effects, particularly for adverse effects of dietary excess NPAAAs in fishes. The explanations of oxidative stress or enzyme inhibition

are traditional and reflect the knowledge of some 20 years ago; and it can be argued that this covers only part of the underlying modes of action. Consequently, this outdated knowledge has to be upgraded by actual “omics” techniques, all the more as with Mim contradictory results with growth and immunity response after pathogen challenge are reported (Verma et al. 2018). An updated evaluation of these effects will solve such contradictions. Furthermore, improved understanding of adverse effects and their modes of action will provide a platform to countermeasures.

Organisms treat some NPAAAs as xenobiotic chemicals. This means that these AAs are subject of the biotransformation pathway (Steinberg 2012). The xenobiotic effect, however, is not an intrinsic, constant property of the chemical itself, such as vapor pressure or melting point; rather it is the result of the interaction between the chemical and the organism. Moreover, the organisms show distinct circadian rhythmicity of the biotransformation activity with resulting rather high and rather low sensitivity to the chemical exposed to, depending on the daytime. If a so-called anti-nutritional compound is fed during the period of high biotransformation activity and less sensitivity, the adverse effect should be minimized (→AANI “Chrononutrition” (Steinberg 2018)). Unfortunately, fishes and aquatic invertebrates are not well studied in this respect; therefore, appropriate time points, when to provide NPAA-containing feed cannot yet be derived. Although in a recent transcriptomic inventory of genes transcribed during daytime or nighttime, Sun et al. (2020a) identified several differently expressed genes in charge of xenobiotic biodegradation and metabolism in *Pseudobagrus (Pelteobagrus) vachellii*; the authors do unfortunately not discuss these findings.

Also with Mim, indications are accelerating that the intestinal microbiota plays a central role, directly or indirectly, in reducing adverse effects of the so-called anti-nutritional factors. The microbiota can be supported, or even structured, by dietary prebiotics and probiotics, respectively. This aspect of aquatic animal nutrition has not yet been considered in empirical trials, but it deserves future attention. Appropriate role models are available for fishes (Haygood and Jha 2018; Tapia-Paniagua et al. 2019) and invertebrates (Sun et al. 2020b).

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Chapter 13

Carbohydrates with Emphasis on Glucose—‘*Life’s Little Luxury*’



Abstract This chapter is an inventory of several basic features of carbohydrate metabolism mainly in fishes. Carbohydrates are the most abundant class of energy-yielding nutrients. High digestibility of monosaccharides but slow growth is often observed in fishes. The extent to which dietary carbohydrates can be used to meet energy requirements varies greatly between and even within species, depending on feeding habit, exercise, genotype, dietary carbohydrate source and level, feeding strategies, and environmental factors. Several hypotheses about potential modes of action have been put forward which are mentioned in brief. Although no real dietary requirement for carbohydrates has been demonstrated in fishes, certain species exhibit reduced growth rates when fed carbohydrate-free diets. Protein sparing by dietary carbohydrates can be achieved without any adverse effects on growth and physiology. Carnivorous fishes are considered poor in utilizing dietary carbohydrates; oral glucose tolerance tests result in persistent hyperglycemia. Nevertheless, these fishes possess almost all essential biological elements of potential carbohydrate utilization, but with differences in the regulatory mechanism. A compilation in this chapter demonstrates that even carnivorous species can tolerate or even use carbohydrates to some extent. In functional metabolomic studies, evidence is emerging that carbohydrates, including glucose, can enhance innate immune response and disease resistance even in carnivorous fishes and invertebrates. Moreover, it is likely that fishes possess a glucosensing system and the current knowledge is sketched.

Carbohydrates are the most abundant class of energy-yielding nutrients and, therefore, a ubiquitous fuel in biology. They are utilized in most organisms, ranging from bacteria to mammals—and are life’s little luxury for many human beings; but also for fishes and aquatic invertebrates? Quite the contrary, it is generally accepted that fishes and invertebrates do not have a specific requirement for dietary carbohydrates. Regardless of species, they are able to survive and grow on diets without dietary carbohydrates (NRC 2011). Rather, dietary carbohydrate can induce adverse effects on growth and immunity even in herbivores. Even more confusing, some carbohydrates are beginning to be understood as regulatory molecules—an intriguing issue.

Monosaccharides are classified according to three different characteristics:

- The placement of its carbonyl group.
- The number of carbon atoms.
- Its chiral handedness.

If the carbonyl group is an aldehyde, the monosaccharide is an aldose; if the carbonyl group is a ketone, it is a ketose. Monosaccharides with three carbon atoms are called trioses, those with four are tetroses, five are pentoses, six are hexoses, etc. (Campbell et al. 2006). Furanose is a collective term for carbohydrates that have a chemical structure that includes a five-membered ring system consisting of four carbon atoms and one oxygen atom, whereas pyranose has a chemical structure that includes a six-membered ring consisting of five carbon atoms and one oxygen atom.

13.1 Carbohydrate Digestibility

Carbohydrate digestibility in fishes decreases with increasing structural complexity (Furuichi and Yone 1982; NRC 2011). Mono- and disaccharides show high digestibility in all fish species, fairly independent of the dietary habit (Storebakken et al. 1998). Some figures: In red seabream, the percent absorption of dietary carbohydrates 2 h after diet administration is about 95% for glucose, 65% for dextrin, and only 4% for starch (Furuichi and Yone 1982). Comparable in yellowtail: About 90% of glucose is absorbed within 2 h after administration, whereas the absorption of starch is only about 10% (Furuichi et al. 1986). In white sturgeon, a higher uptake rate of glucose than of starch is observed after oral intubation of various carbohydrate forms (Deng et al. 2001). A similar result is obtained in Amur sturgeon (*Acipenser schrenckii*) (Jiang et al. 2014); its apparent digestibility of dietary carbohydrates is fructose, glucose > maltose > dextrin > α -starch > cornstarch. However, the growth of Amur sturgeon fed a glucose diet is poor compared to individuals fed dextrin or α -starch diet. One reason for the poor glucose utilization may be that glucose requires no digestion (Dabrowski and Guderley 2003): glucose is present at gut absorption sites faster and in higher concentrations than more complex carbohydrates and excess absorbed glucose may be cleared from circulation before cells can utilize it (Lin et al. 1997; Dabrowski and Guderley 2003). Another reason may be a low glucose regulation capacity, if the fishes are not adapted to moderate dietary levels. This underlines a need for a relatively moderate glucose intake in order to reach basal levels between feedings (Hemre et al. 2002b). Quick assimilation of dietary glucose can be detrimental to fish growth and health (Stone 2003).

High digestibility but slow growth is also observed in white sturgeon (*A. transmontanus*) fed maltose and fructose diets. This is due to low intestinal absorption and poor conversion of the absorbed fructose and maltose to glucose (Hung 1991), while the other sources can be readily hydrolyzed to glucose in the glycolytic pathway. Low fructose absorption and conversion to glucose is also reported for channel catfish (Wilson and Poe 1987). There are indications that utilization of carbohydrates, at least in the sturgeons, is more affected by the feeding

frequency than by the carbohydrate source itself. Continuous feeding proves superior in beluga (*Huso huso*) (Falahatkar et al. 2019) and Siberian sturgeon (Kolman and Kapusta 2018).

Three decades ago, Hung et al. (1989) summarized that the differences in utilizing different carbohydrate sources among fish species result from the different levels of dietary carbohydrate sources and proteins. This assumption sounds like an early, premature version of the “nutrient-specific foraging” theory which was presented and discussed in Chap. 2 (Mayntz et al. 2005; Sørensen et al. 2010). Simply, glucose goes with protein, whereby dietary protein contents determine the amounts of carbohydrates used. For instance, Bergot (1979) reported that rainbow trout can effectively utilize 30% glucose in a 45% protein diet; however, 30% glucose in a 30% protein diet has adverse effects on growth and feed efficiency.

Farmed carnivorous fishes on high-carbohydrate diets tend to increase adiposity, but it remains unclear if this happens by increased lipid accumulation or promotion of lipogenic pathways, or both (Viegas et al. 2016). In order to determine the response of extrahepatic tissues to dietary starch, Viegas et al. (2019) fed European (*Dicentrarchus labrax*) and Asian (*Lates calcarifer*) seabass a control (low starch; LS) or an experimental (high starch; HS) diet. In both species, fatty acid (FA) profiles differ substantially between muscle and visceral adipose tissues. In European seabass, HS promotes de novo lipogenesis of triacylglycerol-bound FA in both adipose tissues. In Asian seabass, however, HS has no effect on muscle FA composition and lipogenic flux, whereas visceral adipose tissue reveals a strong enhancement of de novo lipogenesis in HS-fed fish along with high triacylglycerol-bound glycerol cycling (Viegas et al. 2019).

These few examples exemplify that the extent to which dietary carbohydrates can be used to meet energy requirements varies greatly between and even within species, depending on a multitude of factors. They can be categorized into those related to biology (feeding habit, exercise, genotype, epigenotype), diet (carbohydrate source—plant origin, molecular complexity, physical state; inclusion level, and feeding strategies; nutrient interactions), and the environment (temperature, salinity, stress, photoperiod). The complex interaction of all these factors plays an important role in determining the capacity of the fishes to use a carbohydrate-rich meal. The maximum dietary inclusion level and appropriate source is generally decided based on the protein sparing effect of carbohydrates without adverse effects on growth and physiology of fishes (Kamalam et al. 2017). The protein sparing issue will be revisited in detail in Chap. 17.

Warmwater fishes are able to utilize higher levels (25–55%) of dietary carbohydrate than cold-water or marine fishes (≤ 10 –20%). Although no real dietary requirement for carbohydrates has been demonstrated in fishes, certain species exhibit reduced growth rates when fed carbohydrate-free diets. An appropriate level of carbohydrate in the diet for fishes prevents other nutrients, such as protein and lipids, from metabolism for energy (Wilson 1994).

Carnivorous fishes are considered to be poor in utilizing dietary carbohydrates mainly due to their low intestinal glucose uptake rates and slow blood glucose clearance; therefore, carbohydrates are by no means “little luxury” to them. They have been subject to many studies focusing on carbohydrate nutrition and metabolism, which overwhelmingly indicate the presence of almost all the essential

biological elements of potential carbohydrate utilization, but with differences in the regulatory mechanism (Kamalam et al. 2017).

As mentioned, the relative use of dietary carbohydrates appears to be associated with the complexity of the carbohydrate as one major reason. Certain fish species utilize simple sugars equally well as, or better than, complex carbohydrates, whereas other species do not utilize simple sugars as an energy source. Most species utilize cooked starch better than raw starch. Glucose tolerance tests with omnivorous fishes show reduced glycemic increases that exist for a shorter period of time than in carnivorous fishes confirming a more efficient use of glucose by these species (Fig. 13.1) (Polakof et al. 2012).

This prolonged hyperglycemia and the relative inability of fishes to utilize high levels of dietary carbohydrates have been assumed the result of low levels of endogenous insulin as one mode of action. In other words, insulin secretion is thought insufficient in fishes and suspected to take place significantly later than absorption of glucose, resulting in poor regulation of glucose metabolism. However, studies show that insulin levels in fish are similar to, or often even higher than, those observed in mammals, thus indicating that fishes are not “diabetic” as previously thought (Wilson 1994). Some more recent examples of carbohydrate effects in species of different trophic habits are collected in Table 13.1. They demonstrate that even carnivorous species can tolerate or even use carbohydrates to some extent.

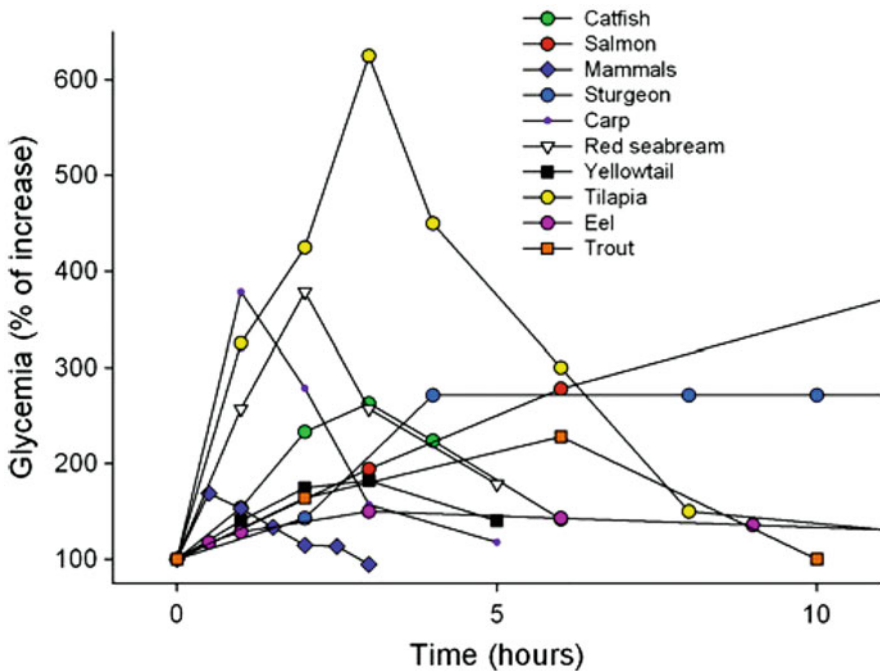


Fig. 13.1 Changes in glycemia in different species of fish and mammals subjected to an oral glucose tolerance test. The X-axis was cut to show better the initial changes in glycemia. The time for values to return to baseline for salmon was more than 40 h and in sturgeon, eel, and tilapia 24 h (From Polakof et al. (2012), with permission from Springer Nature)

Table 13.1 Various dietary carbohydrate forms and doses affect life history traits, from phenotypic to biomolecular level, of selected aquatic animals of different trophic habits

Species, common name, trophic habit	Carbohydrate		Affected trait	References
	Form	Level, %		
Nvertebrates				
<i>Apostichopus japonicus</i> , Japanese sea cucumber, d, o	Cornstarch, replacing macroalgae	0, 5, 10, 20, 30, 40	Replacement ≤30: WG↔	Wen et al. (2018)
<i>Babylonia areolata</i> , spotted babylon, o–h	Wheat starch	5 → 30	Optim WG @ 27, glycolysis↑, lipogenesis↑	Zhang et al. (2009)
<i>Farfantepenaeus duorarum</i> , northern pink shrimp, p, o	Cornstarch, glucose	0 → 40	WG, survival: Starch>glucose	Sick and Andrews (1973)
<i>Haliotis discus</i> , disk abalone, h	Dextrin	41 → 49	Optim WG, SGR, survival @ 47–49C	Lee et al. (2019)
<i>Jasus edwardsii</i> , southern rock lobster, p, c	Dextrin, carboxymethyl cellulose, native wheat starch, gelat cornstarch, native potato, cornstarch	39	Optim growth with carboxymethyl cellulose, wheat starch	Simon (2009)
<i>Macrobrachium nipponense</i> , oriental river prawn, o, c	Cornstarch	5 → 35	Optim WG @ 5–15; <i>hk</i> ↑, <i>pk</i> ↑, hemolymph glucose↑ @ 35	Ding et al. (2017)
	Raw cornstarch, gelat cornstarch, dextrin, maltose, glucose, or cellulose	18	Optim WG @ starch, dextrin, maltose; glycolysis↑, pentose phosphate pathway↑, antioxidant activity↑, immunity↑	Kong et al. (2019)
<i>Penaeus monodon</i> , Asian tiger prawn, p, o	Wheat starch, sucrose, potato starch, cornstarch, dextrin, maltose, glucose	20	Optim WG, ADCs @ wheat starch, sucrose	Niu et al. (2012)
<i>Procambarus clarkii</i> , red swamp crayfish, o	Cornstarch	5 → 35	Optim WG @ 20	Xiao et al. (2014)
<i>Strongylocentrotus purpuratus</i> , purple sea urchin, h		42 → 50	Gonad growth↑	Cuesta-Gomez and Sánchez-Saavedra (2018)

(continued)

Table 13.1 (continued)

Species, common name, trophic habit	Carbohydrate		Level, %	Affected trait	References
	Form				
Fishes					
<i>Acanthopagrus latus</i> , yellowfin seabream, c	Raw and gelat starch	Ratios: 100:100 150:50 50:150 200:0 0:200	Max WG, SGR @ 200:0		Wu et al. (2007a)
	Corn, raw tapioca, or potato starches, raw or gelat	20	Raw: WG↔, SGR↔ Gelat: WG↓, SGR↓		Wu et al. (2007b)
	Raw cornstarch	5, 10, 20, 26	WG↔, SGR↔, FER↔, PER↔ @20C WG↓, SGR↓, FER↓, PER↓ @26C		Wu et al. (2007c)
<i>Acipenser baerii</i> , Siberian sturgeon, b-c	Crude or highly digestible starch	9.9 or 20 as nonprotein energy	Protein sparing L > C		Médale et al. (1991)
<i>Acipenser baerii</i> ♀ × <i>A. schrenckii</i> ♂, hybrid sturgeon, b-c	Raffinose	0.12	WG↑, SGR↑, FCR↔		Xu et al. (2018)
<i>Acipenser schrenckii</i> , Amur sturgeon, b-c	Glucose, fructose, maltose, dextrin, α-starch, or cornstarch	22	WG, SGR: Fructose < maltose < glucose, cornstarch < dextrin, α-starch		Jiang et al. (2014)
<i>Acipenser transmontanus</i> , white sturgeon, b-c	Glucose, fructose, maltose, sucrose, lactose, dextrin, raw cornstarch, or cellulose	27.2	Lipogenesis↑ with glucose or maltose, apparently intolerant to lactose, sucrose, fructose		Hung et al. (1989)
			ADC glucose, galactose, maltose > dextrin > fructose, sucrose > lactose, raw cornstarch > cellulose		Herold et al. (1995)

	Glucose, maltose, corn dextrin, potato dextrin, raw cornstarch, or raw potato starch	1 g kg ⁻¹ bw	Complex carbohydrates delayed plasma glucose concentration peak	Deng et al. (2001)
	Starch or glucose	30	Glucose; SGR [↑] , FER [↑] , PER [↑] (compare hybrid tilapia)	Lin et al. (1997)
<i>Anguilla anguilla</i> , European eel, c	Cooked starch	23 → 40	WG [↑] @ C [↑] , P [↓]	Hidalgo et al. (1993)
	Raw wheat starch, corn maltodextrins, manioc meal, cooked cornstarch	40	WG: Manioc > maltodextrins > raw wheat starch > cooked starch	García Gallego et al. (1994)
<i>Argyrosomus japonicus</i> , dusky kob, c	Cornstarch, gelatin.	4.1 → 24.6	Optim SGR and glucose clearance @ ~16C	Mabasa et al. (2018)
<i>Atractosteus tropicus</i> , tropical gar, c	Cornstarch, gelatin.	0, 6.25, 12.5, 18.8, 25.0	WG [↑] ≤ C22.5, lipid sparing ✓	Guerrero-Zárate et al. (2019)
<i>Brycon orbignyanus</i> , Piracanjuba, o	Dextrin	21 → 51	DWG ↔	Borba et al. (2006)
<i>Carassius gibelio</i> , Prussian (gibel) carp, o	Cornstarch	6 → 38	Optim SGR, FER, PER @ <30C + 37P	Li et al. (2016b)
		45C/2 L; 30C/8 L; 15C/14 L	45C:HSI [↑] , lipogenesis [↑] , lipid uptake [↑] , glycolysis [↑] , <i>glut2</i> [↑] Strain specificity in lipid metabolisms and PER	Li et al. (2019a)
		0, 30, 45	SGR ↔, FER ↔, PER ↔, strain specific	Song et al. (2019)
<i>Chanodichthys erythropterus</i> , topmouth culter (predatory carp), c	α-Starch	0-31	LYZ [↓] , SOD [↓] , <i>hsp70</i> [↑]	Liu et al. (2012)
<i>Coregonus lavaretus</i> , European whitefish, p, c	Cornstarch, wheat meal	0 → 33	Mortality [↑] , blood glucose [↑] , hepatic glycogen [↑] , IgM [↓] , cortisol [↓]	Vielma et al. (2003)
<i>Cromileptes altivelis</i> , humpback grouper, c	Cornstarch, tapioca starch, or dextrin	10, 20	Growth [↑] @ 20	Shapawi et al. (2011)

(continued)

Table 13.1 (continued)

Species, common name, trophic habit	Carbohydrate		Level, %	Affected trait	References
	Form				
<i>Ctenopharyngodon idella</i> , grass carp, h (o)	Cornstarch		20, 35	Optim WG, SGR @ 20C	Liang et al. (2012)
	Starch		17→26	Optim growth @ 17C + 34P	Chen et al. (2012)
	Cornstarch		6→38	SGR↔, FER↔, PER↔	Li et al. (2016b)
<i>Cyprinus carpio</i> , common carp, o	α-Starch, dextrin, or glucose		25, 42	WG: α-starch > dextrin > glucose	Furuichi and Yone (1982)
	Starch, as pea meal or wheat		30, 46	Plasma glucose↔, WG _{pea} > WG _{wheat}	Capilla et al. (2004)
	Cornstarch		0, 15, 30	WG↑, SGR↑, PER↑, FCR↓, lipid↑, liver damage↑	Sun et al. (2018)
<i>Dentex dentex</i> , common dentex, c	Maltodextrin		28	WG↔, FER↔, protein sparing✓	Pérez-Jiménez et al. (2009)
	Gelat starch, dextrin, maltodextrin		12→24	WG↔, best feed utilization @ 18 maltodextrin, protein sparing✓	Pérez-Jiménez et al. (2015)
	Raw or/and gelat starch		25 or 12.5 + 12.5	Optim growth @ 12.5 + 12.5	Peres and Oliveira-Teles (2002)
<i>Dicentrarchus labrax</i> , European seabass, c	Pea seed meal		0→45	WG↔, SGR↔, FCR↔, N utilization↔	Gouveia and Davies (2004)
	Cornstarch + yellow dextrin (2: 1)		25→0	WG↔, PER↑, ADC _{protein} ↓	Enes et al. (2006)
	Native or waxy starch (amylopectin)		10, 20	PER maltose, dextrin, starch > glucose	Enes et al. (2011)
	Gelat cornstarch, dextrin, maltose, glucose		20	WG↔, N utilization↔, protein sparing↔, ADC _s ↔	Moreira et al. (2008)
	Gelat starch		10→30	Protein sparing @ 25 starch	Gatesoupe et al. (2014)

	Gelat starch	0, 20	WG↔, FER↔, PER↑, lipid retention↑	Castro et al. (2015)
	Intraperitoneal glucose load	2 g kg ⁻¹ bw	Sparing of amino acid gluconeogenesis✓	Rito et al. (2018)
	Gelat or raw starch from pea meal	30	Gly↑, Phe↑, pro↓ Muscle lipids↓ only with raw starch	Jarak et al. (2018)
	Starch	Low 0.2 (LS) High 18 (HS)	HS → de novo lipogenesis of triacylglycerol-bound FA in muscle and visceral adipose tissue	Viegas et al. (2019)
<i>Diplodus sargus</i> , white seabream, b-c	Starch, amylopectin (waxy starch)	26, 42	SGR↔, FER↔, PER↑, survival↔, ADC _{protein} ↓	Sá et al. (2008)
<i>Epinephelus coioides</i> , orange-spotted grouper, c	Gelat corn Starch	0→37.5	WG↓, SGR↓, FCR(↓), PER(↑), optim N and energy retention @ 6.8C	Wang et al. (2017b)
<i>Epinephelus lanceolatus</i> , giant grouper, c	Glucose, maltose, or Cornstarch	0, 18	WG: Cornstarch>glucose, maltose, <i>hk</i> ↑, <i>gk</i> ↑	Lu et al. (2018)
<i>Epinephelus lanceolatus</i> ♂ × <i>E. fuscoguttatus</i> ♀, hybrid grouper, c	Cornstarch	0-28	WG↔, FCR↔, PER↑ Protein sparing @ 7-28C	Luo et al. (2016)
	α-Cassava starch, microcrystalline cellulose	8, 12, 16	Insulin↑, <i>atg</i> ↓, <i>foxo</i> ↓, serum lysozyme↓; Optim 10 L + 8C	Li et al. (2018d, 2019b)
<i>Gadus morhua</i> , Atlantic cod, c	Cornstarch	8→20	Broodfish growth↔, FER↔, gonadal development↔	Hemre et al. (1995a)
<i>Gibelion (Catla) catla</i> , Catla, o	Gelat rice or tapioca	40, 50	Mortality↔, hepatocyte hypertrophy↑	Yengkokpam et al. (2005)
<i>Hemibagrus nemurus</i> , Asian redtail catfish, c	Raw cornstarch, broken rice, dextrin, sago flour	7-17	SGR↔, FCR↔	Hamid et al. (2011)
<i>Huso huso</i> , beluga, c	Dextrin	0-20.8	WG↓, SGR↓, PER↓, FER↑	Mohseni et al. (2014)
<i>Ictalurus punctatus</i> , channel catfish, c	Glucose, maltose, fructose, sucrose, cornstarch, or dextrin	33.1	SGR: Dextrin>cornstarch>glucose, maltose, sucrose>fructose	Wilson and Poe (1987)

(continued)

Table 13.1 (continued)

Species, common name, trophic habit	Carbohydrate		Level, %	Affected trait	References
	Form				
<i>Labeo rohita</i> , rohu, o	Cornstarch: Raw (R) or gelat (G)	0→42.4	Best growth @ 80 R; 20 G >20 G → immunity↓, liver glycogen↑, blood glucose↑, glycolytic enzymes↑	Kumar et al. (2007, 2010)	
		43	G + n3-PUFA → immunity↓; R + n3-PUFA → immunity↑	Misra et al. (2006)	
<i>Larimichthys crocea</i> , yellow croaker, c	Wheat starch	0→25	Optim WG, SGR, FER @ 10C	Zhou et al. (2016)	
	Gelat cornstarch	2→32	Optim WG, SGR, FER, PER @ 19.4C	Cheng et al. (2013)	
	α-Cassava starch	6, 9, 12	SGR↓, PER↓ Optim <6C	Li et al. (2019c)	
<i>Lateolabrax japonicus</i> , Japanese seabass, p, c	Cornstarch	0→30	WG↑, optim 18–30C	Zheng et al. (2015)	
<i>Lates calcarifer</i> , Asian seabass, barramundi, c	Wheat flour	20, 46	WG↓ ^a	Peng et al. (2020)	
	Starch	Low 1.6 High 32.5	HS → de novo lipogenesis of triacylglycerol-bound FA only in visceral adipose tissue	Viegas et al. (2019)	
<i>Megalobrama amblycephala</i> , Wuchang (blunt snout) bream, o	Wheat starch, cornstarch, dextrin, maltose, glucose, or cellulose	33	Best WG, SGR, FCR, PER, glycolysis↑ @ dextrin Lowest WG, SGR, FCR, PER @ cellulose	Ren et al. (2015)	
<i>Micropterus salmoides</i> , largemouth bass, c	White dextrin	30, 42	<i>g6pdt</i> ↑, lipogenesis↑	Jiang et al. (2019)	
	Cornstarch	12, 24, 36	Most DEGs↓ @ C↑	Zhang et al. (2019)	
	Starch	0, 5, 10, 15, 20, 25	WG↓, SGR↓, PER↓, oxidative stress↑, LPO↑	Ma et al. (2019)	

<i>Morone saxatilis</i> , striped bass, c	Glucose, maltose, or dextrin	25	WG↑ @ dextrin	Rawles and Gatlin (1998)
<i>Morone chrysops</i> ♀ × <i>M. saxatilis</i> ♂, sunshine bass, c			WG↑, FER↑ @ dextrin<maltose<glucose	
	Glucose, maltose, or dextrin Lipids	20, 40 11, 2	Optim WG, PER @ 20C/11 L (glucose worst); delayed postprandial plasma glucose peak @ dextrin	Hutchins et al. (1998)
	Dextrin, cornstarch, high-amylose cornstarch, glucose	25	Max. WG @ dextrin, high-amylose cornstarch	Rawles and Lochmann (2003)
<i>Mylopharyngodon piceus</i> , black carp, c	α-Starch	20, 40	@40 WG↓, immunity↓	Miao et al. (2016a)
<i>Oncorhynchus mykiss</i> , rainbow trout, c	Sucrose, cornstarch	0→54	≤40 WG↔ starch ≤10 WG (!) sucrose	Pieper and Pfeffer (1980)
	Cornstarch	29	Extrusion↑, starch degradability↑, growth↑	Pfeffer et al. (1991)
	Wheat starch, raw (R) or gelat (G) Lipids	8→24.4 5.7→11.1	Lipogenesis↑, hepatic lipid↑, protein sparing✓ Protein sparing @ L < C	Brauge et al. (1994)
	Expanded wheat	Control: 21 raw C + 22, +37	WG↔, glucose↑, insulin↑; protein sparing✓	Baños et al. (1998)
	Glucose or corn dextrin	10, 20	ADC _{protein} ↓, ADC _{org. matter} , ADC _{carbohydrate} , ADC _{energy} glucose > dextrin	Storebakken et al. (1998)
	Gelat potato starch Dextrin	9, 18, 27, 36 34	WG↔; Protein replacement by L15 + C18 or L11 + C27	Yanamoto et al. (2001)
	Starch (extruded wheat or pea)	0, 20, 40	Insulin↔, IGF-I↔, protein sparing✓	Capilla et al. (2003)
	Precooked cornstarch	7, 23	Mucosal enzymes↑: Disaccharidase, maltase, sucrose, lactase	Krogdahl et al. (2004)
<i>Oncorhynchus tshawytscha</i> , Chinook salmon, c	Gelat starch	28.7	WG↓; programming with glucose✓; strain variability✓	Mazur et al. (1992)

(continued)

Table 13.1 (continued)

Species, common name, trophic habit	Carbohydrate		Level, %	Affected trait	References
	Form				
<i>Oreochromis niloticus</i> , Nile tilapia, o	Wheat starch		16.6, 26.8	N retention↑, enzymes of AA synthesis↑, protein sparing✓	Xiong et al. (2014)
	Cornstarch		20→40	Glucose tolerance↑ @ ≤30C	Liu et al. (2018)
<i>O. niloticus</i> GIFT, genetically improved farmed Nile tilapia, o	Glucose or gelat wheat starch		40	WG↔, SGR↔, feed intake↔, FER↔, HSI↔, liver glycogen↔, insulin↔, IGF-I↔, <i>gk</i> ↔, <i>g6pd</i> ↑, <i>g6pase</i> ↔, lipogenesis↑, protein sparing✓, glucose→GH↓	Qiang et al. (2014b, 2016)
	Glucose or starch		30	Starch: SGR↑, FER↑, PER↑, liver G6PDH↑, 6PGDH↑ (compare white sturgeon)	Lin et al. (1997)
<i>Pagellus bogaraveo</i> , blackspot seabream, o	Wheat or wheat bran		0, 25	WG↔, SGR↔, FCR↔, HSI↔; bran→liver lipid↑	Valente et al. (2010)
<i>Pagrus auratus</i> , Australian snapper, c	Gelat wheat starch		0→45	ADC _{org. matter} ↓, ADC _{energy} ↓ 45 < 35 < 25 < 15C	Booth et al. (2006)
<i>P. major</i> , red seabream, c	α-Starch, dextrin, or glucose		25, 42	WG↔, FER: α-starch, dextrin, glucose	Furuichi and Yone (1982)
<i>Pangasianodon hypophthalmus</i> , striped catfish, o	Starch		0→40	Optim PER @ C20	Hung et al. (2003)
<i>Pangasius bocourti</i> , Mekong catfish, h				Optim PER @ C40	
<i>Paralichthys olivaceus</i> , olive flounder, b-c	Glucose, maltose, or cellulose Dextrin		15 5→25	Best WG or FER @ 25 dextrin, worst @ 15 cellulose	Lee et al. (2003)
	α-Potato starch		15→25	Optim WG @ 20	Lee (2012)
	β-Potato starch		15	FER↓, PER↓	
			20	Optim WG @ α-potato starch or dextrin	Rahman et al. (2016)

	Wheat flour, α -potato starch, α -cornstarch, Na alginate, dextrin, or carboxymethyl cellulose					
	Cornstarch, α -starch	0.9, 16			Glucose sensing-related genes↓	Liu et al. (2019a)
<i>Platichthys stellatus</i> , starry flounder, b-c	Glucose, dextrin	20			Best WG, FER @ 20 dextrin	Lee and Lee (2004)
	Starch	5→25			Lowest WG @ 20 glucose	
<i>Plecoglossus altivelis</i> , ayu sweetfish, p, h	α -Starch	10→40			Best WG @ 30–40C	Nakagawa et al. (2004)
<i>Pseudoplattystoma reticulatum</i> ♀ × <i>Leiarius marmoratus</i> ♂, Amazon hybrid catfish, o	Wheat + corn flour	15→23			WG↔	Bernardes et al. (2016)
<i>Rachycentron canadum</i> , cobia, c	Glucose, sucrose, maltose, dextrin, cornstarch, or wheat starch	20			WG, SGR, PER, survival best: Dextrin, corn, wheat starch; worst: Glucose	Cui et al. (2010)
	Starch	1.3→30.4			Optim SGR, FER, PER @ 18C	Ren et al. (2011)
<i>Rhabdosargus sarba</i> Goldlined (silver) seabream, o	Dextrin	2, 20			$Gk↑$, $g6pase↓$, $gp↓$, $gs↔$, $g6pdh↔$, $gh↔$, $igf-I↔$ translates into: Glycolytic potential↑, gluconeogenic potential↓, glycogenolytic potential↓, hepatic glycogen storage↑, protein sparing✓	Leung and Woo (2012)
	Glucose	0→30			Adults: Hemoglobin↓, serum glucose, protein↔ Juvenile: Plasma glucose↑, protein↑, cholesterol↑	Waagbø et al. (1994)
<i>Salmo salar</i> , Atlantic salmon, c	Wheat starch	0→31			$ACD_{starch}↓$, $ADC_{lipid} @ \geq 9C$, $ACD_{protein}↔$ Protein sparing✓	Hemre et al. (1995b)
					Glycogen in kidney↑, heart↑, gills↑; hematocrit↓, hemoglobin↓, mean cell volume↓, mean cell hemoglobin↓	Hemre et al. (1995c)

(continued)

Table 13.1 (continued)

Species, common name, trophic habit	Carbohydrate		Affected trait	References
	Form	Level, %		
<i>Salvelinus fontinalis</i> , brook trout, c <i>Scophthalmus maximus</i> , turbot, c	Gelat wheat and corn	2.4–23	Large individuals: Glycogen in heart, gills, and kidney↑; plasma glucose(↑), hemoglobin↓, liver function↔, LYZ↔	Hemre et al. (1996)
	Glucose, in vitro	Incubation of head kidney phagocytes	Respiratory burst↓	Lygren and Waagbø (1999)
	Wheat Fish meal	21→10 34→44	ADC _{starch} ↑, ADC _{energy} ↑, growth/protein↓ Protein sparing✓	Hillestad et al. (2001)
	Gelat corn, wheat	10→20	SGR↓; osmoregulatory ability↓; glucose regulation capacity↓; Lipid: Corn>wheat	Hemre et al. (2002a)
	Wheat starch	5, 10	Glycogen↑, hematological parameters↔,	Vangen and Hemre (2003)
	Precooked cornstarch	7, 23	Mucosal enzymes↑: Disaccharidase, maltase, sucrose, lactase	Krogdahl et al. (2004)
	Gelat cornstarch	18, 21, 24, 26	FER↔, ACDs↑, lipid sparing✓	Amin et al. (2014)
	Glucose, sucrose, or dextrin	15	Best WG and FER with dextrin	Miao et al. (2016b)
	α-Potato starch, β-potato starch, β-cornstarch, dextrin	30	ADC _{dry matter} : α-potato starch>dextrin>β-potato starch>β-cornstarch	Lee and Pham (2011)
	Glucose, gelat wheat starch, extruded wheat	10, 40	WG↔, PER↔ ≤ 10C	Booth et al. (2013)
<i>Sebastes schlegeli</i> , Korean rockfish, c <i>Seriola lalandi</i> , yellowtail kingfish, c <i>S. quinqueradiata</i> , Japanese amber-jack, p	α-Potato starch, glucose	10, 20, 30	WG↓ @ 20 glucose	Furuichi et al. (1986)

<i>Silurus meridionalis</i> , Chinese largemouth catfish, c	Raw cornstarch, precooked cornstarch, or glucose	15, 30	WG↔, SGR↔, protein sparing✓ @ 15 C WG↓, SGR↓ @ 30 glucose	Fu (2005)
	Wheat meal, gelat peas, starch	30	Postprandial metabolism↓ @ glucose	Fu (2007)
	Dextrin	26→38	DWG↔, ACDs↔, FCR↑	Conde-Sieira et al. (2016)
<i>Solea senegalensis</i> , Senegalese sole, b-c <i>Sparus aurata</i> , gilthead seabream, (h), c	Gelat cornstarch	14→36	ADC _{protein} ↓	Fountoulaki et al. (2005)
		5, 18, 26	Optim WG, SGR <20C, protein sparing✓	Fernández et al. (2007)
		10, 20, 30	Pyruvate kinase↑, protein catabolism↓, ADC _{protein} ↓	Couto et al. (2008, 2012)
	Native or waxy cornstarch	0, 10, 20	GK↑, G6PD↑, ALAT↓, GDH↓, protein sparing✓, liver glycogen↑ @ 20C, ADC _{protein} ↓ @ 20C	Enes et al. (2008a)
	Starch, dextrin, maltose, or glucose	20	WG↔; PER maltose, dextrin, starch > glucose	Enes et al. (2010)
<i>Tachysurus (Pelteobagrus) fuvdraco</i> , yellow catfish, c	Gelat wheat starch	47, combined with sustained swimming	Acid retention↑, lipid deposition↓, protein sparing✓	Felip et al. (2013)
	Wheat starch	12→28	WG↔, HSI↑, lipogenic activity↑, lipid sparing✓	Bou et al. (2014)
	Fiber replacing wheat starch	1→18	WG↔, HSI↑, lipogenic activity	
	Gelat starch	0, 20	WG↔, PER↑, N retention↔, lipid retention↑, <i>fads2</i> ↑	Castro et al. (2016)
			Abnormal enterocyte architecture, only in combination with vegetable oil	Castro et al. (2019)
	Gelat cornstarch	24→36	SGR↔, FCR↔	Ye et al. (2009)

(continued)

Table 13.1 (continued)

Species, common name, trophic habit	Carbohydrate		Level, %	Affected trait	References
	Form				
<i>Takifugu obscurus</i> , obscure puffer, c	Starch: Varying amylose/amylopectin ratios		25	Max WG, PER @ 6/19	Liu et al. (2014)
<i>Trachinotus ovatus</i> , golden pompano, c	Cornstarch		36	Glycolysis↑, gluconeogenesis↓	Liu et al. (2019b)
			0, 5.6, 11, 17, 22, 28	Carbohydrate metabolism-related genes↓	Zhou et al. (2015)

o omnivorous; *c* carnivorous; *h* herbivorous; *p* planktivorous; *d* detritivorous; *b* benthivorous
bw body weight; *gela* gelatinized; *optim* optimal; *ADC* apparent digestibility coefficient; *ALAT* alanine aminotransferase; *C* dietary carbohydrate content, %; *DEGs* differentially expressed genes; *DWG* daily weight gain; *FCR* feed conversion ratio; *FER* feed efficiency ratio, %; *GDH* glutamate dehydrogenase; *GH* growth hormone; *GK* glucokinase; *HSI* hepatosomatic index; *IGF-I* insulin-like growth factor-I; *L* dietary lipid content, %; *LPO* lipid peroxidation; *LYZ* lysozyme activity; *P* dietary protein content, %; *PER* protein efficiency ratio, %; *SGR* specific growth rate; *SOD*, superoxide dismutase activity; *WG* weight gain
atgl adipose triglyceride lipase selectively hydrolyzes triglyceride the first step of lipolysis; *fads2* fatty acid desaturase2; *foxo1* one forkhead transcription factor central regulator of metabolism; *g6pase* glucose-6-phosphatase enzyme central in the regulation of glycolytic and gluconeogenic pathways; *g6pd* glucose-6-phosphate dehydrogenase initiates the pentose phosphate pathway an alternative route to glycolysis for the degradation of glucose; *g6pdh* glucose-6-phosphate dehydrogenase pentose phosphate pathway; *gk* glucokinase; *gp* glycogen phosphorylase glycogenolysis; *gs* glycogen synthase glycogenesis; *hk* hexokinase; *pk* pyruvate kinase

↑ increase/support; ↓ inhibition/decrease/reduction; ↔ ambiguous or no obvious response; ✓ observed

^aSupplementation of 1% condensed tannins improves growth

13.2 Carbohydrates and Immunity

Fishes fed diets high in carbohydrates for extended periods show poor growth, enlarged livers, and elevated blood glucose. Consequently, it was hypothesized that the resulting accumulation of glycogen in the liver has deleterious effects on the health (Page et al. 1999). These authors were one of the first to study the effects of long-term hyperglycemia in rainbow trouts and reported that no substantial effect of long-term feeding of a high-carbohydrate diet on nonspecific immunity of rainbow trout occurs. However, there is a tendency that dietary carbohydrates have a slight stimulatory effect on phagocytosis at low to moderate levels (Page et al. 1999) pointing out an important additional function of carbohydrates beyond energy supply.

In another carnivorous fish (black carp, *Mylopharyngodon piceus*), Miao et al. (2016a) detected adverse effects of high-carbohydrate diets. Although this species is able to tolerate long-term feeding of 40% dietary carbohydrates, adverse effects on growth and immunity emerge and result in ultrastructural damage of hepatic cells. In omnivorous Wuchang (blunt snout) bream fingerlings, however, Li et al. (2012) found contrary effects. Elevated dietary carbohydrates increase immunity and resistance to *Aeromonas hydrophila* infection of this species.

In carnivorous Japanese flounder (*Paralichthys olivaceus*), dietary carbohydrate is necessary for growth and immune response. Diets with 16% of carbohydrate significantly improve growth, glucose utilization, and immunity. Excessive level (24%) of dietary carbohydrate, however, causes oxidative stress and inflammation and influences glucose transport (Deng et al. 2018). Comparable results are also found in juvenile yellow catfish *Tachysurus (Pelteobagrus) fulvidraco* (Wang et al. 2014).

Recently, Miao et al. (2020) showed that also carnivorous invertebrates require a certain level of dietary carbohydrates. Adequate dietary starch levels increase growth, enhance innate immune response, promote disease resistance, and reduce oxidative stress and inflammatory response as identified in Chinese mitten crab (*Eriocheir sinensis*): 22–27% dietary starch (26–31% dietary carbohydrate) is optimal as digestible energy source in the feed. For more information on polysaccharides as immunostimulants, → Chap. 19 and AAN III “Plant Compounds–Alkaloids and More.”

13.2.1 Glucose

Glucose is the most widely used aldohexose in living organisms. Fishes possess hydrolytic enzymes (amylase, maltase, sucrase, and lactase) necessary to cleave dietary complex carbohydrates into their monosaccharides D-glucose, D-galactose, and D-fructose. These monosaccharides are transported transcellularly by brush border carriers from the intestinal lumen into the cell and then by basolateral carriers

from the cytoplasm into the blood (Collie and Ferraris 1995). However, the control and regulation of carbohydrate/glucose homeostasis vary greatly between different organisms, leading to corresponding fluctuations in blood glucose levels across different classes of vertebrates.

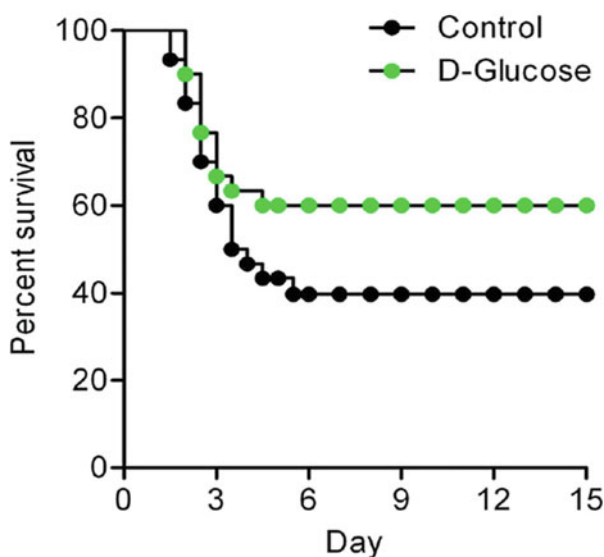
Many papers are stocktaking and the underlying biomolecular mechanisms wait to be discovered (Table 13.1). Some examples: even though dusky kob is a carnivorous species, Mabasa et al. (2018) show that it utilizes 17% dietary carbohydrate for optimal growth without detrimental effects (Table 13.1). At the tested maximum level, physiological breakdown occurs, indicated by reduced growth, reduced feed intake and feed conversion efficiency, prolonged hyperglycemia, liver pathology, and altered microbial communities in the foregut. Interestingly, the dietary carbohydrate tolerance of this carnivorous species does not significantly differ from that of the herbivorous grass carp in the study by Chen et al. (2012). The two studies of grass carp in Table 13.1 are not consistent as the second one did not show any significant response to graded dietary carbohydrate content (Li et al. 2016b). The same inconsistency applies to studies in common carp (Capilla et al. 2004; Sun et al. 2018). These discrepancies are at least partly due to the fact that there is no common experimental standard protocol and catalog of traits to be used in such studies and that different strains, differing in sensitivities and dietary requirements, are tested (→Chap. 40) and fed with feeds that agree only in their gross composition. Overall, it becomes obvious that dietary carbohydrates are utilized, even by carnivorous fish species; however, dietary glucose remains problematic, although one intriguing study has shown that this dietary compound possesses regulatory functions in muscle growth in rainbow trout (Latimer et al. (2019), details →Chap. 19).

Table 13.1 includes also studies with dietary dextrins. These carbohydrates are low molecular weight hydrolysis products of starch or glycogen without a uniform, rather a variable, molecular weight; therefore, consistent results in dietary trials are unlikely. Nevertheless, from this table, it becomes obvious that dextrins are of high nutritional value and belong to the best-utilized saccharides. Furthermore, the European eel appears to be one of the few carnivorous fishes, in which dietary carbohydrates play a more important role as energy-yielding nutrient than for other cultured carnivorous species (Hidalgo et al. 1993; García-Gallego et al. 1995).

An intriguing function of glucose has recently been discovered in Nile tilapia. Exogenous glucose elevates anti-infectious abilities to cope with *Edwardsiella tarda* infection (Zeng et al. 2017). The authors figured out that the survival of tilapia during infection is tightly associated with their metabolome: glucose is the key metabolite to distinguish the survival and dying metabolome after pathogen challenge. Exogenous administration of glucose greatly enhances the survival from the infection (Fig. 13.2), indicating a functional role of glucose in reprogramming metabolomics.

Furthermore, exogenous glucose does not flux into the TCA cycle; instead, it promotes stearic acid and palmitic acid biosynthesis to potentiate the host against the bacterial infection, which is validated by the fact that exogenous stearic acid increases the immune protection in tilapia against *E. tarda* infection. Central in this protection is the Mx protein. The Mx protein has been implicated in antiviral activity (Haller et al. 2007) and obviously also in antibacterial activity. The report by

Fig. 13.2 Increased survival of Nile tilapia in tested groups with intraperitoneally injected 6 mg D-glucose for 6 days and control group with saline. (From Zeng et al. (2017), with permission from Elsevier)



Zeng et al. (2017) is the first to prove that glucose indirectly plays anti-infective actions through the promotion of unsaturated FA biosynthesis (\rightarrow Chap. 26). In a companion paper, the FA-based mechanism of pathogen resistance is confirmed in zebrafish (Xu et al. 2019). In addition to the TCA-independent mechanism, also the boosting of the TCA cycle enhances the survival of zebrafish after *Vibrio alginolyticus* infection (Yang et al. 2018, 2020). For instance, malate triggers a metabolic shift via activating the TCA cycle and elevating taurine production (\rightarrow Chap. 11) (Yang et al. 2020).

13.3 Glucosensing

The main role of nutrient sensing systems is to participate in the control of homeostasis through modulation of feeding behavior or other processes such as energy expenditure or hormone secretion. Glucosensing is the ability of specialized cells to detect changes in the levels of glucose. This ability relates to food intake control and counter-regulatory responses to changes in levels of plasma metabolites in brain areas like hypothalamus and hindbrain. In the pancreatic endocrine cells and intestine, it relates to hormone release, whereas in the liver it relates to the metabolic switch between glucose utilization and production. Evidence obtained in recent years, especially in rainbow trout, supports the presence of nutrient sensing mechanisms, as excellently elaborated and reviewed by Conde-Sieira and Soengas (2017). Here, we shall focus on carbohydrate sensing, since the controlling function of carbohydrates in fishes, including glucose, has been strongly underestimated.

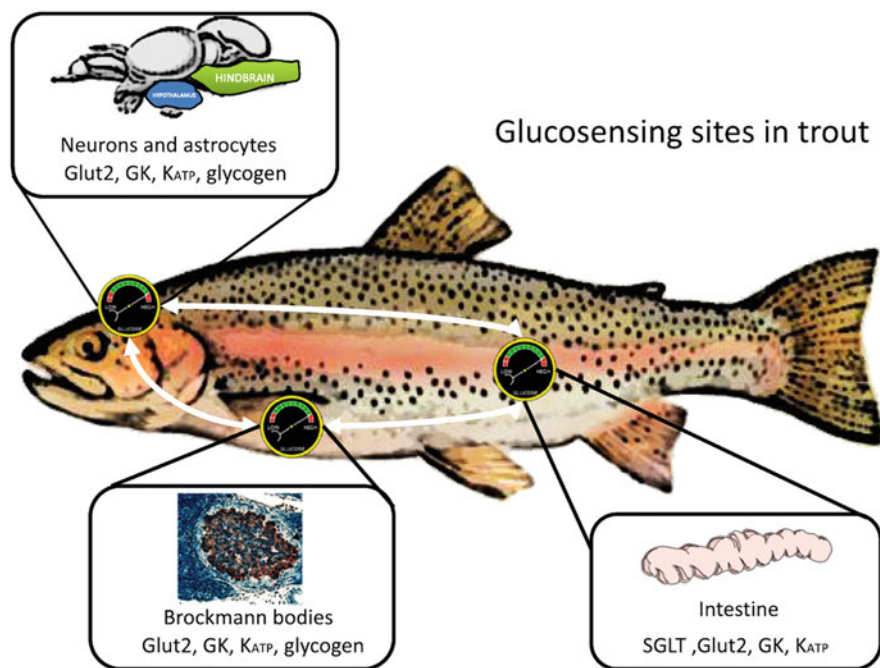


Fig. 13.3 Glucosensing sites and components of the glucosensor systems characterized in rainbow trout to date, including those in the brain regions, intestine, and Brockmann bodies. The different glucosensing markers found in each case are also indicated: *GK* glucokinase; *GLUT2* glucose facilitative transporter type 2; *K_{ATP}* ATP-sensitive inward rectified K⁺ channel; *SGLT* Na⁺-coupled glucose transporters. (From Polakof et al. (2011), with permission from Elsevier)

Comparable papers of glucosensing in aquatic invertebrates are obviously not yet available.

Glucosensing capacity is present in central (hypothalamus and hindbrain) and peripheral (liver, Brockmann bodies, and intestine) locations. Brockmann bodies (Fig. 13.3) are endocrine organs in many teleost fishes and are composed of a collection of islet tissues. The islet tissues are made of endocrine cells, which are the principal sites of insulin synthesis. They are distributed around the spleen and the large intestine. They also secrete other hormones such as glucagon and somatostatin (a growth hormone-inhibiting hormone). Hence, a Brockmann body is a center of control of blood glucose level (Chen et al. 2007).

Although amino acids are the most potent insulin secretagogues in fishes, glucose is able to stimulate the release of insulin (Polakof et al. 2011). Several recent papers provide evidence of glucosensing, so that a hypothetical educational picture emerges (Fig. 13.3). Brain glucose sensors are specialized neurons that respond to fluctuations in local extracellular glucose concentration with changes in their firing rate. Neuronal glucosensing requires glucose uptake by the low-affinity glucose transporter GLUT2, glucose phosphorylation by glucokinase, and the subsequent

metabolism of glucose to increase the intracellular ATP/ADP ratio. This, in turn, leads to the closure of ATP-sensitive potassium channels (K_{ATP} channels), membrane depolarization, calcium entry, and increasing (glucose-excited neurons) neuronal activity and neurotransmitter secretion. This is well understood in mammals and awaits its final proof in fishes (Polakof et al. 2011).

To understand the basic mechanisms, we focus mainly on glucosensing in the brain and follow briefly the review of Polakof et al. (2011).¹ *Glut2* transcription is found in the brain of several species, such as zebrafish (Castillo et al. 2009), rainbow trout (Soengas et al. 2006; Polakof et al. 2007b), and European seabass (Terova et al. 2009). Also the expression of *glut1* is detected in the brain of several species, such as redspotted catshark (*Schroederichthys chilensis*) (Balmaceda-Aguilera et al. 2012a), grass carp (Zhang et al. 2003), zebrafish (Jensen et al. 2006), Atlantic cod (Hall et al. 2005), European seabass (Terova et al. 2009), gilthead seabream (Balmaceda-Aguilera et al. 2012b), orange-spotted grouper (*Epinephelus coioides*) (Liu et al. 2017), and Nile tilapia (Hrytsenko et al. 2010). There is sparse evidence of the involvement of *glut1* in glucosensing. Liu et al. (2017) cloned GLUT1 from *E. coioides* and studied an acute hyperglycemia stress on its expression as well as on glucose tolerance; the authors prove the involvement of GLUT1 in glucosensing. In pearl gentian grouper (*E. fuscoguttatus*♀ × *E. lanceolatus*♂), however, elevated dietary carbohydrate levels do not change the transcription of hepatic *glut1* (Li et al. 2018c). In gilthead seabream, GLUT1 has an important role for osmotic acclimation (Balmaceda-Aguilera et al. 2012b). It is therefore obvious that the knowledge of the regulatory function of *glut1* is still in its infancy.

Glucokinase (GK) is the key enzyme for glucosensing (Niswender et al. 1997) and the utilization of dietary glucose. It phosphorylates glucose in excess in key tissues (pancreas, liver) (Panserat et al. 2014). To date, GK gene transcription and activity are documented in the brain, liver, or intestine of Atlantic salmon (Tranulis et al. 1996), rainbow trout (Panserat et al. 2000), European seabass (Viegas et al. 2013), zebrafish (González-Alvarez et al. 2009), gilthead seabream (Enes et al. 2008b), common carp (Li et al. 2016a), Nile tilapia (Qiang et al. 2014a; Saputra et al. 2016), grass carp (Cai et al. 2018), Wuchang bream (Wang et al. 2017a), turbot (Nie et al. 2015), hybrid grouper (Li et al. 2018b), golden pompano (Zhou et al. 2018), and Japanese flounder (Liu et al. 2019a), but not yet in gibel carp (Li et al. 2018a).

In addition, the K_{ATP} channel is fundamental in glucosensing since it links changes in glucose metabolism to cell membrane electrical activity; however, its function is less well understood than the control by/of GK. K_{ATP} channels have been found expressed in the brain of several teleosts (Polakof et al. 2011). In more detail, Conde-Sieira and Soengas (2017) sketched the integrative responses of glucosensing systems in different fish tissues to an increase or decrease in glucose levels. A GK-dependent glucosensing mechanism is found in the central and peripheral areas of rainbow trouts. Changes in the levels of glucose-induced dietary treatments

¹Extract taken with permission from Elsevier, amended and appropriate references added.

result in changes in glucosensing mechanisms in the hypothalamus and hindbrain. These include changes in *gk* mRNA abundance and activity, glucose, and glycogen levels, *glut2* mRNA abundance, glycolytic and glycogenic potentials, and the activity of ATP-dependent inward rectified potassium channel (K_{ATP} channel).

In another educational cartoon, Conde-Sieira and Soengas (2017) summarized the putative nutrient sensing systems either in the peripheral or central areas of rainbow trout and their metabolic feedbacks (Fig. 13.4). The main role of these systems is to participate in the control of homeostasis as mentioned above. The known mechanisms are comparable to those of mammals in several aspects, but clear differences arise in others, such as the fish capacity of detecting changes in circulating levels of medium-chain FA or polyunsaturated FA (PUFA). These differences between fish and mammals may relate to at least three different reasons. The first reason may relate to the large importance of AAs for metabolic purposes in all fishes. The second reason may be due to the high variety of dietary fish habits resulting in large differences in gastrointestinal morphology and function. The third reason may rely on the existence of multiple gene variants in neuropeptides, hormones, and metabolic effectors resulting from the additional gene duplication.

Later, Conde-Sieira et al. (2018) assessed the response of rainbow trout’s hypothalamus to glucose and oleate by measuring the relative mRNA levels of the transcription factors *brain homeobox transcription factor (bsx)*, *carbohydrate-responsive element-binding protein α (chrebpa)*, *forkhead box O1 (foxo1)*, and *cAMP response element-binding protein (creb)* as well as protein levels of BSX and ChREBP. These parameters, together with the phosphorylation status of CREB and FoxO1,² display changes in hypothalamus of rainbow trout after treatments with glucose or oleate. These changes are known to activate glucose and FA sensing mechanisms and to decrease the orexigenic potential, leading to decreased food intake. This paper provides first information about the association of several transcription factors with the mechanisms involved in linking nutrient sensing systems to expression of neuropeptides involved in food intake regulation.

Recently, Otero-Rodiño et al. (2019) specified that, in rainbow trouts, agouti-related protein (AGRP), but not proopiomelanocortin (POMC), can sense glucose directly through a mechanism involving GK, whose activation by increased levels of glucose leads to modulated activity in specific hypothalamic neurons, ultimately leading to decreased food intake.

²Forkhead box protein O1 (FoxO1) is a transcription factor that plays important roles in regulation of gluconeogenesis and glycogenolysis by insulin signaling and is also central to the decision for a pre-adipocyte to commit to adipogenesis (Nakae et al. 2003).

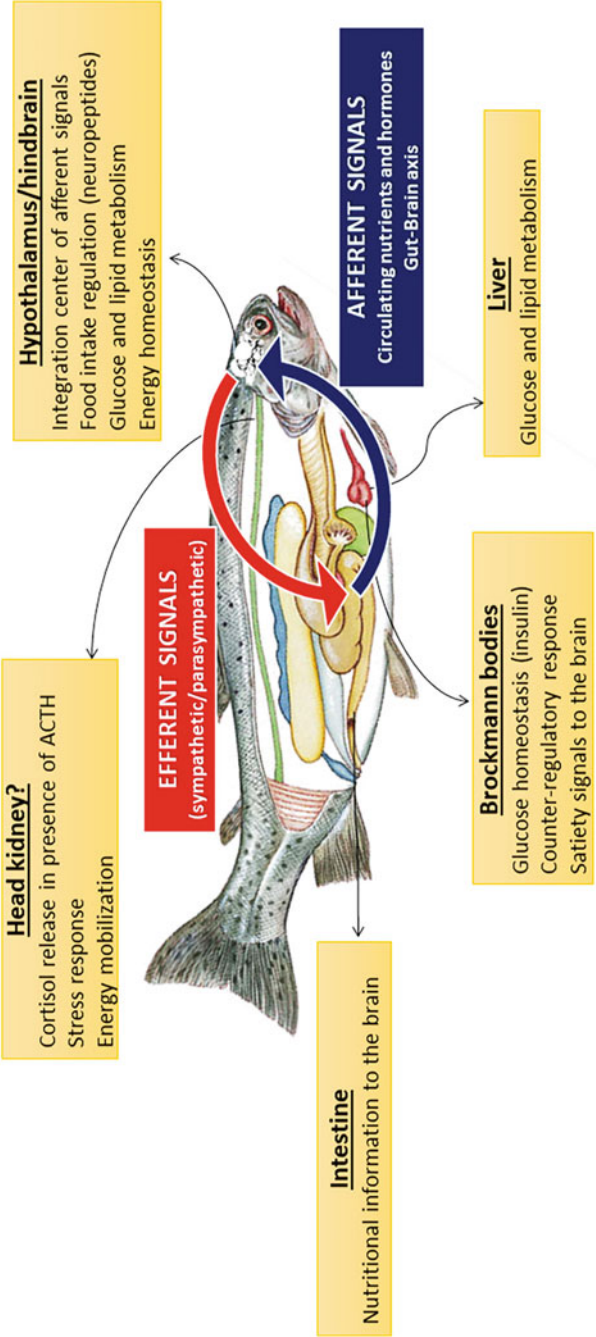


Fig. 13.4 Schematic drawing summarizing functions of nutrient sensing systems in the central and peripheral tissues of fishes. (From Conde-Sieira and Soengas (2017), credit Frontiers Media)

13.4 Rhythmicity

As all organisms on Earth, also fishes and aquatic invertebrates are subject to circadian rhythms triggered by external zeitgebers and controlled by gene transcription. Consequently, all life history traits change in a circadian manner. This rhythmicity also applies to digestive enzyme activity. It is reflected in the monosaccharide contents in blood and other body fluids—even under fasting conditions. Selected studies will highlight this rhythmicity.

In an early study, Holloway et al. (1994) investigated the diurnal patterns of changes in glucose contents in rainbow trouts both during single meal feeding and during a progressive fast of 13 weeks. Glucose exhibits a diurnal pattern in fed rainbow trout, most of which appear to be correlated with the time of feeding (30 – 60 min after onset of light). During fasting, there is a progressive decrease in mean daily glucose concentrations with no peak at all after 13 weeks’ fasting. In contrast to rainbow trout, circadian rhythms of glucose are not evident in Senegalese sole (Oliveira et al. 2013). It is assumed that the circadian rhythm of cortisol stimulates gluconeogenesis and, thereby, regulates glucose homeostasis. Also in olive flounder and sockeye salmon, circadian rhythms of plasma glucose do not occur (Leatherland et al. 1974; Zou et al. 2016).

Circadian variations in plasma glucose, however, have been reported for many fish species: European seabass (Fig. 13.5) (Pavlidis et al. 1997; del Pozo et al. 2013), common dentex (Pavlidis et al. 1999a), red porgy (Pavlidis et al. 1999b), rainbow trout (Polakof et al. (2007a) vs. Reddy and Leatherland (1994) and Hernández-Pérez et al. (2019) who did not identify any rhythmicity), gilthead seabream (Pavlidis et al. 1997; Montoya et al. 2010), tench (de Pedro et al. 2005), Nile tilapia (Fig. 13.5) (Guerra-Santos et al. 2017), yellowtail (Kawanago et al. 2014), and African catfish (*Clarias gariepinus*) (Van Heeswijk et al. 2005).

Noteworthy, del Pozo et al. (2013) reported that the daily rhythms of blood glucose differ in diurnal and nocturnal European seabass: diurnal fishes have the highest glucose levels during the photophase, whereas the nocturnal ones present highest concentrations at the end of the dark phase (Fig. 13.5). These findings provide evidence that internal physiological changes occur in seabass undergoing seasonal phase inversions of their feeding rhythms.

In farmed fishes, the feeding rhythm is controlled by aquaculturists. Feed can be provided any time, for instance, immediately after light on, at the end of the light phase, or during the dark phase. Does the time point of feeding influence the plasma glucose level? This question has experimentally been answered by Guerra-Santos et al. (2017) for omnivorous Nile tilapia. One group of fishes was fed once a day at 11:00 h (zeitgeber time, ZT6) and the other one at 23:00 h (ZT18). The results show a daily rhythmic variation for plasma glucose (Fig. 13.5), with an acrophase³ for fish fed during the light phase at ZT 23:11 ± 03:46 h and for the group fed during the

³Time at which the peak of a rhythm occurs

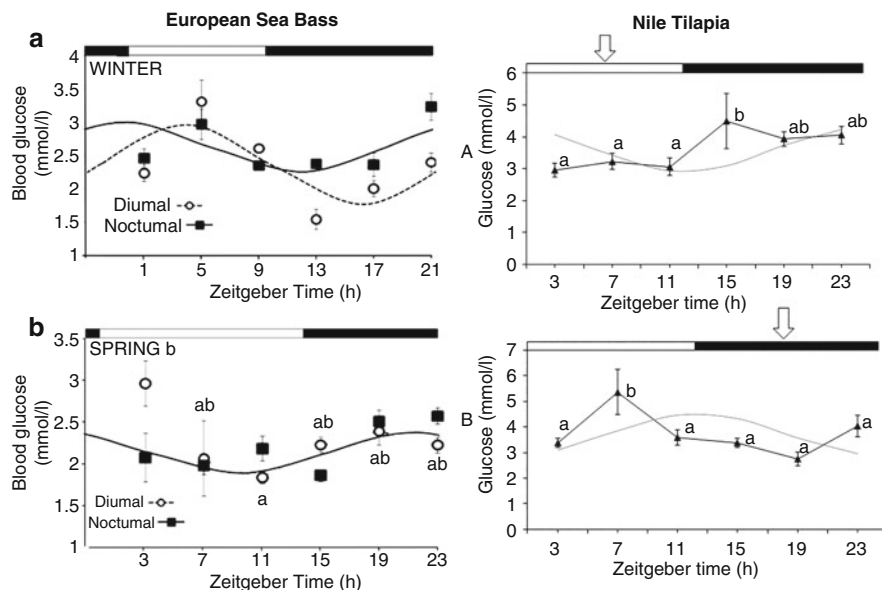


Fig. 13.5 *European seabass* daily cycle of blood glucose levels in winter (a) and spring (b) during both feeding behavior inversions. Each point represents the mean blood glucose value of five seabass showing diurnal (white circles) and nocturnal (black squares) feeding behavior. The cosinor adjustment for diurnal fish is represented by dotted sinusoidal line and continuous sinusoidal line for nocturnal. Different letters denote significant differences between every time point ($P < 0.03$). Vertical axis shows the blood glucose level ($\text{mmol L}^{-1} = \text{mM}$) and horizontal axis the zeitgeber time (in hours). (From del Pozo et al. (2013), with permission from Springer Nature). *Nile tilapia* blood glucose levels in Nile tilapia under 12:12 h LD cycles. (a) group fed at 11:00 h; zeitgeber time, ZT6; (b) group fed at 23:00 h (ZT18). Data represent means \pm SEM. The white and black bars at the top show the photophase and dark phase, respectively. The vertical axis represents blood glucose levels in mM, while the horizontal axis shows the zeitgeber time in hours. The arrows at the top of the actograms indicate the feeding time for the groups. Significant variations were found by one-way ANOVA, $P < 0.05$, followed by the Duncan test. Different letters denote significant differences between time points for each feeding condition. (From Guerra-Santos et al. (2017), with permission from Elsevier)

dark phase at ZT $11:33 \pm 04:07$ h. These results indicate a plasticity of the circadian system and its synchronizers in Nile tilapia.

Fishes have a greater behavioral plasticity than terrestrial animals. This may be due to factors related to the time of year, food availability, or even the presence of predators. Individual factors may also explain this rhythmic plasticity. For instance, goldfish (*Carassius auratus*) has a daily rhythm of swimming activity in some individuals during daytime, while other individuals display their activity during the night. Feeding is considered a powerful synchronizer of fish locomotor activity (Guerra-Santos et al. 2017).

13.5 Concluding Remarks

Due to the emergence of new, fast, and relatively inexpensive “omics” techniques, carbohydrates are no longer considered only from the nutritional and energetic perspective. From this perspective, they have been termed not essential nutrients. However, aquaculturists begin to get rid of this bias, since it is becoming clear that, contrary to these classical beliefs, even carbohydrates carry out controlling functions, interact on biomolecular levels, or strengthen immunity as critical metabolite. This applies even to glucose, which has been found to indirectly improve immunity and increase pathogen resistance by triggering the production of unsaturated FAs.

In many species, clear rhythmicity of serum carbohydrate concentrations occurs. Therefore, the question arises of can this rhythmicity be used to improve carbohydrate utilization particularly of carnivorous aquatic animals?

While convincing evidence is emerging that carbohydrate diet can influence intestinal microbiota, the impact of these changes on the host remains to be evaluated in depth (→Chap. 15). The functional flexibility of the gut microbiome is likely to play an important role in the digestive adaptability and increased immunity of fishes, particularly with respect to dietary carbohydrates (Kamalam et al. 2017).

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Chapter 14

Glucose Homeostasis—‘*Life’s Little Luxury Balanced*’



Abstract This chapter continues the inventory of carbohydrates in nutrition of aquatic animals and puts emphasis on homeostatic mechanisms of glucose in serum and tissues. A balanced blood glucose level is central in normal physiological activities in all animals. Central processes of glucose homeostasis, such as glycolysis, gluconeogenesis, and glycophagy, are sketched. The beneficial control of the gut microbiota in glucose metabolism is beginning to be understood; this regulatory process deserves future scientific attention.

A balanced blood glucose¹ level is paramount in maintaining normal physiological activities in all animals, and insulin is central in this process. Insulin occurrence in the gastroenteropancreatic system has been documented in mollusks, annelids, arthropods, echinodermata, urochordates (tunicates), cyclostomes, elasmobranches, and teleosts (Sower et al. 2000; Sherwood et al. 2005). Whereas the regulation in fishes is relatively well understood—but less well than in higher vertebrates—carbohydrate homeostasis in invertebrates only begins to be studied. Instead, the focus of past invertebrate studies has mainly been put on insulin-(like) hormones and their role in sexual determination, development, and reproduction (van Minnen et al. 1989; Ventura et al. 2012; Ma et al. 2013; Vázquez-Islas et al. 2014; Cherif-Feildel et al. 2019).

14.1 Homeostasis: Fishes

Glucose homeostasis is maintained as an insulin-mediated closed feedback loop involving the Brockmann bodies in teleosts (→ Chap. 13), liver, and peripheral tissues including the brain, muscle, and adipose tissues. A significant number of studies have reported on the control of glucose homeostasis in selected farmed fishes (Plagnes-Juan et al. 2008; Panserat et al. 2009; Polakof et al. 2011). The site of

¹Glucose means D-glucose if not otherwise noticed

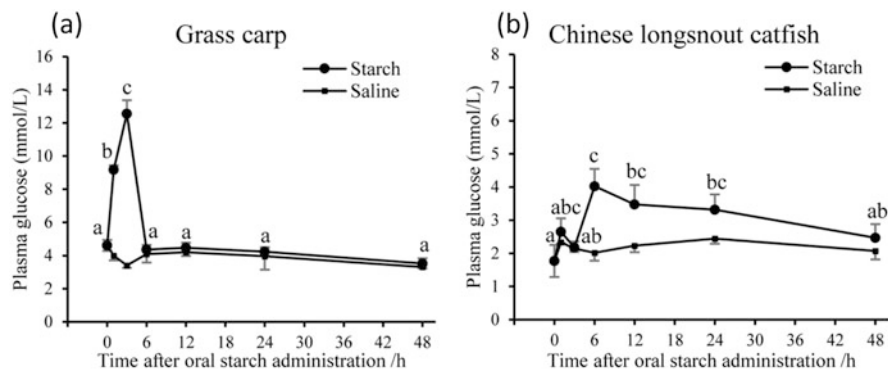


Fig. 14.1 Changes in the plasma glucose levels of (a) grass carp and (b) Chinese longsnout catfish following the oral administration of starch. Each point represents the mean of six replicates. Different letters indicate significant differences ($P < 0.05$). (From Su et al. (2020), with permission from the Cambridge University Press)

terminal carbohydrate digestions are the brush borders of the intestinal lining. Fishes possess the same suite of endogenous digestive enzymes to cleave polysaccharides as other vertebrates, and it can be assumed that fish digestive-enzyme activities are maintained at levels for optimal digestive rate of energy or nutrients from their diet. For carbohydrates, this largely appears to be true: herbivores and detritivores usually have higher carbohydrase activities (including polysaccharidase and disaccharidase activities) than carnivores, even when raised in the laboratory on a common low-starch diet (German 2011). This can easily be demonstrated by comparing herbivorous grass carp (*Ctenopharyngodon idella*) and carnivorous Chinese longsnout catfish (*Tachysurus dumerili* (*Leiocassis longirostris*)) after oral starch administration (Su et al. 2020). Grass carp, but not Chinese longsnout catfish, exhibits a rapid and strong increase in plasma glucose (Fig. 14.1) (→ Chap. 21). Moreover, grass carp exhibits a faster clearance of plasma glucose than the catfish. This difference is based on inhibition of gluconeogenesis and enhanced glycolysis, hepatic glycogen metabolism, and glucose-induced lipogenesis in grass carp. Taken together, grass carp and Chinese longsnout catfish are species with significantly different demands for dietary carbohydrate and exhibit clear differences in glucose (Su et al. 2020).

14.1.1 Metal Supplementation

Several metals, such as chromium (Cr) or cobalt (Co), can improve the utilization of carbohydrates in fishes. Cr is considered a cofactor for insulin activity and part of an organic tolerance factor (Anderson 1981). CrCl_3 enhances glucose tolerance, increases lipogenesis rate, and affects glycogen accumulation in the presence of insulin (Rosebrough and Steele 1981). Dietary Cr_2O_3 , however, does not affect

Table 14.1 Effect of dietary chromium on glucose or starch metabolism (selected studies)

Fish species	Cr; mg kg ⁻¹	Effect	Reference
<i>Cyprinus carpio</i> , common carp	Cr methionine; 1.09	Serum glucose↓	Cui et al. (2018)
	Cr chloride; ≤1.5	Serum glucose↓	Ahmed et al. (2013)
<i>Labeo rohita</i> , rohu	Cr picolinate; 0.3→0.6 Cr chloride; 0.3→0.6	Glucose utilization↑ Glucose utilization↔	Asad et al. (2019)
<i>Larimichthys crocea</i> , large yellow croaker	Cr polynicotinate; 5.0→10.0	Starch utilization↑	Wang et al. (2014)
<i>Megalobrama amblycephala</i> , Wuchang bream	Cr picolinate; ~0.3	Serum glucose↓	Ren et al. (2018)
<i>Oncorhynchus mykiss</i> , rainbow trout	Cr picolinate; ≥0.8	Serum glucose↓	Küçükbay et al. (2006)
<i>Oreochromis niloticus</i> × <i>O. aureus</i> , hybrid tilapia	Cr picolinate; 2.0	↔	Pan et al. (2003)
<i>Oreochromis niloticus</i> , Nile tilapia	Cr picolinate; 0.4	Serum glucose↓	Mehrim (2014)
	Cr picolinate; 1.0 Or Cr yeast; 2.0	Glucose utilization↑	Rakhmawati et al. (2018)

↓reduction; ↔ no obvious effect; ↑ increase

glucose utilization as shown in channel catfish (*Ictalurus punctatus*) (Ng and Wilson 1997); the oxide is obviously a badly utilizable chemical species.

In carps, Cr salts improve glucose utilization and inhibit gluconeogenesis, likely by modulating the endogenous insulin activity (Hertz et al. 1989). In tilapia fed glucose, dietary Cr increases the weight gain, energy deposition, and liver glycogen content (Shiau and Lin 1993). The same applies to dietary treatment with Co salts, which improves glucose utilization and inhibits gluconeogenesis (Hertz et al. 1989). In contrast to glucose, tilapia's starch metabolism does not increase with Cr supplementation (Shiau and Lin 1993).

Some more studies on dietary Cr supplementation with mixed, but mostly serum glucose reducing effects, are listed in Table 14.1.

14.1.2 Physiological Details

The *microvilli* constituting the brush border have enzymes for the final part of digestion anchored into their apical plasma membrane as integral membrane proteins. Monosaccharides cross the brush border membrane by simple diffusion or by active transport via specific transporters (Chap. 16). The piscine glucose transporters of the brush border show characteristics similar to those found in mammals, i.e., they are electrogenic and dependent on energy and Na⁺. The glucose transporters show

varying characteristics along the intestinal tract. Glucose affinity appears to increase from the proximal to the distal parts of the intestine, while the pyloric *ceca*² display the lowest affinity. Variations in Na⁺ dependence may indicate that the transporters of the distal parts of the intestine are more efficient in capturing glucose molecules from the chyme than the pyloric *ceca*. It is possible that differences in transporter characteristics are because of the differences in the molecular environment of the transporter (Krogdahl et al. (2005) and references therein).

While in mammals, blood circulating glucose concentrations never drop below a minimal level of approximately 5 mM (Verri et al. 2001), it can be in fishes as low as <0.5 mM (in silver pomfret, *Pampus argenteus*). Regular levels in fishes are in the low mM range but can reach even values up to ~17 mM (Table 14.2).

Enes et al. (2009) compiled several hypotheses to explain the low dietary glucose utilization in fishes:

- Stronger effect of dietary amino acids (AAs) than glucose as stimulators of insulin secretion.
- Relative low number of insulin receptors (*insr*)³ in fish muscle as compared to mammals.
- Low glucose phosphorylation capacity.
- Low peripheral utilization of glucose (Moon 2001) and therefore an imbalance between hepatic glucose uptake (glycolysis) and production (gluconeogenesis).

Until recently, the imbalance hypothesis appeared to be the most widely accepted one (Krogdahl et al. 2005; Enes et al. 2009). Nevertheless, it is still not possible to derive a cohesive picture on the integrated process of carbohydrate hydrolysis and absorption and interaction with diet composition for any of the fish species under cultivation. The physiological mechanisms behind the species differences are not completely known (Krogdahl et al. 2005). Applying updated biomolecular techniques, such as high-throughput DNA sequencing, more comprehensive and more complex pictures of affected pathways are going to be elucidated (→ Chaps. 15 and 19).

In brief: Glycolysis is the only route of glucose catabolism in all organisms, including fishes. It consists of a progressive oxidation of one molecule of glucose (C₆) into two pyruvate (C₃) molecules. The regulation of the glycolytic pathway depends on the activity of key enzymes, such as hexokinases, 6-phosphofructo-1-kinase, and pyruvate kinase. Central in gluconeogenesis, fructose-1,6-bisphosphatase (FBPase) is rate-limiting, catalyzing the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate. Along with phosphofructokinase, FBPase regulates the flux of glucose metabolism and is involved in the formation of glucose

²Pyloric *ceca* are blind appendages attached to the proximal intestine of many fishes that increase the intestinal surface area without increasing the length or thickness of the intestine itself.

³Alternate splicing during transcription results in either *insra* or *insrb* isoforms: *insra* and *insrb* demonstrate a similar role in glycolysis, but *insra* is also involved in inhibiting gluconeogenesis via downregulating the expression of *foxo1a* (Gong et al. 2018).

Table 14.2 Reported minimal and maximal glucose levels in the hemolymph and blood systems of selected aquatic invertebrate and fish species

Species	Glucose, mM	Reference
Invertebrates		
<i>Anodonta anatina</i> late spring, late summer Mid-summer	0.1 0.05	Pekkarinen (1997)
<i>Artemesia longinaris</i>	0.4	Velurtas et al. (2011)
<i>Cancer borealis</i>	0.05–0.5	In Verri et al. (2001)
<i>Cancer pagurus</i>	0.8	
<i>Carcinus maenas</i>	~1.1	Powell and Rowley (2008)
<i>Eriocheir sinensis</i>	~0.42	Li et al. (2017)
<i>Haliotis fulgens</i>	1.9	Viana et al. (2007)
<i>Homarus americanus</i>	1.1–1.4	In Verri et al. (2001)
<i>Litopenaeus vannamei</i>	0.4 to ~4.5	In Verri et al. (2001), Maggioni et al. (2004)
<i>Macrobrachium nipponense</i>	2.5	Ding et al. (2017)
<i>M. rosenbergii</i>	2.4	Manush et al. (2005)
	0.8	Chang et al. (2006)
<i>Neohelice granulata</i>	0.4	Marqueze et al. (2011)
<i>Nephrops norvegicus</i>	10	Stentiford et al. (2001)
<i>Orconectes limosus</i>	0.03–0.2	In Verri et al. (2001)
<i>Panulirus longipes</i>	1.64	Dall (1975)
<i>Paranephrops zealandicus</i>	0.21	Broughton et al. (2017)
<i>Penaeus monodon</i>	0.8–1.4	In Verri et al. (2001)
<i>Pleoticus muelleri</i>	0.4	Velurtas et al. (2011)
<i>Procambarus clarkii</i>	0.9	In Verri et al. (2001)
Fishes		
<i>Acipenser sinensis</i>	2.5	Shi et al. (2006)
<i>Acipenser schrenckii</i>	3.5	Shi et al. (2006)
<i>Barbonymus schwanenfeldii</i>	~3.3	Eslamloo et al. (2017)
<i>Bidyanus bidyanus</i>	3.4	Stone et al. (2003)
<i>Carassius auratus</i>	0.7–5.1	Chavin and Young (1970)
<i>C. gibelio</i>	3.8–4.3	Shi et al. (2016)
<i>C. gibelio</i> A strain; DT strain	1.7; 2.3	Jin et al. (2017)
<i>Clarias gariepinus</i>	4.7	Al-Dohail et al. (2009)
<i>Colossoma macropomum</i>	4.4	Soares et al. (2016)
<i>Cyprinus carpio</i>	~1.8 to 8.3	Blasco et al. (1992); Saravanan et al. (2011)
<i>Danio rerio</i>	2.2 to ~7.1	Fang et al. (2014); Dalmolin et al. (2015)
<i>Danio rerio</i> 0.2 day post fertil.; 1.0 dpf	0.06; 0.40	Rocha et al. (2014)
<i>Dicentrarchus labrax</i>	~3.3 to ~5.5	Martins et al. (2013); Enes et al. (2011b)

(continued)

Table 14.2 (continued)

Species	Glucose, mM	Reference
<i>D. labrax</i> nocturnal, winter; noct, spring Diurnal, winter; diurnal, spring	2.7; 2.2 ~2.3; ~2.3	del Pozo et al. (2013)
<i>Epinephelus coioides</i>	~4.0	Liu et al. (2017a)
<i>Gadus morhua</i>	9.4	Hall et al. (2006)
<i>Galaxias maculatus</i>	~1.7	Davies et al. (1994)
<i>Glyptoperichthys gibbiceps</i>	~16.9	MacCormack et al. (2003)
<i>Huso huso</i>	~2.8 to ~4.1	Yousefi et al. (2012); Jalali et al. (2010)
<i>Labeo victorinus</i>	~4.7	Ngugi et al. (2015)
<i>Lates calcarifer</i>	~6.3	Talpur and Ikhwanuddin (2013)
<i>Megalobrama amblycephala</i>	3.60 to ~10.5	Li et al. (2016); Pan et al. (2017)
<i>O. mykiss</i>	~2.2 to ~10.7	Güllü et al. (2014); Sheikhzadeh et al. (2012)
<i>Opsanus tau</i>	~1.4	Mensing et al. (2005)
<i>Oreochromis mossambicus</i>	~2.4 – ~6.1	Riley Jr et al. (2009)
<i>O. niloticus</i> low/high stocking density	~3.9; ~7.6	Aketch et al. (2014)
<i>O. niloticus</i> , incl. GIFT	~1.9 to 6.7	Liu et al. (2017b); Hohlenwerger et al. (2016)
<i>Pagellus bogaraveo</i> 45% dietary protein	~5.2	Figueiredo-Silva et al. (2010)
<i>Pagrus major</i>	<10	Furuichi and Yone (1981)
<i>P. major</i> @ varying dietary Arg	~1.2... ~2.8	Rahimnejad and Lee (2014)
<i>Pampus argenteus</i>	~0.2 – ~0.3	Peng et al. (2013)
<i>Pangasianodon hypophthalmus</i>	~5.6	Prabu et al. (2016)
<i>Paralichthys olivaceus</i>	~1.7	Kim et al. (2010)
<i>Piaractus mesopotamicus</i>	~1.75	Takahashi et al. (2014)
<i>Potamotrygon</i> cf. <i>histris</i>	~1.7	Brinn et al. (2012)
<i>Prochilodus lineatus</i>	~1.8	Winkler et al. (2007)
<i>Pseudaphritis urvillii</i>	~2.4	Davies et al. (1994)
<i>Pterophyllum scalare</i> , whole body	~3.0	Norouzitallab et al. (2009)
<i>Salmo caspius</i>	~3.7	Kenari et al. (2013)
<i>S. salar</i>	~4.0 to ~5.2	González et al. (2016); Olsen et al. (2006)
<i>S. trutta</i>	~3.2	Wells et al. (2006)
<i>Scophthalmus maximus</i>	~2.5	Fuchs et al. (2017)
<i>Sebastes schlegelii</i>	~2.7	Hwang et al. (2013)
<i>Seriola quinqueradiata</i>	<11.8	Furuichi and Yone (1981)
Silver catfish	~3.5	Saccol et al. (2013)
<i>Solea senegalensis</i>	~1.5 – ~3.5	Conde-Sieira et al. (2015)
<i>Solea senegalensis</i> constant light Light-dark 12:12	~0.5 – ~1.0 ~0.6 – ~1.3	Oliveira et al. (2013)
<i>Solea solea</i>	~4.64	Palermo et al. (2013)

(continued)

Table 14.2 (continued)

Species	Glucose, mM	Reference
<i>Sparus aurata</i>	1.8 to ~10.6	Ortuño et al. (2003); Pérez-Jiménez et al. (2012)
<i>Systomus sarana</i>	~3.3	Das et al. (2011)
<i>Tinca tinca</i> spring; summer Autumn; winter	~2.9; ~4.7 ~4.6; ~3.2	de Pedro et al. (2005)
<i>Trachinotus ovatus</i>	~3.3	Zhou et al. (2015)

from non-carbohydrates (Enes et al. (2009)). Many of the enzymes involved are diet-responsive, not only quantitatively, but also qualitatively (Table 14.3); they respond to the amount of dietary carbohydrate offered as well as to the carbohydrate type. However, the responses are not uniform; rather they appear to be species-specific and merit future detailed studies. Although the database is much smaller than for fishes, the same conclusion can be drawn for invertebrates (Table 14.3).

Fishes and aquatic invertebrates possess all carbohydrate regulating and metabolizing enzymes (→ Chap. 18). **Glycolysis**, hexokinases (HK), 6-phosphofructo-1-kinase (PFK-1), and pyruvate kinase (PK), and **gluconeogenesis**, phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase). Under aerobic conditions, glucose is catabolized through the glycolytic pathway, Krebs cycle, and respiratory chain for ATP (adenosine triphosphate) production or through the pentose phosphate pathway, leading to the production of NADPH for lipid biosynthesis and ribose 5-phosphate required for nucleotide synthesis. Excess glucose can be stored as glycogen (via glycogenesis) or converted to lipids. Under fasting conditions, glucose requirements for metabolic purposes can be satisfied by glycogen degradation into glucose (via glycogenolysis) or by de novo glucose synthesis via gluconeogenesis from certain non-carbohydrate substrates. Thus, a balance between glucose storage and glucose production, which is accomplished by nutritional and hormonal factors, is of utmost importance for maintaining glucose homeostasis, and it is mainly dependent on the regulation of expression and activity of key enzymes (Enes et al. 2009).

This picture is going to be refined by biomolecular studies. Bertucci et al. (2019) investigated the interaction of the anorexigenic neuropeptide nesfatin-1 and glucose in rainbow trout and show that nesfatin-1 reduces the expression of genes involved in glucose uptake in the gut but increases their expression in the liver and muscle. Additionally, nesfatin-1 treatment increases the transcription of genes involved in glucose metabolism and distribution such as *insulin* and *igf-II*. These results indicate that nesfatin-1 can decrease the intake of dietary glucose and increase the glucose uptake in tissues such as liver and muscle, in which it can be rapidly anabolized or stored.

In the hindbrain and hypothalamus, an increase in gene expression of the glucosensing machinery and transporter *sglt-1* occurs in vivo in response to

Table 14.3 Dietary regulation of glycolytic and gluconeogenetic enzymes in selected aquatic invertebrates and fishes

Enzyme	Species	Carbohydrate type	Response	Reference
Invertebrates				
Hexokinase	<i>Apostichopus japonicus</i>	Gelat cornstarch	↑	Xia et al. (2015)
	<i>Cherax quadricarinatus</i>	Dextrin	↑	Zhu et al. (2013)
	<i>Litopenaeus vannamei</i>	Wheat starch	↓	Rosas et al. (2001)
	<i>L. vannamei</i> low salinity high salinity	Not mentioned	↔ ↑	Gaxiola et al. (2005)
	<i>Macrobrachium nipponense</i>	Cornstarch	↑	Ding et al. (2017)
	<i>Neohelice granulata</i>	Boiled rice	↔ ^a	Marqueze et al. (2011)
	<i>Penaeus monodon</i>	Wheat starchsucrose Potato starch Cornstarch Dextrin	↑ ↑ (↑) ↑ ↔	Niu et al. (2012)
Pyruvate kinase	<i>Apostichopus japonicus</i>	Gelat cornstarch	↑	Xia et al. (2015)
	<i>Cherax quadricarinatus</i>	Dextrin	↑	Zhu et al. (2013)
	<i>Macrobrachium nipponense</i>	Cornstarch	↑	Ding et al. (2017)
	<i>Neohelice granulata</i>	Boiled rice	↑ ^a	Marqueze et al. (2006)
			↔ ^a	Marqueze et al. (2011)
Fructose-1,6-bisphosphatase	<i>Apostichopus japonicus</i>	Gelat cornstarch	↓	Xia et al. (2015)
Fishes				
Hexokinase	<i>Cyprinus carpio</i>	Pea starch	(↓)	In Enes et al. (2009)
		Wheat starch	(↓) (↑)	
	<i>Dicentrarchus labrax</i>	Cornstarch, native	↓	In Enes et al. (2011a)
		Cornstarch, waxy	↔	Enes et al. (2006)
		Glucose	↑	In Enes et al. (2011a)
		Cornstarch, gelat	↓	
		Cornstarch, waxy or native	↑	
		Glucose	↑	
	<i>Labeo rohita</i>	Cornstarch	↑	Kumar et al. (2010)

(continued)

Table 14.3 (continued)

Enzyme	Species	Carbohydrate type	Response	Reference
	<i>Oncorhynchus mykiss</i>	Pea starch	↑	Panserat et al. (2000a)
		Wheat starch, extruded	↑	In Enes et al. (2009)
	<i>Oreochromis niloticus</i> × <i>O. aureus</i>	Waxy cornstarch, glucose	↔	Tung and Shiau (1991)
	<i>Sparus aurata</i>	Cornstarch, waxy, native, or gelat	(↑)	Enes et al. (2008a, 2011a)
	<i>Takifugu obscurus</i>	Cornstarch	↔	Liu et al. (2015)
Glucokinase	<i>Dicentrarchus labrax</i>	Cornstarch, waxy or native	↑	Enes et al. (2006)
		Glucose	↑↑	Enes et al. (2008c)
		Cornstarch, gelat	↑	In Enes et al. (2011a)
	<i>Labeo rohita</i>	Cornstarch	↑	Kumar et al. (2010)
	<i>Oncorhynchus mykiss</i>	Pea starch	↑	Panserat et al. (2000a)
		Wheat starch, extruded	↑	In Enes et al. (2009)
		Fructose	↔	Panserat et al. (2001b)
		Glucose	↑↑	Panserat et al. (2001a)
	<i>Perca fluviatilis</i>	Digestible carbohydrates	↑	In Enes et al. (2009)
	<i>Salmo salar</i>	Starch	↑	
	<i>Sparus aurata</i>	Pea starch	↑↑	In Enes et al. (2011a)
		Cornstarch, gelat	↔; ↑	Enes et al. (2008b)
		Cornstarch, native	↑	In Enes et al. (2011a)
		Cornstarch, waxy	↑	Enes et al. (2008a)
		Glucose	↑↑	Enes et al. (2008c)
	<i>Takifugu obscurus</i>	Cornstarch	↑	Liu et al. (2015)
Pyruvate kinase	<i>Dicentrarchus labrax</i>	Cornstarch, native	↑	In Enes et al. (2011a)
		Glucose	↑↑	
	<i>Labeo rohita</i>	Cornstarch	↑	Kumar et al. (2010)

(continued)

Table 14.3 (continued)

Enzyme	Species	Carbohydrate type	Response	Reference
	<i>Oreochromis niloticus</i> × <i>O. aureus</i>	Waxy cornstarch, glucose	↔	Tung and Shiau (1991)
	<i>Perca fluviatilis</i>	Digestible carbohydrates	↑	In Enes et al. (2009)
	<i>Sparus aurata</i>	Cornstarch	↑	In Enes et al. (2011a)
		Cornstarch, gelat	↑, ↑↑	
		Cornstarch, waxy	↓	Enes et al. (2008a)
		Cornstarch, pre-gelat	↑	In Enes et al. (2011a)
	<i>Takifugu obscurus</i>	Cornstarch	↑	Liu et al. (2015)
6-Phosphofructo-1-kinase	<i>Sparus aurata</i>	Cornstarch, gelat	↑	In Enes et al. (2011a)
	<i>Takifugu obscurus</i>	Cornstarch	↔	Liu et al. (2015)
Fructose-1,6-bisphosphatase	<i>Dicentrarchus labrax</i>	Cornstarch, waxy or native	↓	Enes et al. (2006)
		Glucose	(↓)	In Enes et al. (2011a)
		Cornstarch, gelat	↓	
	<i>Labeo rohita</i>	Cornstarch	↓	Kumar et al. (2010)
	<i>Perca fluviatilis</i>	Digestible carbohydrates	↓	In Enes et al. (2009)
	<i>Salmo salar</i>	Starch	↔	
	<i>Sparus aurata</i>	Cornstarch, gelat	↑	In Enes et al. (2011a)
		Cornstarch, waxy or native	(↓)	
		Cornstarch, gelat	↑	
	<i>Takifugu obscurus</i>	Cornstarch	↔	Liu et al. (2015)
Glucose-6-phosphatase	<i>Dicentrarchus labrax</i>	Cornstarch, waxy or native	↔	Enes et al. (2006)
		Glucose	↓, ↔	In Enes et al. (2011a)
	<i>Labeo rohita</i>	Cornstarch	↓	Kumar et al. (2010)
	<i>Oncorhynchus mykiss</i>	Pea starch	(↑)	Panserat et al. (2000b)
		Glucose	(↓)	Panserat et al. (2001a)
	<i>Oreochromis niloticus</i> × <i>O. aureus</i>	Waxy cornstarch, Glucose	↔↑	Tung and Shiau (1991)
	<i>Sparus aurata</i>	Cornstarch, gelat or waxy	↔	Enes et al. (2008b, a)

(continued)

Table 14.3 (continued)

Enzyme	Species	Carbohydrate type	Response	Reference
	<i>Takifugu obscurus</i>	Cornstarch	↔	Liu et al. (2015)

↑ increase, ↓ decrease in activity/transcription; ↔ ambiguous or no apparent response; *gelat* gelatinized
^aanimals were subject to additional environmental stresses, such as anoxia or hyposalinity

intracerebroventricular injections of nesfatin-1 (Blanco et al. 2017). Simultaneously, injected nesfatin-1 decreases the expression of hepatic glucotransporters *glut4* and *sglt-1*. As glucose is highly metabolized in the brain as energy source, the results indicate that the effect of nesfatin-1 at central level is to decrease the glucose storage in peripheral tissues and increase its utilization in the brain. This demonstrates two distinct effects of nesfatin-1 on glucose homeostasis in central vs. peripheral tissues and highlights its role as a metabolic regulator peptide (Bertucci et al. 2019).

A bridge to invertebrates, Galloway and Cutfield (1988) provided biochemical evidence for the presence of an insulin-like molecule in the digestive tract of a tunicate and reinforced the view that, during evolution, the pancreatic hormones originated in the gastrointestinal mucosa of early chordate organisms.

14.2 Homeostasis: Invertebrates

In crustaceans and other invertebrates, hemolymph glucose levels are usually low (Table 14.2). However, values up to 10 mM can be found (*Nephrops norvegicus*). Furthermore, the data of the same species are sometimes not consistent, as shown in *Carcinus maenas* and *Litopenaeus vannamei*, in which the glucose levels differ by one order of magnitude or more (Table 14.2).

As early as at the level of unicellular eukaryotes, namely, in the ciliate *Tetrahymena pyriformis*, there occur both the insulin-like substances and their receptors immunologically similar to those in higher vertebrates (Pertseva and Shpakov 2002) (for metabolic imprinting in *Tetrahymena* with insulin, →AAN I “Transgenerational Effects” (Steinberg 2018)).

In bivalves, glucose metabolism appears to be regulated via production of insulin-like substances by the gut epithelium as found in two freshwater bivalves (*Anodonta cygnea*, *Unio pictorum*). Insulin-like substance secretion is modulated by hemolymph glucose concentrations (Plisetskaya et al. 1978). The action of insulin-like substances to control the blood glucose content applies also to the edible freshwater bivalve *Lamellidens marginalis* (Subramanyam 1984).

In the pond snail (*Lymnaea stagnalis*), a group of insulin-like peptides occur, called MIP (molluscan insulin-like peptides) (Smit et al. 1993). By their primary structure, MIPs are 20–40% identical to those of other peptides of the insulin

superfamily. They perform regulations on growth, carbohydrate metabolism, calcium balance in tissues, and reproduction (Pertseva and Shpakov 2002).

In crustaceans, glucose levels are stimulated through the release of crustacean hyperglycemic hormone (CHH) neuropeptides from the sinus gland into the hemolymph. This hyperglycemic mechanism is verified in many species (Chung et al. 2010; Chung 2014). Furthermore, endogenous insulin is likely to be involved in the regulation of carbohydrate metabolism in *Litopenaeus vannamei* and *Homarus americanus* (Sanders 1983; Gutiérrez et al. 2007).

In a detailed study, Jiang et al. (2020) characterized the *insulin-like peptides (ilp)* gene from the giant river prawn (*Macrobrachium rosenbergii*) and its relationship to carbohydrate metabolism. ILP contributes to oligosaccharide and polysaccharide accumulation from glucose with a delayed response to the glucose concentration. CHH stimulates the gluconeogenesis process to elevate the glucose level, and it is regulated by the expression of ILP and negative feedback mechanism. This means, ILP and CHH counteract to regulate glucose levels in *M. rosenbergii*.

14.3 Regulation

14.3.1 Glycolysis

Glycolysis is the process that metabolizes one glucose molecule to two pyruvate molecules with the concomitant net production of two high-energy molecules ATP and NADH (reduced nicotinamide adenine dinucleotide). Under anaerobic conditions, pyruvate can be further processed to lactate or ethanol; under aerobic conditions, it can be completely oxidized to CO₂ (Berg et al. 2015).

14.3.2 Gluconeogenesis

Gluconeogenesis generates glucose from certain non-carbohydrates to maintain blood glucose levels and avoid low levels (hypoglycemia). Maintaining levels of glucose is important because the brain depends on glucose as its primary fuel, and red blood cells use glucose as their only fuel (Nelson and Cox 2005). From breakdown of proteins, these substrates include glucogenic AAs (Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Met, Pro, Ser, Val, Phe, Ile, Thr, Trp, Tyr (only Leu and Lys are not glucogenic (Berg et al. 2015)) (→ Chap. 5). From breakdown of lipids (such as triglycerides), the substrates include glycerol and odd-chain FAs; and from other metabolic steps, they include pyruvate and lactate.

The gluconeogenic-specific enzyme glucose-6-phosphatase (G6Pase) first appeared in cnidarians but is also present in echinoderms, mollusks, and vertebrates. Expression data from *Drosophila* indicates that G6Pase and, hence, gluconeogenesis initially had a neuronal function. Therefore, Miyamoto and Amrein (2017)

hypothesize that most likely also in other Arthropoda (e.g., *Crustacea*) and possibly, in some vertebrates, gluconeogenesis is used as a means of neuronal signaling.

In fishes, gluconeogenesis takes place mainly in the liver and kidney (Knox et al. 1980). This process occurs during periods of fasting, starvation (Pastoureaud 1991), low-carbohydrate diets, intense exercise, or environmental stress situations, such as hypoxia or change in salinity (Guillaume et al. 1984; Reyes-Ramos et al. 2018). Acclimation to changes in salinity applies particularly to estuarine animals (Oliveira and Da Silva 2000).

Carnivorous fishes are highly dependent on gluconeogenesis from dietary AAs to maintain glycemia. Glucose recycling (glucose→C₃-intermediate→glucose) may potentiate the effects of glucose administration in sparing AA gluconeogenesis. Furthermore, farmed carnivorous fishes under high-carbohydrate diets tend to increase adiposity (Viegas et al. 2016). Diets for European seabass and gilthead seabream juveniles should include no more than 20% of digestible carbohydrates (Enes et al. 2011a). At higher inclusion levels, fish growth and feed utilization tend to be depressed. Protein sparing by digestible dietary carbohydrate is still relatively controversial, as some studies point out such an effect while other works failed to demonstrate it. Digestibility of native starch is high (>70%) in seabass and seabream and can be improved by processing starches (>90%). European seabass apparently performs better with starch than with glucose, in contrast to gilthead seabream. Glucose tolerance tests show that the duration of postprandial hyperglycemia is similar in both species, indicating that glucose is rather rapidly metabolized. In both species, dietary carbohydrate intake enhances the enzymes of the glycolytic pathway as well as increases liver glycogen content to store excess glucose and to maintain glucose homeostasis (Enes et al. 2011a).

Subsequently, Gatesoupe et al. (2014) confirmed that the European seabass can be fed plant carbohydrates at 25% without detrimental effects. The authors studied the transcription of selected genes (Fig. 14.2): Glucokinase (*gk*) is the enzyme that phosphorylates glucose to glucose-6-phosphate, the first limiting step of glucose storage in the liver. Amylopectin-rich diet (waxy corn, WM) increases the transcription of the corresponding gene. Conversely, the gene coding for phosphoenolpyruvate carboxykinase (*pepck*) is repressed on starchy diets. This enzyme converts oxaloacetate into phosphoenolpyruvate, the first step of hepatic gluconeogenesis, and decreases when the animals are fed carbohydrates. The bidirectional glucose transporter GLUT2 (→ Chaps. 13 and 16) is required in hepatic cells to uptake or to secrete glucose for storage or production, respectively. The transcription of the corresponding gene is not significantly different between diets. The observed fecal production of acetate indicates microbial contribution to the digestion of the high dietary carbohydrate shares. The functional flexibility of the microbiome likely plays an important role in the digestive adaptability of fishes (Karasov et al. 2011) but remains to be investigated further.

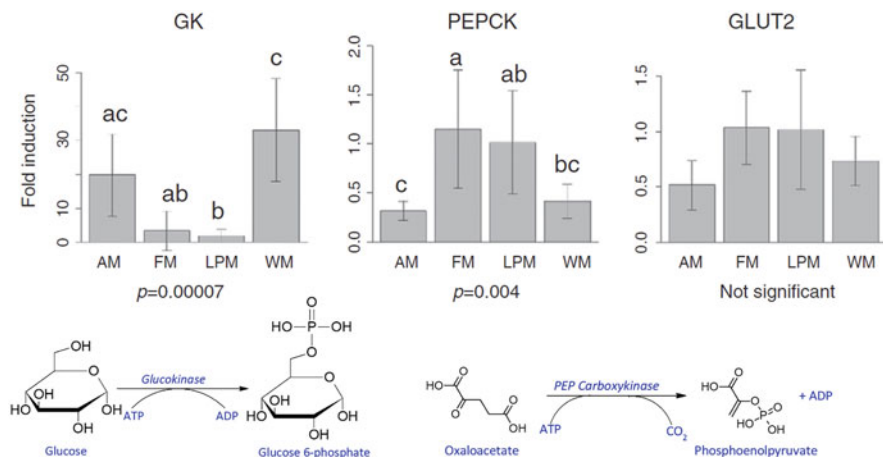


Fig. 14.2 Relative expression levels of the genes coding for *glucokinase* (*gk*), *phosphoenolpyruvate carboxykinase* (*pepck*), and *glucose transporter* (*glut2*) in the liver of seabass fed four diets, 7 ± 1 h after the last meal. The standard deviation of mean level is represented as a vertical segment, and different letters indicate significant differences between groups. (From Gatesoupe et al. (2014), with permission from Elsevier). *FM* fish meal + cellulose as single carbohydrate additive; *WM* highly digestible (waxy corn, containing 99% amylopectin); *AM* slowly digestible (amylcorn, resistant starch containing 70% amylose and 30% amylopectin); *LPM* lupin meal that is rich in non-starch polysaccharides

14.3.3 Glycophagy

Another process in maintaining glucose homeostasis plays an important role: glycophagy in many tissues including the heart, liver, and skeletal muscles. Glycophagy is part of autophagy,⁴ a catabolic process that degrades cytoplasmic constituents and organelles in the lysosome. It is a highly conserved self-digestion process. Many kinds of selective autophagy occur in response to various physiological cues such as mitophagy, reticulophagy, and glycophagy.⁵ Moreover, glycophagy is highly regulated by many signal pathways like the cyclic AMP protein kinase A/protein kinase A, PI3K-Akt/PKB-mTOR, and calcium (Xia et al. 2015).

Starvation-induced protein degradation is a salient feature of autophagy, but recent progress has illuminated how autophagy, during both starvation and nutrient-replete conditions, can mobilize diverse cellular energy and nutrient stores such as lipids, carbohydrates, and iron. Processes such as lipophagy (→ Chap. 24),

⁴The literal meaning of “autophagy” is to “eat oneself.” Autophagy is subject to circadian rhythmicity, and this rhythmicity, in turn, is impacted by nutritional deficiency as recently shown in Nile tilapia (Wu et al. 2020).

⁵Selective degradation of mitochondria, endoplasmic reticulum, or cellular glycogen by autophagy in response to changing cellular conditions.

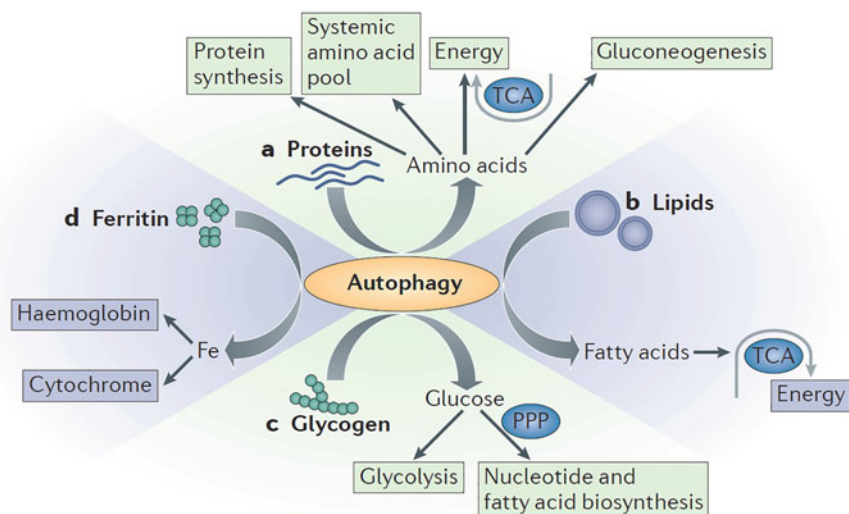


Fig. 14.3 Autophagy-derived metabolites support diverse anabolic functions. The autophagic degradation of proteins, lipids, glycogen, and ferritin via autophagy enables multiple anabolic and biosynthetic pathways in cells. **(a)** Under low nutrient conditions, autophagy-mediated protein catabolism results in the production of free amino acids that provide structural units for protein synthesis and serve to maintain intracellular amino acid pools. These amino acids can also be converted to substrates that are utilized by the tricarboxylic acid (TCA) cycle for energy production or used as substrates for glucose production by gluconeogenesis. **(b)** Fatty acids produced via lipophagy are converted into acetyl-CoA, which fuels the TCA cycle and energy production. **(c)** Glycogen stores in the liver and skeletal muscles are broken down to produce glucose that is utilized by glycolysis for energy (ATP) production as well as for the production of substrates, such as citrate, used for new lipid synthesis. In addition, glucose is diverted into the oxidative pentose phosphate pathway (PPP), a side branch of glycolysis important for nucleotide and fatty acid biosynthesis as well as the antioxidant response. **(d)** Iron released from ferritin stores is utilized for the synthesis of metalloproteins such as hemoglobin and cytochromes. (From Kaur and Debnath (2015), with permission from Springer Nature)

glycophagy, and ferritinophagy enable cells to salvage key metabolites to sustain and facilitate core anabolic functions (Fig. 14.3).

Glycophagy delivers cellular glycogen to the vacuole where it is degraded in response to changing cellular conditions. In mice, this process is necessary to sustain life during the period of postnatal hypoglycemia. Although the mechanisms involved in this process are still largely unknown, recent findings demonstrate that the starch-binding domain-containing protein1 (STBD1) binds to glycogen and delivers it to autophagosomes (Panserat et al. (2019) with references therein).

So far, the physiological functions of autophagy in fish metabolism have rarely been studied. Recently, however, Han et al. (2019) showed in Nile tilapia that autophagy inhibition affects growth of fishes. Moreover, autophagy is conducive to maintaining the homeostasis, the inhibition of which will worsen the metabolic profiles of otherwise healthy fish, as demonstrated by lipid accumulation, weaker antioxidant capacity, and increased inflammation. Furthermore, autophagy

influences other forms of nutrient metabolism, such as glycometabolism and proteometabolism, and plays an important role in fueling energy and nutrient stores. The more intricate mechanism is possibly associated with the AMPK and mTOR signaling pathways (→ Box: Yin and Yang of Energy Regulation, Chap. 5).

In particular, Han et al. (2019) found that the glycogen content is lower in the group treated with an autophagy inhibitor, indicating that glycogen utilization is improved by the inhibitor treatments. Biomolecular data from the tissues show that glycolysis takes place mainly in muscles, and the inhibitor improves glycolysis in adipose tissues; however, the inhibitor mostly reduces the capacity of gluconeogenesis in the liver. Therefore, inhibitor-induced autophagy inhibition reduces the portion of energy sourced from lipid metabolism yet enhances glycolysis overall. This indicates that glucose metabolism can be regulated by autophagy. The overall significance of this process in carbohydrate metabolism in fishes and invertebrates with different feeding habits is still to be studied in detail.

14.4 Concluding Remarks

For a long time, the role of the intestinal microbiota in glucose metabolism has been overlooked and is still more neglected than considered. This applies also to carbohydrate metabolism of fishes. The abovementioned “classical” bias of fishes being diabetic has delayed the progress of identifying biomolecular and biochemical processes that control serum glucose levels. Obviously, the regulation is more complex than anticipated. The function of metals, such as Cr and Co, as prebiotic-like compounds needs verification by “omics” techniques. Furthermore, being the first ones, Falcinelli et al. (2016) detected that probiotic (*Lactobacillus rhamnosus*) treatment reduces the serum glucose level in zebrafishes by upregulation of genes related to glucose level reduction. This study provides insight into how probiotics interact in regulating a novel gene network involved in glucose metabolism. It points out a possible role for *L. rhamnosus* in the treatment of impaired glucose tolerance and food intake disorders by gut microbiota manipulation. Moreover, feeding glucose and *Escherichia coli* to zebrafish produces a prophylactic effect against *Vibrio cholerae* infection (Nag et al. 2018). These intriguing examples of gut microbiome significance in glucose metabolism allow finally putting forward the challenging hypothesis: improving the composition of the gut microbiota makes even carnivores tolerate increased carbohydrate diets.

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Chapter 15

Glucose Intolerance—‘*Life’s Real Luxury?*’



Abstract This chapter consolidates mechanisms of glucose intolerance in carnivorous fishes and presents mechanistic studies of impairments. Hyperglycemic fishes suffer from diabetic impairment of bone tissues and scales and from anxiety-like behavior. In addition to classical modes of action of glucose tolerance in herbivorous and omnivorous species, such as activity of glucose transporters and symporters, evidence is emerging that hepatic transcript levels of key glucose metabolism genes are regulated by dietary carbohydrate levels via epigenetics. Further hypotheses of glucose intolerance in carnivores are sketched. In rainbow trouts, glucose intolerance can be explained by the inhibiting effects of insulin on glucose disposal. Recent field experiments and feeding trials in brown trouts indicate that the capacity for glucoregulation is actually higher than generally assumed. The brown trout issue requires reevaluation to check its generalizability. Finally, this chapter points out issues still worth being studied extensively.

Generally, carnivorous teleosts are considered (D)-glucose-intolerant. However, this phenomenon is species-specific and depends on the ontogenetic developmental stage, physiological conditions such as starvation or nutritional programming (→AAN III “Nutritional Programming”), or external factors, such as temperature (Table 15.1). To recall common knowledge, species at a low trophic level (such as omnivorous and herbivorous ones) are able to use higher levels of dietary carbohydrates (up to 50%). This contrasts fishes at higher trophic levels, namely, piscivorous/carnivorous ones, such as salmonids and many marine species tolerating maximal 20–25% (Wilson 1994; Krogdahl et al. 2005; Enes et al. 2011a). Consequently, this chapter tries to unravel modes of action of glucose tolerance and intolerance, respectively.

Table 15.1 Occurrence of glucose tolerance in selected fishes

Name, common name	Glucose tolerance	Reference
Herbivores and omnivores		
<i>Ameiurus melas</i> , black bullhead catfish	++	Legate et al. (2001)
<i>Carassius gibelio</i> , gibel carp	+ / ++ strain-dependent	Jin et al. (2018)
<i>Ctenopharyngodon idella</i> , grass carp	++ non-adapted +++ adapted	Li et al. (2018)
<i>Danio rerio</i> , zebrafish	+ / ++ @ <i>lactobacillus rhamnosus</i>	Falcinelli et al. (2016)
<i>Cyprinus carpio</i> , common carp	++	Furuichi and Yone (1981)
<i>Megalobrama amblycephala</i> , Wuchang bream	++ non-adapted +++ adapted	Li et al. (2016)
<i>Oreochromis niloticus</i> , GIFT tilapia	+	Chen et al. (2018)
<i>O. niloticus</i> , Nile tilapia	(+)	Wright Jr et al. (2000)
	++	Liu et al. (2018)
<i>O. niloticus</i> × <i>O. aureus</i> , hybrid tilapia	+ / ++ size-dependent	Tung and Shiau (1993)
<i>Piaractus mesopotamicus</i> , pacu, fruit-eating	+++	Takahashi et al. (2018)
Carnivores		
<i>Anguilla rostrata</i> , American eel	(+)	Legate et al. (2001)
<i>Dicentrarchus labrax</i> , European seabass	(+)	Enes et al. (2011b)
<i>Epinephelus coioides</i> , orange-spotted grouper	(+)	Liu et al. (2017)
<i>Gadus morhua</i> , Atlantic cod	(+)	Hemre et al. (1991)
<i>Oncorhynchus mykiss</i> , rainbow trout	(+)	Palmer and Ryman (1972); Bergot (1979); Harmon et al. (1991); Legate et al. (2001)
	+ after rapamycin treatment	Dai et al. (2014)
<i>O. tshawytscha</i> , Chinook salmon	(+) non-adapted + adapted	Mazur et al. (1992)
<i>Paralichthys olivaceus</i> , olive flounder	+	Lee et al. (2003)
<i>Salmo salar</i> , Atlantic salmon	(+) winter + summer	Hemre et al. (1995, 2002a)
<i>S. salar</i> , starch-fed	(+) farmed + wild	Betancor et al. (2018)
<i>Scophthalmus maximus</i> , turbot	(+)	Garcia-Riera and Hemre (1996)

(continued)

Table 15.1 (continued)

Name, common name	Glucose tolerance	Reference
<i>Seriola lalandi</i> , yellow-tail kingfish	(+)	Booth et al. (2013)
<i>S. quinqueriata</i> , yellowtail	(+)	Furuichi and Yone (1981)
<i>Solea senegalensis</i> , Senegalese sole	++	Conde-Sieira et al. (2015, 2016)
<i>Squalus suckleyi</i> , North Pacific spiny dogfish	++	Deck et al. (2017)

+++ high; ++ medium; + low; (+) very low, doubtful, or lacking

15.1 Tolerance

A classical paper on hybrid tilapia reports that carbohydrate utilization is influenced by weight with bigger fishes showing better glucose tolerance and weight gain than smaller ones (Tung and Shiao 1993). Furthermore, plasma glucose level of tilapia fed glucose diet peaks at 2 and 4 hour after the meal in small and large fishes, respectively. When starch diet is fed, no peak is observed in plasma glucose. In addition, Table 15.1 shows that the glucose tolerance of (carnivorous) elasmobranchs and carnivorous teleosts—except Senegalese sole—seem to differ with elasmobranchs being apparently glucose-tolerant.

Starvation affects glucose tolerance. It induces a gradual decrease in the glucose clearance rate in carnivorous fishes, at the same time as the insulin/glucagon ratio is skewed in favor of glucagon (Hemre et al. 2002b). Fasting decreases the use of exogenous glucose in glucose-loaded brown trout, resulting in sustained hyperglycemia, possibly controlled by glucagon and glucagon-like peptide effects on the liver, in the face of declining insulin titers during this time. Concurrently, fasting also reduces glucose tolerance (Hemre et al. 2002b).

The TOR (target of rapamycin) signaling pathway has emerged as a potential mediator of the regulation of glucose homeostasis (Cornu et al. 2013) (→Box: Yin and Yang of Energy Regulation, Chap. 5). It has been shown in rainbow trout that the inhibition of the TOR pathway by rapamycin can increase the glucose tolerance through the inhibition of hepatic gluconeogenesis (Lansard et al. 2010; Dai et al. 2013, 2014).

In addition, Qin et al. (2018) pointed out the importance of transporters, namely, the Na⁺-dependent glucose transporter SGLT1 and the passive glucose transporters GLUT2 (→ Chap. 16), in the glucose tolerance of common carp. The authors find that high mRNA levels of *splt1* and *glut2*, high glucose transport capacity of brush border membrane vesicles, and high intestinal *villus* are detected in the proximal and mid-intestine (Fig. 15.1). Food intake greatly enhances the expressions of intestinal glucose transporters and thereby improves glucose absorption. This study confirms

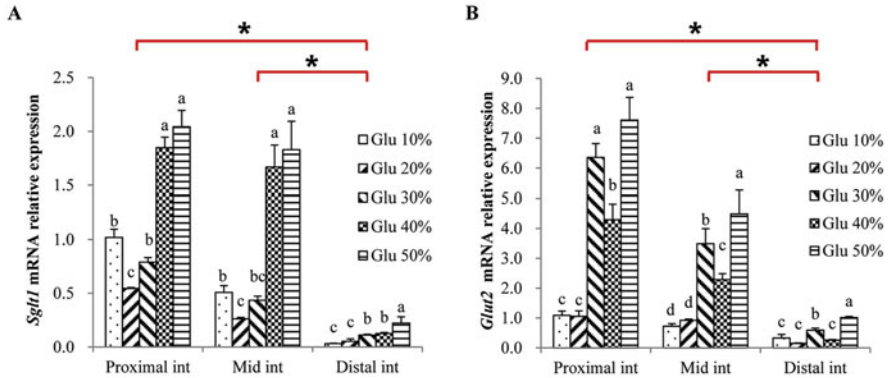


Fig. 15.1 Effects of dietary glucose on intestinal glucose absorption of common carp. (a) Intestinal *sGLT1* mRNA expression. (b) Intestinal *glut2* mRNA expression. Results are represented as mean \pm SEM. Different lowercase letters above the columns of the same intestinal segment indicate significant differences ($P < 0.05$). *Proximal int* proximal intestine; *Mid int* mid-intestine; *Distal int* distal intestine. * Means significant differences between the intestinal segments under all treatments. (From Qin et al. (2018), with permission from Elsevier)

that increasing levels of dietary glucose and NaCl promote the mRNA and protein levels of intestinal SGLT1 and GLUT2, facilitate brush border membrane vesicles glucose transport, and therefore enhance intestinal glucose absorption and the glucose tolerance in common carp. It supports one of the classical hypotheses.

Is epigenetics involved in carbohydrate tolerance?

Very recently, Cai et al. (2020) provided a hepatic whole genome DNA methylation analysis in grass carps fed with moderate- or excessive-carbohydrate-level diet. Although the mRNA expression levels of genes involved in glucose metabolism are significantly changed in grass carps fed with diets of different carbohydrate contents, there is no significant difference in the global genomic DNA methylation levels. Despite that, the hepatic transcript levels of key glucose metabolism genes are regulated by dietary carbohydrate levels via DNA methylation (Fig. 15.2): transcription levels of *glycogen synthase (gs)* and *pyruvate kinase (pk)* decrease, whereas those of *phosphoenolpyruvate carboxykinase (pepck)* and *glucose-6-phosphatase a.1 (g6pca.1)* increase in the high-carbohydrate (HC) diet group. As a result, also the serum glucose concentrations increase.

Glucose regulates orexigenic hormones, *ghrelin* and *neuropeptide Y* (expressed in the brain Fig. 15.3), and the GH/IGF-I axis (Riley Jr et al. 2009). Surprisingly, brain *ghrelin* level is reduced, indicating that the elevated *npv* mRNA levels are responding to changes in glucose levels and not *ghrelin*. Therefore, NPY containing neurons obviously are a “glucosensor” as understood from mammals (Levin et al. 2004).

Fig. 15.2 Hepatic mRNA expression levels of different methylated genes (DMGs) involved in carbohydrate metabolism in grass carp fed with different carbohydrate levels. (From Cai et al. (2020), credit Frontier Media). *HC* high-carbohydrate diet; *gs* glycogen synthase; *pk* pyruvate kinase; *pepck* phosphoenolpyruvate carboxykinase; *g6pca.1* glucose-6-phosphatase a.1

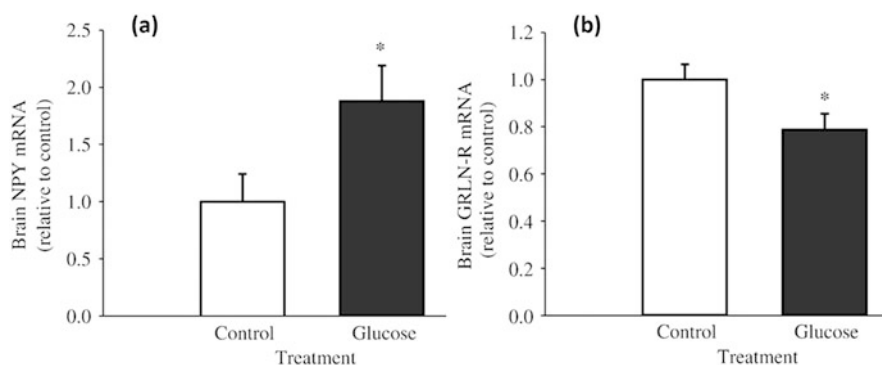
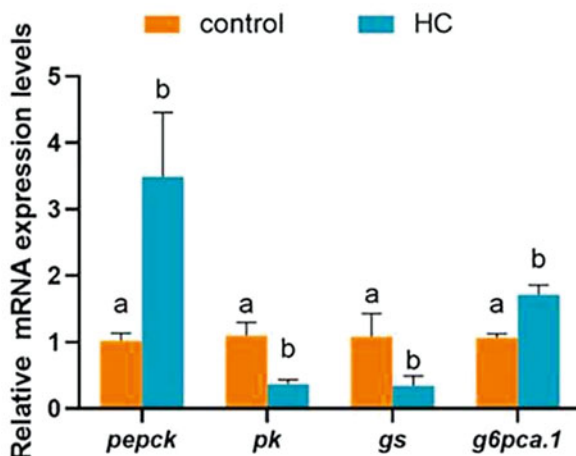


Fig. 15.3 Total brain mRNA levels of *npv* (a) and *ghs-r1a* (b) 6 hours following a single glucose injection load in *Oreochromis mossambicus*. Columns represent mean \pm SEM ($n = 8-10$). * Significantly different from control, $P < 0.05$. (From Riley Jr et al. (2009), with permission from Elsevier). Growth hormone secretagogue receptor, or ghrelin receptor, is a ghrelin-binding receptor and plays a role in energy homeostasis and regulation of body weight

15.2 Intolerance

Carnivorous teleosts are generally considered glucose-intolerant, indicating that glucose load results in persistent hyperglycemia and, in many cases, reduced growth. In teleosts, persistent hyperglycemia generally coincides with transient hyperinsulinemia. Vice versa, resting glucose turnover rates for fish species are in general 20–100 times lower than values reported in mammals of equivalent body mass, consistent with their lower body temperatures and metabolic rates (Moon 2001).

Glucose intolerance has far-reaching consequences.

Rainbow trout is a well-studied carnivorous (piscivorous) species that shows many particularities in its nutrition. While glucose is a principal source of cellular energy in mammals, it is a relatively minor energy source in trouts; rather this species is a poor user of dietary carbohydrates illustrated by a clear glucose intolerance phenotype after carbohydrate intake. Moreover, trouts have a high requirement for protein, with amino acids (AAs) being largely used for energy production by catabolism or through the production of fatty acids (FAs) and glucose (Panserat et al. 2013). In other words, glucose metabolism and glucose homeostasis are highly dependent of the feeding status. In fed as well as unfed animals, glucose will be used directly as an energy source in many tissues through glycolysis. However, in fed animals, dietary glucose in excess will be either stored as glycogen mainly in the muscle and liver or converted into FAs in the liver and fat tissue in order to be used later (during the fasted state) for energy purposes. However, it is generally accepted that rainbow trouts do not have a specific requirement for dietary glucose (NRC 2011), because they are able to survive and grow on diets devoid of carbohydrates. Instead, carbohydrate inclusion level in pellets is limited due to a significant decrease in growth parameters associated with a postprandial persistent hyperglycemia (up to 20 mM) in rainbow trout fed with more than 20% carbohydrates. Occasionally, detrimental effects on the liver (“fatty liver” = steatosis hepatis) are also observed, indicating a problem in metabolic use of dietary glucose (Panserat et al. 2013). This is the well-established knowledge. Later, the same laboratory, however, carried out an intriguing trout experiment with surprising results (see Fig. 15.3).

As representatives of an early stage in vertebrate evolution, sturgeons possess several metabolic peculiarities (Singer and Ballantyne 2005); however, these peculiarities do not include glucose tolerance (→ Chap. 13). Rather, as benthic-carnivores, sturgeons show distinct glucose intolerance as shown in white sturgeon (*Acipenser transmontanus*) (Hung 1991) and characterized by a long-term duration of high plasma glucose concentration after feeding high levels of digestible carbohydrates (Fig. 15.4a).

As mentioned, glucose tolerance/intolerance depends, to a certain degree, on the actual physiological status of an individual. This status changes not only during fasting and refeeding periods but also on a circadian basis (→ AAN I “Chrononutrition” (Steinberg 2018)). Consequently, López-Olmeda et al. (2009) raised the question of whether glucose tolerance in fish depends on the daily feeding time and frequency. The authors describe for the first time a daily rhythm in tolerance in omnivorous goldfish. After a glucose meal, blood glucose kinetics differs depending on the mealtime, between middle of the light phase (ZT6)¹ and middle of the dark phase (ZT18). Glucose concentrations are higher at the middle of the dark phase than at the middle of the light phase as soon as 15 min after feeding (Fig. 15.4b).

¹By convention in studies of circadian rhythms, lights on is taken as the beginning of the *zeitgeber* (synchronizer) time (ZT) and correspond to ZT0; thus, lights off correspond to ZT12.

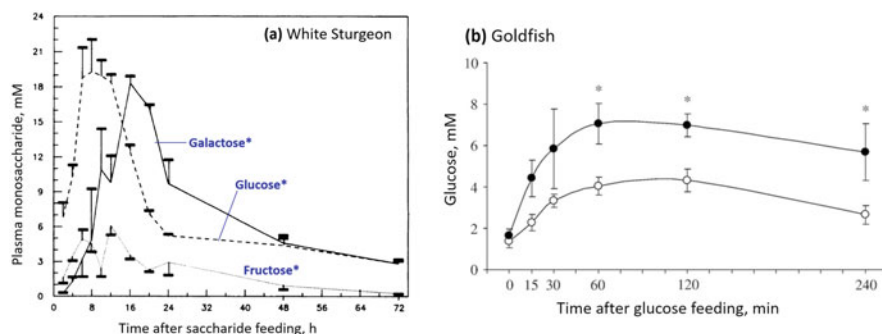


Fig. 15.4 (a) Plasma glucose, fructose, and galactose concentrations in white sturgeon fed glucose, fructose, and galactose, respectively. Each carbohydrate was fed orally at a dose of $167 \text{ mg (100 g body weight)}^{-1}$ in gelatin capsules to 33 fishes with 1 capsule per fish. Each point represents the mean \pm SEM of three fishes. *Significant ($P < 0.05$) difference by one-way ANOVA. A trace amount of fructose ($<0.3 \text{ mM}$) was found in the plasma of the glucose- and galactose-fed sturgeon. No galactose (0.1 mM) was found in the plasma of the glucose- and fructose-fed sturgeon. Zero hour was at 0930 h; plasma glucose concentration at 0 h was $4.3 \pm 0.1 \text{ mM}$ (mean \pm SEM, $n = 3$). (From Hung (1991), with permission from the Oxford University Press). (b) Blood glucose kinetics in goldfish after a complete meal with glucose ($3.8 \text{ g glucose kg}^{-1} \text{ fish}$) as carbohydrate source. Glucose kinetics were measured in animals fed periodically once a day at two different times of the light-dark cycle: ZT6 (middle of the light phase, ML; white circles) and ZT18 (middle of the dark phase, MD; black circles). Glucose values at 0 min belong to starved animals. Data from each sampling time (0, 15, 30, 60, 120, and 240 min) were compared between ML and MD. * indicate significant differences between ML and MD. (From López-Olmeda et al. (2009), with permission from Elsevier)

Although this study appears to be the only available one showing a circadian changes of glucose tolerance, daily rhythmicity of digestive physiology and metabolism has been demonstrated also in European eel (López-Olmeda et al. 2012), Nile tilapia (Costa et al. 2016), or gilthead seabream (Montoya et al. 2010; Felip et al. 2015) (\rightarrow AAN I, (Steinberg 2018)). Therefore, it can be postulated that the modulation of the general sensitivity to dietary glucose happens on a circadian basis and deserves detailed studies.

Numerous hypotheses have been proposed to explain the differences in glucose regulation and persistent hyperglycemia in different aquatic animals, which include that:

- Glucose is not an effective stimulator of insulin secretion (Polakof et al. 2012).
- The number of muscle insulin receptors is low or they are rather insensitive (Parrizas et al. 1994).
- Glucose transporters are absent in muscles (Wright et al. 1998).
- Glucose phosphorylation is poorly required for glucose metabolism and utilization (Dabrowski and Guderley 2003).
- The regulation of endogenous glucose produced by gluconeogenesis is poor (Panserat et al. (2000b, 2001)).

The poor utilization of dietary carbohydrates may also be due to hormonal and nutritional regulation, because of evolutionary adaptation (Polakof et al. 2011, 2012). To date, the physiological and molecular basis of glucose intolerance in fish is not fully understood and subject to biomolecular studies with innovative “omics” approaches (Zhang et al. 2018).

Reviewing the scientific success triggered by the question of glucose tolerance in carnivorous fishes asked by Moon (2001), Polakof and Panserat (2016) pointed out the interaction of glucose and other major nutrients, such as AAs, at both signaling and metabolic levels. Unlike in mammals, arginine reveals in fishes a powerful insulin secretion. On the other hand, methionine seems to improve, as in mammals, the glucose tolerance in carnivorous fishes. However, the precise underlying mechanisms of both AAs remain to be elucidated.

Interesting results emerge from comparative studies of lines of one species with different feeding physiology. The favorite species is again rainbow trout. Kamalam et al. (2012) hypothesized that two rainbow trout lines divergently selected for lean (L) or fat (F) muscle differ in their ability to metabolize glucose. The authors investigated whether genetic selection for high muscle fat content leads to a better capacity to metabolize dietary carbohydrates. Juveniles from the two lines are fed diets with or without gelatinized starch for 10 weeks. Growth rate, feed efficiency, and protein utilization are lower in the F than in the L line; in both lines, intake of carbohydrates is associated with a moderate postprandial hyperglycemia, a protein sparing effect, an enhancement of nutrient (TOR-S6) signaling cascade, and a decrease of energy-sensing enzyme (AMPK) (→Box: Yin and Yang of Energy Regulation, Chap. 5). Gene expression of hepatic glycolytic enzymes is higher in the F line fed carbohydrates than in the L line, but concurrently transcripts for the gluconeogenic enzymes are also higher in the F line, possibly impairing glucose homeostasis. However, the F line shows an elevated gene expression of hepatic enzymes involved in lipogenesis and fatty acid (FA) bioconversion, in particular with an increased dietary carbohydrate intake. Enhanced lipogenic potential coupled with higher liver glycogen content in the F line indicates better glucose storage ability than in the L line.

Moreover, this feeding results not only in hyperglycemia but also in global and promoter-specific changes of histone marks in the liver (Marandel et al. 2016). Permissive H3K9ac histone marks globally increase in rainbow trouts refed with a protein-rich diet, while H3K36me3 marks display greater enrichment around the transcription start sites of specific gluconeogenic gene loci of trout fed a protein-rich diet compared to trout fed a carbohydrate-rich diet. Furthermore, trouts on carbohydrate-rich diet exhibit global hypomethylation of DNA (Fig. 15.5).

This study indicates that the hepatic epigenetic landscape can be affected by both nutritional status and dietary carbohydrates at global and target gene levels. The authors expect that understanding the mechanism underlying the setting up of these epigenetic modifications will improve understanding of its impact on the glucose-intolerant phenotype in carnivorous teleosts.

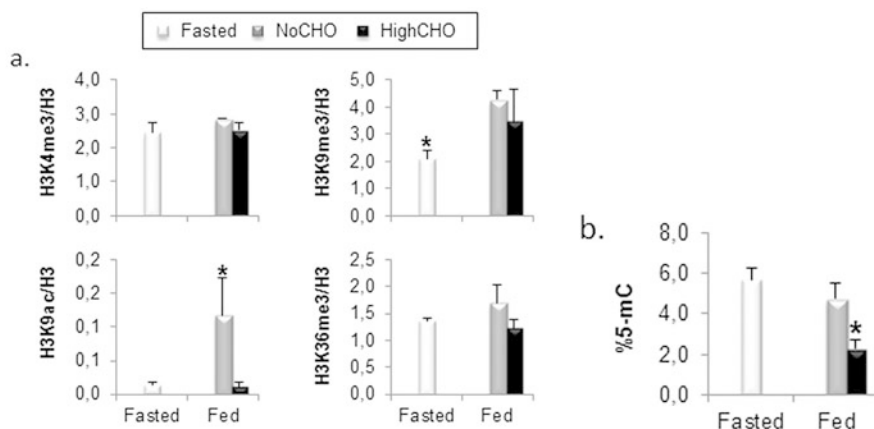


Fig. 15.5 Global epigenetic modification in fasted trout (white bars) and those fed the NoCHO (gray bars) or the HighCHO (black bars) diet. Analyses of global histone modifications (a), global DNA methylation (b). Data are expressed as mean \pm SD, stars indicate significant differences between conditions ($P < 0.01$). (From Marandel et al. (2016), credit Springer Nature)

Box: Rainbow Trout and Insulin

A strange picture: the rainbow trout is going to lift its veil.

The peptide hormone glucagon is one key endocrine signal involved in the regulation of glucose metabolism, and it increases glycemia in fish (Polakof et al. 2012). Forbes et al. (2019a) tested this issue by glucagon administration and found that glucagon only leads to modest changes in glucose fluxes that partly explain why trout seems to be unable to control glycaemia very tightly. In particular, after 4 h of glucagon administration, the glucose fluxes (production and disposal) fall below baseline. This late reduction of glucose fluxes may be caused by a counter-regulatory response of insulin, potentially linked to a decrease in glycogen phosphorylase, muscle-associated (*pygm*) transcript abundance. This gene encodes a muscle enzyme involved in glycogenolysis. Highly similar enzymes encoded by different genes are found in the liver and brain.

In a companion paper, Forbes et al. (2019b) characterized therefore the impacts of insulin on the glucose kinetics in vivo. The authors presented unexpected results. They show that rainbow trouts have a unique response to insulin: the inhibition of glucose disposal. This unexpected effect of insulin has not been documented in other animals, especially mammals that stimulate glucose disposal by several-fold instead. These interspecific differences may be explained by the contrasting effects of insulin on the gluco/hexokinases of trout (inhibition) versus mammals (activation). Insulin also reduces hepatic glucose production in trouts, whereas mammals can achieve complete

(continued)

suppression. This partial reduction of hepatic glucose production may be because insulin does not affect glycogenolysis in trouts and only inhibits gluconeogenesis, whereas mammals shut down both pathways. The integrated actions of insulin that lead to reducing glucose fluxes in trout only provide them with a very limited capacity to decrease glycemia. Therefore, the glucose intolerance classically exhibited by rainbow trout can be explained by the inhibiting effect of insulin on glucose disposal. The partial inhibition of hepatic glucose production may be because insulin only affects gluconeogenesis, but not glycogen breakdown. This atypical regulation of specific gluconeogenic gene paralogs indicates that the “glucose-intolerant” phenotype in rainbow trout is under posttranscriptional control (Kostyniuk and Mennigen 2020). This evidence shows that miRNAs have evolved specific roles in complex salmonid genome regulation, and assessment of metabolic consequences about *de novo* gluconeogenesis awaits functional conformation in transfected trout hepatocytes *in vitro* and in other salmonids to test whether rainbow trout is unique.

The above finding by Marandel et al. (2016) in rainbow trout intrigued Zhang et al. (2021), who demonstrated in Chinese perch juveniles that HC diets can upregulate the DNA methylation level of *g6pca* to inhibit the expression level of *g6pca*, thereby affecting glucose metabolism. *g6p* genes are central in the gluconeogenic pathway; the key role still needs to be verified. The authors put forward an interesting hypothesis, namely, that the poor utilization of carbohydrate in Chinese perch is related to the lack of comprehensive regulation of carbohydrate metabolism due to the loss of another *g6p* homolog in the genome.

Moreover, differences in glucose tolerance are noted also between Chinook salmon strains, which indicate that genotype is an important determinant of glucose utilization also in this species (Mazur et al. 1992). Overall, diet and genotype interactions observed in glucose metabolism indicate that it is likely to obtain trout genotypes with improved ability for lipid biosynthesis in the presence of dietary carbohydrates and based on improved understanding of underlying epigenetics. This allows the conclusion that genetic selection is a way to modify the poor capability of glucose homeostasis in carnivorous fish species, primarily by influencing the regulation of key enzymes involved in glucose and lipid metabolism.

However, is the capability of trouts to use carbohydrates really that poor?

15.3 Feeding Habit Reevaluation

Two laboratories conducted the reassessment of feeding habits of trouts. Salmonids, including brown trout and rainbow trout, are considered as strictly carnivorous species (Jonsson and Gravem 1985). Indeed, when fishmeal is substituted to more

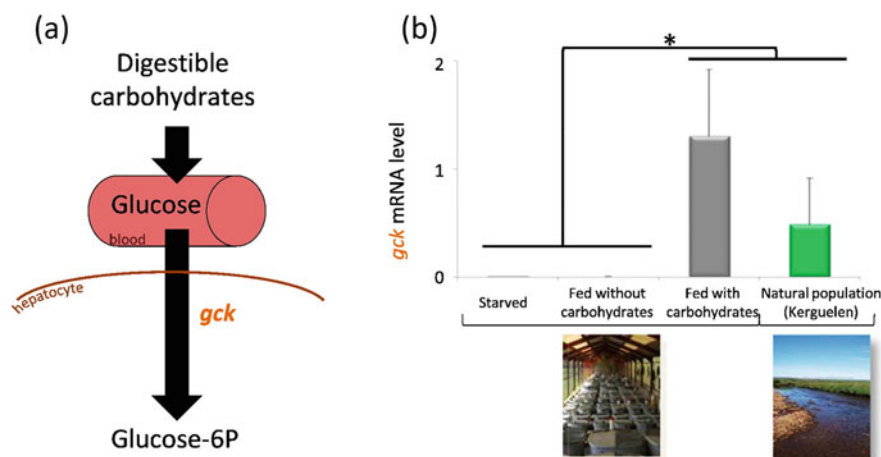


Fig. 15.6 (a) Sketch of glucose utilization via glucokinase (*gck*) in rainbow trout. (b) Kerguelen trout and carbohydrate-fed trout possess elevated *gck* mRNA levels as compared to farmed ones either starved or fed without carbohydrates. (From Marandel et al. (2018), with permission from Elsevier)

than 20% by digestible carbohydrates, rainbow trout display a persistent postprandial hyperglycemia. One hypothesis put forward to explain this persistent hyperglycemia is the absence of an inducible hepatic glucokinase (*gck*) activity and thus the inability of the fishes to convert efficiently the intracellular glucose into glucose-6-phosphate. However, the full-length cDNA sequence encoding for a *gck* is cloned in rainbow trout (Panserat et al. 2000a). Based on this discovery, additional investigations reveal that hepatic *gck* transcription is mainly regulated by dietary carbohydrates (Fig. 15.6a) (Panserat et al. 2014; Marandel et al. 2015).

Consequently, Marandel et al. (2018)² tested the hypothesis that wild salmonids make use of their *gck* regulatory system following ingestion of carbohydrates through natural feeding. Therefore, several species of salmonids, including brown trout (*Salmo trutta*), have been introduced into oligotrophic originally fish-free freshwater ecosystems on the sub-Antarctic Kerguelen Islands since the 1950s. The usual trophic resources were limited to terrestrial or aquatic invertebrates. Moreover, a significant fraction of the population does not migrate to the marine environment for their growth phase. Such a behavior thus limits the diet of these fishes to freshwater invertebrates. These resident brown trout frequently display hepatic alterations similar to steatosis; ingestion of dietary carbohydrates is shown to increase hepatic lipid depositions through lipogenesis. In fact, the authors found that Kerguelen trout are mainly feeding on prey containing digestible carbohydrates. Furthermore, glycemia and *gck* mRNA transcription demonstrate that trout

²Extract taken with permission from Elsevier, appropriate references added.

metabolize dietary glucose (Fig. 15.6b). Therefore, Marandel et al. (2018) conclude that brown trouts in a natural environment consumes dietary carbohydrates. This means that their omnivory depends on environmental circumstances and conditions. Under the condition of missing appropriate prey, these salmonids appear conditionally omnivorous, rather than strictly carnivorous.

In the same line of evidence, Choi and Weber (2015) explored the limits of glucose kinetics in resting and swimming rainbow trouts by using hyperglycemic animals and the infusion of exogenous glucose at twice the baseline rate of hepatic production. The authors found that resting hyperglycemic trouts maintain high enough rates of glucose production and disposal to account for 36% of metabolic rate but reach 100% when exogenous glucose is supplied for a few hours. With their baseline glucose fluxes chronically elevated, they can completely suppress hepatic production and boost disposal by 160% to minimize the effects of a massive glucose challenge. Such responses are typical of mammals, but unexpected for an ectotherm. They are probably mediated by the effects of insulin on GLUT2 and GLUT4, as well as key enzymes of carbohydrate metabolism. Without these large and rapid changes in glucose fluxes, trout glycemia would have increased four times faster than observed here to reach dangerous levels exceeding 100 mM within hours (Choi and Weber 2015). Almost nothing is known about the endocrine regulation of glucose fluxes. Sorting out the roles of insulin, hyperglycemia, and GLUTs represents an important challenge for future work.

The same applies to the swimming performance of rainbow trouts. Choi and Weber (2016) reported that fishes show perfect glucoregulation at all exercise intensities, because the rates of glucose production and disposal change in synchrony. When fishes are given exogenous glucose during swimming, they have the capacity to boost disposal while they deal with the high total glucose production caused by exogenous supply and residual hepatic production.

To recall, rainbow trouts are considered glucose-intolerant with growth delay and persistent postprandial hyperglycemia observed in juveniles fed diets containing more than 20% of carbohydrates. However, the broodstock is hypothesized to use carbohydrates more efficiently than juveniles do. Callet et al. (2020) tested this hypothesis and showed that broodstocks are able to use diets containing digestible carbohydrates (pregelatinized starch) as high as 35% and can then grow and reproduce normally over an entire reproductive cycle for females and at least at the beginning of the cycle for males. These results are highly promising and indicate that dietary carbohydrates can, to a higher extent than anticipated, replace proteins in aquafeed of trout broodstock.

Together, these trout studies show that the capacity for glucoregulation appears to be much higher than previously described and requires broad reevaluation (Weber et al. 2016). This issue will be revisited in AAN III “Nutritional Programming.”

15.4 Impairments

Zebrafish is a convenient model for studying diabetes-related impairments in vertebrates and the number of papers about hyperglycemia-caused malformation and diseases is exploding in the past years. A few striking examples serve here to explain the severity of hyperglycemia.

In hyperglycemic zebrafish, scales demonstrate diabetic impairment of bone tissue resulting in enzymatic alterations of bone-specific markers with severe osteoporotic phenotypes (Fig. 15.7) (Carnovali et al. 2016). In addition, the onset of chronic hyperglycemia leads to impaired peripheral glucose metabolism and to retinal alterations. The retinal blood vessels show abnormalities resembling the human diabetic retinopathy (Singh et al. 2019).

On the cellular level, chronic hyperglycemia can lead to dysfunctions that can become irreversible over time, a process that is termed glucose toxicity. In its initial stages, it is characterized by defective insulin gene expression (Robertson et al. 2003). Under high glucose conditions, increased formation of reactive oxygen

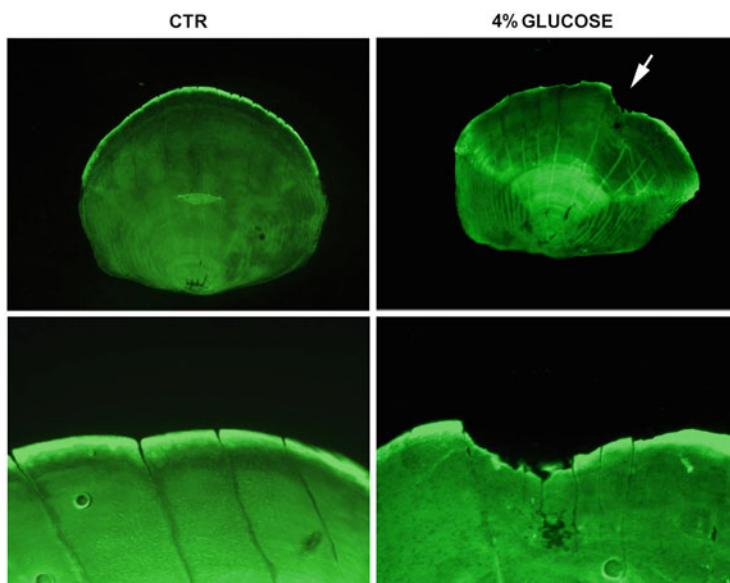


Fig. 15.7 Scale visualized through calcein vital staining. Control zebrafish scales show normal morphology while scales of individuals treated with 4% glucose show extended mineralized matrix resorption along the borders (upper panel) with osteoporotic lacunae (with arrow). The bone loss is better visible with higher magnification (lower panel). (From Carnovali et al. (2016), with permission from Springer Nature)

species (ROS) leads to upregulation of MMP-13,³ which promotes enhanced matrix remodeling and degradation of the extracellular matrix; this process damages sensory axons and leads to their degeneration (Waldron et al. 2018).

Moreover, multiple neurotransmitter pathways can be affected by hyperglycemia. Exposure of zebrafish to high levels of glucose elevates acetylcholinesterase (AChE) activity and impairs long-term avoidance memory (Capiotti et al. 2014). Consequently, treatment with an AChE enzyme inhibitor reverses this adverse effect, demonstrating that the observed cognitive worsening is directly related with AChE activity. Furthermore, hyperglycemia-induced alterations consistently comprise anxiety-like behavior (dos Santos et al. 2018).

15.4.1 Antidotes

Many of the approaches against adverse effects of hyperglycemia use ROS quencher from terrestrial and marine plants (Kim et al. 2015; Saito et al. 2018; Carnovali et al. 2019). Further modes of action have been identified, such as antidiabetic action (Kim et al. 2016; Benchoula et al. 2019), promotion of the nerve growth factor pathway (Nam et al. 2019), or regulation of glucose and lipid metabolic adaptation (Xu et al. 2019). In addition, Kaur et al. (2019) applied cinnamon to combat adverse effects in overfed zebrafish and found lowered blood glucose, lipid, triglyceride levels, and modulated metabolic genes. Furthermore, dos Santos et al. (2018, 2020) successfully administered dietary diphenyl diselenide to overcome hyperglycemia-mediated anxiety-like behavior (avoidance of lit environments) in zebrafish, and Oyelaja-Akinsipo et al. (2020) reported about the protective role of diosgenin, a phytosteroid sapogenin, against hyperglycemia-mediated cerebral ischemic brain injury in zebrafish. Further antidote actions of plant compounds can be expected in future studies.

Using the alkaloid berberine (→AAN III Plant Compounds—Alkaloids and More), He et al. (2021) demonstrate that this alkaloid (50 mg kg⁻¹) can improve glucose metabolism in Wuchang bream via enhancing hepatic glycolysis and inhibiting hepatic glycogen synthesis and gluconeogenesis. Furthermore, berberine reduces the metabolic burden of the liver by inhibiting fat synthesis, promoting lipid decomposition, and enhancing fat uptake in peripheral tissues.

15.5 Concluding Remarks

This chapter points out that, despite progress in identifying basic mechanisms of glucose intolerance in teleosts, even in 2018 the physiological and molecular basis of this phenomenon is not yet fully understood (Zhang et al. 2018). The authors

³Matrix metalloproteinase 13 or collagenase 3 is involved in the breakdown of extracellular matrix, such as collagen.

conducted a comparative genomics study of carbohydrate/glucose metabolic genes from fishes to mammals. More than 70% of the carbohydrate/glucose metabolic genes are conserved in the studied species; therefore, the authors hypothesize that, compared to conserved fundamental glucose metabolic enzymes, insulin regulators clearly contribute to variances in glucose metabolism between the species.

The glucose metabolism is still worth being studied extensively with open issues being:

- Circadian rhythmicity of glucose utilization and digestion.
- Glucose tolerance during the ontogenetic development.
- Involvement of and improvement by the intestinal microbiota in glucose tolerance.
- Role of epigenetics in glucose tolerance.
- Heredity of possibly acquired increased glucose tolerance to succeeding generations.

Moreover, it has to be checked whether the recently detected mechanism glucose intolerance of the rainbow trout applies also to other salmonids in particular or carnivores in general. On the other hand, the feeding habit reevaluation of brown trouts shows that the capacity for glucoregulation appears to be much higher even in carnivorous fishes than generally described in the literature. This intriguing issue requires broad reevaluation.

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Chapter 16

Carbohydrate Transport—‘Life’s Useful Luxury Distributed’



Abstract Central in one hypothesis for limited carbohydrate in carnivorous fishes, carbohydrate transport from the intestine to peripheral tissues is suboptimal. Therefore, this chapter focuses on the mechanism of carbohydrate in fishes and aquatic invertebrates. Transport of glucose is regulated by transport proteins that are modulated in their expression, cellular distribution, synthesis, and half-lives. Two major transporter protein families exist: passive glucose transporters and energy-demanding sodium/solute symporters. Between carnivorous and omnivorous fish species, there exist differences in the adaptability, activity, and affinity of the glucose transporters, which appear to be able to explain a major part of the differences. Recent studies show that nutrition history of the aquatic animals plays a significant role in tolerating carbohydrate-rich diets. An inventory in grass carps shows that miRNAs play a central role in this process. Moreover, the function of glucose transporters is more comprehensive, since they participate in pathogen infections as well as modulating resistance against hypoxic stress and salinity changes with symporters facilitating the latter. Evidence is accumulating that, beside monosaccharide transporters, disaccharide transporters exist in aquatic animals. In conclusion, the chapter points out open issues.

Glucose¹ transport supplies fuel that is needed for energy metabolism by all cells. Moreover, glucose is used also as a signaling molecule. The supply of glucose is especially important for brain neurons, which have a high metabolic rate supported by an obligate consumption of glucose. Transport of glucose is regulated by transport proteins that are modulated in their expression, cellular distribution, synthesis, and half-lives (McCall 2019).

There exist three classes of transporters mediating the transmembrane glucose (fructose) or sucrose transport in fishes and aquatic invertebrates (Berg et al. 2015), whereby the study of the sucrose transporters is still in its infancy (Meyer et al. 2011; Scheffler and Ahearn 2017):

¹Glucose means D-glucose if not otherwise noticed.

- Passive glucose transporters (protein symbol, GLUT; gene symbol also, *slc2a* – solute carrier). They convey the thermodynamically downhill movement of glucose across the plasma membranes, depending on hexose concentration, from mucosal to serosal or serosal to mucosal direction. GLUT1 and GLUT3 continually transport glucose into cells at an essentially constant rate. GLUT2 ensures that glucose rapidly enters the liver or hepatopancreas cells only in times of plenty. GLUT4 transports glucose into muscle and fat cells. GLUT5 functions primarily as fructose transporter (Berg et al. 2015).

More than one decade ago, about 18 facilitated diffusion glucose transporters (GLUT1a to GLUT13b) have been identified in zebrafish ionocytes (Tseng et al. 2009). More recently, Jiménez-Amilburu et al. (2015) found that GLUT12 is a novel insulin-regulated GLUT expressed in insulin-sensitive tissues, such as cardiac muscle, skeletal muscle, and adipose tissue. This paper also indicates that *glut12* is an essential GLUT in the heart where the reduction in glucose uptake due to *glut12* deficiency leads to heart failure.

- Energy-demanding sodium/solute symporter family (Na^+ -dependent glucose transporter, SGLT). “Symporter” means the thermodynamically unfavorable flow of one species of ion or molecule up a concentration gradient is coupled to the favorable flow of a different species down a concentration gradient (Berg et al. 2015). SGLTs use the electrochemical gradient of monovalent cations, such as Na^+ , Li^+ , or H^+ . For example, the SGLT1 is a high-affinity glucose transporter that couples glucose transport into the cell to an inwardly directed Na^+ gradient. This Na^+ gradient is maintained by Na^+/K^+ -ATPase in the basolateral membrane. The stoichiometry of the process dictates that two Na^+ cross the membrane with each glucose molecule (Raja 2004). The activity of these transport systems is regulated primarily by diet and ontogenetic development.

The two types of glucose transporters are sketched in Fig. 16.1a, and Table 16.1 provides an overview of glucose transporters studied in aquatic invertebrates and fishes.

In the recent past, a new family emerged, called the *slc45* gene family of putative sugar, presumably disaccharide, transporters (Fig. 16.1b) (Vitavska and Wieczorek 2013) that will be presented in detail below.

Glucose transporters are not interchangeable. Na^+ -independent glucose transporters delivering glucose among different tissues comprise especially the low-affinity transporter GLUT2 as identified in rainbow trout (Marty et al. 2007). Several glucose transporters are found in Atlantic salmon given high plant diets: *glut1*, *glut2*, and *glut3* are highly expressed in the liver and *glut4* in fast muscles. *glut1* and *glut3* are expressed in the brain, but at low levels, and *glut1* in the midgut (Sissener et al. 2013).

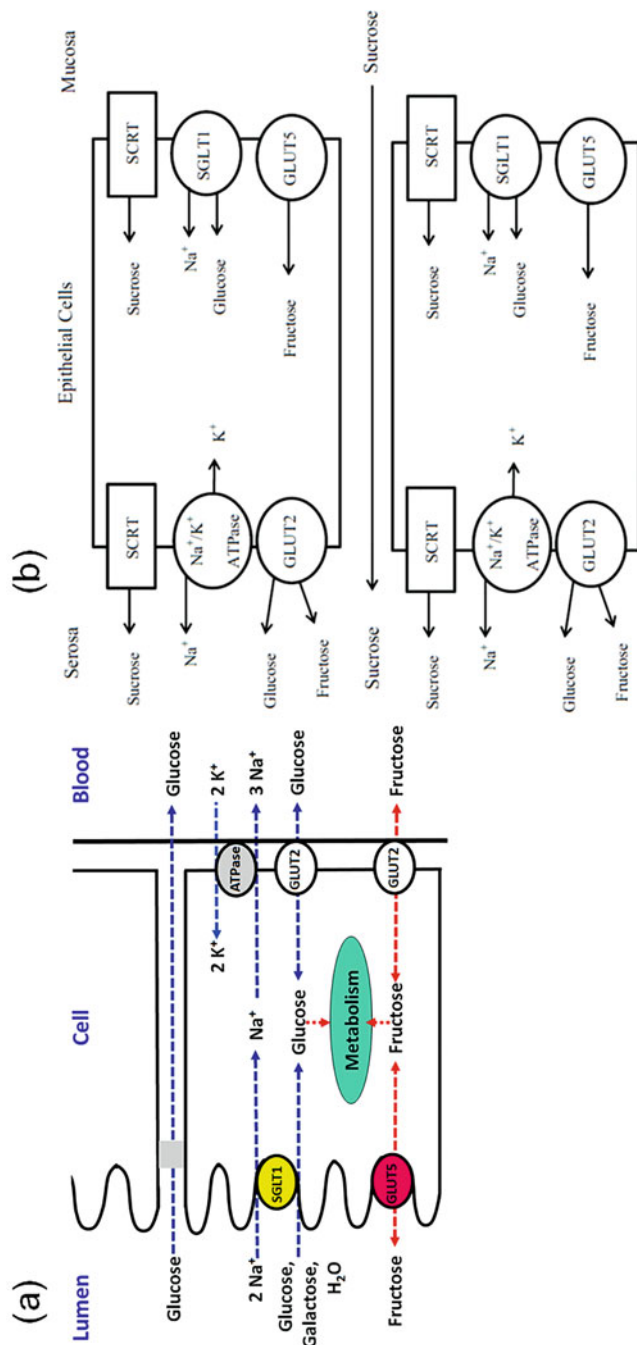


Fig. 16.1 (a) Sketch of monosaccharide (glucose) transport in fishes and aquatic invertebrates. The Na^+ -dependent glucose transporter (SGLT1) is located in the apical membrane and transports (two) Na^+ coupled with glucose or galactose and water. This transport is driven by the inwardly directed Na^+ gradient, which is maintained by the activity of Na^+/K^+ -ATPase in the basolateral membrane. The facilitative transporter GLUT2 transports sugars across the basolateral membrane, whereas the facilitative transporter GLUT5 transports fructose across the apical membrane (redrawn and modified from Wright (1993) and Raja (2004)). (b) Proposed disaccharide (sucrose) transport system (SCRT) alongside glucose (SGLT1) and fructose (GLUT5) transporters in American lobster intestinal cells. The model includes a transcellular transport of sucrose from lumen to blood by a brush border SCRT-like transporter and a presumed basolateral analog of this disaccharide transporter. Paracellular diffusional sucrose transport is suggested to contribute to total transmural transport. (From Likely et al. (2015), with permission from Springer Nature)

Table 16.1 Intestinal glucose transporters in selected aquatic invertebrate and fish species

Species		
Scientific name, common name	Transporter studied	Selected reference
Invertebrates		
<i>Biomphalaria glabrata</i> , ram’s horn snail	GLUT-like	Thomas (1989)
<i>Chlamys farreri</i> , scallop	SGLT1	Wang et al. (2015)
<i>Crassostrea gigas</i> , Pacific oyster	SGLT	Hanquet et al. (2011)
<i>Eriocheir sinensis</i> , Chinese mitten crab	GLUT4	Li et al. (2017)
<i>Hediste diversicolor</i> , ragworm	GLUT-like	Ahearn and Gomme (1975)
	SGLT	Albrechtsen and Gomme (1984)
<i>Homarus americanus</i> , Atlantic lobster	GLUT2, GLUT5, SGLT1	Duka and Ahearn (2014)
<i>Limulus polyphemus</i> , horseshoe crab	GLUT-like, SGLT-like	Sterling and Ahearn (2011)
<i>Litopenaeus setiferus</i> , Atlantic white shrimp	GLUT2, SGLT1, SGLT4	Obi et al. (2013)
<i>L. vannamei</i> , Pacific white shrimp	GLUT1, GLUT2	Wang et al. (2017c)
<i>Mytilus edulis</i> , blue mussel	SGLT	Pajor et al. (1989)
<i>M. galloprovincialis</i> , Mediterranean mussel	SGLT, GLUT(?)	Louzao et al. (1993)
<i>Ostrea edulis</i> , European flat oyster	SGLT-like	Riley (1981)
<i>Perna viridis</i> , green mussel	GLUT-like	Pan and Wang (2004)
<i>Tridacna squamosa</i> , fluted giant clam	SGLT	Chan et al. (2019)
Fishes		
<i>Ameiurus melas</i> , black bullhead catfish	GLUT-like	Legate et al. (2001)
<i>Anguilla anguilla</i> , European eel	GLUT-like	Reshkin et al. (1988)
<i>A. rostrata</i> , American eel	GLUT-like	Legate et al. (2001)
<i>Carassius auratus</i> , goldfish	GLUT2-, SGLT1, SGLT2	Blanco et al. (2017)
<i>Ctenopharyngodon idella</i> , grass carp	GLUT1, GLUT2, GLUT3 ^a SGLT1, SGLT4 ^b	Liang et al. (2020)
<i>Cyprinus carpio</i> , common carp	GLUT1, SGLT1 GLUT2	Syakuri et al. (2019) Deng et al. (2020)
<i>Danio rerio</i> , zebrafish	GLUT1a – GLUT13b GLUT1, GLUT2, GLUT3 GLUT10, GLUT12 SLC2A1, SLC2a	Dalmolin et al. (2018)
<i>Dicentrarchus labrax</i> , European seabass	GLUT2	Terova et al. (2009)
<i>Epinephelus coioides</i> , orange-spotted grouper	GLUT4	Zhang et al. (2016)

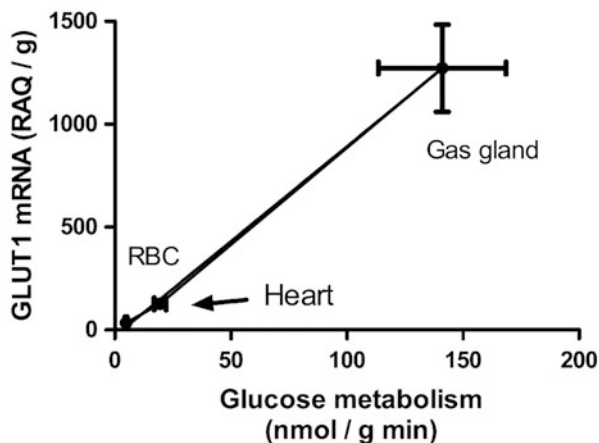
(continued)

Table 16.1 (continued)

Species		
<i>E. fuscoguttatus</i> ♀ × <i>E. lanceolatus</i> ♂, pearl gentian grouper	GLUT1, GLUT2, GLUT4	Li et al. (2018c)
<i>E. lanceolatus</i> , giant grouper	GLUT1, GLUT4	Lu et al. (2018)
<i>Eptatretus stoutii</i> , Pacific hagfish	GLUT, SGLT	Weinrauch et al. (2018)
<i>Gadus morhua</i> , Atlantic cod	GLUT1 – GLUT3, GLUT4	Clow et al. (2016)
<i>Leucoraja erinacea</i> , little skate	SGLT1, SGLT2	Kipp et al. (1997)
<i>Megalobrama amblycephala</i> , Wuchang (blunt snout) bream	GLUT2, SGLT1	Zhang et al. (2018)
<i>Micropterus salmoides</i> , largemouth bass	GLUT1	Yang et al. (2017)
<i>Misgurnus anguillicaudatus</i> , pond loach	SGLT1	Gonçalves et al. (2007)
<i>Oncorhynchus mykiss</i> , rainbow trout	GLUT1a, GLUT1b, GLUT2, GLUT4, SGLT1, SGLT1	Bertucci et al. (2019)
<i>Oreochromis niloticus</i> , Nile tilapia	GLUT1, GLUT4, SGLT1	Subramaniam et al. (2019)
<i>Oryzias latipes</i> , medaka	SGLT2	Goto et al. (2019)
<i>Rachycentron canadum</i> , cobia	GLUT4	Li et al. (2019)
	GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, GLUT9	Ye et al. (2020)
<i>Raja erinacea</i> , skate	SGLT1, SGLT2	Kipp et al. (1997)
<i>Salmo salar</i> , Atlantic salmon	GLUT4, SGLT1	Bakke-McKellep et al. (2008)
<i>S. trutta</i> , brown trout	GLUT4	Díaz et al. (2007)
<i>Schroederichthys chilensis</i> , redspotted catshark	GLUT1	Balmaceda-Aguilera et al. (2012a)
<i>Sparus aurata</i> , gilthead seabream	GLUT1, SGLT1	Balmaceda-Aguilera et al. (2012b) ^c
<i>Squalus acanthias</i> , spiny dogfish	SGLT1, SGLT2	Althoff et al. (2006)
<i>S. suckleyi</i> , North Pacific spiny dogfish	GLUT1, GLUT4 ^d	Deck et al. (2016, 2017)
<i>Tachysurus (Pelteobagrus) fulvidraco</i>	SGLT1, SGLT2	Zhao et al. (2020a)
<i>Takifugu rubripes</i> , fugu	GLUT1a, GLUT6	Munakata et al. (2012)
<i>Trachinotus ovatus</i> , pompano	GLUT4	Li et al. (2019)

^aA hypoxia-responsive glucose transporter^bCrowding stress affected mRNA expression of these transporters, especially along the intestine, indicating that increased stocking density increases glucose absorption^cIn *Sparus aurata*, *glut1* also contributes to osmotic acclimation of this euryhaline fish (Balmaceda-Aguilera et al. 2012b)^dDeck et al. (2016) hypothesized that, in elasmobranchs, GLUT4 has taken on the role of GLUT2 in other vertebrates. This hypothesis is worth being challenged

Fig. 16.2 The relationship between GLUT1 transcript levels and glucose metabolism in the gas gland, heart, and red blood cells (RBC) of Atlantic cod. (From Hall et al. (2014), with permission from Springer Nature)



In Atlantic cod, Hall et al. (2014) comprehensively described the distribution and function of GLUTs: GLUT1 is ubiquitous; GLUT2 is abundant in tissues that release glucose; GLUT3 expression is high in the brain; and GLUT4 is high in the heart and muscle. Furthermore, there are extremely high levels of GLUT1 in the gas gland; this is consistent with high lactate production rates in this tissue. The rate of glucose metabolism in the gas gland, heart, and red blood cells correlates well with relative transcript levels of GLUT1 (Fig. 16.2). This provides a strong support for the contention that GLUT1 expression relates to the rates of glucose metabolism. GLUT3 is relatively high in the brain, gill, and head kidney. GLUT4 expression in the gill is elevated relative to other tissues implying the use of glucose as a metabolic fuel and indicating a weak insulin responsiveness in Atlantic cod tissues (Hall et al. 2014).

Another example of the varying abundance of transcripts in different tissues comes from grass carp. The highest expression of *glut1* occurs in the heart; that in the brain is moderate, while lower expression levels are observed in the muscle, gill, intestine, kidney, spleen, and liver (Fig. 16.3) (Li et al. 2018a).

Studying GLUT2 also in juvenile grass carp (Fig. 16.4), Zhao et al. (2020b) identified results differing from those of *glut1* (Fig. 16.3). The expression of *glut2* is highest in the liver, followed by the intestine and kidney (Fig. 16.4). Furthermore, oral administration of glucose reveals a rapid regulatory effect on the expression of *glut2* in the intestine, but its expression in the liver and kidney is hardly affected. In addition, both insulin and glucagon increase the expression of *glut2* in the primary hepatocytes. This study indicates that the expression of *glut2* in the liver of grass carp is not sensitive to glucose load but to insulin and glucagon. The upregulation of *glut2* by these hormones is involved in the bidirectional transport of glucose in the liver. Moreover, Qin et al. (2020) recently showed that glucagon receptors in the liver of grass carp are not only involved in the glucose metabolism but also in immunity. They are also affected by viral and bacterial challenges. This indicates

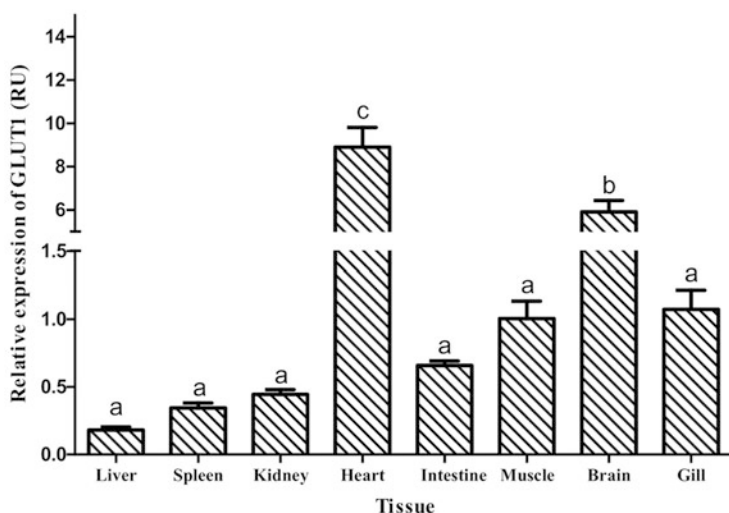


Fig. 16.3 Relative expressions of ciGLUT1 in various tissues of *Ctenopharyngodon idella*. Data are relative units with reference to the muscle. Data are means of six replicates. Different superscripts indicate significant differences ($P < 0.05$). (From Li et al. (2018a), with permission from Elsevier)

further that the textbook classification of glucose transporters, at least in fishes, has to be updated.

Comparing the expression of *glut4* of carnivores (golden pompano, cobia) with one omnivore (tilapia), Li et al. (2019) hypothesized that decreased *glut4* mRNA expression in the heart and white muscle explains the poor utilization of carbohydrate in carnivores (Fig. 16.5). By exclusion of arguments, the authors conclude that none of the parameters examined clearly delineate the differences in the ability to use dietary carbohydrate between carnivore and omnivores, except the transcription of *glut4* caused by feeding high-carbohydrate/low-lipid diets (HCD/LLD).

In a recent study, the electrogenic glucose absorption in pigs, rainbow trouts, and tilapia was compared (Subramaniam et al. 2020). The study reveals similar Michaelis constants (K_m) in both fish species, with a super high-affinity, high-capacity system in tilapia and a super high-affinity/super low-capacity system in trout. Glucose absorption in tilapia and trout most likely takes place through SGLTs, with a lack of apical GLUT2 involvement, indicating a dominating one-absorptive pathway for total glucose absorption. In contrast, glucose absorption in the pig jejunum may be attributed to SGLTs, with functional apical GLUT2 involvement, indicating two absorptive pathways for total glucose absorption. Given these differences in transporter involvement, this study demonstrates continued glucose absorption at higher concentrations in the porcine jejunum, whereas this is not observed in tilapia and trout. These transport pathways observed in the mammal may be an evolutionary adaptation different from fish. The latter may not be exposed to a diet high enough in free glucose to allow facilitative apical glucose absorption.

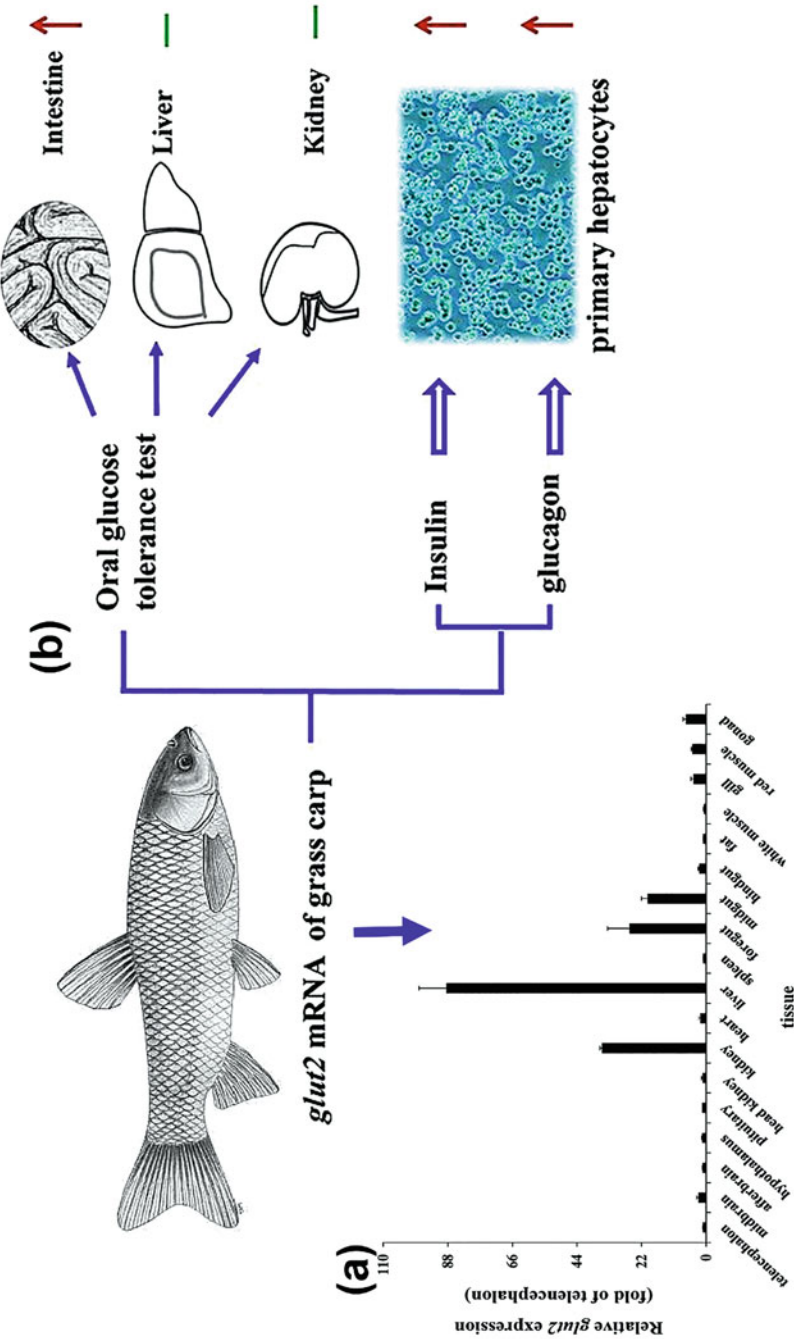


Fig. 16.4 (a) High levels of *glut2* mRNA was detected in the liver, intestine, and kidney of juvenile grass carp. (b) *glut2* expression in the liver and kidney was insensitive to blood glucose; insulin and glucagon enhanced *glut2* expression in the hepatocytes. (From Zhao et al. (2020b) with permission from Elsevier; image credit [archive.usgs.gov](https://www.usgs.gov))

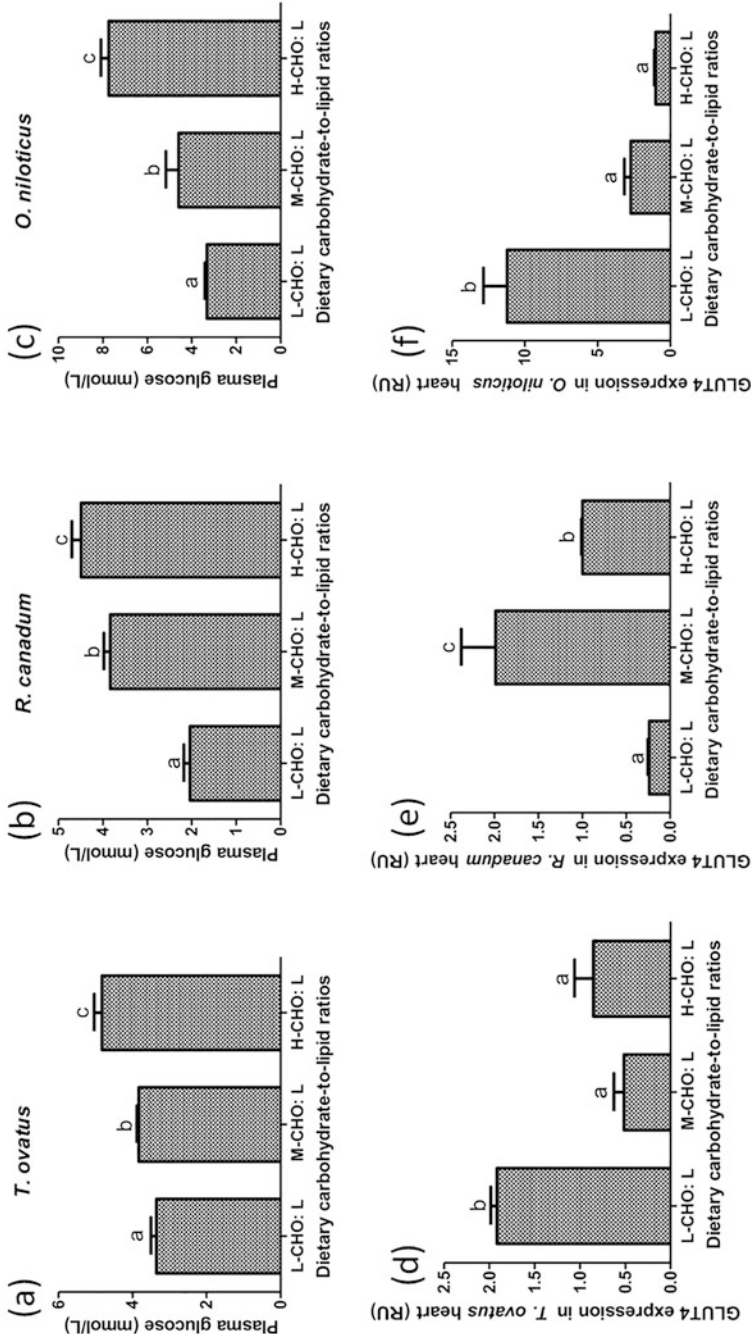


Fig. 16.5 Effect of different dietary carbohydrate-to-lipid ratios on plasma glucose concentration of *Trachinotus ovatus* (a), *Rachycentron canadum* (b), and *Oreochromis niloticus* (c) after 8 weeks of feeding trial. The expression of *glut4* in the heart of *T. ovatus* (d), *R. canadum* (e), and *O. niloticus* (f) after 8 weeks of being fed different carbohydrate-to-lipid ratio diets. Carbohydrate levels: L 20, M 30, H 40%; data are means \pm SD (n = 6). Different letters indicate significant differences ($P < 0.05$). (From Li et al. (2019), with permission from Elsevier)

16.1 Intestinal Transport

16.1.1 Invertebrates

The best studied intestinal sugar transporters in marine invertebrates are involved with dietary sugar uptake, such as SGLT1, SGLT4, GLUT2, and GLUT5 (Martínez-Quintana and Yepiz-Plascencia 2012), and most reports focus on crustaceans.

16.1.1.1 Crustaceans

In crustaceans, the hormonal plasma glucose levels are regulated not only by insulin but also by a specific crustacean hyperglycemic hormone (CHH). The first CHH was isolated from eyestalks of the sand fiddler crab (*Uca pugilator*) by Abramowitz et al. (1944); it significantly upregulates the hemolymph glucose level.

Vilella et al. (2003) demonstrated the energy- and Na⁺-dependent glucose transport in the hepatopancreas in the kuruma prawn (*Marsupenaeus japonicus*). The glucose cotransport is inhibited by phoridizin, a competitive inhibitor of SGLT1 and SGLT2 (Rossetti et al. 1987). The authors assume a low homology at the nucleotide level among mammalian SGLT1 and SGLT2 and crustacean Na⁺/glucose symporter, because no SGLT1- or SGLT2-specific products are amplified from kuruma prawn hepatopancreatic RNA using primers designed on highly conserved motifs of the mammalian transporters.

In Chinese mitten crab (*Eriocheir sinensis*), the passive transporter GLUT4 can be regulated by CHH (Li et al. 2017). EsCHH promotes glucose release from hepatopancreatic cells. It is very likely that the regulation of the glucose homeostasis bears still surprises; for instance, Wang et al. (2017b) demonstrate that in *L. vannamei* two CHHs are involved in glucose metabolism and that even host immune response can be directly modulated by the LvCHH family.

Usually, *glut1* is expressed in all organs; however, not evenly as shown in *L. vannamei*. High expressions are found in muscle, pleopods, the epidermis, and the stomach. This is due to the fact that these organs need high energy: muscle and pleopods are locomotive organs and are involved with procuring food; epidermis cells secrete materials to form the exoskeleton, and the stomach grinds the food and delivers the food into midgut (Huang et al. 2015).

Differences to glucose uptake in fishes do exist at least in American lobster (*Homarus americanus*). Behnke et al. (1998) described that the Na⁺-dependent carrier does not respond to gradients of Li⁺ and K⁺. Furthermore, the transport proteins appear to have a more general function than carrying only glucose; they seem to be involved in response to bacterial and viral infections as well as osmoregulation. Later, Sterling Jr et al. (2009) identified a novel Na⁺-dependent fructose transporter in the hepatopancreas, and Obi et al. (2011) a transepithelial glucose and fructose transport across the intestine. Duka and Ahearn (2014) specified the action of the symporter: glucose and fructose are transported by a single SGLT-type carrier

in each organ. For both sugars, Na^+ is the preferred, high affinity, cation in the hepatopancreas, and K^+ the preferred, high affinity, cation in the intestine.

16.1.1.2 Mollusks

In the freshwater snail *Biomphalaria glabrata*, El-Shaikh et al. (1993) showed that glucose transport is an active process, because Na^+/K^+ -ATPase plays a major role. The same applies to the glucose transport in the octopus *Eledone moschata* (Tritar and Peres 1976) and the pond snail *Lymnaea stagnalis* (Kits et al. 1991). Hanquet et al. (2011) confirmed the involvement of SGLT symporter in the Pacific oyster (*Crassostrea gigas*). Furthermore, food availability affects the *sgt* transcription: under high food conditions, *sgt* expression is higher than under food deprivation.

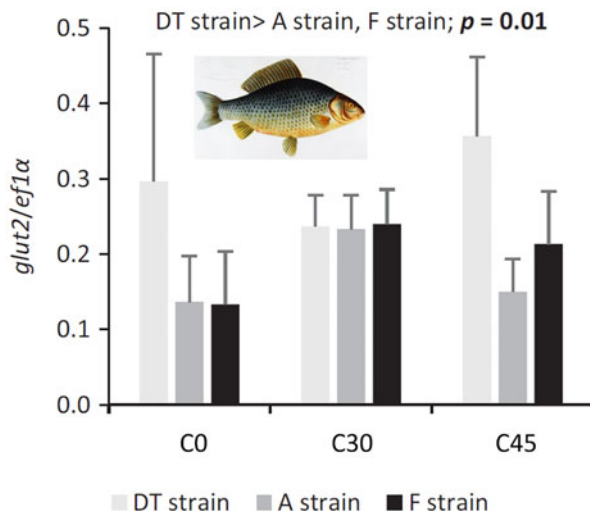
An extraordinary function of a glucose transporter has recently been identified in the fluted giant clam (*Tridacna squamosa*) (Chan et al. 2019). This clam transcribes a *sgt1*-like gene in its *ctenidia*,² and the abundance of the SGLT1-like protein is upregulated by exposure to light with subsequent increase of glucose uptake from the ambient seawater. Therefore, the *ctenidium* of *T. squamosa* is not simply a respiratory organ. The reason is likely based on the symbiotic association of the clam host with phototrophic zooxanthellae, as the light-dependent properties of these enzymes and transporters allow the host to react to light in synchrony with the photosynthetic activity of its symbionts.

16.1.2 Fishes

As mentioned above, glucose absorption is generally lower in fishes than that in mammals (Collie and Ferraris 1995). Lower densities of symporters and smaller amounts of absorptive tissue may be one reason for the lower capacity of glucose uptake in the intestine (Krogdahl et al. 2004). Among different species, transport of glucose across the basolateral membrane of the intestine is adaptive to glucose/carbohydrate levels in herbivorous and omnivorous species by either changing the density of the transporter or altering tissue mass (Collie and Ferraris 1995; Krogdahl et al. 2004). No adaptation of glucose uptake capacity, however, is found in carnivorous fishes (Buddington and Hilton 1987). This can partially explain why some carnivorous fish species have poor ability to utilize carbohydrate; and Buddington and Hilton (1987) consequently hypothesized that the lower levels of carbohydrate in the diet of rainbow trout and other carnivores throughout their evolution has resulted in a reduced ability to phenotypically regulate the digestion of carbohydrates. In its exclusivity, this statement appears somewhat outdated as the brown trout field experiment introduced to previous fish-less lakes on the Kerguelen

²*Ctenidia* are comb- or feather-like shaped respiratory organs found in many mollusks.

Fig. 16.6 mRNA level of hepatic glucose transporter in three strains of gibel carp fed with C0, C30, or C45 diets at 6 hour after the last meal. *glut2*, glucose transporter 2. Data are presented as mean \pm SD ($N = 3$ tanks), statistical differences of *glut2* were evaluated by two-way ANOVA ($P < 0.05$, value in bold). (From Song et al. (2019), with permission from Wiley; image credit Bloch (1782-1784))



Islands shows, in which carbohydrate-rich diets induce *glucokinase* transcription (Marandel et al. (2018), for more details, \rightarrow Chap. 15). It is also likely that the glucose uptake capacity changes, but information is not available.

Evaluating the intraspecific variability (\rightarrow Chap. 40), Song et al. (2019) identified interesting strain-specific variability in gibel carp on HCDs. The authors tested the unselected Dongting strain (DT strain) and the selected A and F strains. The fishes were fed no cornstarch (C0), 30% cornstarch (C30), or 45% cornstarch (C45). Gibel carps tolerate even 45% dietary carbohydrates, and selected F strain shows better growth than the other two by increasing glycolysis and decreasing glycogenesis. The strain specificity can be seen in the expression of *glut2*, which is higher in DT strain than in the other two strains (Fig. 16.6).

16.1.2.1 Herbivorous and Omnivorous Fishes

The capability of grass carps to clear a glucose load can be associated with its nutrient history and is facilitated through glycogen synthesis and glucose metabolic key enzymes. To test this hypothesis, Li et al. (2018b) provided diets with low lipid content and increasing carbohydrate contents from 1.5 (low) to 34.8% (high), before a glucose tolerance test was conducted. The authors show that long-term adaptation to HCDs increases the glucose tolerance through the promotion of glycogen synthesis and glucokinase (GK) activity (Fig. 16.7) and hexokinase activity. GK facilitates phosphorylation of glucose to glucose-6-phosphate, the first glycolysis step of the eventual conversion of glucose (C_6) into pyruvate (C_3). The energy released in this process is used to form the high-energy molecule ATP.

Increasing the carbohydrate content of the diet further and feeding it to non-adapted grass carps can, however, cause adverse effects. Wang et al. (2017a)

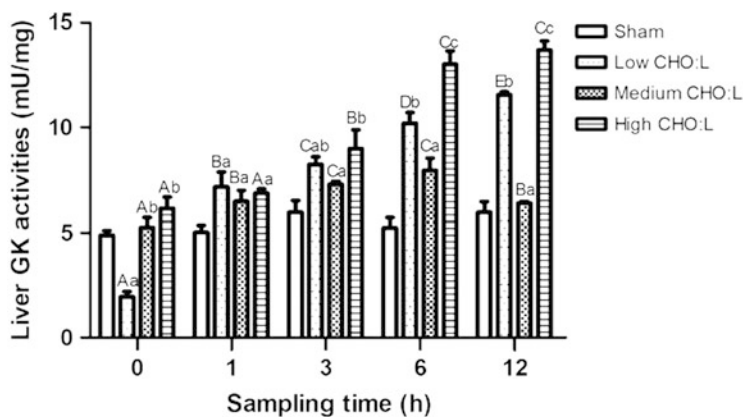


Fig. 16.7 Hepatic glucokinase (GK) activities of *Ctenopharyngodon idella* subjected to a glucose load after adaptation to different carbohydrate-to-lipid ratio diets. Each data represents the mean of six replicates. Significant differences ($P < 0.05$) among sampling times within each treatment (diets) and three treatments at each sampling time are indicated by different letters, respectively (upper case for sampling times within each treatment and lower case for three treatments at each sampling time). A 0.65% saline solution served as sham. (From Li et al. (2018b), with permission from Wiley)

showed that the intake of HCD (452 g kg^{-1}) causes excess fat deposition in the liver, which predisposes the animals to liver steatosis or other metabolic disorders. Various lipid metabolism-related factors, including miRNAs, transcription factors, and related enzymes and proteins, contribute to the processes of fatty liver formation. A number of miRNAs, including *miR-122*, *miR-370*, *miR-33*, *miR-355*, *miR-27*, *miR378/378**, and *miR-125a-5p*, regulate lipid metabolism and lipogenesis. *miR-122* regulates also the expression of many mRNAs, including *glycogen synthase 1*, *fas*, and *acc1* (Wang et al. 2017a).³

To improve the glucose metabolism in Wuchang bream fed HFDs, Zhang et al. (2018) amended the diet with ~1% resveratrol (a stilbenoid polyphenol) and found that the hepatic glucose homeostasis is restored through downregulation of *gck* and *sglt1* and upregulation of *glut2* (Fig. 16.8).

Using a glucose tolerance test, Liu et al. (2018) observed a delayed gene transcription of *glut4* in *Oreochromis niloticus*: the expressions in all groups fed different dietary carbohydrate levels remain stable during the first 3 hours and

³Functions of the individual microRNAs: a potential lipogenic role of *miRNA-122* has been hypothesized in zebrafish, in which an increased expression of *miRNA-122* coincides with increased expression of the lipogenic gene *fas*. Furthermore, overexpression of *miR-33* in hepatocytes decreases cholesterol efflux to apolipoprotein A1, a key step in the generation of HDL. *miR-370* directly targets *cpt-1a* gene and consequently reduces FA β -oxidation in cultured hepatic cell lines. In addition to directly regulating genes involved in FA metabolism, *miR-370* regulates the expression of *miR-122*. Overexpression of *miR-370* leads to an upregulation of *miR-122*, and a resulting upregulation of lipogenic genes such as *srebp-1c* and *dgat2* (Wang et al. 2017a).

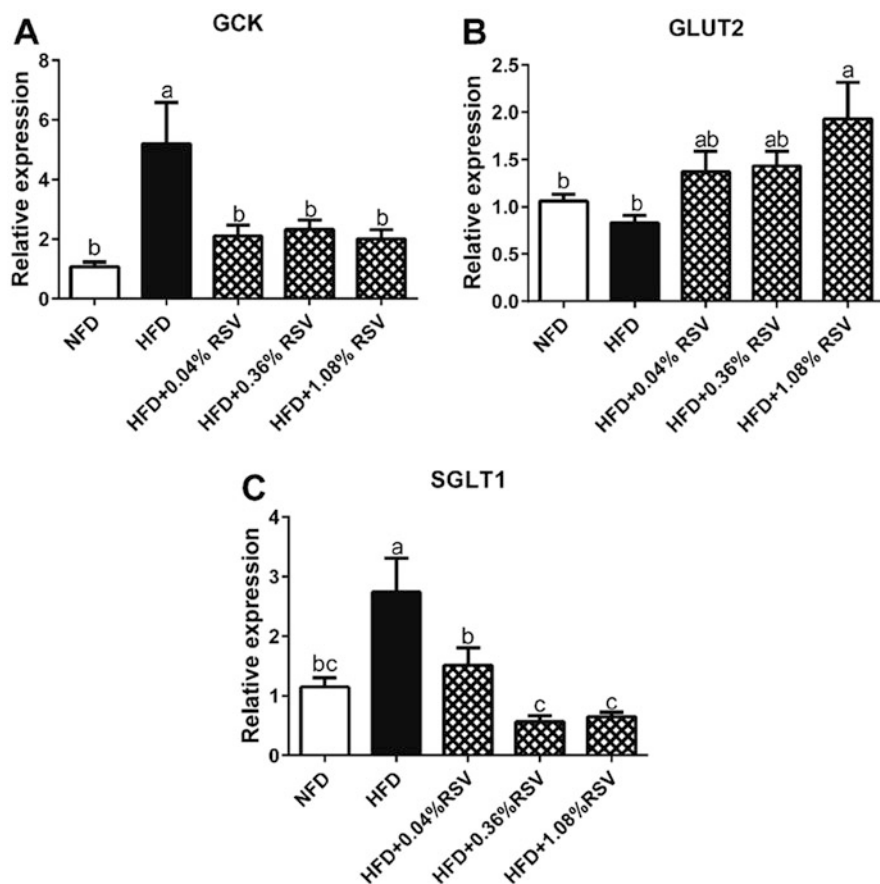


Fig. 16.8 High-fat diet (HFD) and resveratrol control the hepatic transcription of genes involved in glucose flux in Wuchang bream. **a** *Glucokinase* (*gck*), **b** *glucose transporter 2* (*glut2*), and **c** *sodium-dependent glucose cotransporter 1* (*splt1*) mRNA. The expression level of each gene was normalized to β -actin expression. Data are means \pm SD ($n = 16$). Different letters indicate significant differences among all groups ($P < 0.05$). (From Zhang et al. (2018), with permission from Springer Nature)

increase significantly from 6 to 12 h after glucose injection (Fig. 16.9). It reaches the maximum at 6 h in the CH and CL groups, while in the CM group, it reaches it at 12 h. The authors assume that this delay is one reason for the relative glucose intolerance in this omnivorous species.

However, this assumption has to be refined, since Nile tilapia can adapt its metabolic responses to a diet with carbohydrates (Boonanuntanasarn et al. 2018). Carbohydrate feed intake is associated with decreased hepatic gluconeogenesis, an induction of hepatic lipogenesis, and a regulation of the last step of glycolysis in

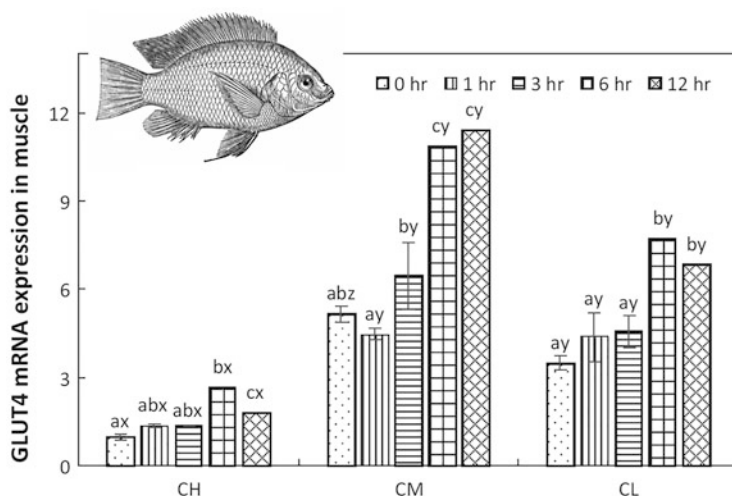


Fig. 16.9 Effects of glucose injection on *glut4* expression in *Oreochromis niloticus* fed with different dietary carbohydrate levels. The values are means \pm SE ($n = 3$); different letters (a–c) in the same carbohydrate levels denote significant differences at different times; and different letters (x–z) in the same time point denote significant differences in the different carbohydrates levels ($P < 0.05$). CH 40%; CM 30%; CL 20% carbohydrate levels. (From Liu et al. (2018), with permission from Wiley; image credit Galleries of Reptiles and Fishes of the British Museum)

muscle tissue. The optimal balance between dietary proteins and carbohydrates for best growth of Nile tilapia is 45% protein and 33% carbohydrate.

16.1.2.2 Carnivorous Fishes

Also in carnivorous fishes, a control of glucose levels exists in tissues relying on glucose supply, such as the in brain. Accordingly, decreased food intake occurs in fishes fed HCD or after hyperglycemic treatments. On the other hand, increased food intake takes place in fishes fed diets with reduced carbohydrate levels or after hypoglycemic treatments (Soengas et al. 2018).

The carbohydrate utilization after export from the gut appears to be one major bottleneck. In Atlantic salmon, the regulation of gut transporters has been observed in response to different diets, but the capacity to further utilize carbohydrate after absorption is limited (Sissener et al. 2013). It is therefore hypothesized that the carbohydrate utilization for this species is regulated after absorption. By applying metformin, a medication for the treatment of diabetes, Panserat et al. (2009) found that the poor utilization of glucose by peripheral tissues is likely one of the reasons for hyperglycemia. Supportingly, Sala-Rabanal et al. (2004) found only one Na^+ -dependent glucose carrier in brush border membrane vesicles from proximal and distal intestine of gilthead seabream. Apparent affinity constants of glucose transport

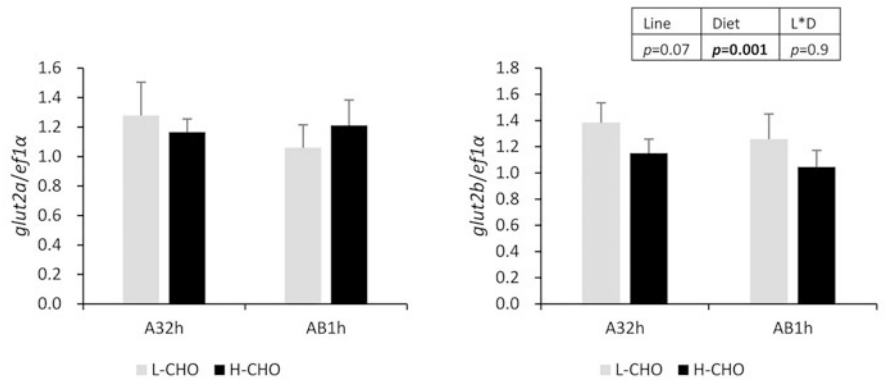


Fig. 16.10 mRNA levels of selected glucose transporter in the liver of two lines of rainbow trout fed with low-carbohydrate diet and high-carbohydrate diet. *Glut2a* and *glut2b*, glucose transporter 2 paralogs. Data are presented as mean \pm SD ($n = 6$), the statistical differences of *glut2a* and *glut2b* were evaluated by two-way ANOVA ($P < 0.05$, value in bold). (From Song et al. (2018), credit Company of Biologists)

are 0.24 mM in proximal and 0.18 mM in distal intestine – comparable with those of the SGLT1 of higher vertebrates.

As the first actor involved in glucose transport in and out of the liver, Song et al. (2018) studied the gene encoding GLUT2 in two isogenic lines of rainbow trout (A32h, AB1h). No difference in the expression of *glut2a* is found between lines and diets. However, *glut2b* is expressed at a lower level in trouts on HCD (Fig. 16.10). This is surprising, because this gene is not strongly regulated by dietary glucose (Kirchner et al. 2008; Polakof et al. 2010; Jin et al. 2014), and a plausible explanation is lacking so far (for more details, \rightarrow Chap. 18).

Increasing numbers of comparative studies between omnivorous and carnivorous fishes reinforce the above hypothesis. Subramaniam et al. (2019) compared Na⁺-dependent glucose transport along the length of the intestine between Nile tilapia and rainbow trout. In tilapia, a high-affinity, high-capacity kinetic system accounts for the transport throughout the proximal intestine, mid-intestine, and hindgut segments. In contrast, trout’s glucose absorption is 20–35 times lower and segregated into 3 significantly different kinetic systems found in different anatomical segments: high-affinity, low-capacity system in the pyloric ceca; super high-affinity, low-capacity system in the midgut; and low-affinity, low-capacity system in the hindgut. In other words, tilapia and trout demonstrate differences in transport kinetics and the intestinal segmental contribution.

Focusing on the transcription of passive glucose transporters in cobia (*Rachycentron canadum*), Ye et al. (2020) studied the relative expressions of *glut2*, *glut3*, *glut5*, and *glut9* in the liver after glucose injection. The expressions reach peaks at 24, 24, 12, and 24 hpi, respectively. *Glut1* and *glut4* do not respond. The slow increase of *gluts* is one main reason of the low glucose utilizing capacity in

R. canadum. Further reasons are insufficient insulin secretion and low activities of key glycolytic enzymes.

16.1.3 The Multiple Functions of GLUTs: A Double-Edged Sword?

16.1.3.1 Crustaceans

Studies from the past two decades show that GLUT and SGLT transporters possess more comprehensive functions than only carbohydrate transport. On infection with *Vibrio harveyi*, *glut1* is upregulated in hemocytes of tiger prawn (*Penaeus monodon*) (Fig. 16.11) (Somboonwiwat et al. 2006). Likewise, the infection in *L. vannamei* with white spot syndrome virus (WSSV) occurs via interaction between WSSV envelope proteins and a transmembrane protein. This transmembrane protein is hypothesized to belong to the GLUT family (Huang et al. 2012). As a proof of concept, Huang et al. (2015) silenced *glut1* with double-stranded RNA (dsRNA), and the survival of infected *L. vannamei* increases significantly (Fig. 16.11).

Carbohydrate transporters, particularly Na⁺-dependent ones, are central also in osmoregulation. To gain insight into this compensatory mechanism that accompanies acclimation to hyposalinity, changes in transcripts in gill tissue in green crab (*Carcinus maenas*) were analyzed. Many genes are upregulated including transport proteins and, not astonishing, specifically an energy-dependent Na⁺/glucose symporter (Towle et al. 2011). Likewise in Atlantic white shrimp (*Litopenaeus setiferus*), glucose uptake in hepatopancreas brush border vesicles is stimulated in the presence of inwardly directed gradients of either Na⁺ or K⁺ (Obi et al. 2013). In addition, in mud crab (*Scylla paramamosain*) postlarvae, the disaccharide transporter facilitated trehalose⁴ transporter (*tret1*) belongs to most significantly differentially expressed genes in postlarvae transferred to low-salinity water (Zhang et al. 2020).

Martínez-Quintana et al. (2014) characterized GLUT1 in the Pacific sister species (*L. vannamei*). The protein is highly similar to its homologous proteins in other species and has the sequence signatures present in all the members of the glucose transporter family. Hypoxia regulates the expression of *glut1* mRNA in hepatopancreas and gills (Fig. 16.12). The hypoxia stress provokes an acute increase in the glucose transport and induces a shift from aerobic to anaerobic metabolism. The accelerated anaerobic glycolysis pathway needs to be fueled with glucose (Soñanez-Organis et al. 2011). This requirement is reflected by elevated transcription of *glut1* after 3 h of hypoxia in both, gills and hepatopancreas (Fig. 16.12). A similar function and response to hypoxia has been confirmed for *glut2* (Martínez-Quintana et al. 2015). In addition to *sgl1* (Obi et al. 2013), also *glut1* responds to other

⁴Trehalose = two α-glucose units with 1,1-glycosidic bond

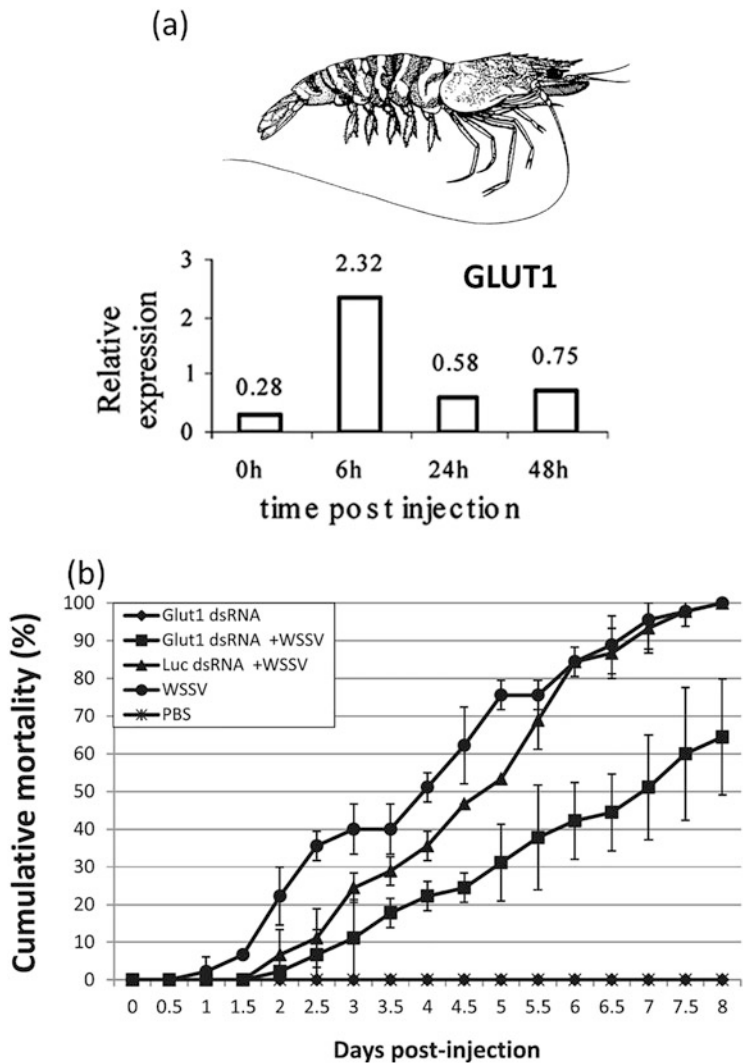


Fig. 16.11 (a) Relative expression of *glucose transporter 1* (*glut1*) in hemocytes of *Penaeus monodon* at 0, 6, 24, and 48 hours after *Vibrio harveyi* injection determined by real-time PCR. (From Somboonwiwat et al. (2006), credit KoreaScience; image credit Motoh (1985)). (b) Gene silencing of *glut1* of *Litopenaeus vannamei* decreases mortality after WSSV injection. Individuals (1 g) were injected with 2 mg *glut1* dsRNA, 2 mg *glut1* dsRNA plus WSSV, 2 mg *Luc* dsRNA plus WSSV, PBS (negative control), or WSSV alone (positive control) as indicated. The experiment was done for three times of 15 shrimps in each group. Each point represents mean \pm SD. (From Huang et al. (2015), with permission from Elsevier)

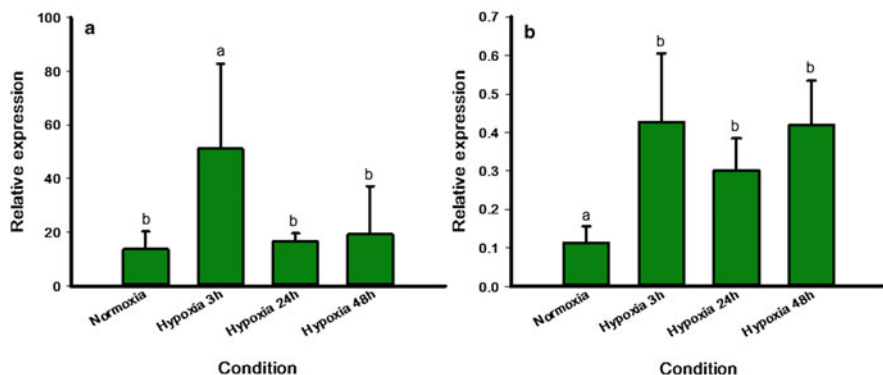


Fig. 16.12 Relative expression level of *glut1* in *Litopenaeus vannamei* exposed to normoxia ($5.3 \pm 0.3 \text{ mg L}^{-1}$ dissolved oxygen, DO) and hypoxia ($1.45 \pm 0.3 \text{ mg L}^{-1}$ DO) in gills (a) and hepatopancreas (b). Columns are means \pm SD. Different letters indicate significant differences ($P < 0.05$). (From Martínez-Quintana et al. (2014), with permission from Springer Nature)

environmental stressors, such as changes in salinity (Wang et al. 2017c), however triggering different pathways.

16.1.3.2 Fishes

For many years, the teleost intestine has been known to be a major center for osmoregulation, and recent work has clarified many properties of monovalent ion transport across intestinal epithelia of marine teleost fishes, comparable to the process described for *C. maenas* above. Reshkin and Ahearn (1987) were one of the first to study osmoregulation in the euryhaline *Oreochromis mossambicus* with respect to feedback of salinity adaptation on glucose transport. This transport takes place in brush border membrane vesicles of the upper and lower intestines. The authors showed that adaptation to different environmental ion concentrations markedly influences carrier kinetics of sugar uptake into epithelial cells of both intestinal regions.

Again comparable to crustaceans, Clow et al. (2004) reported for isolated Atlantic cod hearts under hypoxia that (1) activation of glucose transport is required to support hypoxic performance, (2) the rate-limiting step for glucose utilization is glucose transport, and (3) glucose uptake in cod hearts does not involve an Na^+ -dependent mechanism, indicating that the transporter belongs to the GLUT family. Supporting evidence is provided by a companion study in short-horned sculpin (*Myoxocephalus scorpius*) (MacCormack and Driedzic 2007) as well as in gilthead seabream (Balmaceda-Aguilera et al. 2012b).

16.1.4 Disaccharide Transporter

Evidence is accumulating that, beside monosaccharide transporters, disaccharide transporters exist in aquatic animals. In *H. americanus*, Likely et al. (2015) studied the transepithelial absorption of dietary sucrose, a disaccharide, composed of glucose and fructose. Sucrose is transported largely intact across the intestine supporting the occurrence of a functional disaccharide membrane transporter. Intrigued by the discovery of a gene for a distinct sucrose transporter (SLC45–1, originally named SCRT)⁵ in *Drosophila melanogaster* (Meyer et al. 2011), Likely et al. (2015) and Scheffler and Ahearn (2017) functionally characterized the putative disaccharide transporter analog in the hepatopancreas of American lobster. They found that H⁺ and Na⁺ are each capable of driving the uptake of the sucrose characterizing this transporter as symporter. Furthermore, maltose and trehalose inhibit the carrier-mediated sucrose transport. In terms of natural feeding ecology, the use of a putative lobster SCRT by both maltose and trehalose appears appropriate as these crustaceans commonly digest glycogen and chitin, polymers of maltose and trehalose, respectively. In addition to Fig. 16.1a, Likely et al. (2015) drafted a model of sucrose and monosaccharide transport in American lobster (Fig. 16.1b) that incorporates results from several studies of this animal.

Studies on disaccharide transporters in aquatic animals are sparse. However, three more hints on such a structure do exist. Vitavska and Wieczorek (2013) listed an SLC45 family-like protein in *Daphnia pulex*, its amino acid sequence clustered close to SLC45–1 of *Drosophila melanogaster* – both are arthropods. According to KEGG BRITE, it is an H⁺/sugar symporter⁶ and most likely transports also disaccharides; however, the functional verification is yet lacking.

The copepod *Calanus finmarchicus*, like many other species in the Calanidae family, can enter into a facultative diapause during the last juvenile phase to enable survival during unfavorable periods. Transcriptional profiling has begun to provide insight into metabolic changes occurring as *C. finmarchicus* prepares for and enters into diapause or skips diapause to prepare for the terminal molt. A transcriptomics inspection reveals several enriched Gene Ontology terms associated with mono- or disaccharide transport (Tarrant et al. 2016), however, with yet undisclosed function.

First indications of the existence of a disaccharide transporter in finfishes have been reported for zebrafish: Sander et al. (2019) found that the proximal tubule expresses two genes belonging to the *slc45* family with homology to sucrose transporters in plants and, again, the H⁺/sucrose symporter in *Drosophila* (Meyer et al. 2011). However, the functional role of the SLC45-related transporters in vertebrates remains to be characterized.

⁵Slc45–1 exhibits a significant similarity to plant sucrose transporters as well as to the members of the human SLC45 family (Vitavska and Wieczorek 2013).

⁶https://www.genome.jp/kegg-bin/get_htext?dpx02000+DAPPUDRAFT_47061

16.2 Concluding Remarks

Until recently, the functional characterization of glucose transporters in fishes and aquatic invertebrates appeared to be more or less completed. However, recent evidence points out that several topics deserve future studies to clarify contradictions or to refine existing knowledge. The following questions continue to be answered:

- Do oligosaccharide transporters exist also in fishes and invertebrates, other than crustaceans?
- Studies in penaeid shrimps indicate that glucose transporter proteins can serve as gate for bacterial and viral pathogen entry. Do also fish GLUTs function as such double-edged sword?
- In zebrafish brain, Dalmolin et al. (2018) found much more than 5 GLUTs and several isoforms of individual GLUTs. Some GLUTs were already identified in fishes, for example, GLUTs 1, 2, and 4 (Polakof et al. 2012); however, it is still unclear whether other GLUT isoforms do exist, and little is known about the GLUTs and their functional roles in the energy supply for the fish brain.
- Dalmolin et al. (2018) also described an antagonism between growth hormone (GH) and GLUTs in zebrafish. The presence of GH downregulates the transcription of most GLUTs analyzed. Therefore, chronic GH exposure or an acute injection of GH hinder the energy status and impair blood glucose uptake in brain tissue of zebrafish.

Although the latter fish model is rather artificial, the paper points out that there is an interaction of carbohydrate transporters and hormones (not only GH, but also insulin, glucagon, and most likely others). This interaction deserves future attention.

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Chapter 17

Protein Sparing by Carbohydrates—‘Life’s Useful Luxury’



Abstract This chapter comprises information about protein sparing by dietary carbohydrates. It confirms the well-understood limits of substitution: carnivores tolerate max. ~20% and omnivores roughly double this value. Due to their prevailing omnivorous, rather than pure carnivorous, feeding habit, invertebrates tolerate ~30% and occasionally even more. The tolerance of omnivorous fishes can be increased by modulating the intestinal microflora. Epigenetic modes of action are not yet comprehensively studied in aquatic animals but deserve a closer look.

Since protein is the most expensive macronutrient in aquafeeds, a great deal of research has been conducted on the protein-sparing potential of nonprotein ingredients. Protein sparing is the process by which an animal derives energy from sources other than protein. Such sources comprise dietary lipids and carbohydrates.

Carbohydrate utilization as an energy source to spare protein is determined by carbohydrate origin, inclusion content, physical state, and molecular complexity (Stone 2003). There appears to be potential for the use of supplemental enzymes to enhance the carbohydrate utilization; however, care must be exercised when using such products as some of the breakdown products, particularly galactose and xylose from non-starch polysaccharides, are not tolerated by most fishes; the increased availability of these monomers may be detrimental to fish growth and health.

17.1 Sparing Details

The protein-sparing effect is species- and strain-dependent and varies according to species’ feeding habitat (cold vs. warmwater) or feeding habit (carnivorous vs. omnivorous). Most warmwater fishes, such as Nile tilapia, utilize higher levels of dietary carbohydrates (occasionally up to 40% of diet dry matter) than cold-water fish, such as rainbow trout ($\leq 20\%$ of diet dry matter). The limited ability of carnivores (such as salmonids) compared to omnivores (such as tilapias or carps) to efficiently utilize dietary carbohydrates as energy source is illustrated by

the prolonged hyperglycemia after oral administration of glucose or high-carbohydrate (HC) meals (>20% of diet dry matter) in carnivores (Figueiredo-Silva et al. 2013).

One reason for intraspecific variability is the action of the gut microbiota. It can be hypothesized that the composition of this micro-ecosystem is central in utilizing carbohydrates, producing short-chain fatty acids (SCFAs) and strengthening host mucosal immunity (Hoseinifar et al. 2017). Furthermore, SCFAs support gut health by decreasing epithelial permeability and modulating cytokines in the intestine (Macia et al. 2012) (→ Chap. 26). The microbiota aspect, however, has been addressed only in a few papers. We shall revisit this topic.

Another major reason for intraspecies variability is the individual nutritional history (Stone 2003). Individuals fed carbohydrate-rich diets in a specific period of early development exert long-term dietary tolerance (→ Chap. 13 and AAN III “Nutritional Programming”). Table 17.1 provides a survey of aquatic invertebrates and fishes with respect to protein sparing by different forms of dietary carbohydrates. In terms of feed utilization, protein sparing, and growth stimulation, omnivorous species tend to outdo carnivores. However, even several carnivorous species do show improved growth if fed a low-starch diet compared to a diet totally devoid of starch. It is obvious that dietary protein sparing is feasible in omni- and herbivorous as well as juvenile carnivorous invertebrate and fish species: *Homarus americanus*, *Litopenaeus vannamei*, *Macrobrachium rosenbergii*, *Penaeus monodon*, as well as *Acanthopagrus latus*, *Acipenser baerii*, *Clarias macrocephalus* × *C. gariepinus*, *Dentex dentex*, *Epinephelus malabaricus*, *Heterobranchus longifilis*, *Huso huso*, *Oncorhynchus mykiss*, *Pseudoplatystoma reticulatum*, *Salvelinus fontinalis*, *Scophthalmus maximus*, *Seriola lalandi*, *Solea senegalensis*, *Sparus aurata*, and *Tachysurus (Pelteobagrus) fulvidraco*. Noteworthy, a few omnivorous fishes are doubtful or appear even unable to spare dietary protein on carbohydrate diets: *Tinca tinca* and *Ictalurus punctatus*. The underlying mechanisms have to be figured out in studies to come.

Furthermore, the reports of the nominally herbivorous *Ctenopharyngodon idella* are not consistent with three out of four studies reporting lacking protein sparing effects. This is surprising since the natural diets of this fish contain high portions of plant materials rich in carbohydrates; a convincing explanation for the lack of protein sparing by carbohydrates in this species has not yet been provided.

In a recent study, Li et al. (2020) pointed out the significance of gut microbes in increasing the tolerance of HC diets. The authors showed that HC (45%) diet increases weight in Nile tilapia, however, combined with elevated mortality (Fig. 17.1a,b). Furthermore, HC diets reduce the acetate content and induce intestinal inflammation combined with decreased resistance against *Aeromonas hydrophila* challenge. Such inflammation can be overcome by modulating the gut microbiota. Probiotics can be used to reduce pro-inflammatory cytokines via the NF-κB pathway which is likely related to the production of SCFAs (Nawaz 2018) (→ Chap. 26). As a proof of concept, the addition of sodium acetate in HC diets

Table 17.1 Protein sparing by carbohydrate supplementation in selected aquatic species. Trials without unsupplemented controls are not included

Species, trophic habit	Dietary carbohydrate, %	Tested trait	Protein sparing	Reference
Invertebrates				
<i>Apostichopus japonicus</i> juv, d (o)	26→66, starch	Max SGR @ C49	✓ @ C45→56	Xia et al. (2015)
<i>Homarus americanus</i> postlarva, p, c	0→40, starch	Replacement of macroalgae with ≤C11	✓	Wen et al. (2018)
	9→17, starch	Max WG @ C9	✓	Capuzzo and Lancaster (1979)
<i>H. americanus</i> juv	7.5, starch	WG↔	(✓)	Brown (2006)
<i>Liopenaeus vannamei</i> juv, p, d – c	14→30 starch	Max WG @P34 + C19	✓ @ P30 + C25 @ P34 + C19	Wang et al. (2015)
<i>Macrobrachium idae</i> juv, p, c	23→44 starch	WG↑ @ C < 32 WG↓ @ C41→55	✓ @ C32–37	Sundaravadivel and Sethuramalingam (2017)
<i>M. rosenbergii</i> juv–sub, p, c	24–64, starch Fiber cellulose	WG↑ @ C↑ WG↓	✓ None	Gabriel et al. (1988)
	0–30, fiber cellulose	WG↑ @ C ≤ 20	✓	Fair et al. (1980)
	10–30	Max sparing @ L:C = 1:4	✓	Clifford III and Brick (1979)
<i>Penaeus monodon</i> juv, c	20, 25, 30, glucose, dextrin, starch	FER, PER Starch>dextrin>glucose	✓ with starch	Shiau and Peng (1992)
<i>Pinctada fucata martensii</i> (sub)adult, o	30→50	Optim WG @ C45 + P25	✓	Yang et al. (2019)
<i>Samastacus spinifrons</i> juv, o	16→24; 35→36	Max WG @ P30 + C low	✓ @ C low	Salgado-Leu and Tacon (2015)
Fishes				
<i>Acanthopagrus latus</i> juv, c	5→26 raw starch	WG↔ @ C10–20 WG↓ @ C26	✓ @ ≤20	Wu et al. (2007)

(continued)

Table 17.1 (continued)

Species, trophic habit	Dietary carbohydrate, %	Tested trait	Protein sparing	Reference
<i>Acipenser transmontanus</i> sub-yearlings, c	27 glucose, fructose, maltose, sucrose, lactose, dextrin, raw cornstarch, or cellulose	WG: Maltose, glucose > dextrin, raw cornstarch, sucrose diets > lactose, fructose, cellulose	Not obvious	Hung et al. (1989)
<i>Anguilla anguilla</i> juv, c	Wheat meal, 20→56 Fish and poultry meal 70→30 Cooked starch	Optim WG @ P50/C38 WG↑, optim @ P40→25, C23→40 WG↑	✓ ✓ ✓ best @ manioc meal ✓ P30→25	Degani and Viola (1987) Hidalgo et al. (1993) García Gallego et al. (1994) Mohanta et al. (2007)
<i>Barbonymus gonionotus</i> fry, h – o	Raw wheat starch, corn maltodextrins, manioc meal, cooked cornstarch 30→38	WG↔ @ C34	✓	Stone et al. (2003)
<i>Bidyanus bidyanus</i> juv, o	15→60, wheat meal, raw wheat starch, gelat wheat starch, or dextrin 26→60	WG↔ ≤C30 WG↔	✓ ≤ C30 ✓	Alexis and Papaparaskeva-Papoutsoglou (1986) Singh et al. (2006)
<i>Cirrhinus mrigala</i> , fry, o – p	30, 35, 40 various forms	Max WG @ C40 dextrin+P30	✓ @ P30–35	Jantrarotai et al. (1998)
<i>Clarias macrocephalus</i> × <i>C. gariepinus</i> juv, c	13→57, dextrin	Max WG @ P35, C30	✓ @ P35, C30	Sandre et al. (2017)
<i>Colossoma macropomum</i> juv, p – o	41→51	Optim WG @ C46 + L4 WG↓ @ C51	✓ @ C46	Chen et al. (2012)
<i>Ctenopharyngodon idella</i> juv – sub, h – o	17, 22, 26 starch 16, 22, 45 starch 17 cellulose+8 starch; 40 starch	WG↔ @ C↑ Max WG @ P34 WG↓ @ C45 WG↔	None ✓ @ C ≤ 22 None	Cai et al. (2018) Guo et al. (2015)

<i>Cyprinus carpio</i> juv, o – b	42, α-starch, dextrin, glucose	WG α-starch > dextrin > glucose	✓	Furuichi and Yone (1982)
<i>C. carpio</i> sub	5, 10, 15	CF↑ @ C10*	✓	Fan et al. (2018)
	30 starch, glucose, fructose, galactose	Growth reduction; Glucose, fructose depress gluconeogenesis; glucose promotes lipogenesis	✓	Shikata et al. (1994)
	4–58, dextrin + potato starch	WG↑ @ C ≤ 48	✓	Shimeno et al. (1995)
<i>Cyprinus gibelio</i> juv, o	0, 15, 30, cornstarch	WG, SGR↑ @ C30; PER↑ @ C15,30	✓	Li et al. (2017)
	7, 13, 20, 26 raw cornstarch	WG↔	✓	Cheng et al. (2017)
	0, 30, 45, cornstarch	WG, glucose regulation: Strain-specific	✓ strain-specific	Song et al. (2019)
<i>Dentex dentex</i> juv, c	12, 18, 24 three forms	WG↔	✓ @ C ≤ 18	Pérez-Jiménez et al. (2015)
<i>Diplodus sargus</i> juv., o	0, 20, 36 waxy cornstarch	WG↔	✓	Sá et al. (2007)
	C 26, 42 two starch forms; P 36, 48	WG↔ normal starch more effective than waxy starch	✓ P48→36	Sá et al. (2008)
	14→25 starch, glucose	WG↓ @ C↑	Limited ✓ @ starch + low P	Shiau and Lin (2001)
<i>Epinephelus malabaricus</i> juv, c	12, 15, starch	Per↔	None	Lie et al. (1988)
<i>Gadus morhua</i> , c	0, 10, 20, 30 starch	WG↔, PER↔		Hemre et al. (1989)
<i>Gibelion (Catla) catla</i> finger, o	15, 25, 35, dextrin	Max WG @ 35 survival↔, FCR↔, PER↔	✓	Seenappa and Devaraj (1995)
<i>Heterobranchius longifilis</i> , c	17–21 <i>sorghum</i> sp. starch	SGR↔, FCR↔, max PER@13	✓ @ P:L 1.58 + P:C 2.22	Ovie et al. (2005)
<i>Heteropneustes fossilis</i> fry, c – o	25, 30, 35 Glucose/dextrin	Max WG @ C35 + P35	✓	Rahman et al. (2017)
<i>Huso huso</i> sub, c	11→27	WG↔	✓	Mohseni et al. (2011)
<i>Ictalurus punctatus</i> finger, o	33, glucose, fructose, sucrose, maltose, cornstarch, dextrin	WG, FE: Dextrin < cornstarch < glucose, maltose, sucrose < fructose	None	Wilson and Poe (1987)

(continued)

Table 17.1 (continued)

Species, trophic habit	Dietary carbohydrate, %	Tested trait	Protein sparing	Reference
<i>Laboe fimbriatus</i> juv, h – o	26, 32, 37	WG↔ @ P ≥ 23	√ P27→23	Kathane et al. (2017)
<i>Laboe rohita</i> finger, h	30, 35, 40, dextrin, sucrose, glucose	WG↑	√ dextrin>sucrose/ glucose	Erfanullah (1995)
	15, 25, 35	Optim WG @ P40 + C35 dextrin	√ @ P40 + C35	Satpathy and Ray (2009)
	35–42, wheat bran	Optim FCR @ C35	√ @ C35	Jan et al. (2013)
<i>Lates calcarifer</i> juv, c	5–17, gelat starch	<17	√	Nankervis et al. (2000)
<i>Megalobrama amblycephala</i> juv, o	0→47, cassava starch	Max WG, SGR @ 31	√	Zhou et al. (2013)
<i>Oncorhynchus mykiss</i> juv, c	32, glucose, Maltose, dextrin, raw comstarch	SGR, WG, FE: Glucose>maltose>starch	√	Hung and Storebakken (1994)
	5–50, gelat comstarch	Muscle growth↑	√ only below digestible protein requirement	Alami-Durante et al. (2019)
	0, 25, gelat comstarch	SGR↑, [WG↑, WG↓] two isogenic lines	√	Song et al. (2018)
<i>O. mykiss</i> sub, c	Sucrose, comstarch, 10–54	Max PER @ C35.8 sucrose	√	Pieper and Pfeffer (1980)
	30, crude starch, gelat starch	WG↑	√ gelat > crude	Kaushik and de Oliva (1985)
	38, five forms	WG↑	√ even long-term	Kaushik et al. (1989)
	0, 23 digestible carbohydrates	Muscle growth↑	√ to avoid protein degradation	Peragón et al. (1999)
	17, gelat starch	WG↑	√	Kamalam et al. (2012)

<i>O. tshawytscha</i> , c	8, 16, dextrin	SGR↑	√ @ 8 (√) @ 16	Buhler and Halver (1961)
<i>Oreochromis niloticus</i> sub, o – h	3–48	GR↑ @ C↑	√	Shimeno et al. (1993)
<i>Oreochromis niloticus</i> juv	16→40 starch	WG↑ @ C ≤ 32	√ P25 replacement	Azaza et al. (2015)
	0→50	Max WG @ C30	√ even long-term (40 weeks)	Boonanuntanasarn et al. (2018)
	0→40 starch	Optim WG @ C20	√ @ low P (24)	Wang et al. (2017)
<i>O. niloticus</i> GIFT juv, o – h	20→40 starch	Optim WG @ C30	√	Liu et al. (2018)
<i>O. niloticus</i> × <i>O. aureus</i> , o – h	33→41 three forms	WG↔ @ C↑+P↓	√ starch, dextrin > glucose	Shiau and Peng (1993)
<i>Pagrus major</i> juv, c	25 starch, dextrin, glucose	SGR↔	√	Furuichi and Yone (1982)
<i>Pangasianodon</i> <i>hypophthalmus</i> finger, o	20, 40 dextrin+starch	Max WG @ C40	√	Bhumarkar et al. (2017)
<i>Piaractus</i> <i>mesopotamicus</i> juv, o, f	41→50 starch	WG↔ C utilization depends on dietary lipids	√ max @ P25	Abimorad and Carneiro (2007)
<i>Pseudoplatystoma</i> <i>reticulatum</i> juv, c	17, 30 starch	SGR↓ @ C30	√ @ C17 + P44 + L6.6	Gonçalves et al. (2018)
<i>Rachycentron</i> <i>canadum</i> juv, c	1, 7, 13, 18, 24, 30, starch	SGR↑ @ C ≤ 18–21	√	Ren et al. (2011)
<i>Rhamdia quelen</i> , c – o	8→17, dextrin	WG↔ @ C ≤ 16	√ @ C ≤ 16	Moro et al. (2010)
<i>Salmo salar</i> juv, c	0–31, wheat starch	Hematocrit↓, hemoglobin↓, mean cell volume↓, mean cell hemoglobin↓; critical @ C > 22	√	Hemre et al. (1995)
	41–53, gelat wheat, corn, oat	FCR: Wheat > corn, oat	√ season- dependent ^a	Hemre and Hansen (1998)

(continued)

Table 17.1 (continued)

Species, trophic habit	Dietary carbohydrate, %	Tested trait	Protein sparing	Reference
<i>Salvelinus fontinalis</i> juv, b – c	0–28, gelat starch	Optim WG @ P53 + C8 + L24	✓	Amin et al. (2014)
<i>Sciaenops ocellatus</i> juv, c	24–39,	Optim WG, FE @ C24 ^b	None	Ellis and Reigh (1991)
<i>Scophthalmus maximus</i> juv, c	5, 15, 25, starch	WG↑ @ C ≤ 15	✓	Zeng et al. (2015)
	0, 5, 15, 28, dextrin	WG↓	✓ @ C ≤ 15	Miao et al. (2016)
<i>Seriola lalandi</i> juv, c	10–40, starch	WG↔	✓ @ C ≤ 10	Booth et al. (2013)
<i>S. quinqueradiata</i> juv, c	3–24, dextrin	WG↑ @ C↓	None	Shimeno et al. (1996)
	20, pot. Starch	SGR↔	✓	Furuichi et al. (1986)
	Glucose		None	
<i>Solea senegalensis</i> juv, b – c	9–20, starch	WG↔	✓ @ P55→45 + L16	Guerreiro et al. (2014)
	27–38, mainly starch	WG↔	✓ @ P60→48 + L6	Salas-Leiton et al. (2018)
<i>Sparus aurata</i> juv, c (h)	40, high-digestible C	WG↔	✓ if fed in the morning	García-Meílán et al. (2014)
<i>S. aurata</i> finger	5, 18, 26, gelat starch	WG↔	✓ @ C ≤ 20	Fernández et al. (2007)
<i>Tachysurus (Pelteobagrus) fulvidraco</i> juv, b – c	24, 30, 36, gelat starch	WG↑ @ P30	✓	Ye et al. (2009)
<i>Tinca tinca</i> juv, d – o	18–55, dextrin	GR↓ @ C55 + P < 35	None	de Pedro et al. (2001)

b benthivorous, *c* carnivorous, *d* detritivorous, *f* frugivorous, *h* herbivorous, *o* omnivorous, *p* planktivorous, *juv* juvenile; *sub* subadult; *finger* fingerling; *CF* condition factor; *WG* weight gain; *GR* growth rate; *SDA* specific dynamic action (metabolic cost of processing food); *SGR* specific growth rate; *FE* feed efficiency; *PER* protein efficiency ratio; *C* carbohydrate; *P* protein; *L* lipid; ↑ increase/support; ↓ decrease/reduction; ↔ no apparent effect; affected by feeding frequency

^aAtlantic salmon kept at 12.5 °C show better protein sparing and energy utilization from gelatinized starch than at 2 °C, together with substantially improved glucose tolerance at the higher temperature

^bWG, FE, apparent net retention of protein, and energy in red drum are inversely related to dietary carbohydrate content

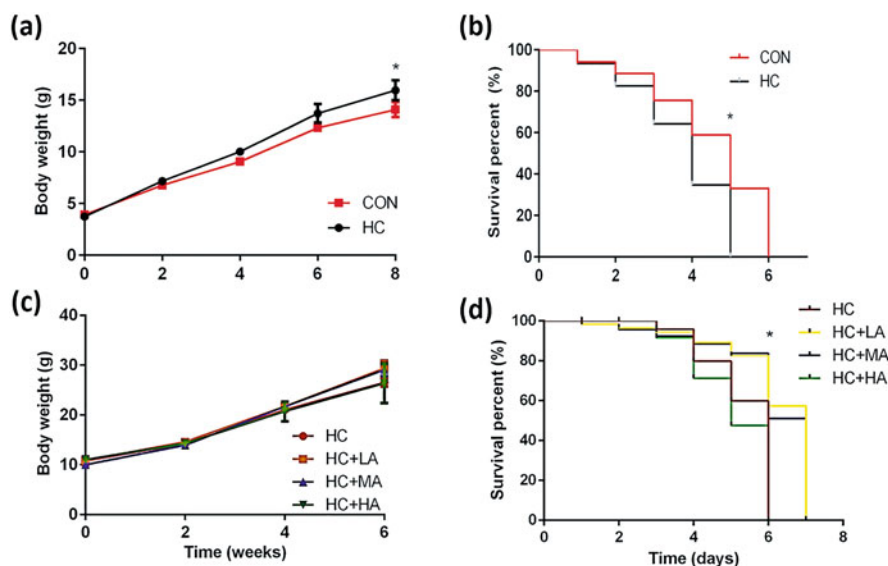


Fig. 17.1 High-carbohydrate (HC) diet promotes growth of Nile tilapia but increases mortality. **(a)** Body weight of control and HC diet group. **(b)** Survival rate. *CON*, fishes fed 25% carbohydrate diet; *HC*, fishes fed 45% carbohydrate diet. Addition of sodium acetate in HC diet increases resistance of Nile tilapia to *Aeromonas hydrophila*. **(c)** Body weight of HC diet group and HC diet group supplemented with different concentrations of sodium acetate (NaAc). **(d)** Survival rate of four groups. *HC + LA*, HC diet +922.5 mg kg⁻¹ NaAc; *HC + MA*, HC diet +1845 mg kg⁻¹ NaAc; *HC + HA*, HC diet +3690 mg kg⁻¹ NaAc. Values are means \pm SEM, * significant difference ($P < 0.05$) using Student's *t*-test. (From Li et al. (2020), with permission from Elsevier)

alleviates intestinal inflammation and increases tilapia's resistance to *Aer. hydrophila* (Fig. 17.1c,d). The addition of 1.9 g kg⁻¹ sodium acetate inhibits p38 mitogen-activated protein kinase and NF- κ B phosphorylation and, eventually, decreases the transcription of pro-inflammation cytokines (*il-8*, *il-12*, *tnf- α* , *il-1 β*) in the intestine.

From recent *Drosophila* studies, remarkable results about effects of HC diets are emerging. Studying inheritance of induced metabolic traits through the germline, Teltumbade et al. (2020) show highly significant overlap of differentially expressed genes between ancestral generation, sperm, embryos, and individuals of succeeding generations. These genes are associated with lipid and carbohydrate metabolism as well as immune response. Paternal diet-induced metabolic traits are delivered to the oocyte via mRNA and influence the embryonic development. They are inherited across generations. This paper firmly establishes the existence of germline-mediated epigenetic inheritance. Since available mammalian data on inheritance of diet-induced metabolic traits reveal similar correlations (Cropley et al. 2016), studies on fishes and aquatic invertebrates are overdue to close this gap.

17.2 Concluding Remarks

Protein-sparing effects, measured as increased protein retention, are found when carbohydrates are given in available (gelat) forms; and better results are obtained for complex than for simple sugars. Furthermore, carbohydrates lead to growth promotion when kept within limits which support the metabolic need (reduced gluconeogenic activity) and do not induce adverse effects (Hemre et al. 2002).

The efficiency of dietary carbohydrate on protein sparing varies even within a single species. This variability may be because of the particular strain of fishes used (more information is available → Chap. 40); feeding history; environmental factors, including temperature, light regime, and season (→ Chap. 16); or feed matrix, the type (mono-, di-, polysaccharide), and amount of carbohydrate supplied in the diet. Long-term effects of feeding history are applied in nutrient programming, and underlying modes of action will be presented in AAN III.

Studies of the gut microbiota as modulator of the protein-sparing effectiveness are rare and warrant future attention. The paper by Li et al. (2020) provides strong evidence that optimizing the composition of the gut microbiota by dietary prebiotics or probiotics will decrease the susceptibility to HC feeds and subsequent diseases. So far, the beneficial effect has been shown only in omnivorous Nile tilapia and the assumption that this approach applies even to carnivores warrants empirical tests.

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Chapter 18

Carbohydrate Preference and Metabolism—‘*Life’s Little Luxury Digested*’



Abstract This chapter focuses on the ability of aquatic invertebrates and fishes to handle increased dietary glucose or starch. Sea urchins appear to have a regulated intake target for dietary protein but not for carbohydrate (→Nutrient-Foraging Hypothesis, Chap. 2). Marine shrimps can be reared at low salinities if additional carbohydrates provide extra energy for osmoregulation. Promising fish studies exist showing improved immunity via modulation of the intestinal microbiota by dietary pre- and probiotics. In carnivorous fishes, almost all essential biological elements of carbohydrate metabolism are present, but with differences in the regulatory mechanism due to low adaptability (→ Chap. 15) in digestive and glucose transport capacities, poor glucose homeostasis, complex hormonal regulation, distinctive glucose, energy and nutrient sensing mechanisms, regulatory deficits in hepatic intermediary metabolism, and weak glucose utilization in peripheral tissues. Moreover, feeding history is essential in tolerating and utilizing carbohydrates by carnivores. The ability of the intestinal microbiome in carnivorous fishes to utilize dietary carbohydrate as energy and as substrate for fermentation of short-chain fatty acids remains an interesting avenue for future research. A variety of hypotheses explaining the differences of carnivores and omnivores/herbivores in tolerating dietary carbohydrates could be verified more or less only partly and are discussed. One difference comprises the intriguing fact that omnivores on carbohydrate diets can improve the innate immunity. Finally, miRNAs acting as negative posttranscriptional regulators affecting target mRNA stability and translation have emerged as critical regulators of hepatic control of glucose-homeostasis and deserve future research.

Carbohydrates are central to many essential metabolic pathways. Although dietary consumption comprises a variety of carbohydrates, digestion breaks down complex carbohydrates into a few monosaccharides for metabolism: glucose, fructose, and galactose (Fig. 18.1). Glucose constitutes the major part of the products and is the primary structure that is distributed to cells in the tissues, where it is broken down or stored as glycogen (→ Chap. 14). In aerobic respiration, glucose is metabolized to release energy, with carbon dioxide and water as by-products. Most of the fructose and galactose travel to the liver, where they can be converted to glucose.

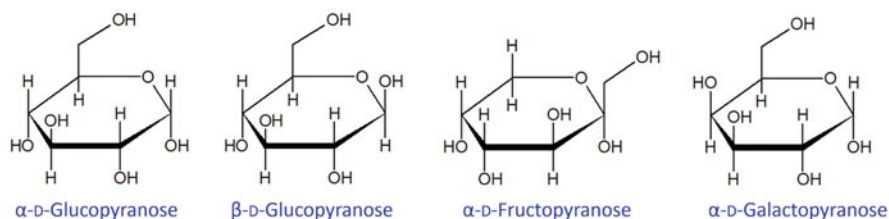


Fig. 18.1 Major hexoses in Haworth projection

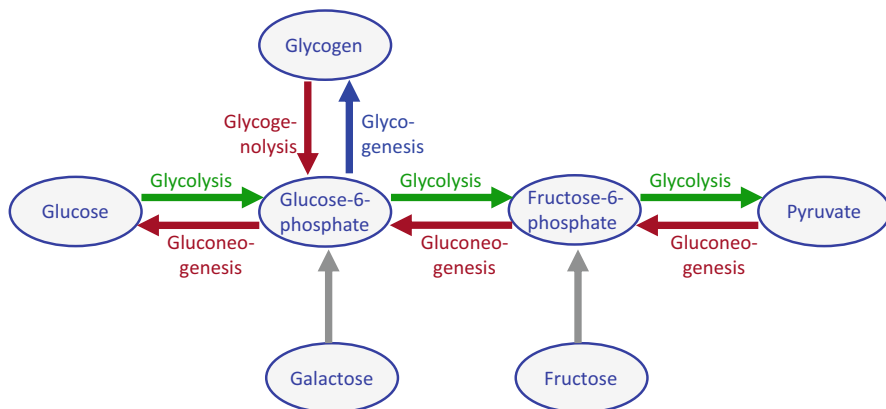


Fig. 18.2 Diagram of the relationship between the processes of carbohydrate metabolism, including glycolysis, gluconeogenesis, glycogenesis, glycogenolysis, fructose metabolism, and galactose metabolism

A simplified diagram of carbohydrate metabolism is sketched in Fig. 18.2 (Berg et al. 2015). In particular:

- *Glycolysis* is the sequence of reactions that metabolize one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP. Pyruvate can be further processed anaerobically to lactate (lactic acid fermentation) or ethanol (alcoholic fermentation).
- *Gluconeogenesis* is the synthesis of glucose from certain non-carbohydrate substrates, such as pyruvate, lactate, or amino acids (AAs).
- *Glycogenesis* is the formation of glycogen from monosaccharides via glucose-6-phosphate.
- *Glycogenolysis* is the biochemical breakdown of glycogen to glucose.

Selected studies are going to highlight carbohydrate preference and metabolism in farmed and wild aquatic animals.

18.1 Invertebrates

Comprehensive studies of carbohydrate metabolism are available for crustaceans, herbivorous mollusks, and echinoderms.

18.1.1 Mollusks

In the great pond snail (*Lymnaea stagnalis jugularis*), Friedl (1975) observed a protein-sparing effect by dietary rice starch fed after starvation. In contrast to this basic approach, carbohydrate metabolism in freshwater snails have mainly been studied to combat these invertebrates in their function as vectors for human parasites, particularly trematodes (*Schistosoma* sp., *Fasciola* sp.) (Agarwal and Singh 1988). The genera *Bulinus*, *Lymnaea*, *Biomphalaria*, and *Pomacea* are of special interests. It is assumed that these animal vectors are attracted to molucidal baits by dietary carbohydrate supplementation. In fact, starch emerges as the strongest individual carbohydrate attractant for *Lymnaea acuminata*, followed by maltose; less attractive are sucrose, glucose, or fructose (Tiwari and Singh 2004).

18.1.1.1 *Haliotis*

Abalones (*Haliotis* spp.) are slow feeders and their natural diet (macroalgae) consists of 40–50% carbohydrates. Consequently, they have various enzymes capable of hydrolyzing complex carbohydrates (Fleming et al. 1996). In donkey's ear abalone (*H. asinina*), Thongrod et al. (2003) showed that high levels of dietary lipid negatively affect abalone growth, whereas high levels of carbohydrate (43–48%) support it. Abalones are able to adjust their intestinal enzyme levels in order to maximize the acquisition of dietary protein and carbohydrates, but not lipids. In fact, Garcia-Esquivel and Felbeck (2006) confirmed that lipase activity is not affected by the type of food in another abalone (*H. rufescens*). Similarly, Uriarte et al. (2006) reported that postlarval growth of this abalone is positively related to dietary diatom protein and carbohydrate, but not to lipids; and Lee et al. (2017) identified that growth performance is best in *H. discus* on α -cellulose.

In farmed abalones, gut-bacterial populations are shaped by ontogeny, macroalgal diet substrates, bacteria present in the environment, and other environmental factors. The hypothesis emerges that weaning post-settlement diatom-fed abalone onto artificial feed alters the natural succession of bacterial establishment in their guts with macroalgae-rich diet reducing gut microbiotic diversity. In fact, Nel et al. (2018) showed that the gut-bacterial composition of *H. midae* weaned onto fresh kelp *Ecklonia maxima* display a relatively low diversity and dominated by fermentative *Clostridium* bacteria. The large share of fermentable polysaccharides present in macroalgae induces this dominance, whereas energy-rich formulated

feeds result in elevated bacterial diversity. This paper opens the perspective that manipulating the gut microbiota can improve the digestibility of carbohydrate-rich diets.

18.1.2 Echinoderms

Heflin et al. (2012) fed adult green sea urchin (*Lytechinus variegatus*) formulated diets with graded protein (12→36%) and carbohydrate (21→39%) levels and detected that dietary carbohydrate is a poor predictor for growth. However, the protein-carbohydrate interaction explains best the protein efficiency ratio (PER). PER decreases with increasing dietary protein level, more so at higher carbohydrate levels (Fig. 18.3). In a succeeding study, Heflin et al. (2016b) reported that large adult *L. variegatus* held in culture have a tightly regulated intake target for dietary protein but not for carbohydrates indicating that this species regulates feed intake to satisfy its protein requirement. Elevated dietary protein levels result in decreased feed intake (Heflin et al. 2012). Regardless of nutrient ratios or macronutrient concentration, individuals adjust intake patterns to defend this target. This finding supports the nutrient-specific foraging hypothesis presented in Chap. 2.

An overview of dietary carbohydrates controlling life history traits in echinoderms is presented in Table 18.1.

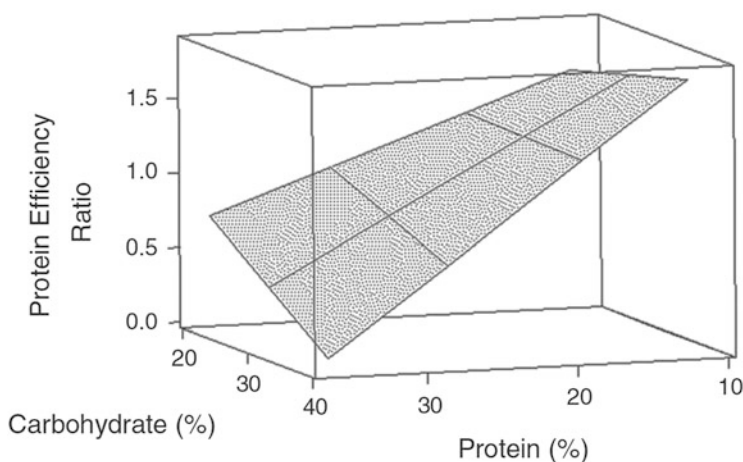


Fig. 18.3 Relationship between protein efficiency ratio and protein×carbohydrate interaction effect of individual *Lytechinus variegatus* fed diets with varying protein and carbohydrate contents for 9 weeks. (From Heflin et al. (2012), with permission from Elsevier)

Table 18.1 Effect of combined carbohydrates on life history traits in selected echinoderms

Scientific name, common name	Carbohydrate, dietary level, %	Affected life history trait	Reference
<i>Apostichopus japonicus</i> , Japanese sea cucumber	Starch, 20: Corn, sweet potato, tapioca, potato	WG: Corn>tapioca, sweet potato, potato	Wen et al. (2017)
<i>Crassostrea gigas</i> spat, Pacific oyster	<i>Pavlova pinguis</i> , <i>Rhodomonas salina</i> , <i>Tetraselmis</i> sp., <i>Nannochloropsis</i> -like	SGR best on algae high in carbohydrates (<i>P. pinguis</i>)	Brown et al. (1998)
<i>Holothuria (Metriatyla) scabra</i> , sandfish	Bulk carbohydrates (nitrogen-free extract) of feed algae	Larval development↑	Duy et al. (2016)
<i>Lytechinus variegatus</i> , green or variegated sea urchin	Wheat starch 19→38	WG↓	Taylor et al. (2017)
	Wheat starch 0→37 P 31→9	Gamete growth and development↓	Hammer et al. (2006)
	Wheat starch 21→39 P 36→21	PER↓ with C↑ (→Fig. 18.3)	Heflin et al. (2012)
	Wheat starch 12→18 P 43→11	Optim WG @ C18 and P 30	Heflin et al. (2016a)
<i>Paracentrotus lividus</i> , purple sea urchin	<i>Rhodomonas</i> sp. rich in carbohydrates	Growth↑, development↑, survival↑	Castilla-Gavilán et al. (2018)
<i>Strongylocentrotus purpuratus</i> , purple sea urchin	Cornstarch C42→58; P30→17	Optim development @ C50→58 and P17→23	Cuesta-Gomez and Sánchez-Saavedra (2017)
	C42, 50 P30→17	Gonads↑ @ C50 and P30	Cuesta-Gomez and Sánchez-Saavedra (2018)

↓ low content/reduction; ↑ high content/support/increase; ↔ no apparent effect; WG weight gain; PER protein efficiency ratio; SGR specific growth rate; FCR feed conversion ratio; C carbohydrate; L lipid; P protein

18.1.3 Crustaceans

The glucose transport and utilization process in crustaceans is similar to that of mammals and fishes. Most crustaceans are omnivorous and polysaccharides are hydrolyzed into oligosaccharides, branched-chain α -dextrin, and maltose in the digestive gland. These compounds are then completely hydrolyzed into monosaccharides, transported as glucose, and absorbed by other organs. The chemical reactions involved in the tricarboxylic acid (TCA) cycle mainly occur in hepatopancreas and muscle. The activity of enzymes involved in the TCA pathway is closely related to the glucose content and changes in relation to glucose concentration. The pentose phosphate pathway, which is negligible in fishes, is the main carbohydrate metabolism pathway in decapod crustaceans during the molting stage. Most crustaceans have some key enzymes of the gluconeogenesis pathway in the

hepatopancreas. Dietary carbohydrates not used in metabolism may accumulate as lipid and glycogen. The glycogen in the crustacean hepatopancreas is an important precursor for chitin synthesis, and it serves a critical role during the molt cycle (Wang et al. (2016a) and references therein).

Crustaceans are well equipped to synthesize carbohydrates from proteins and other proteinaceous material (Rosas et al. 2002; Oliveira et al. 2004). Therefore, dietary carbohydrate requirement is not strongly expressed; however in terms of energy consumption, the use of proteinaceous material for gluconeogenesis is disadvantageous, since crustaceans using proteins to channel energy to growth have to produce a big loss of energy through ammonia excretion (Rosas et al. 2002).

Inferring ecological niches of six crabs via digestive enzyme activities, Johnston and Freeman (2005) compared stomach contents and key enzymes. Red rock crab (*Nectocarcinus integrifrons*) consumes large quantities of seagrass and has high cellulase activity to digest the constituent cellulose. New Zealand porcelain crab (*Petrolisthes elongatus*) ingests brown and green algae and has considerable laminarinase and β -glucosidase activities to digest the laminarin in its diet. Omnivorous swiftfooted shore crab (*Leptograpsus variegatus*) has high activities of protease, α -glucosidase, and α -amylase and appears well equipped to utilize protein and carbohydrate. Stomach contents in velvet crab (*Nectocarcinus tuberosus*) and green (shore) crab (*Carcinus maenas*) indicate that these species are omnivorous. *N. tuberosus* has high cellulase and chitinase for digesting the cellulose in plants and the chitin in invertebrate shells, respectively. *C. maenas* has intermediate digestive enzyme levels and uses more of a generalist feeding strategy than other species. Speedy crab (*Guinusia* [*Plagusia*] *chabrus*) is carnivorous, consuming encrusting bryozoans, hydroids, crustaceans, and fishes. Thus, it possesses high protease activity, particularly trypsin, to digest the protein in its animal prey (Johnston and Freeman 2005).

Due to its carnivorous feeding habit, *Panulirus argus* is supposed to use preferentially proteins and lipids in energy metabolism, while carbohydrates are poorly utilized. With increasing dietary inclusion of wheat flour, lobsters show high levels of free glucose and stored glycogen in different tissues (Rodríguez-Viera et al. 2017). Modifications in intermediary metabolism reveal a decrease in AA catabolism coupled with a higher use of free glucose as carbohydrates rise up to 20%. Lobsters are not able to tightly regulate α -amylase transcription according to dietary carbohydrate levels but exhibit a marked difference in secretion of this enzyme into the gut (Fig. 18.4).

Typical effects of various carbohydrate diets are summarized in Table 18.2. Of special interest are studies in *Litopenaeus vannamei* reared at low salinities, which show that an optimal carbohydrate supply (~20%) enables the animals to live at inland low salinities by providing extra energy for osmoregulation (Wang et al. 2014). Moreover, it alleviates adverse effects of concomitant ammonia stress. Wheat starch reveals the best growth performance of the tested carbohydrate sources (Wang et al. 2016b).

For osmoregulation during acute hypo-osmotic stress, *L. vannamei* first uses muscle glycogen as energy resource and then AAs in the muscle through

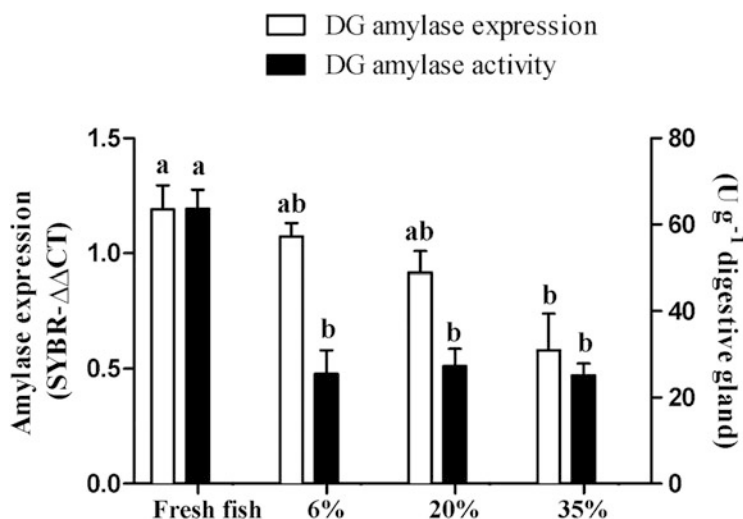


Fig. 18.4 α -Amylase activity and gene expression in the digestive gland (DG) of *Panulirus argus* feeding. Diets were named according to their carbohydrate level (6%, 20%, 35%) and a control with fresh fish muscle. Values are means \pm SEM ($N = 6$ lobsters per diet). Different letters indicate statistical differences according to the Tukey's test ($P < 0.05$). Amylase activity in the DG is highly impacted by formulated diets feeding. (From Rodríguez-Viera et al. (2017), credit PeerJ)

gluconeogenesis. AAs are released and transported to the hepatopancreas and gills for gluconeogenesis to cope with the salinity stress. Glucose metabolism occurs in muscles and gills after 24 h of the stress to allocate energy for osmoregulation and physical activities (Wang et al. 2017).

Complex dietary carbohydrates, however, are not ideal for shrimps in low-salinity environments. Qiao et al. (2017) reared *L. vannamei* hypo-osmotically with glucose, sucrose, and cornstarch as dietary carbohydrate sources, respectively. Dietary sucrose and glucose result in better growth performance than cornstarch. In the intestinal microbiota, *Proteobacteria* are the most prevalent members, and *Actinobacteria* decrease in abundance, while *Firmicutes* increase in cornstarch-fed animals (Fig. 18.5). Furthermore, bacteria related to complex carbohydrate degradation are in low abundance, whereas the abundance of opportunistic pathogenic bacteria increases in cornstarch-fed group (Fig. 18.5), indicating again that the diet imposes selective pressure on the intestinal microbiota. The degradation efficiency of complex carbohydrates by the modified gut microbiota appears to be limited.

Finally, the question arises whether an appropriate manipulation of the gut microbiota by supplying prebiotics or/and probiotics can improve carbohydrate utilization of carnivorous/omnivorous shrimps or shrimps in hypo-osmotic conditions. Li et al. (2018) documented that various prebiotics and probiotics strengthen the immunity of *L. vannamei* in farms. Prebiotic inulin benefits immune response in this shrimp at low salinity via modulated intestinal microbiota: the relative abundance of the *Firmicutes* phylum and *Bacillus* genus increase (Zhou et al. 2020).

Table 18.2 Effect of combined carbohydrates on life history traits in selected crustaceans

Scientific name, common name	Carbohydrate, dietary level, %	Affected trait	Reference
<i>Cherax quadricarinatus</i> , Australian red claw crayfish	Dextrin, 0→27	WG↓, SGR↓ @ C↑L↓ or C↓L↑	Zhu et al. (2013)
<i>Farfantepenaeus duorarum</i> , northern pink shrimp	Starch, 0, 10, 40	WG and SRV best @40	Sick and Andrews (1973)
<i>Litopenaeus vannamei</i> , Pacific white shrimp	Flour from wheat, corn, sor- ghum, millet, or rice, ~20	Best WG @ wheat FCR 1.2–1.4	Cruz- Suárez et al. (1994)
	Cornstarch, 5→30	Max WG and SRV @ 20 and low salinities	Wang et al. (2014)
	Glucose, sucrose, wheat starch, cornstarch or potato starch, 20 each	Best WG @ wheat starch @ low salinities	Wang et al. (2016b)
	Cornstarch, 5→30	mRNA of carbohydrate metabolism↑ @ 20	Wang et al. (2017)
	Fructose, 10→30	Osmoregulation↑, SRV↑ @ 20 and low salinities	Xiao et al. (2017)
	Glucose, sucrose, cornstarch, 20 each	Best WG and SRV with glucose and sucrose @ low salinities	Qiao et al. (2017)
<i>Macrobrachium nipponense</i> , oriental river prawn	Cornstarch, 5→35	Optim WG < 15	Ding et al. (2017)
	Raw cornstarch, gelat corn- starch, dextrin, maltose, glu- cose, cellulose, 18 each	WG: Starch > glucose, cellulose	Kong et al. (2019)
<i>Marsupenaeus japonicus</i> , kuruma shrimp	Glycogen, starch, dextrin, sucrose, 10	Best WG @ sucrose	Deshimaru and Yone (1978)
<i>Neohelice granulata</i> , burrowing crab	Boiled rice, 35	Glyceroneogenesis ^a ↓ after summer starvation	Sarapio et al. (2017)
<i>Palaemon serratus</i> , common prawn	Wheat starch Glycogen	Assimilation↑ Assimilation↑	Forster (1972)
<i>Pandalus platyceros</i> , Califor- nia spot prawn	Dextrin Potato starch, 45	Assimilation↑ Assimilation↓	
<i>Panulirus argus</i> , Caribbean spiny lobster	Cornstarch, rice starch, 30	No protein sparing, Cornstarch less digestible	Rodríguez- Viera et al. (2014)
	Wheat flour, 5, 20, 35	Protein sparing @ <20 (→Fig. 18.4)	Rodríguez- Viera et al. (2017)

(continued)

Table 18.2 (continued)

Scientific name, common name	Carbohydrate, dietary level, %	Affected trait	Reference
<i>Penaeus monodon</i> , black tiger prawn	Wheat starch, sucrose, potato starch, cornstarch, dextrin, maltose, glucose, 20 each	Best WG @ wheat starch or sucrose	Niu et al. (2012)

↓ low content/reduction; ↑ high content/support/increase; ↔ no apparent effect; *gelat* gelatinized; WG weight gain; SGR specific growth rate; FCR feed conversion ratio; SRV survival; C carbohydrate; L lipid

^aMetabolic pathway synthesizing glycerol 3-phosphate or triglyceride from precursors other than glucose (Nye et al. 2008)

Similarly, dietary supplies of yeast, yeast extract, or lactic acid bacteria beneficially shift the microbiota composition (Ringø et al. 2020; Zheng et al. 2021). However, strengthening of immune response, rather than improved carbohydrate utilization, has been in the focus of these papers, and it remains open whether these dietary manipulations also increase carbohydrate digestion.

18.2 Fishes

Carbohydrates in food for fishes in the wild comprise glycogen from the muscle and liver of prey animals, glucose in prey blood, and trehalose and chitin in blood and exoskeleton of crustaceans and insects. Plant sources of carbohydrates, such as phytoplankton, grasses, and macroalgae, ingested by herbivorous and omnivorous species, may contain some digestible mono- and disaccharides, such as glucose, fructose, and sucrose. However, most carbohydrates in plants eaten by fishes are polysaccharides including cellulose, hemicelluloses, alginates, pectins, mucilages, or chitin. Carbohydrates in formulated feeds range from highly digestible mono-, di-, and oligosaccharides to insoluble and indigestible hemicelluloses and cellulose. Starch, as amylose or amylopectin, is the main carbohydrate in formulated feed serving both as energy source and as pellet binder. Amylose is a single-stranded molecule with several thousand glucose molecules that form a helix not easily attacked by α -amylase, whereas amylopectin is highly branched and easily hydrolyzed by enzymes (Krogdahl et al. 2011).

Fishes have a lower ability to utilize dietary carbohydrate than mammals (Polakof et al. 2012; Kamalam et al. 2017). Existing literature on regulatory mechanisms of glucose homeostasis mainly focuses on carnivorous fishes, especially salmonids. Carbohydrate utilization depends on the feeding habit, with lower glucose tolerance in carnivorous fishes than in omnivorous or herbivorous ones (Moon 2001; Hemre et al. 2002). Also the developmental stage determines this tolerance; for instance, Pen-Hsing and Shi-Yen (1993) identified that hybrid tilapia shows better glucose tolerance in large than in small individuals.

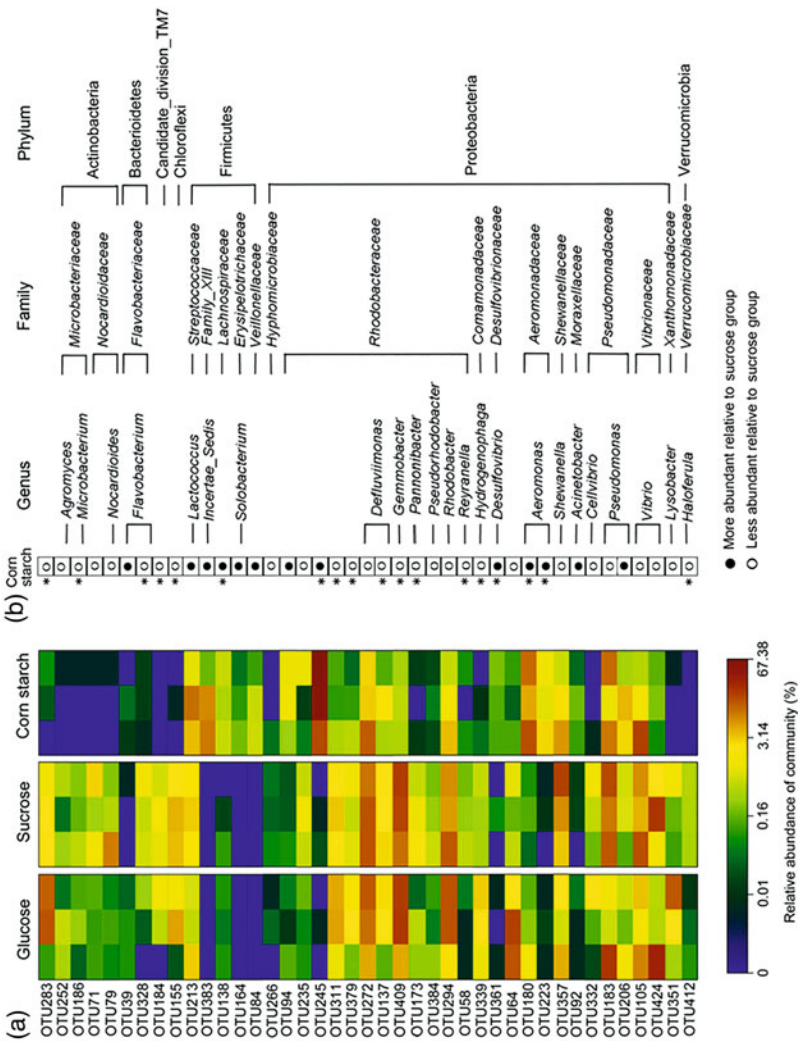


Fig. 18.5 Heatmap of bacterial abundance in the intestines of *Liopentaeus vammamei* fed with different carbohydrate sources. According to redundancy analysis and operational taxonomic units (OTU) proportions, 41 OTUs were selected to produce the figure. (a) Heatmap of the abundance of 41 OTUs. (b) The changing direction of the OTUs. Dots and open circles indicate the abundance of OTUs in the cornstarch-fed group that were more or less abundant than in the sucrose-fed group, respectively. The taxonomy of the OTUs (genus, family, and phylum) is depicted on the right. Asterisks (*) represent significant differences between Group S and Group C, based on Student's t-test. (From Qiao et al. (2017), with permission from Wiley)

Many omnivores and herbivores can utilize dietary carbohydrates, and glucose is indeed an important energy source for certain tissues such as the brain. In herbivores, digestion of carbohydrate-rich food often depends on symbiotic microorganisms in the gut, which use anaerobic fermentation to acquire energy for their own use. The fermentation breakdown products are mainly short-chain fatty acids (SCFAs), such as butyric, propionic, and acetic acid (for more details about SCFA effects, see below and particularly Chap. 26), which easily diffuse across the epithelium. Of the SCFAs, only propionic acid can be converted to glucose. This and the observations that several herbivores depend on lipid-rich food sources such as diatoms for growth and reproduction indicate that glucose probably does not meet the total energy demand throughout the life cycle of herbivorous species (Sundell and Rønnestad 2011).

18.2.1 *Carnivores*

Recent reviews (Polakof et al. 2008; Conde-Sieira et al. 2018) indicate the presence of almost all essential biological elements of carbohydrate metabolism in carnivorous fishes, but with differences in the regulatory mechanism. These differences comprise the inherently limiting steps beginning with low, nevertheless existing, adaptability¹ in digestive and glucose transport capacities, poor glucose homeostasis, complex hormonal regulation, distinctive glucose, energy and nutrient sensing mechanisms, regulatory deficits in hepatic intermediary metabolism, and weak glucose utilization in peripheral tissues (Figs. 18.6 and 18.7) (Kamalam et al. 2017).

The European eel is able to utilize efficiently cooked cornstarch at levels of 40% with good growth and food conversion (Hidalgo et al. 1993). To study the capacity of this species to adapt its intermediary metabolism to variations in diet concentrations of protein, Suárez et al. (1995) measured representative enzymes involved in glycolysis, pentose phosphate pathway, gluconeogenesis, and AA deamination, respectively. The highest weight gain and feed efficiency are obtained on diets with the highest energy content and with the greatest carbohydrate level. High carbohydrate levels enhance glucose utilization to yield energy and lipid formation, and gluconeogenesis increases in response to a lower dietary carbohydrate content.

Inside the liver, excess glucose is stored in the form of glycogen. Song et al. (2018b) studied this process in two isogenic lines of rainbow trout (A32h, AB1h) and find that liver glycogen increases in fish fed with the high-carbohydrate (H-CHO) diet, but no differences between the two lines are observed. In the next

¹Refer to Chap. 15: Marandel et al. (2018) showed that wild brown trouts can make use of their *gck* regulatory system following ingestion of carbohydrates through natural feeding. Under the condition of missing appropriate prey, these salmonids appear conditionally omnivorous, rather than strictly carnivorous.

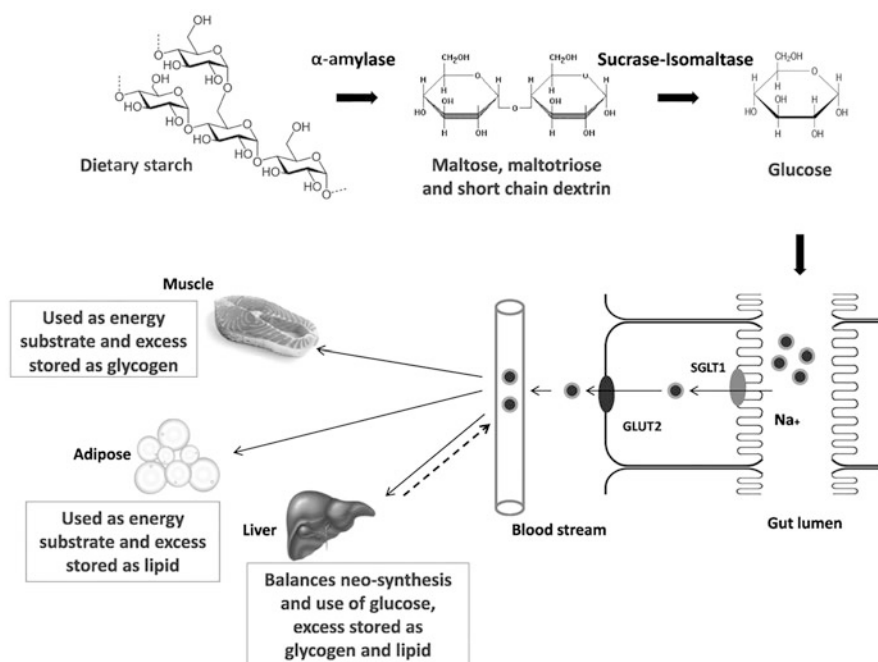


Fig. 18.6 Schematic representation of dietary carbohydrate utilization in fishes illustrating digestion, intestinal glucose transport, and glucose metabolism in the major insulin-sensitive tissues. SGLT1, Na⁺-dependent glucose symporter; GLUT2, Na⁺-independent facilitative glucose transporter type 2. (From Kamalam et al. (2017), with permission from Elsevier)

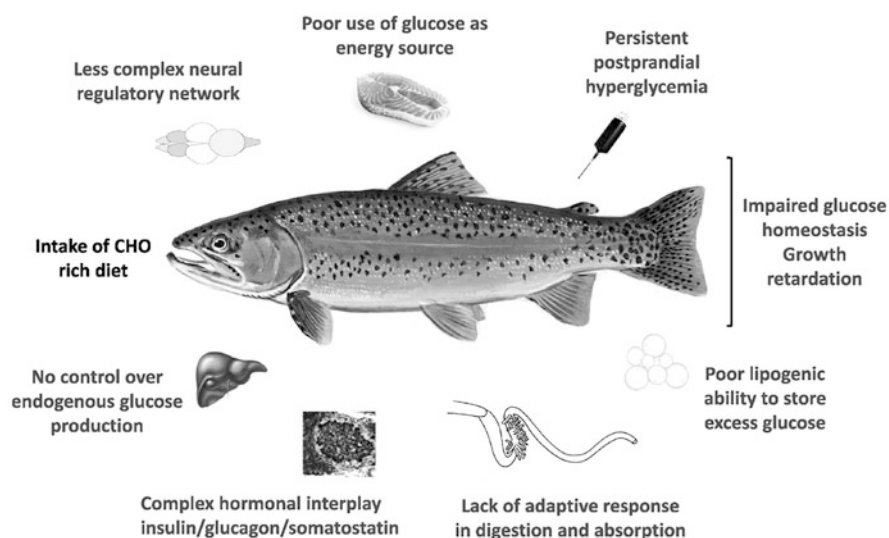


Fig. 18.7 Schematic summary of biological limitations for carbohydrate utilization in carnivorous fishes. (From Kamalam et al. (2017), with permission from Elsevier)

step, glycolytic genes, *gcka* and *gckb*, are markedly higher expressed in fishes fed the H-CHO diet than in those on L-CHO diet (Song et al. 2018b).

The dietary carbohydrate intake in rainbow trout is associated with hyperglycemia and downregulation of the energy sensor AMPK (→Box: “Yin and Yang of Energy Regulation,” Chap. 5) but also with atypical regulation of glycolysis and gluconeogenesis in the liver. The first steps of glycolysis and gluconeogenesis catalyzed by glucokinase and phosphoenolpyruvate carboxykinase are regulated at the molecular level by dietary carbohydrates as expected (i.e., induction of the glycolytic *gck* (Fig. 18.8) and repression of the gluconeogenic *pck*, Fig. 18.9). By contrast and surprisingly, for two other key glycolytic enzymes (*phosphofructokinase* (*pfkl*) and *pyruvate kinase* (*pk*)), some paralogs (*pfklb* and *pklr*) are inhibited by carbohydrates (Fig. 18.9), whereas some of the genes coding gluconeogenic enzymes (the glucose-6-phosphatase enzyme *g6pcb1b* and *g6pcb2a* gene and the *fructose 1,6-bisphosphatase* paralog *fbp1a*) are induced (Fig. 18.9). Furthermore, differences for metabolic genes are found between the two isogenic lines (*gcka*, *gckb* *pklr* (Fig. 18.9); *pck2*, *fbp1a*, *g6pcb2a* (Fig. 18.9)), confirming the existence of genetic polymorphisms for nutritional regulation of intermediary metabolism in rainbow trout (Song et al. 2018b).

In two other isogenic rainbow trout lines, Song et al. (2018c) demonstrated the effects of dietary carbohydrate on glucose, lipid, and mitochondrial metabolism in the liver and muscle. This paper confirms the atypical regulation by dietary carbohydrates of hepatic glycolysis and gluconeogenesis described above and the absence of regulation for muscle glucose transport and phosphorylation (Polakof et al. 2012). High mRNA levels for genes involved in hepatic lipogenesis, cholesterol metabolism, and energy metabolism as well as for those involved in glycolysis in the muscle are found after feeding carbohydrates. In compliance with the results of the two other isogenic lines (Song et al. 2018b), this paper presents evidence of the existence of genetic variability for the expression of metabolic genes, especially for the biosynthesis of long-chain polyunsaturated fatty acids (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA; → Chaps. 27 and 28).

Insulin is able to activate the protein kinase, a key element in the insulin signaling pathway, and to regulate hepatic metabolism-related target genes (Plagnes-Juan et al. 2008). As in mammals, insulin decreases mRNA expression of gluconeogenic genes, including *glucose 6-phosphatase* (*g6pase*), *fructose 1,6-bisphosphatase* (*fbpase*), and *pepck*. Insulin also limits the expression of *carnitine palmitoyltransferase 1* (*cpt1*), a limiting enzyme of fatty acid β -oxidation. Again as in mammals, glucose is an important regulator of insulin target genes, such as the glycolytic enzyme *pk* and the lipogenic enzyme *fatty acid synthase* (*fas*). Glucose also stimulates expression of *glucokinase* (*gck*), which has no equivalent in mammals. This study demonstrates that insulin possesses the intrinsic ability to regulate hepatic gene expression in rainbow trout and indicates that other hormonal or metabolic factors may counteract some of the documented postprandial actions of insulin, namely, the absence of postprandial regulation of hepatic gluconeogenic gene expression. In turn, the pattern of insulin and insulin receptor encoding gene expression in selected tissues is regulated by nutritional state (fed vs. fasted) and glucose (Caruso and Sheridan 2012).

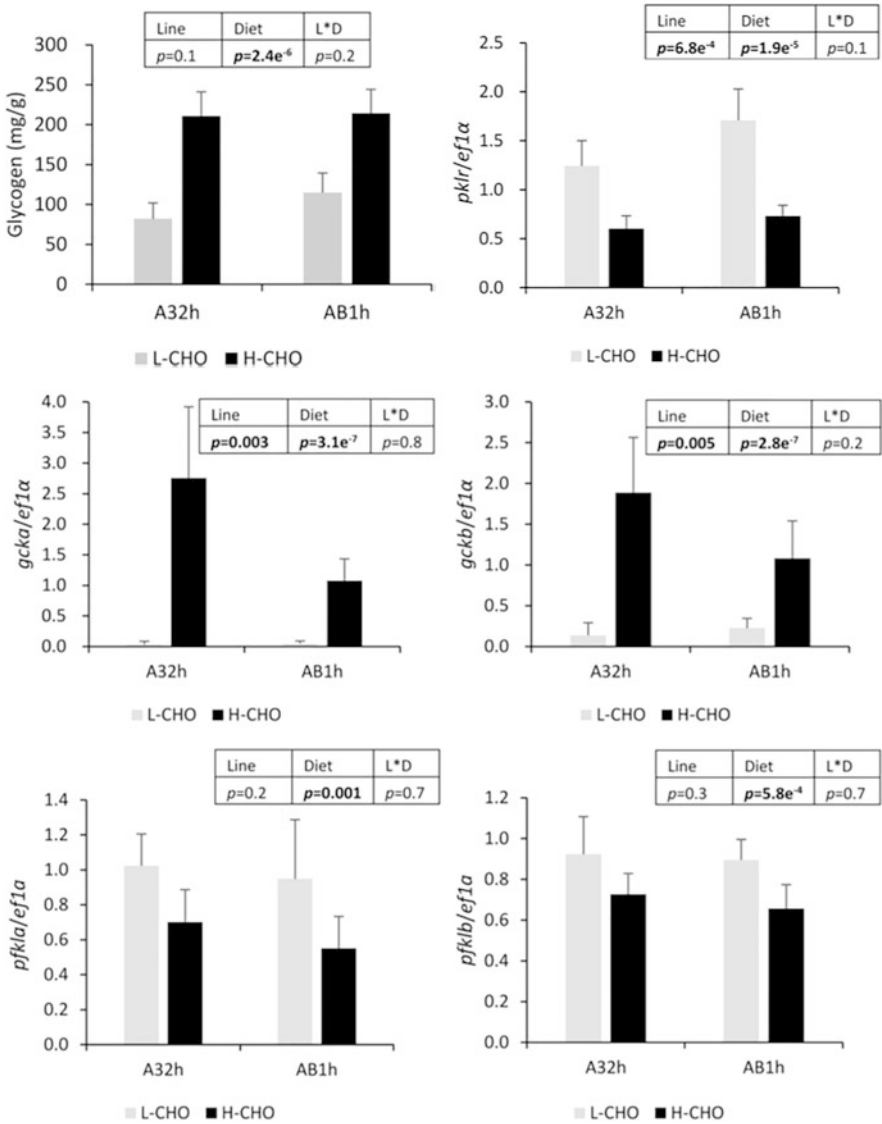


Fig. 18.8 Liver glycogen and mRNA levels of selected glycolytic enzymes in the liver of two lines of rainbow trout fed with low-carbohydrate diet and high-carbohydrate diet. *Gcka*, *gckb* glucokinase paralogs; *pfkl* 6-phosphofructokinase, liver type; *pkf* pyruvate kinase, liver type. Data are presented as mean ± SD ($n = 6$), statistical difference of liver glycogen was evaluated by two-way ANOVA ($P < 0.05$, values in bold). (From Song et al. (2018b), credit Company of Biologists)

Through inhibition of hepatic gluconeogenesis, rapamycin treatment improves glucose tolerance in rainbow trout (Dai et al. 2014). Transcription of *gck*, *g6pase I* and *II*, and *fbpase* is reduced by rapamycin after glucose administration with

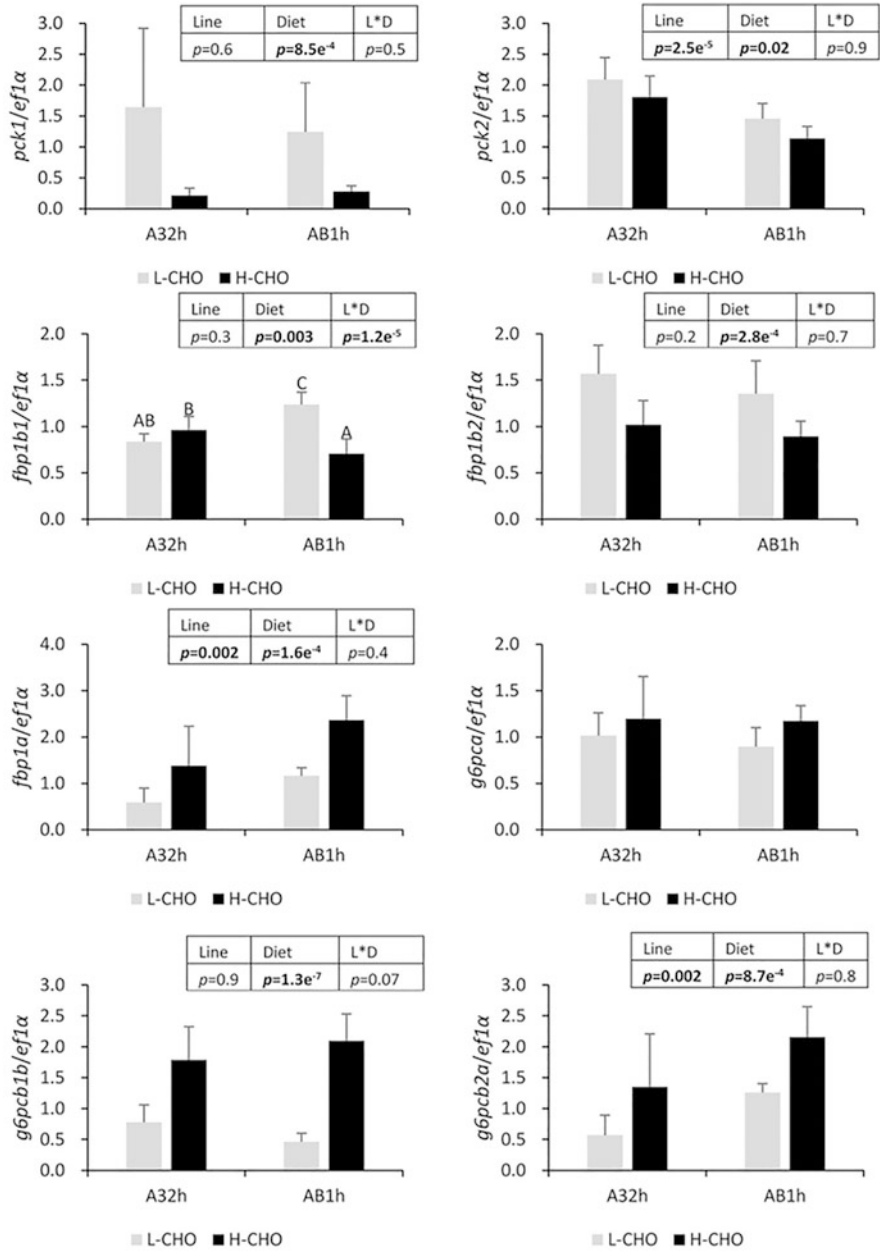


Fig. 18.9 mRNA levels of selected gluconeogenesis enzymes in the liver of two lines of rainbow trout fed with low-carbohydrate diet and high-carbohydrate diet. *pck1*, *pck2* phosphoenolpyruvate carboxykinase paralogs; *fbp1b1*, *fbp1b2*, *fbp1a* fructose 1,6-bisphosphatase paralogs; *g6pca*, *g6pcb1b*, *g6pcb2a* glucose 6-phosphatase paralogs. Data are presented as mean \pm SD ($n = 6$), the statistical differences of *pck1*, *pck2*, *fbp1b2*, *fbp1a*, *g6pca*, *g6pcb1b*, and *g6pcb2a* were evaluated by two-way ANOVA ($P < 0.05$, values in bold). Because of interaction, a post hoc Tukey's test was performed for *fbp1b1*. Different superscripts indicated significant differences between treatments. (From Song et al. (2018b), credit Company of Biologists)

subsequent reduced activity of the corresponding enzymes. This is the first in vivo evidence in fishes that glucose regulates hepatic *gck* expression and, in rainbow trouts, activity through a TOR-dependent pathway (→Box: “Yin and Yang of Energy Regulation,” Chap. 5). This approach deserves a broader consideration in terms of more general application in aquafeed for carnivores in order to decrease glucose susceptibility (Dai et al. 2014).

Even the social status shapes liver metabolism and, in particular, glycogen metabolism in rainbow trouts, favoring accumulation of glycogen reserves from incoming energy in dominant fishes and reliance on onboard fuels in subordinate fishes (Gilmour et al. 2012). The hepatic glycogen content of dominant fish exceeds that of control fishes, indicating enhanced glycogen accumulation in response to the acute stress of hierarchy formation aided by downregulation of hepatic glycogen phosphorylase activity and ample incoming energy from food intake. Hepatic glycogen reserves in subordinate fishes likely fall during the acute stress of hierarchy formation; they cannot be fully replenished owing to low food intake.

Typical effects of various dietary carbohydrates are collected in Table 18.3. It becomes obvious that carnivorous species tolerate at least 20% dietary carbohydrates. Above this grade of inclusion, oxidative stress and reduced immunity have to be expected with subsequent enteritis, reduced growth, and reduced survival after pathogen challenge.

Examining the use of endogenous glucose during fasting provides a new perspective of assessing glucose metabolism. Yang et al. (2019) applied this approach and starved Japanese flounder for 3, 9, 24, and 48 h and 3, 7, 14, 21, and 28 days, respectively, after a 10-week feeding trial with 12% and 20% of dietary carbohydrates (α -starch + cornstarch). The authors sketch the hepatic glucose and lipid metabolic adaptations during postprandial starvation (Fig. 18.10). Based on the mRNA levels of related genes and biochemical compositions of tissues, the processes of glycogenesis, glycolysis, pentose phosphate pathway, and lipogenesis in the liver are activated once after feeding. Subsequently, the process of gluconeogenesis is induced. Then, the supply of glucose needed by the body’s energy metabolism gradually changes from exogenous (feeding) to endogenous (gluconeogenesis and glycogenolysis). With proceeding starvation, primary substrate of energy sources in Japanese flounder switches from glycogen to hepatic lipid. During starvation, intake of a carbohydrate-enriched diet is found to activate gluconeogenesis and lipolysis. These observations demonstrate that previous feeding with different levels of dietary carbohydrates influences glucose and lipid metabolic responses of Japanese flounder during starvation.

In carnivorous fishes, the gut microbiome actively carries out fermentative processes (Geurden et al. 2014). Members of the phylum *Firmicutes* and *Spirochaetes* are central in the fermentation of dietary carbohydrates and transporting nondigestible sugars across their cellular membranes (Corrigan et al. 2015). The most commonly produced molecule from this process is pyruvate. Therefore, the elevation of the glycolysis/gluconeogenesis and pyruvate metabolism pathways is an indication of the fermentative potential of the intestinal microbiome of trouts. The fermentation of dietary carbohydrates by the intestinal microbiota results in the

Table 18.3 Dietary carbohydrates impact metabolism and immunity in selected carnivorous fishes

Species	Carbohydrate, level (%)	Metabolic or biomolecular trait	References
<i>Dentex dentex</i>	Gelat cornstarch, dextrin, maltodextrin, 12, 18, 24	Starch → activity↑ of hepatic and muscle hexokinase and pyruvate kinase	Pérez-Jiménez et al. (2015)
<i>Dicentrarchus labrax</i>	Gelat cornstarch, 0, 20	Plasma cholesterol↑, phospholipids↑, lipids↑, glycogen↑, cholesterol metabolism genes↑, LC-PUFA biosynthesis↔	Castro et al. (2015)
<i>Diplodus sargus</i>	Gelat cornstarch, 35 Feeding frequency↑→	α-Amylase↓, hepatic GCK↓, FBPase↑, hepatic dehydrogenases↔	Enes et al. (2015)
<i>Epinephelus akaara</i>	Cornstarch, 0, 18, 30	Pro-inflammatory genes overexpressed @30, growth↓	Yang et al. (2018)
<i>E. lanceolatus</i>	Glucose, maltose, cornstarch, 18	Hepatic <i>glut4</i> ↑ Starch: <i>Pk</i> ↑, <i>hk</i> ↑	Lu et al. (2018)
<i>Micropterus salmoides</i>	Wheat starch, cassava starch, pea starch, high-amylose cornstarch, 10	High-amylose starch: Oxidative stress↓, gluconeogenesis↑	Song et al. (2018a)
	Wheat starch, 5, 10, 20	Oxidative stress↑, innate immunity↓, gluconeogenesis↓	Lin et al. (2018)
	Cornstarch, 0→25	WG↓, antioxidant enzymes↓, innate immunity↓, gluconeogenesis↓	Ma et al. (2019)
<i>Oncorhynchus mykiss</i>	Extruded wheat, gelat starch, 28	Glucagon↓, insulin(↓)	Del Sol et al. (2004)
<i>Paralichthys olivaceus</i>	α-Starch + cornstarch 0, 12, 20	<i>Gck</i> ↑, <i>pfk</i> ↑, <i>hk</i> ↑ @12, <i>pepck</i> ↓@12 (→Fig. 18.10)	Yang et al. (2020)
<i>Salmo trutta</i>	Comparison of wild and farmed animals	Wild: <i>gck</i> ↑, GCK↑	Marandel et al. (2018)
<i>Solea senegalensis</i>	Pea starch, 18–27	Final GCK↑	Conde-Sieira et al. (2016)
<i>Sparus aurata</i>	Gelat cornstarch, 10–32	Hepatic G6Pase↔, GCK↑	Caseras et al. (2002)
	Pea starch, 0, 20	<i>g6pasel/fbpase</i> ↓	Panserat et al. (2002)
	Gelat cornstarch, 0, 20	Cholesterol absorption and transport↓	Castro et al. (2016a)
		Hepatic LPO↓, intestinal LPO↔	Castro et al. (2016b)

(continued)

Table 18.3 (continued)

Species	Carbohydrate, level (%)	Metabolic or biomolecular trait	References
<i>Trachinotus ovatus</i>	Cornstarch, 0→28 raw	PRO↔; AMY↑, LIP↑, AKP↑, ATPase↑ @ < 11; gh↑@17–28	Zhou et al. (2015)

↑ increase/support; ↔ no apparent effect; ↓ decrease/reduction; *gelat* gelatinized; *fbpase* fructose 1,6-bisphosphatase; *gck* glucokinase; *g6pase* glucose-6-phosphatase; *glut* (passive) glucose transporter; *pk* pyruvate kinase; *hk* hexokinase; *pfk* phosphofructokinase; *pepck* (gluconeogenic) phosphoenolpyruvate carboxykinase; *PRO* protease; *AMY* amylase; *LIP* lipase; *AKP* alkaline phosphatase; *ATPase*, Na⁺, K⁺-ATPase; *FCR* feed conversion ratio; *TAG* triacylglycerol; *LPO* lipid peroxidation

formation of SCFAs (→Chap. 26), which can serve as energy source and promote health of intestinal enterocytes (Louis and Flint 2009). The ability of the rainbow trout intestinal microbiome to utilize dietary carbohydrate as an energy-yielding substrate remains an interesting avenue for future research and may improve the understanding of carbohydrate digestibility in fish (Lyons et al. 2017).

18.2.2 Omnivores

Based on studies in common carp, it was hypothesized that high dietary carbohydrate utilization of omnivorous and herbivorous fish can be partly ascribed to the strong inhibition of gluconeogenesis after the intake of carbohydrate-rich meals with fructose 1,6-bisphosphatase being central in this process (Panserat et al. 2002). This enzyme converts fructose-1,6-bisphosphate to fructose 6-phosphate in the gluconeogenesis pathway.

Actually, Li et al. (2017) reported that the fructose 1,6-bisphosphatase 1b identified from the liver of Wuchang bream shares a high similarity with that of higher vertebrates. The long-term feeding of a carbohydrate-rich diet inhibits its transcription and activity, indicating the downregulation of the gluconeogenic pathways. This is in line with results observed in common carp and reinforces the abovementioned hypothesis.

18.2.2.1 Zebrafish

Robison et al. (2008) were the first to observe substantial sexual dimorphism in the hepatic transcriptome of zebrafish fed diets composed of graded carbohydrate contents from the larval stage through sexual maturity. Males upregulate genes associated with oxidative metabolism, carbohydrate metabolism, energy production, and amelioration of oxidative stress, while females show increased transcription of genes associated with translation. With increasing dietary carbohydrates, males

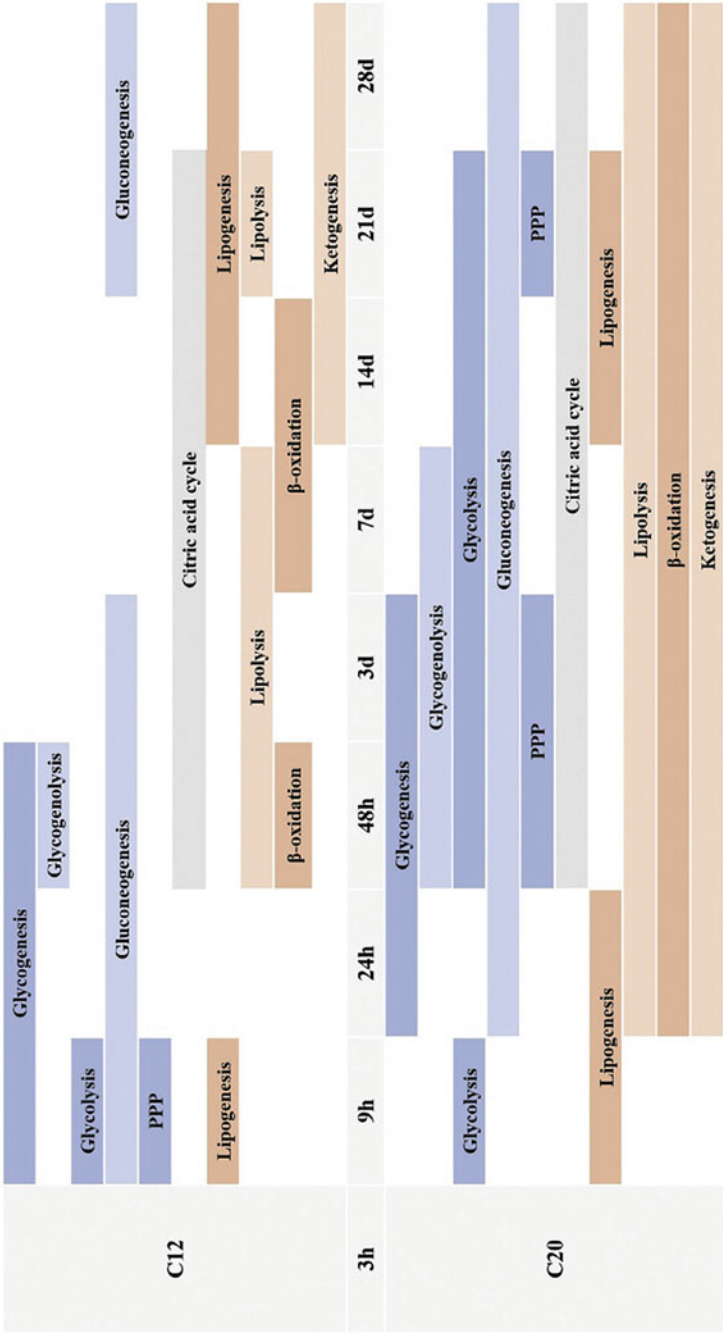


Fig. 18.10 The glucose and lipid metabolic adaptations at the molecular level in the liver of Japanese flounder during postprandial starvation. *PPP*, pentose phosphate pathway. (From Yang et al. (2019), with permission from Elsevier). **C12** 12% α -starch+cornstarch, 49.49% crude protein, 9.65% crude lipid, gross energy of 18.98 kJ g⁻¹; **C20**, 20% α -starch+cornstarch, 49.99% crude protein, 9.75% crude lipid, gross energy of 19.11 kJ g⁻¹

upregulate genes associated with oxidative metabolism (e.g., *hadh β* , *ucp2*)² while females upregulate genes associated with glucose phosphorylation (e.g., *glucokinase*). Sexual dimorphism in the hepatic transcriptome has the potential to alter nutrient partitioning and utilization in a sex-specific manner in mature fishes, since male and female zebrafishes respond differently to dietary carbohydrate manipulation in terms of growth. So far, this intriguing paper appears to be a solitaire.

18.2.2.2 Common Carp

To identify interrelationships of macronutrients in optimized dietary nutrient supplies for common carp, Heinitz et al. (2018) formulated isoenergetic diets based on digestible protein contents (DP) and digestible fat-to-digestible carbohydrate energy ratios (DEF/DEC). All diets result in excellent performance with feed conversion ratios lower than 1.0 and high digestible protein efficiency ratios. Highest growth and feed utilization efficiency are graphically determined for a diet containing 209 g kg⁻¹ DP/1.2 DEF/DEC (Fig. 18.11). Body lipid increases for 251 g kg⁻¹ DP/0.8 DEF/DEC in comparison with 209 g kg⁻¹ DP/1.2 DEF/DEC (not shown). Overall, a tendency of more dietary lipid energy on low DP side and a tendency of more dietary carbohydrate energy on high DP side are visible for best growth performance.

18.2.2.3 Nile Tilapia

Unraveling mechanistic details of glucose resistance of omnivores, Nile tilapia on carbohydrates exhibits an increase in lipid deposition (Boonanonuntanasarn et al. 2018a). This is due to increased lipogenic capacity, as reflected by higher hepatic *fas* transcription and activity, as well as higher levels of plasma triglyceride. This paper shows that the well-known capacity of omnivorous fishes to use dietary carbohydrates is linked to gluconeogenesis and lipogenesis. Nile tilapia can effectively adapt to the intake of carbohydrates, as reflected by:

- Weak postprandial hyperglycemia (5.3–6.1 mM).
- Higher level of hepatic glycogen.
- Higher glycolytic pyruvate kinase (mRNA level and activity) in muscle.
- Inhibition of the gluconeogenic pathway for glucose-6-phosphatase and phosphoenolpyruvate carboxykinase enzymes (at molecular and enzymatic levels).

²Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein) beta subunit is a subunit of a multienzyme complex that is involved in mitochondrial fatty acid oxidation; mitochondrial uncoupling proteins (ucps) are members of the larger family of mitochondrial anion carrier proteins which separate oxidative phosphorylation from ATP synthesis with energy dissipated as heat.

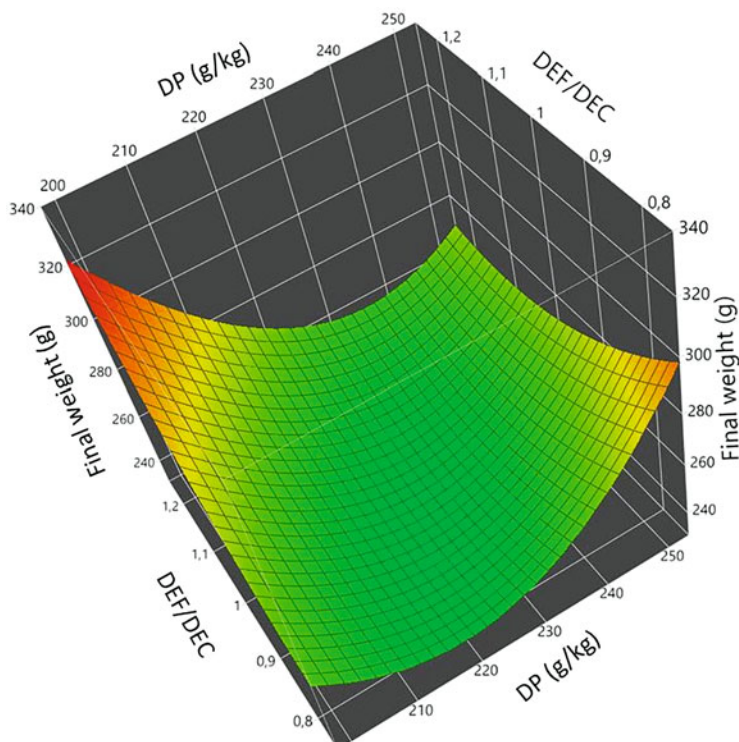


Fig. 18.11 Response surface plot of final weight in common carp (DEF/DEC, digestible fat-to-digestible carbohydrate energy ratio; DP, digestible protein). (From Heinitz et al. (2018), with permission from Wiley)

In a companion paper, Boonanuntanasarn et al. (2018b) tested the molecular adaptation of the glucose metabolism in this species. Fishes were fed three different levels of carbohydrates 0% (CHO-F), 30% (CHO-M), and 50% (CHO-H) dextrin. After 45 days and 90 days of feeding, the authors analyzed plasma parameters and mRNA levels for genes involved in glycolysis, gluconeogenesis, lipogenesis, and glucose transport in the liver and muscle. The best growth appears on CHO-M diet and the worst on CHO-H diet. Increases of hepatic and muscle glycogen (Fig. 18.12), hepatic somatic index, and plasma metabolites (glucose, triglycerides, and cholesterol) are linked to increased dietary carbohydrates. Differences in growth responses are mainly due to the protein contents in experimental diets. However, no hyperglycemia and no change of body compositions are found indicating that dietary carbohydrates are efficiently used as energy source. Moreover, no clear molecular adaptation for glycolysis, gluconeogenesis, and lipogenesis in the liver and glycolysis in muscles is detected. Based on these findings, the authors hypothesize that these metabolic pathways at a molecular level are not the main actors explaining the efficient use of glucose in tilapia.

In fishes fed the CHO-H diet, no downregulation of hepatic gluconeogenesis and no induction of hepatic lipogenesis and of muscle glycolysis occur to explain the

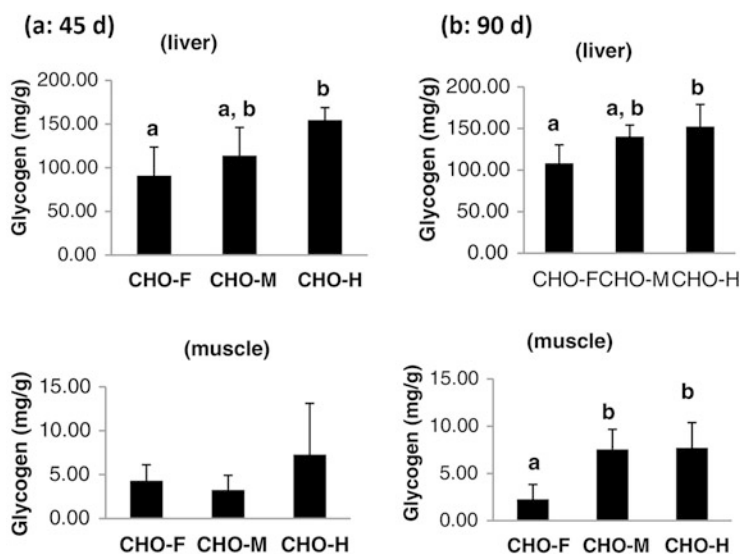


Fig. 18.12 Glycogen levels after intake of diets with no (0%; CHO-F), intermediary (30%; CHO-M), and high levels (50%; CHO-H) of carbohydrates in the liver and muscle of Nile tilapia. Glycogen was performed on the muscle and liver and sampled 5 h after the last meal, following 45 days (a) and 90 days (b) of rearing. Data represent means \pm SD. Statistical differences in glycogen between samples were evaluated in group means by one-way ANOVA statistical test: different letters indicate significant differences ($P < 0.05$). (From Boonanuntanasarn et al. (2018b), with permission from Elsevier)

absence of hyperglycemia in tilapia. Therefore, the authors developed interesting ideas: usually, diets for tilapia are low in lipids (5%) compared to rainbow trout feeds (15–20%). This can be one of the main reasons for

- High levels of lipids inducing glucose intolerance in rainbow trout (Figueiredo-Silva et al. 2012).
- Low-lipid diets leading to increased glucose tolerance in Senegalese sole (Borges et al. 2014; Conde-Sieira et al. 2016).

Finally, Boonanuntanasarn et al. (2018b) consider that (i) higher glycogen storing capacities in tilapia fed the CHO-H diet in the liver and muscle are sufficient for this fish to control glucose homeostasis or/and (ii) there is high potential to use glucose as an energy source in different tissues (such as the brain or heart). Taken together, these papers show that there are many testable and promising hypotheses left for studies to come.

In GIFT tilapia, Chen et al. (2018) showed that glucose is an effective insulin secretagogue and the liver plays an important role to clear the glucose load through stimulation of glucose uptake, glycolysis, glycogenesis, and lipogenesis. Interestingly, only *glucose transporter 1 (glut1)*, but not *glut2*, responds to glucose uptake one hour after the administration.

As in crustaceans, the question arises whether a beneficial manipulation of the gut microbiota is feasible. Wang et al. (2020) tested a prebiotic (inulin at 0.5% inclusion) and found that it alleviates the adverse metabolic syndrome and regulates the intestinal microbiota composition in Nile tilapia on high-carbohydrate diet (HCD, 45% inclusion). Furthermore, dietary inulin improves also the survival of fishes on HCD diet challenged with *Aeromonas hydrophila*.

18.2.2.4 Wuchang (Blunt Snout) Bream

To identify mechanisms of glucose tolerance in Wuchang bream, Li et al. (2016) monitored the activities of glucokinase (GCK, facilitating the phosphorylation of glucose to glucose-6-phosphate) in glucose-tolerant Wuchang bream. The authors determined plasma glucose levels and liver GCK activities and expressions in fishes subjected to a glucose load after being fed two dietary carbohydrate levels (30% and 42%). Plasma glucose levels decrease after high-carbohydrate adaption. Glucose load induces a remarkable increment of glycemia in both groups with maximum level attained at 1 h. Thereafter, it decreases to the basal value and remains constant (Fig. 18.13a). Hepatic GCK expression in both groups increases with the maximum value at 2 h after the glucose load (Fig. 18.13c), whereas no statistical difference is observed in enzymatic activities during the first 4 h (Fig. 18.13b). Then, both the activities and transcription of GCK decrease with time (Fig. 18.13 b,c). This paper shows that hepatic GCK expressions and activities of this species are highly inducible by glucose administration and high dietary carbohydrate improves the glucose tolerance through enhanced GCK expressions and activities.

Major dietary carbohydrate forms, namely, glucose or wheat starch, affect life history as well as biochemical and biomolecular traits differently. To demonstrate this, Xia et al. 2018 evaluated the effects of different types and levels of carbohydrates on growth performance, apparent digestibility, and skin-associated mucosal nonspecific immune parameters. Fishes are randomly fed four diets containing two carbohydrate (glucose or starch) diets and two carbohydrate levels (330 or 440 g kg⁻¹). High carbohydrate levels remarkably increase weight gain, apparent digestibility of dry matters, protein and carbohydrates, body crude protein content, plasma levels of aspartate transaminase, and skin-associated mucosal levels of immunoglobulin M (IgM). Moreover, it increases high-density lipoprotein cholesterol and lysozyme and advances the transcription of *mucin 2 (muc2)*, *mucin 5b (muc5b)*,³ and *apolipoprotein A-I (apoA-I)*. The opposite is true for feed conversion ratio, plasma levels of IgM, skin-associated mucosal levels of major

³Mucins are a family of high molecular weight, heavily glycosylated proteins. They are key component in most gel-like secretions and have serving functions from lubrication to cell signaling to forming chemical barriers (Marin et al. 2007). Apolipoprotein A-I (Apo A-I) is pivotal in the cholesterol homeostasis and has anti-inflammatory properties. In addition to its role in innate immunity and inflammation, this protein participates in the removal of damaged and apoptotic cells (Kravitz et al. 2005).

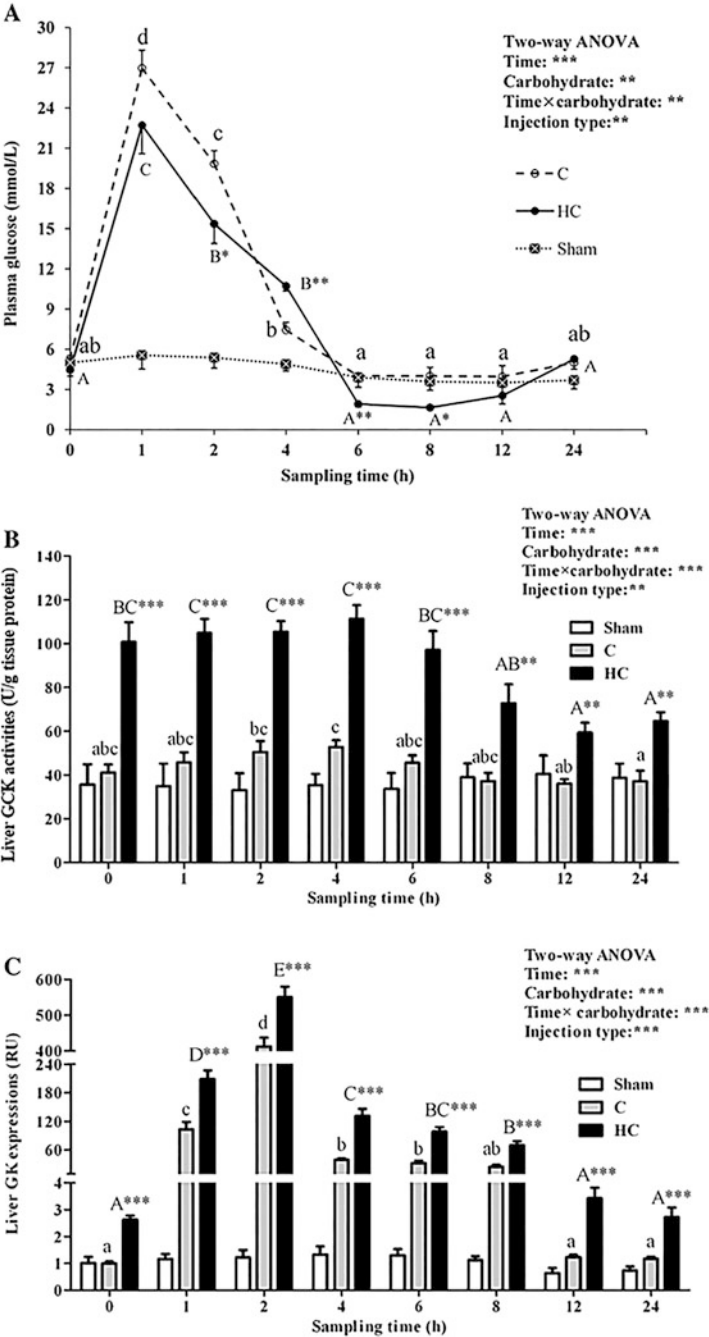


Fig. 18.13 Plasma glucose levels (a) and liver glucokinase (GCK) activities (b) and expressions (c) of Wuchang bream subjected to a glucose load after the adaptation to dietary carbohydrate levels. RNA expressions (relative units, RU) refer to the values obtained in the liver of fishes fed control diet at 0 h. Each data represents the mean of eight replicates. C, control; HC, high carbohydrate.

histocompatibility complex and β -defensins, and the transcriptions of *heat shock protein 60* (*hsp60*).

Different effects of dietary glucose and dietary wheat starch in Wuchang bream do occur; they are summarized in Table 18.4. Glucose increases the survival rate, apparent digestibility of dry matters, protein and carbohydrates, body crude ash, plasma levels of total protein, globulin, immunoglobulin M, complement C3, complement C4, and the transcriptions of *muc5b*. Starch, particularly high-starch diet, increases viscerosomatic and hepatosomatic indices, condition factor, abdominal fat percentage, and apparent digestibility of lipid and advanced the transcriptions of *muc2*, *apoA-I*, and *hsp70*. In sum, the inclusion of high level of glucose in the diet of Wuchang bream improves growth performance and nonspecific immunity and increases the efficiency of dietary protein (Xia et al. 2018).

18.2.3 Herbivores

18.2.3.1 Grass Carp

In juvenile grass carps, increased dietary carbohydrates do not improve growth but increase lipid deposition (Guo et al. 2015). The mesenteric adipose tissue is the major lipid deposition site; the muscles and liver are secondary. This agrees well with almost classical findings in common carp and other omnivores, in which dietary carbohydrate stimulates glycolysis and lipogenesis and depresses gluconeogenesis and AA degradation in the hepatopancreas. This comprehensive regulation of nutrient metabolism may account for the protein-sparing effect exerted by dietary carbohydrate (Shimeno et al. 1995), which is effective in grass carp only in the low dietary carbohydrate range (\rightarrow previous Chapters).

In addition, Cai et al. (2018) reported that, when grass carps are faced with different dietary carbohydrate levels, the strategies of maintaining homeostasis are different (Fig. 18.14). Lower dietary carbohydrate intake causes slight modulations of the lipid metabolism. Insufficient carbohydrate intake can induce poorer utilization of glucose or lipid and more proteolysis for supplying energy. As a result, it has no adverse effect on feed utilization and growth. In contrast, excessive dietary carbohydrate intake promotes efficient glucose metabolism that provides energy and metabolic intermediates for lipid synthesis and eventually increases accumulation of lipids. Moreover, increased lipid contents induce appetite suppression by



Fig. 18.13 (continued) Significant differences ($P < 0.05$) among sampling times within each treatment (diets) are indicated by different letters (lower case for control, upper case for high carbohydrate). Significant difference between the two treatments at each sampling time: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: $P > 0.05$. (From Li et al. (2016), with permission from Elsevier). **Sham treatment**, the remaining fishes of the control group were injected with equal volumes of 0.9% saline solution ($16.7 \text{ mL kg}^{-1} \text{ BW}$) in order to assess the effects of handling and injection stress on plasma glucose levels and hepatic GCK activities and expressions

Table 18.4 Effect of dietary glucose or starch on traits in Wuchang bream (Xia et al. (2018))

Trait/variable	Glucose		Wheat starch	
	33%	44%	33%	44%
Survival	↑	↔	↔	↔
Weight gain	↔	↑	↔	↔
Feed conversion ratio	↔	↔	↑	↑
Viscerosomatic index	↔	↔	↑	↑
Hepatosomatic index	↔	↑	↑	↑
Condition factor	↔	↔	↑	↑
Abdominal fat percentage	↔	↔	↑	↑
ADC dry matter	↑	↑	↔	↑
ADC protein	↑	↑	↔	↑
ADC lipid	(↑)	↔	↓	↑
ADC carbohydrate	↔	↑	↓	↔
Plasma				
Glucose	↔	↔	↔	↔
Total cholesterol	↔	↔	↔	↑
Triglyceride	↔	↔	↔	↔
High-density lipoprotein	↔	↑	↓	↓
Low-density lipoprotein	↔	(↑)	↔	↑
Total protein	(↑)	↑	↔	↔
Albumin	↔	↑	↔	↔
Globulin	(↑)	↑	↔	↓
Aspartate transaminase	↔	↔	↔	↑
Alanine transaminase	↔	↔	↔	↔
Lactate dehydrogenase	↔	↑	↑	↑
Insulin	↔	↔	↔	↔
Growth hormone	↔	↔	↔	↔
Complement 3	↑	↑	↓	↓
Complement 4	↑	↑	↓	↓
Immunoglobulin M	↑	↓	↓	↓
Skin mucosa				
Complement 3	↔	↔	↔	↔
Complement 4	↔	↔	↔	↔
Immunoglobulin M	↑	↑	↔	↑
Major histocompatibility complex	↑	↑	↑	↓
High-density lipoprotein	↓	↔	↔	↑
Lysozyme	↔	↔	↔	↔
β-D-N-acetylglucosamine	↔	↔	↔	↔

↑ increase, ↓ reduction; ↔ no apparent effect; ADC apparent digestibility coefficient

modulating the *leptin* transcription. This hormone regulates appetite to achieve energy homeostasis by inhibiting hunger. Reduced food intake brings about lower growth (Fig. 18.14).

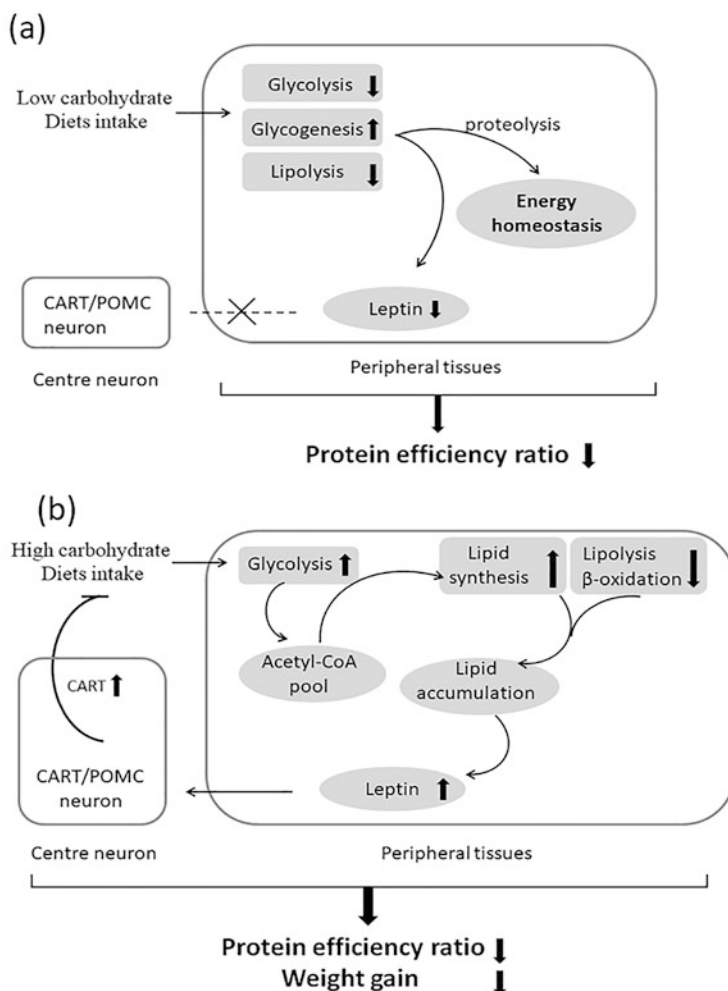


Fig. 18.14 The strategies of grass carp responding to insufficient or excessive dietary carbohydrate levels. (a) The strategy of grass carp responding to low-carbohydrate diets. (b) The strategy of grass carp responding to high-carbohydrate diets. (From Cai et al. (2018), with permission from Elsevier)

18.2.4 Action of miRNAs

To date, various molecules are involved in the posttranscriptional regulation of gene expression, including microRNAs (miRNAs) (Bartel 2004). Herkenhoff et al. (2018) reviewed that in fishes, numerous miRNAs have been identified as such, but only a small fraction have known functions. Considering that miRNAs have important functions in shaping morphological and physiological phenotypes, and its function is highly conserved within vertebrates, analysis of miRNA profiles from other

species can potentially be useful to understand and to provide wide applications in fish breeding.

Box: Targets of MicroRNAs

In their meta-analysis, Kostyniuk and Mennigen (2020) provide an educational survey of how to identify the potential targets of miRNAs. This Box presents permitted excerpts of their paper.

Since their discovery in *Caenorhabditis elegans* (Lee et al. 1993), miRNAs have emerged as important posttranscriptional regulators of gene expression (O’Brien et al. 2018). In animals, miRNA-dependent regulation of targeted protein-coding mRNA transcripts is principally mediated via complementary base pairing between the mature miRNA’s seed region and short elements within the targeted mRNA’s 3’UTR sequence⁴ (McGeary et al. 2019). Initially considered a peculiarity in *C. elegans*, it has rapidly become clear that miRNAs are deeply conserved in evolution (Moran et al. 2017). Generally, increases in organismal complexity in animals are considered to scale with the quantity and complexity of the miRNA repertoire, and lineage-specific acquisition of novel miRNAs with losses of only a few specific miRNA families has been reported. Functionally, miRNAs have been linked to several key biological functions, including the regulation of energy metabolism (Rottiers and Näär 2012).

Since the advent of genome sequencing, major strides have been made in many traditional research models, including teleost fishes and rainbow trout in particular (Mennigen 2016), for which annotated miRNA repertoires (Juanchich et al. 2016) and in silico target prediction algorithms (Mennigen and Zhang 2016) have been published following the sequencing of its genome (Berthelot et al. 2014). Because rainbow trouts have been used as a comparative research model due to its “glucose-intolerant” phenotype (Forbes et al. 2019) and the evolution of its genome (Berthelot et al. 2014), significant research efforts have been geared toward the elucidation of molecular regulation of energy metabolism in this species. It is therefore not surprising that with newly available genomic resources, comparative research of epigenetic molecular mechanisms involved in transcriptional and posttranscriptional control of energy metabolism has increased substantially over the last several years in this species (Best et al. 2018). In addition to transcriptional level investigations, which addressed potential roles of chromatin and DNA methylation level regulation of hepatic expression of genes with roles in energy metabolic pathways (Marandel et al. (2016), Kostyniuk et al. (2018, 2019))

(continued)

⁴In molecular genetics, the three prime untranslated region (3’UTR) is the section of mRNA that immediately follows the translation termination codon. The 3’UTR often contains regulatory regions that posttranscriptionally influence gene expression (Barrett et al. 2012).

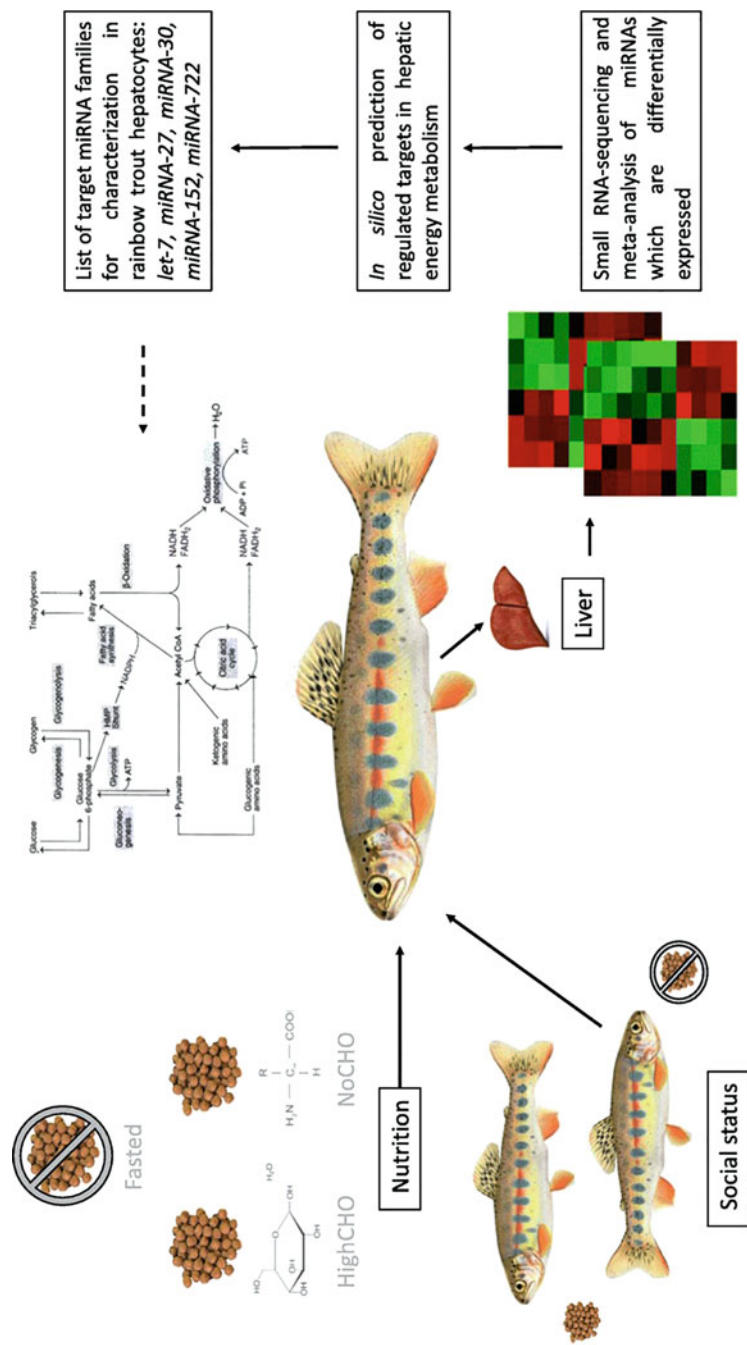
have conducted a range of studies to investigate regulation and potential posttranscriptional roles for miRNAs in hepatic energy metabolism in rainbow trout. A sketch of metabolic challenges in the form of changes of nutritional quantity and quality as a function of diet and/or social context is provided in Box Fig. 1 (Kostyniuk et al. 2019).

In a pioneering study, Mennigen et al. (2012) investigated hepatic miRNA expression, activation of the insulin pathway, and insulin-regulated metabolic target genes in rainbow trout. Postprandial activation of the hepatic insulin signaling pathway correlates with miRNA expression potentially implicated in negative feedback and/or metabolic target gene regulation (Mennigen et al. 2012). Later, a role for insulin-regulated *miR-122* in controlling postprandial glucose homeostasis was identified (Mennigen et al. 2014a). Two isoforms of *miR-122*, *omy-miR-122a* and *omy-miR-122b*, exist in rainbow trout, contrary to the situation in humans, where only one isoform exists. Hepatic expression of *omy-miR-122b*, but not *omy-miR-122a*, exhibits significant postprandial changes. A higher expression of *omy-miR-122b* compared to the time of feeding occurs 4 h after refeeding (Fig. 18.15). While *miR-122* is highly conserved, the role of species-specific miRNAs on endocrine signaling activity and function involved in the postprandial “glucose-intolerant” phenotype in rainbow trout warrants further study (Mennigen and Zhang 2016).

MiR-34a plays a critical role in tumor suppression and cellular senescence. The liver and pancreas are main target organs for *miR-34a*. Furthermore, *miR-34a* expression disorder is related to a consequence of multiple dysfunctions, such as insufficient secretion of insulin, cell injury, and insulin resistance. Liu et al. (2019) studied the action of this miRNA in Wuchang bream. Exposed to HCD, *miR-34a* is central in maintaining glucose homeostasis. Downregulated *miR-34a* treated with antisense oligonucleotide in vivo leads to enhanced glucose tolerance and insulin sensitivity and reduces liver fat accumulation (Miao et al. 2018; Liu et al. 2019). Furthermore, *miR-34a* regulates the hepatic glycolipid metabolism by targeting the SIRT1 protein,⁵ since *miR-34a* silencing in HCD-fed individuals reduces glycogen accumulation and increases hepatic lipolysis by activating the SIRT1 protein expression (Miao et al. 2019).

In GIFT, another miRNA is central in glucose utilization: *miR-1338-5p* (Qiang et al. 2017). The authors showed that the inhibition of *miR-1338-5p* promotes the *growth hormone-inducible transmembrane* protein (*ghitm*) expression in the pituitary and liver. *Ghitm* interferes in the growth hormone-growth hormone receptor-insulin-like growth factor signaling pathway by competing with the growth hormone receptor for combination with growth hormone and then reduces the growth of fishes. Moreover, the serum reduction of growth hormone may regulate insulin secretion and result in the increasing sugar and fat storage in serum and liver.

⁵SIRT1 is an enzyme that deacetylates proteins that contribute to cellular regulation (reaction to stressors, longevity) (Sinclair and Guarente 2006).



Box Fig. 1 MicroRNAs (miRNAs) are small non-coding RNAs which act as posttranscriptional regulators by decreasing targeted mRNA translation and stability. Principally targeting small 3'UTR elements of protein-coding mRNAs through complementary base pairing, miRNAs are promiscuous regulators of the transcriptome. Changes of specific energy metabolism-related transcript steady-state abundances can be assessed, allowing correlative analysis between differentially regulated miRNAs and their *in silico* predicted mRNA targets. (From Kostyniuk and Mennigen (2020), with permission from Elsevier)

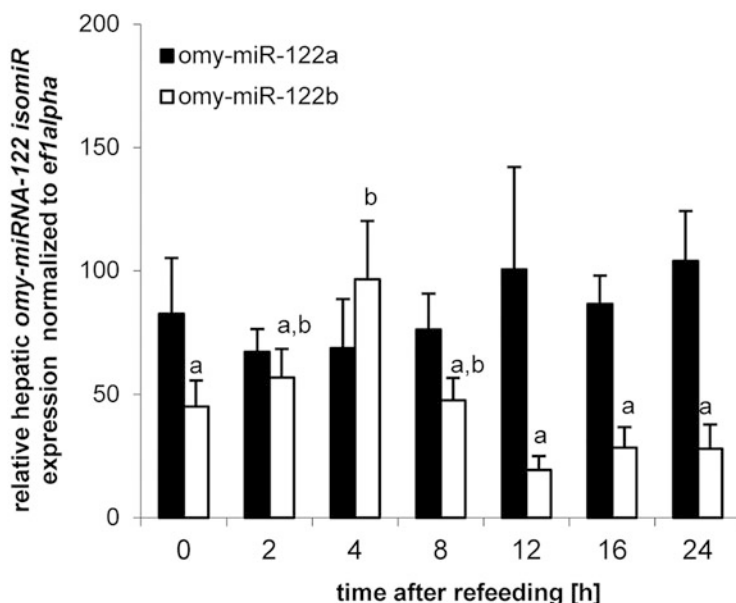


Fig. 18.15 Postprandial expression profiles of hepatic *miRNA122* in rainbow trout. (From Mennigen et al. (2012), credit the Public Library of Science)

Synoptically, Rasal et al. (2020) identified liver-specific miRNAs linked to carbohydrate metabolism in farmed *Labeo rohita*. 138 conserved and 161 novel miRNAs are identified: *miR-22*, *miR-122*, *miR-365*, *miR-200*, and *miR-146* are involved in carbohydrate metabolism. Further analyses depict mature miRNA and their predicted target sites in genes that are involved in development, cellular activities, and transportation. This study confirms and completes earlier ones in rainbow trout, which identified liver-specific *miR-33* and *miR-122* associated with glucose metabolism (Mennigen et al. 2014a, 2014b).

Kostyniuk et al. (2019) induced prolonged postprandial hyperglycemia in rainbow trout by using a HCD and profiled genome-wide hepatic miRNAs. The authors identified differentially regulated hepatic miRNAs and used an in silico approach to predict bona fide mRNA targets and pathways. Diet-induced hyperglycemia results in differential regulation of hepatic miRNAs. Some of the identified miRNAs, such as *miR-27b-3p* and *miR-200a-3p*, are known to be responsive to hyperglycemia in the liver of hyperglycemic glucose-tolerant fish (see above, one form is also found in *L. rohita*) and mammals, indicating an evolutionary conserved regulation. Using Gene Ontology term-based enrichment analysis, Kostyniuk et al. (2019) identified intermediate carbohydrate and lipid metabolism and insulin signaling as potential targets of posttranscriptional regulation by hyperglycemia-regulated miRNAs. Specifically, metabolic pathways (gluconeogenesis, de novo lipogenesis), cell signaling pathways (insulin signaling), and organelle dynamics (autophagy → Chap. 14,

mitochondrial fusion) are subject to posttranscriptional regulation by miRNAs in response to HCDs. Together, these results provide a robust framework for testable hypotheses of functional involvement of specific hepatic miRNAs in glucose intolerance in rainbow trout.

Recently, Kostyniuk and Mennigen (2020) provided a meta-analysis of differentially expressed hepatic miRNAs in rainbow trout with a “glucose-intolerant” phenotype. Following exposure to nutritional and social context-dependent metabolic challenges, the authors analyzed differential miRNA expression from small RNA-sequencing datasets generated with a consistent bioinformatics pipeline in conjunction with an *in silico* target prediction of metabolic transcripts and pathways. The authors provide evidence for evolutionary conserved (*let-7*, *miR-27* family) and rewired (*miR-30* family, *miR-152*, *miR-722*) miRNA-metabolic target gene networks in the context of the salmonid genome.

Since DNA level and posttranscriptional epigenetic molecular mechanisms are likely to act in concert to regulate gene expression across species including rainbow trout (Kuc et al. 2017), Kostyniuk and Mennigen (2020) used *in silico* predictions to assess the potential for such interaction. Taking advantage of the profiling of multiple molecular epigenetic mechanisms in the same rainbow trout liver samples (Marandel et al. 2016; Kostyniuk et al. 2019), the analysis raises the possibility that miRNAs up- and downregulated in response to a HCD may contribute to global hypomethylation via repression of transcripts of *dnmt*⁶ transcripts (e.g., *let-7* family members) and de-repression of TET enzyme⁷ transcripts (e.g., *miR-29b-3p* and *miR-722-5p*). Future studies will therefore explore the potential interaction of molecular epigenetic factors in hepatic energy metabolism in rainbow trout. Finally, Kostyniuk and Mennigen (2020) propose that the identified miRNA families should be prioritized for future comparative functional investigation in the context of hepatic energy and glucose metabolism in rainbow trout and other fish species.

Besides miRNAs, sequence-specific RNA-binding proteins are involved in the posttranscriptional regulation of gene expression. Among the RNA-binding proteins, quaking (QKI) proteins are evolutionarily conserved and known to be vital factors for oligodendrocytes, monocytes/macrophages, endothelial cell, myocyte function, organogenesis, cell differentiation, and embryonic development (Radomska et al. 2016). To identify its functions in fishes, Shi et al. (2018) characterized QKI-a in Wuchang bream and tested the response to HCD feeding and glucose/insulin/glucagon treatment. Glucose and insulin administration, as well as HCD feeding, downregulate the transcriptions of *qkia* in the brain, muscle, and liver (Fig. 18.16), whereas the opposite is true after a glucagon load. These findings

⁶DNA methyltransferases, a family of enzymes catalyzing the transfer of a methyl group to DNA. DNA methylation serves a wide variety of biological functions. All the known DNA methyltransferases use S-adenosyl methionine (SAM) as the methyl donor (Nelson and Cox 2005).

⁷The TET enzymes are a family of ten-eleven translocation (TET) methylcytosine dioxygenases. They are instrumental in DNA demethylation. 5-Methylcytosine is a methylated form of the DNA base cytosine that often regulates gene transcription and has several other functions in the genome (Wu and Zhang 2017).

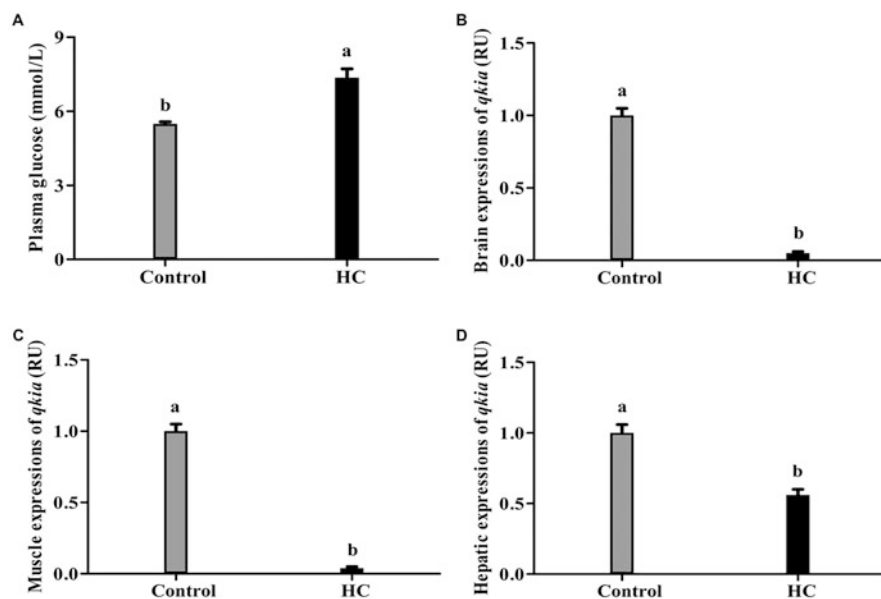


Fig. 18.16 Plasma glucose levels (a) and relative expressions of *qkia* in the brain (b), muscle (c), and liver (d) of *Megalobrama amblycephala* fed different 12% (control) and 25% cornstarch (high-carbohydrate HC) diets. Data refer to the control diet (relative units RU) and are means of four replicates. Different lowercase letters indicate significant differences ($P < 0.05$). (From Shi et al. (2018), credit Public Library of Science)

point out that *qkia* plays an important role in the glucose metabolism of fishes, but details remain unclear and wait for further studies.

18.3 Concluding Remarks

From the host's perspective and disregarding the role of the gut microbiota, the leading consortia of research into carbohydrate digestion in carnivorous fishes perfectly express the dilemma of elevated carbohydrate shares in aquafeed for carnivorous fishes. It briefly refers to the state of the art and points out future perspectives: "Carnivorous rainbow trout exhibit prolonged postprandial hyperglycemia when fed a diet exceeding 20% carbohydrate content. This poor capacity to utilize carbohydrates has led to rainbow trout being classified as 'glucose-intolerant'. The metabolic phenotype has spurred research to identify the underlying cellular and molecular mechanisms of glucose intolerance, largely because carbohydrate-rich diets provide economic and ecological advantages over traditionally used fishmeal, considered un-sustainable for rainbow trout aquaculture operations" (Kostyniuk et al. 2019). Consequently, a variety of hypotheses are put forward which could be

verified more or less only partly. Several of these hypotheses have been mentioned above and in preceding chapters.

“Evidence points to a contribution of hepatic intermediary carbohydrate and lipid metabolism, as well as upstream insulin signaling. Recently, miRNAs acting as negative posttranscriptional regulators affecting target mRNA stability and translation, have emerged as critical regulators of hepatic control of glucose-homeostasis in mammals (Melkman-Zehavi et al. 2011), revealing that dysregulated hepatic miRNAs might play a role in organismal hyperglycemia in metabolic disease” (Kostyniuk et al. 2019). There emerges a new set of testable hypotheses about glucose intolerance in carnivorous fishes, particularly, based on the reassessment of brown trouts as conditionally omnivorous, rather than strictly carnivorous (Marandel et al. 2018).

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Chapter 19

Regulatory Potential of Carbohydrates—*‘Life’s Little Luxury Controls’*



Abstract This chapter compiles recent findings of the regulatory potential of mono- and polysaccharides. The regulation comprises mainly growth, immunity, and subsequent increased resistance against pathogens but also shows modulations of gene expressions. In carnivores, glucose modulates the activity of hepatic glucokinase, stimulates brain-gut peptide expressions, and potentially triggers growth- and immunity-related genes. Even adipose cells take part in the carbohydrate metabolism by producing insulin in a glucose-responsive manner. In myogenic precursor cells, glucose availability acts as a regulator of protein synthesis. Finally, the significance of gut microbiota in carbohydrate-mediated regulation is increasingly considered since it can produce short-chain fatty acids, which improve innate immunity.

In addition to their nutritional potential and energy provision, as sketched in previous chapters, carbohydrates interact on more sophisticated or even hidden levels; they exert regulatory functions in glucosensing (\rightarrow Chap. 13), immunostimulation (mainly polysaccharides) and interact with genes. Furthermore, growth modulations, but also diseases, such as liver damages elicited by carbohydrates, have been detected.

19.1 Growth Modulation

Dietary glucose has modulatory functions on growth performances, even in carnivorous aquatic animals. For instance, on a carbohydrate-free diet, rainbow trouts show decreased growth. This is due to muscular hypotrophy but not to a change in the level of muscular hyperplasia, indicated by changes in the protein:DNA ratio without variations in total DNA content (Fig. 19.1a) (Peragón et al. 1999). In addition, the absence of carbohydrates increases protein degradation and decreases the protein synthesis in white muscles. This behavior in the protein turnover also explains reduced growth and white muscle weight gain. Furthermore, the protein degradation induces an important fraction of amino acids (AAs) to be used for gluconeogenic purposes and not for protein synthesis or growth. The absence of

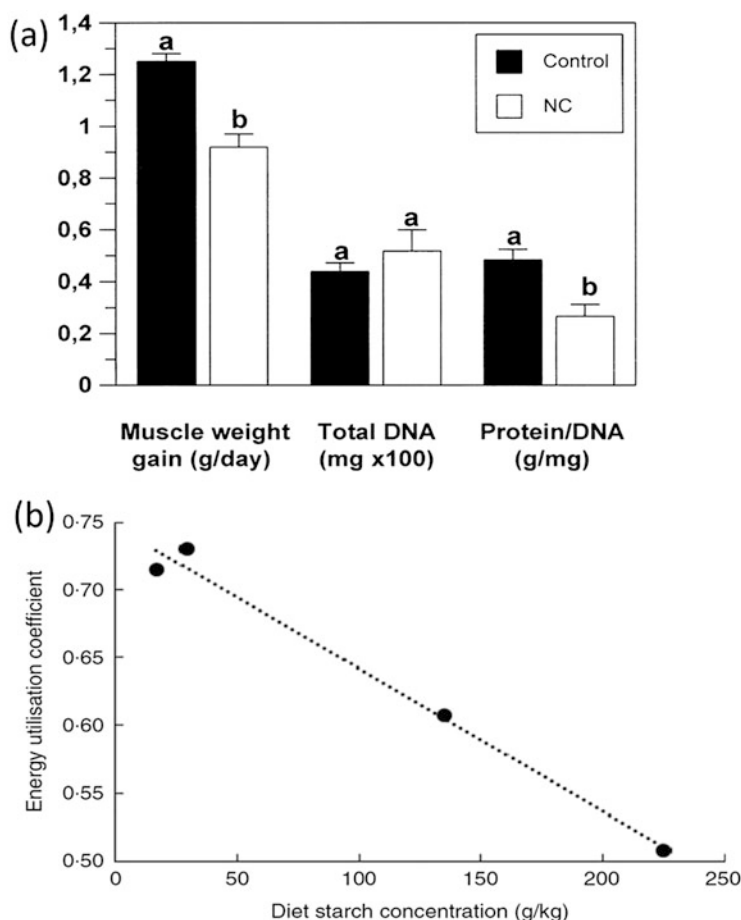


Fig. 19.1 (a) Nature of white muscle growth in rainbow trout fed a control or a carbohydrate-free (NC) diet. Results are expressed as means \pm SEM of 12 individual fishes. Results were tested with a one-way ANOVA following a student's t-test using means. Different superscripts indicate significant differences ($P < 0.05$). (From Peragón et al. (1999), with permission from Elsevier). (b) Relationship between diet starch concentration and energy utilization coefficient values in barramundi. (From Glencross et al. (2017), with permission from the Cambridge University Press)

dietary carbohydrates also downregulates myogenic genes (Chapalamadugu et al. 2009; Alami-Durante et al. 2019).

In barramundi and contrary to other fishes, the efficiency of energy utilization is reduced if fed starch diets (Fig. 19.1b) (Glencross et al. 2017), indicating that the inclusion of starch causes problems with the protein utilization in this species.

RNA-binding proteins are critical posttranscriptional regulators of RNA and influence pre-mRNA splicing, RNA localization, and stability. In mammals, the RNA-binding protein quaking (QKI) has been shown to be involved in diabetes (Nutter and Kuyumcu-Martinez 2018). Recently, Shi et al. (2018) translated this

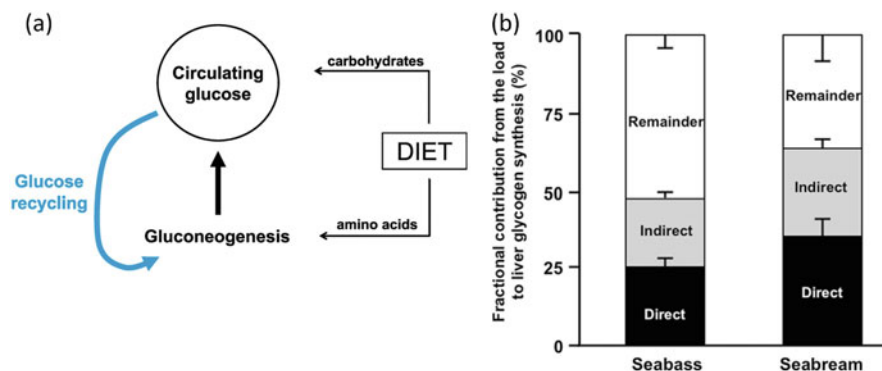


Fig. 19.2 (a) Metabolic model in carnivorous fishes representing the possible sources of plasma glucose appearance during feeding with carbohydrates. Dietary carbohydrate digestion contributes to glucose directly via absorption. Gluconeogenesis is fueled by amino acids derived from diet as well as by pyruvate equivalents from peripheral tissue glycolysis of circulating glucose. This latter process is defined as glucose recycling. (From Rito et al. (2017), with permission from Elsevier). (b) Fraction of hepatic glycogen derived from an intraperitoneal glucose load at 48 hours post-load in fasted sea bream and sea bass. The contributions of direct and indirect pathways of glycogen synthesis to the synthesized fraction are also shown. The remainder represents preexisting glycogen and/or glycogen synthesized from unlabeled precursors. (From Rito et al. (2018), credit Springer Nature)

approach to omnivorous Wuchang bream and found that glucose administration, as well as high-carbohydrate (HCD) feeding, remarkably downregulates the transcription of *qkia* in brain, muscle, and liver, indicating that *qkia* plays an important, but still obscure, role in the glucose metabolism of fishes (\rightarrow Chap. 18).

In addition to the myogenic function and the interference in translational regulation of mRNA, significant novel regulatory activities of glucose have been detected in carnivorous fishes. Following a glucose challenge, Rito et al. (2017) found a hitherto unexplored mechanism for the sparing of dietary protein in sea bass (sketched in Fig. 19.2a). This mechanism is effective under conditions where gluconeogenesis is poorly suppressed by carbohydrate feeding—as it is usually the case for many carnivorous fishes. Subsequently, Rito et al. (2018) compared the share of the different pathways of glycogen synthesis for two carnivorous marine fish species (Fig. 19.2b): For fasted sea bass and sea bream, $47 \pm 5\%$ and $64 \pm 10\%$ of glycogen is synthesized from the glucose load, respectively. Direct and indirect pathways contribute equally (25% direct, 21% indirect for sea bass; 36% direct, 29% indirect for sea bream).

In addition, Liu et al. (2018) proved that oral administration of glucose affects the gene expressions of preprovasoactive intestinal peptide, pituitary adenylate cyclase activating polypeptide, and gastrin in intestine in Japanese flounder (*Paralichthys olivaceus*) (Fig. 19.3). High oral glucose loads increase the activity of hepatic glucokinase (GCK), which phosphorylates glucose to glucose-6-phosphate, the first, limiting step of glucose storage in the liver. This supports previous findings

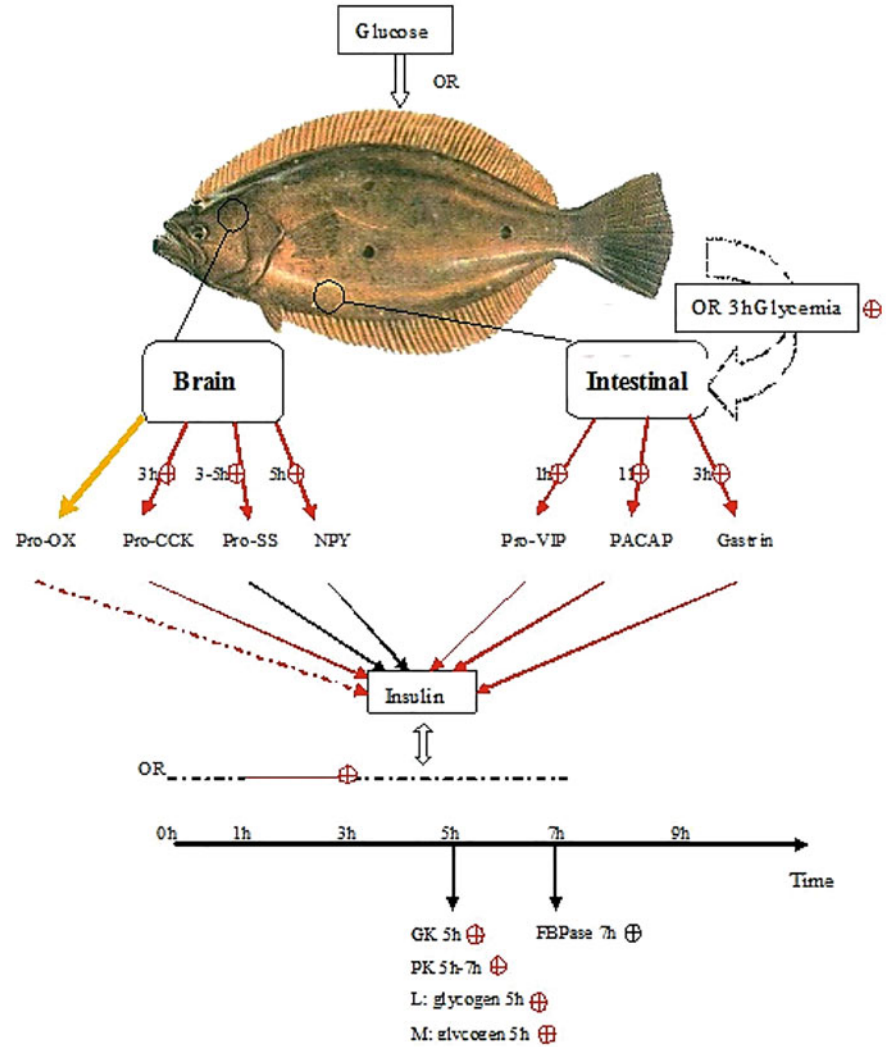


Fig. 19.3 Regulations of glucose metabolism in brain and intestine as affected by oral (OR) administration of glucose in Japanese flounder (*Paralichthys olivaceus*). Red line stands for stimulation, and black line means inhibition. The red circle represents the significant promotion effects. The black circle represents the significant inhibition effects. Black dotted line means inhibition but did not show significant difference. The yellow line stands for there is no significant difference in all time points. Point in time after glucose loads with red and black symbol means significant difference was found at that time point. (From Liu et al. (2018), with permission from Elsevier). *L*: liver; *M*: muscle; *FBPase* fructose-1,6-bisphosphatase; *GK* (GCK) glucokinase; *Pro-OX* orexin precursor (stimulates food intake); *Pro-CCK* cholecystokinin precursor (regulates digestion and satiety); *Pro-SS* prosomatostatin (affects neurotransmission and cell proliferation); *NPY* neuropeptide Y; *Pro-VIP* preprovasoactive intestinal peptide; *PACAP* pituitary adenylate cyclase activating polypeptide; *gastrin* a brain-gut peptide. Gastrin has a function similar to that of VIP and PACAP in glucose metabolism, and it can also stimulate insulin production (Dockray et al. 2001)

in European sea bass (Enes et al. 2006), rainbow trout (Polakof et al. 2008), and turbot (Nie et al. 2015).

Furthermore, also the pyruvate kinase (PK) activity is induced in the flounder by oral administration of glucose (Liu et al. 2018). PK is the last step in glycolysis and catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one molecule of ATP. Fructose-1,6-bisphosphatase¹ (FBPase) activity continuously decreases during the first period after oral glucose administration, indicating a reduced gluconeogenesis. This results in lowered blood glucose levels and indicates that Japanese flounder has the definite adaptability to high glucose loads.

Further evidence of the signaling function of starch and glucose on growth metabolism and feeding behavior of omnivorous fishes has been presented by Unniappan's laboratory in several papers. Sundarrajan et al. (2016) identified that HCD reduces nucleobindins² (*nucb1*) mRNA expression in the hypothalamus of goldfishes (Fig. 19.4a,b). A similar reduction of *nucb1* transcription is found in the gut (Fig. 19.4c, d) without clear changes in the feeding behavior. This contrasts findings in Wuchang bream (\rightarrow Chap. 18) with reduced transcription of *nucb1* leading to increased feeding activity (Sundarrajan et al. 2016). This regulatory pathway deserves clarification.

In companion papers, Bertucci et al. (2017a, b) reported direct actions of glucose in growth control and feed uptake. Somatic growth is coordinated by the GH-IGF axis. Growth hormone (GH) is released from the adenohypophysis in response to signals from the hypothalamus. However, in organ cultures of goldfish, Bertucci et al. (2017a) found that glucose upregulates the mRNA expression of receptors (*ghr-I*, *ghr-II*) and growth factors (*igf-I*, *igf-II*) (Fig. 19.5a–d), independently of circulating growth hormone. Therefore, it is likely that glucose directly regulates ghrelin and NUCB2/nesfatin-1³ in the intestine and hepatopancreas in vitro. In fact, Bertucci et al. (2017b) are able to show that glucose modulates *preproghrelin* and *nucb2/nesfatin-1* mRNA expression in a concentration-dependent manner in the intestine, a result that needs to be rechecked and expanded to other species (Fig. 19.5e–h). In addition, Blanco et al. (2020) characterized goldfish adipocytes as cells with properties similar to that of pancreatic β -cells (Fig. 19.6): These cells express considerably high levels of *preproinsulin* mRNAs, possess the necessary machinery for processing preproinsulin and responding to extracellular glucose

¹Fructose-1,6-bisphosphatase (FBPase) is a rate-limiting enzyme in gluconeogenesis, catalyzing the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate. Along with phosphofructokinase, FBPase regulates the flux of glucose metabolism and is involved in the formation of glucose from noncarbohydrates (Li et al. (2017) with references therein).

²Nucleobindins are multi-domain Ca^{2+} and DNA-binding proteins that play an important role in cell signaling (Gonzalez et al. 2012). Furthermore, they are precursors of the appetite-reducing (anorexic) peptide nesfatin-1 (Dore et al. 2017).

³Novel anorexigen; increased nesfatin-1 in the hypothalamus contributes to diminished hunger, a “sense of fullness.”

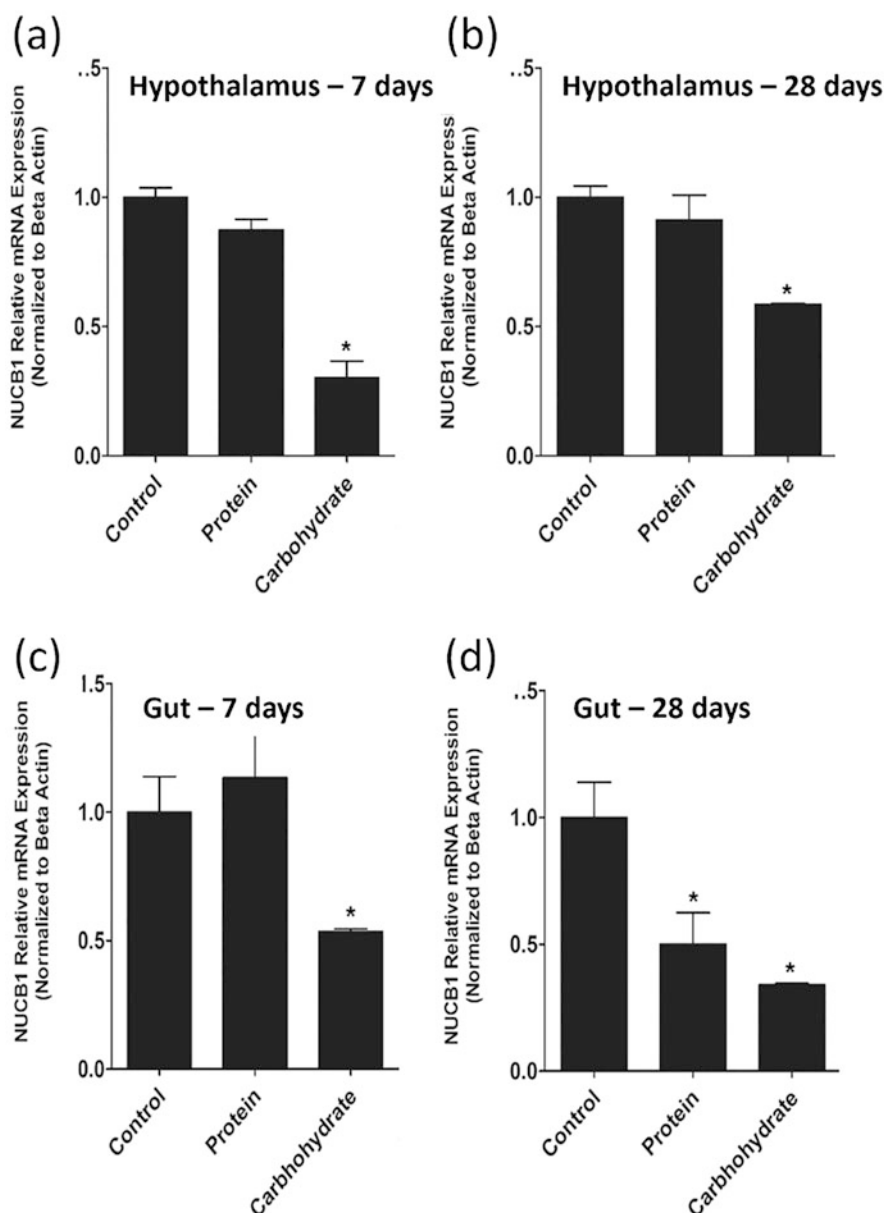


Fig. 19.4 Nucleobindin-1 (*nucb1*) mRNA expression in goldfish hypothalamus after 7 days (a) or 28 days (b) of feeding special diets containing varying amounts of macronutrients. *Nucb1* mRNA expression in goldfish hypothalamus was downregulated in response to high-carbohydrate feeding for 7 days (a). *Nucb1* mRNA expression was significantly reduced in goldfish hypothalamus post-high-carbohydrate feeding for 28 days (b). In gut, *nucb1* mRNA expression was attenuated after high-carbohydrate after 7 days (c). In addition, *nucb1* mRNA expression was decreased in the gut of goldfish fed on high-protein, high-carbohydrate diet after 28 days of feeding (d). *Nucb1* mRNA expression were normalized to β -actin. * denotes significant differences between treatment groups

(GCK, glucose transporters 1, 2, 4), and produce insulin in a glucose-responsive manner.

Combining glucose supply and muscle growth, Latimer et al. (2019) recently identified a surprising regulatory function of glucose in rainbow trout. Muscle growth (protein accretion) responds in a coordinated way to changes in both nutrient availability and composition. Insulin⁴ and IGF1 promote muscle accretion by stimulating muscle glucose and AA uptake, the former partly due to stimulation of GLUT4 (Díaz et al. 2009). Protein synthesis is reduced by lack of glucose rather than the protein degradation enhanced (Fig. 19.7) (Latimer et al. 2019). The study highlights the need for further research into glucose-related mechanisms regulating protein retention and growth, particularly as aquafeeds transition toward higher inclusion of plant-based carbohydrate-rich feedstuffs.

Also for invertebrate, a new regulatory function of glucose is going to be identified. It is well understood that serotonin is involved in mediating hunger-related changes in behavioral state (Steinberg (2018)). To figure out how hunger signals cooperate with serotonergic cells, Dyakonova et al. (2015) found in the pond snail (*Lymnaea stagnalis*) that glucose signaling plays a direct role in mediating the hunger-related behavioral state.

19.2 Gut Microbiota

Gut microbiota participates in the metabolism of carbohydrates and affects the nutritional metabolic processes. Vice versa, quality, complexity, and structure of dietary carbohydrates affect the composition of the gut microbiota. Changes of the microbiome enable hosts to utilize diet components that could not well be used before. Qiao et al. (2017) showed that the carbohydrate diet imposes selective pressure on the intestinal microbiota in *Litopenaeus vannamei* at low salinity (Fig. 19.8). Sucrose, glucose, or cornstarch diets result in different compositions of the gut microflora. Fifty-six operational taxonomic units comprise the core microbiome. *Proteobacteria* are the most prevalent microbes; the abundance of *Actinobacteria* decreases while that of *Firmicutes* increases in cornstarch-fed animals. Furthermore, bacteria related to complex carbohydrate degradation are in low abundance, whereas the abundance of opportunistic pathogenic bacteria increases in the cornstarch-fed group.



Fig. 19.4 (continued) ($P < 0.05$). Data are means + SEM. (From Sundararajan et al. (2016), credit Nature Publishing Group)

⁴For more unexpected effects of insulin, also refer to Box: Rainbow Trout and Insulin in Chap. 15.

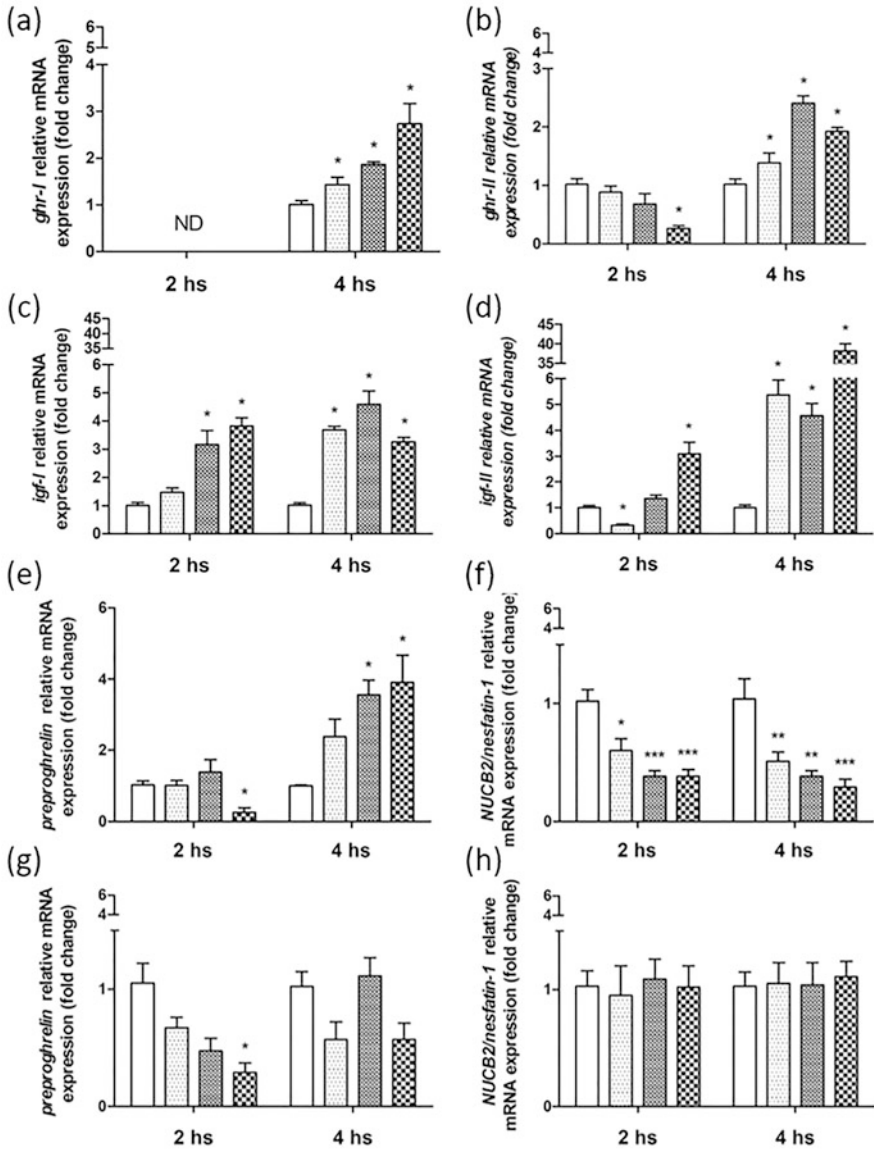


Fig. 19.5 (a) *Ghr-I*, (b) *ghr-II*, (c) *igf-I*, and (d) *igf-II* mRNA expression in goldfish hepatopancreas culture after 2 h and 4 h of graded glucose treatments. Data are shown as mean \pm SEM. *Preproghrelin* and *nucb2/nesfatin-1* mRNA expression in intestine (e, f) and hepatopancreas (g, h) of goldfish after 2 h and 4 h of 25 mM, 50 mM, and 100 mM glucose treatments. Data obtained by RT-qPCR are shown as mean \pm SEM. * denotes statistical differences between control and treated groups: * P < 0.05, ** P < 0.01, *** P < 0.001. (From Bertucci et al. (2017b, a), with permission from Elsevier). White control; glucose additions: light gray 25 mM; gray 50 mM, checked 100 mM

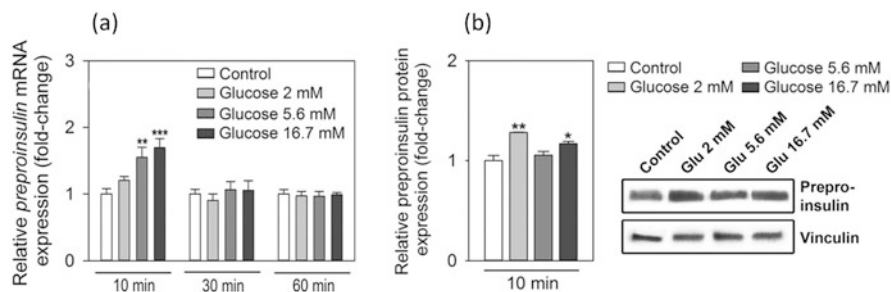


Fig. 19.6 Glucose responsiveness of preproinsulin expression in goldfish adipose tissue. **(a)** Concentration and time-dependent effects of glucose on *preproinsulin* mRNA expression in goldfish adipose tissue *in vitro*. Cultured adipose tissue was incubated alone (control, no glucose) or containing different concentrations of glucose (2, 5.6, or 16.7 mM). Data is shown as mean + SEM ($n = 12$ fish). **(b)** Effects of glucose on the protein expression levels of preproinsulin in goldfish cultured adipose tissue at 10 min postincubation. Data are presented as band density of preproinsulin/vinculin ($n = 3$ fishes). Representative blots are shown in the image. (From Blanco et al. (2020), with permission from Wiley). Asterisks denote statistical differences between control and treated groups assessed by analysis of variance and Student-Newman-Keuls post hoc test (** $P < 0.01$, *** $P < 0.001$)

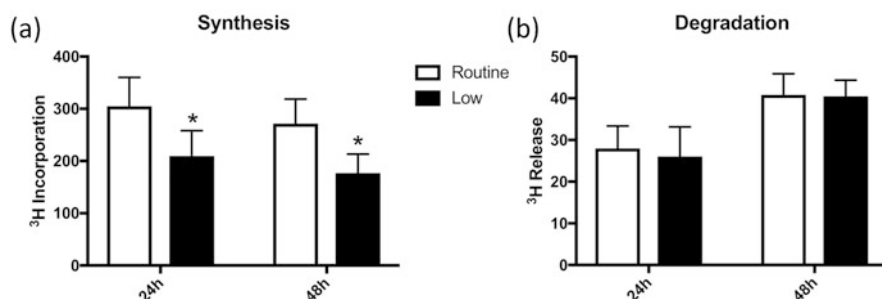


Fig. 19.7 Effects of low glucose media on rates of protein synthesis (^3H incorporation) and protein degradation (^3H release) in 5- and 6-day-old cells, 24 h and 48 h after treatment, respectively. Cells were incubated in media containing 2% FBS supplemented with routine glucose (25 mM) or low glucose (5.5 mM). Means \pm SE ($n = 3$ experiments); * significantly different ($P < 0.05$). (From Latimer et al. (2019), with permission from Elsevier)

Different results are obtained in Atlantic salmon. Villasante et al. (2019) fed medium-carbohydrate (15% wheat starch)/medium-protein (MC/MP) diet or high-carbohydrate (30% wheat starch)/low-protein (HC/LP) diet. *Firmicutes*, *Actinobacteria*, and *Proteobacteria* are the major phyla determined in either experimental group. Phylum *Planctomycetes*, class *Planctomycetia*, order *Planctomycetales*, and genus *Lactococcus* are significantly more abundant in fishes on HC/LP diet than in fishes on MC/MP diet (Fig. 19.9). This study indicates that feeding a carbohydrate-rich meal to salmon exerts a low impact on the structure of gut microbial communities, affecting mostly low-abundance bacteria capable of metabolizing anaerobically carbohydrates as major energy-yielding substrate.

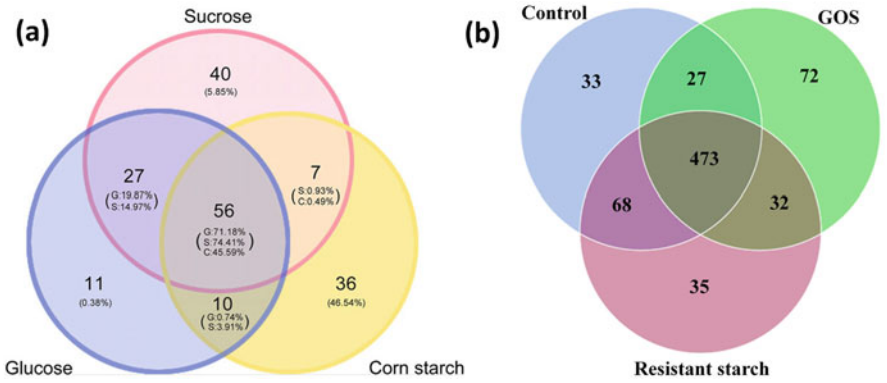


Fig. 19.8 Venn diagrams showing the distribution of all operational taxonomic units (OTUs). (a) OTUs shared by *Liopenaeus vannamei* fed different carbohydrate sources. The percentages in the Venn diagram indicate the ratios of the sequences that are associated with the OTUs in total sequences in each group. (From Qiao et al. (2017), with permission from Wiley). (b) Shared and unique OTUs (phylum level) of gut microbiota of galactooligosaccharides and resistant starch (using the combined data set) in *Scylla paramamosain*. (From (Tran et al. 2020), credit Frontiers Media)

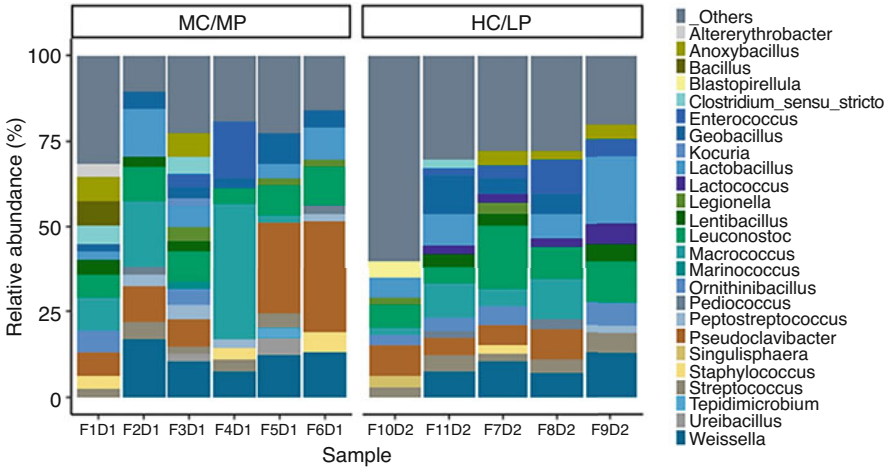


Fig. 19.9 Relative abundance (%) at genus level for each sample in distal intestine digesta microbiota from salmon ($n = 6$; F1D1, F2D1, F3D1, F4D1, F5D1) fed the medium-carbohydrate/medium-protein diet (MC/MP) and from salmon ($n = 5$; F10D2, F11D2, F7D2, F8D2, F9D2) fed the high-carbohydrate/low-protein diet (HC/LP). (From Villasante et al. (2019), credit MDPI, Basel, Switzerland)

From anaerobic degradation of carbohydrates, short-chain fatty acids (SCFAs) can be released (→ Chap. 26), provided an appropriate microbial composition exists in the intestine. To optimize the composition, feed can be supplemented with pre- or probiotics (→AAN III Chapters on Prebiotics and Probiotics). In a trial with mud

crabs (*Scylla paramamosain*), Tran et al. (2020) fed galactooligosaccharides (GOS) and resistant starch (RS).⁵ Both prebiotics alter gut microbiota (Fig. 19.8b) and elicit the production of SCFAs. Particularly, the production of butyric acid is related to the increased abundance of many bacterial genera in both prebiotic-supplemented groups. Most of these genera coincide with others, indicating that the carbohydrate-driven bacteria interact syntrophically and change the metabolic pathways to produce butyric acid.

19.3 Immunity

19.3.1 Adverse Effects

Excess of dietary carbohydrates can reduce growth, interferes with the utilization of other nutrients, and adversely affects immunity with eventual enteritis. Zhou et al. (2017) compared the carbohydrate tolerance in omnivorous and highly carbohydrate-tolerant Wuchang bream and carnivorous black carp and found that HCDs are detrimental to immune response and health in both species (Fig. 19.10—even in Wuchang bream.

Feeding lower levels, Li et al. (2017) documented underlying mechanisms of the carbohydrate tolerance in Wuchang bream. Glucose administration induces a remarkable decrease of hepatic *fructose-1,6-bisphosphatase 1b* (*fbp1b*) mRNA expression, but the enzymatic activity remains constant during the first 4 h. Furthermore, the long-term feeding of a carbohydrate-rich diet inhibits its mRNA expression and activity; gluconeogenesis is reduced or even inhibited. This supports the hypothesis that noncarnivorous fishes usually have a stronger inhibition of hepatic gluconeogenesis after HCD feeding than carnivorous fishes, leading to higher-carbohydrate tolerances in noncarnivorous fishes (Panserat et al. 2002).

Consistent with the comparison between Wuchang bream and black carbon, high-starch diets (HSDs) induce chronic stress in carnivorous *Micropterus salmoides*, which include food intake and metabolic parameters (Lin et al. 2018). Moreover, this fish readjusts its glucosensing under high-starch stress but exhibits relatively limited capacity to metabolize dietary glucose. Excessive dietary starch also influences liver function, increases oxidative stress, and suppresses innate immunity.

Phenotypically known since long, HCDs lead to poor growth and inflammatory immune response as recently reported from juvenile Hong Kong grouper (*Epinephelus akaara*). Yang et al. (2018) fed diets containing few (0% cornstarch, C1), moderate (18%, C2), and high (30%, C3) levels of carbohydrate to this carnivore. After 8 weeks, *E. akaara* on C3 diet exhibits poor growth. Immune-related genes, including *il-8*, *tlr9*, *cxc4*, and *ccl4*—all are pro-inflammatory

⁵RS is starch, including its degradation products, that escapes from digestion in the small intestine of healthy individuals and has a prebiotic potential (Topping et al. 2007).

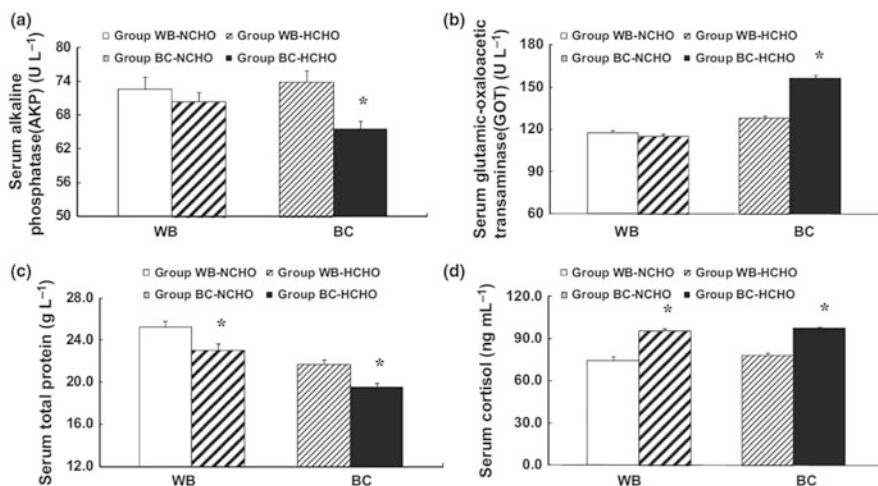


Fig. 19.10 Effects of high- or normal-carbohydrate diet on serum alkaline phosphatase (a), glutamic-oxaloacetic transaminase (b), total protein (c), and cortisol (d) levels of Wuchang bream (*Megalobrama amblycephala*) and black carp (*Mylopharyngodon piceus*). Data are expressed as mean \pm SEM ($n = 9$). Significant differences ($P < 0.05$) between values obtained from normal and high-carbohydrate groups are marked by asterisks above histogram bars in paired samples t-tests for each species. WB-NCHO Wuchang bream-fed diet containing 30.4% carbohydrate; WB-HCHO Wuchang bream-fed diet containing 52.9% carbohydrate; BC-NCHO Black carp-fed diet containing 20.5% carbohydrate; BC-HCHO black carp-fed diet containing 40.5% carbohydrate. (From Zhou et al. (2017), with permission from Wiley)

cytokines—and *nfkB inhibitor alpha* (inhibiting the immune response to infection), are overexpressed in individuals on C3 diet (Fig. 19.11), indicating an inflammatory process in the liver. This issue will be revisited in detail in AAN III (Fish Meal Replacement).

Another adverse effect of HCDs was detected in hybrid grouper (*E. fuscoguttatus* ♀ \times *E. lanceolatus* ♂): The increase of dietary starch promotes the secretion of insulin, downregulates the expression of *atgl* and *foxo1*, and accounts for lipid accumulation (Li et al. 2018). Adipose triglyceride lipase (*atgl*) is considered as the rate-limiting enzyme involved in lipolysis, and *foxo1* (forkhead box O1) is a transcription factor involved in the regulation of lipid metabolism.

Feeding graded levels of starch to Atlantic cod, Tingbø et al. (2012) studied proteoglycans in skeletal muscle and showed that the muscular extracellular matrix is affected by this diet. Proteoglycans are extracellular matrix proteins with unique carbohydrate side chains. HSD leads to elevated transcription of *biglycan* and *decorin*, small leucine-rich proteoglycans regulating collagen fibrillogenesis (Fig. 19.12). Diet-induced changes in the tissue expression of these molecules likely influence the ability to resist infection, wound healing, and scarring. It leads to deformities in muscle, heart, and bone (Tingbø et al. 2012). The most intriguing result of this study, however, is that the HSD increases the transcription of the

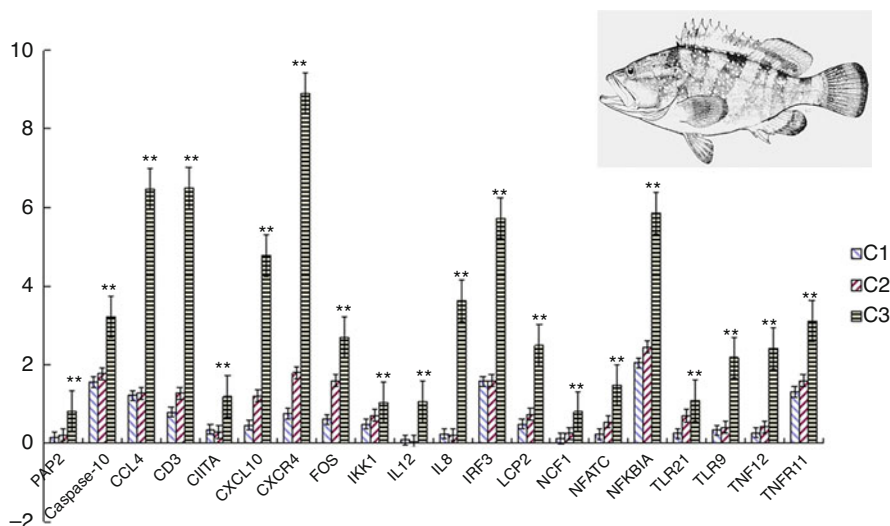


Fig. 19.11 RT-qPCR confirmation of genes expressed at different carbohydrate level in C1, C2, and C3 groups of juvenile Hong Kong grouper (*Epinephelus akaara*) after the 8 weeks feeding trial. Columns represent mean \pm standard error of three fish. Asterisks indicate significant ($P < 0.01$) differences. The genes chosen for immune system are *apt2* antigen peptide transporter 2; *cccl4* C-C motif chemokine 4; *cd3* T-cell surface glycoprotein CD3 delta chain; *cIIta* MHC class II transactivator; C-X-C motif chemokine 10; *cxcr4* C-X-C chemokine receptor type 4; *fos* proto-oncogene *c-fos*; *ikk1* inhibitor of nuclear factor kappa-B kinase subunit alpha; *il12* interleukin-12 subunit beta; *il8* interleukin-8; *irf3* interferon regulatory factor 3; *lcp2* lymphocyte cytosolic protein 2; *ncf1* neutrophil cytosol factor 1; *nfatc* nuclear factor of activated T cells; *nfkbia* NF-kappa-B inhibitor alpha; *tlr21* toll-like receptor 2 type-1; *tlr9* toll-like receptor 9; *tnf12* tumor necrosis factor 12; *tnfr11* tumor necrosis factor receptor superfamily member 11. (From Yang et al. (2018), credit Springer Nature and FAO)

pro-inflammatory gene *il-1 β* in the muscles, eventually suppressed by the upregulated anti-inflammatory cytokine *il-10* (Fig. 19.12).

This advantage of being able to tolerate HCDs, however, can be combined with severe disadvantages as recently shown even in Wuchang bream. Pan et al. (2017) identified that high oral glucose administration impairs immune functions, leading to oxidative stress and liver injury. Persistent hyperglycemia inhibits the hepatic expression of the PI3K/Akt⁶ signaling pathway and *heme oxygenase-1*.⁷ The subsequent upregulation of PI3K/Akt signaling and *nrf2*⁸ without inducing the

⁶The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (PKB, also known as Akt) signaling pathway was reported to be associated with the regulation of Nrf2, which confers protection against cell death induced by oxidative stress due to hyperglycemia insult in mammals (Varma et al. 2005).

⁷Enzyme catalyzing oxidation of heme to biologically active molecules (Maines 1997)

⁸Nuclear factor erythroid 2-related factor 2 usually regulating the gene expressions of antioxidant enzymes (Kobayashi et al. 2002)

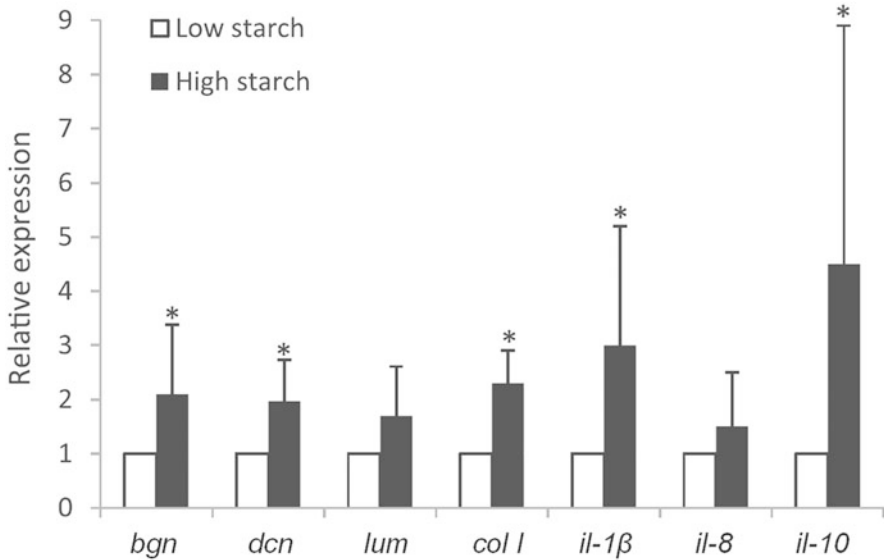


Fig. 19.12 Real-time PCR data showing the relative expression of small leucine-rich proteoglycans (SLRPs), *collagen I*, *il-1β*, *il-8*, and *il-10* mRNA in skeletal muscle of Atlantic cod. *Col I* collagen I; *il* interleukin; small leucine-rich proteoglycans: *bgn* biglycan (regulating collagen fibrillogenesis); *dcn* decorin; *lum* lumican. * $P < 0.05$. (From Tingbø et al. (2012), with permission from Elsevier)

antioxidant system is recognized as an unknown compensatory role in response to oxidative stress. By using transcriptomics and metabolomics, Prisingkorn et al. (2017) and Prathomya et al. (2017) found that high-starch diet results in early symptoms of liver damage in Wuchang breems. A sketch of the hypothetical pathway of this damage is presented in Fig. 19.13. Individuals fed the HCD also exhibit low levels of betaine (Xu et al. 2018), which is a metabolite crucial for maintaining healthy liver functions. Furthermore, betaine enhances the intestinal immunity via TOR and NF-κB signaling pathways (as shown in on-growing grass carps) (Sun et al. 2020).

Noteworthy, the high number of upregulated genes associated with several neurodegenerative diseases is a strong indication of neurodegenerative changes caused by the HCD in Wuchang bream: Alzheimer’s (145 genes), Huntington’s (131 genes), and Parkinson’s diseases (127 genes) (Prisingkorn et al. 2017).

19.3.2 Beneficial Effects

Applied in Traditional Chinese Medicine, dietary supplementation of female ginseng (*Angelica sinensis*) polysaccharides from rhizomes enhances resistance in Malabar grouper (*Epinephelus malabaricus*) against *Edwardsiella tarda* and improves

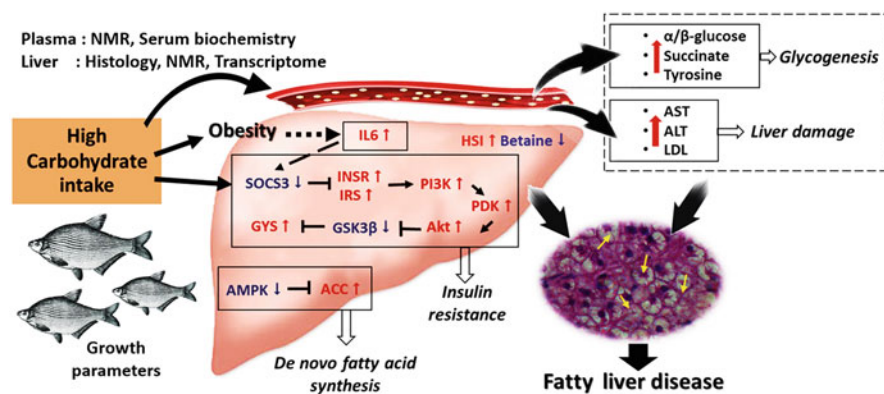


Fig. 19.13 A hypothetical mechanism through which the observed changes in transcriptome, serum biochemistry, and serum and liver metabolomics caused by high-carbohydrate diet can lead to fatty liver disease in Wuchang bream. (From Prisingkorn et al. (2017), credit BioMed Central). *IL6* interleukin 6 (acting as both pro-inflammatory cytokine and anti-inflammatory myokine); *HSI* hepatosomatic index; *SOCS3* suppressor of cytokine signaling 3; *INSR* insulin receptor; *IRS* insulin receptor substrate; *PI3K* phosphoinositide-3-kinase; *PDK1* phosphoinositide-dependent protein kinase 1; *GYS* glycogen (starch) synthase; *GSK3β* glycogen synthase kinase 3 beta; *AKT* RAC-gamma serine/threonine-protein kinase; *AMPK* AMP-activated protein kinase; *ACC* acetyl-CoA carboxylase; *AST* aspartate transaminase; *ALT* alanine transaminase; *LDL* low-density lipoprotein

phagocytosis, proliferation, and respiratory burst activity of head kidney leukocytes (Wang et al. 2011).

Later, Wang et al. (2016) showed that polysaccharides of various plants can be used as immunostimulants to enhance innate immune response and disease resistance against *Aeromonas hydrophila* infection in fishes. The authors fed polysaccharides from *Ficus carica*, *Radix isatidis*, and *Schisandra chinensis* to crucian carps (*Carassius carassius*) and documented increases of leukocyte phagocytosis activity, serum bactericidal activity, lysozyme activity, total protein level, complement C3, and superoxide dismutase activity in the blood of treated fishes. Consequently, an *Aeromonas* challenge progresses significantly less lethal than in untreated individuals. There is a tendency of *F. carica* being the most effective immunostimulant. Supporting this finding, Yang et al. (2015) found on the biomolecular level that *F. carica* polysaccharide upregulate *il-1β*, *tnf-α*, two pro-inflammatory cytokines and downregulated *hsp70* (Fig. 19.14), thereby eliciting humoral innate immune response as well as resistance of grass carp to *Flavobacterium columnare* (Fig. 19.14).

An intriguing example of immune strengthening by the disaccharide cellobiose has recently been reported by Cao et al. (2020) and will be discussed in Chap. 20. For further information on polysaccharides as well as complex oligosaccharides (mannanoligosaccharides, fructooligosaccharides, or sulfated polysaccharides), and other plant compounds as immunostimulants, please refer to AAN III (chapters on Prebiotics).

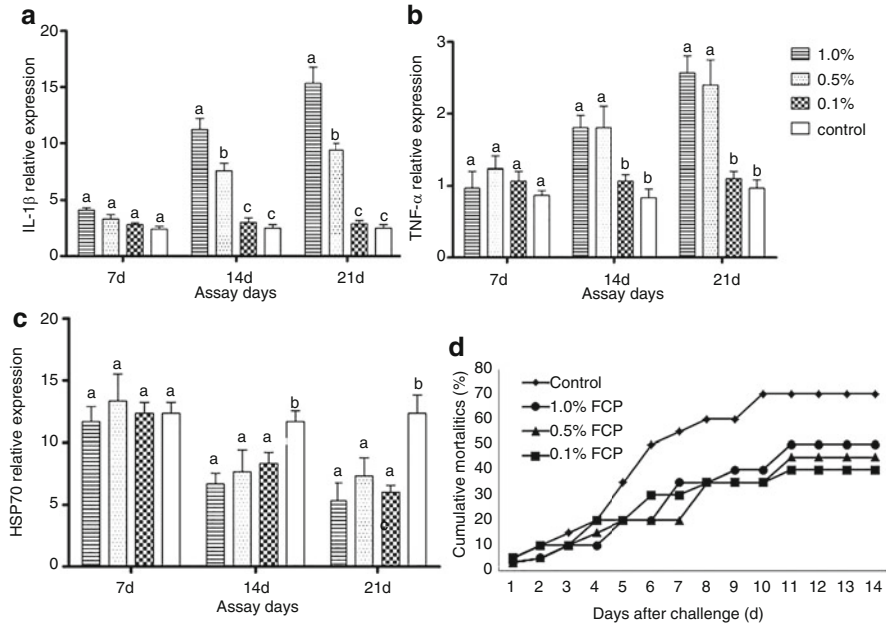


Fig. 19.14 The relative expression of pro-inflammatory *il-1 β* (a) and *tnf- α* (b) as well as *hsp70* (c) in blood of grass carp-fed *Ficus carica* polysaccharide. Data represent the average \pm SD at the same sampling time with different letters as significantly difference ($P < 0.05$). (d) The cumulative mortality of grass carp-fed *Ficus carica* polysaccharide during 15 days post challenge with *Flavobacterium columnare*. (From Yang et al. (2015), with permission from Elsevier)

19.4 Concluding Remarks

In isolated myogenic precursor cells and organ cultures, it has been proven that glucose directly beneficially affects muscle cell growth and modulates the physiological status of organs. Since these findings contrast existing paradigms about physiological and biomolecular effects of glucose, we have to figure out whether the same regulatory function applies to glucose in intact fishes and if this function has been overlooked so far. Furthermore, feeding studies present a variety of indications that, and sometimes how, dietary glucose or starch, directly or indirectly via gut microbiota, elicit gene regulation and transcription. These hints point out that the regulatory function particularly of glucose with beneficial results has to be reconsidered. Moreover, the question arises: Can a goal-oriented modulation of the gut microbiota increase the regulatory potential of dietary carbohydrates by production of SCFAs (\rightarrow Chap. 26)?

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Chapter 20

Oligosaccharides—‘Sweet or Healthy Promises’



Abstract This chapter focuses on oligosaccharides and their nutritional and controlling function in aquatic animals. Oligosaccharides serve as energy source and support growth best with decreasing molecular weight. Based on metabolomics, evidence is growing that di- and trisaccharides beneficially affect immunity. Immunity strengthening applies also to fructo-, xylo-, and konjac oligosaccharides, which serve as well-established prebiotics. Even stachyose, known as antinutritional factor, elicits a hormetic response with beneficial effects in the low-dose range. The axis gut microbiome-host is beginning to be understood and results show that short-chain fatty acids are central in improving health. Finally, this chapter points out the missing consideration of epigenetics in the action of oligosaccharides.

Oligosaccharides are saccharide polymers containing a small number, typically two to six, of monosaccharides. The most common oligosaccharides in nature are disaccharides, two monosaccharide units joined by a glycosidic linkage (Brody 1999). Oligosaccharides are hydrolyzed into their constituent monosaccharides by various brush-border enzymes, such as maltase, sucrase, or trehalase. Brush-border maltase activity is often much higher than of the other enzymes, for example, 10 times higher in salmonids. Glucose, galactose, and fructose reach the blood by specific transporters in the brush border and basolateral membrane or by diffusion (Krogdahl et al. 2011).

On carbohydrate-rich feeds, plasma glucose levels often increase with increasing dietary glucose concentrations; however, less strong with condensed saccharides, such as maltose or dextrin (Fig. 20.1). In addition, the effect of different carbohydrate sources on fish growth often follows inversely carbohydrate molecular weight. When Buhler and Halver (1961) compared dietary glucose, maltose, dextrin, and potato starch, growth rate of Chinook salmon (*Oncorhynchus tshawytscha*) decreases with increasing carbohydrate molecular weight.

Comparable to sunshine bass, juvenile red rock lobsters (*Jasus edwardsii*) show that different carbohydrate diets elicit a rise in hemolymph glucose concentration; the more complex the saccharides are, the lower the effects (Fig. 20.2).

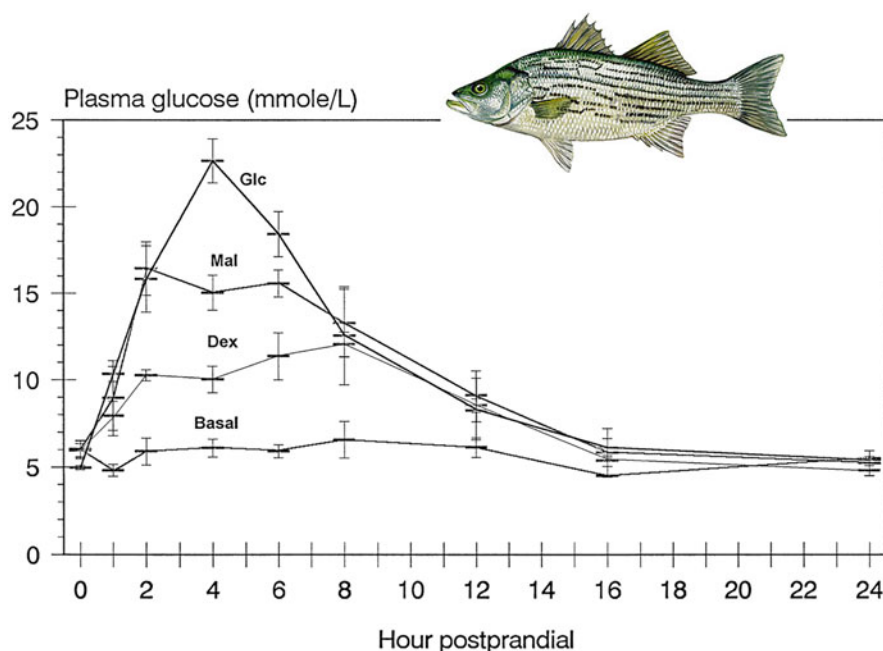


Fig. 20.1 Postprandial plasma glucose concentration in juvenile sunshine bass (*Morone chrysops* ♀ × *M. saxatilis* ♂)-fed diets with different kinds of carbohydrates at 40% of diet. Values are means ± SEM of duplicate analyses on two to three fishes from each dietary treatment. *Glc* glucose; *Mal* maltose; *Dex* dextrin. (From Hutchins et al. (1998), with permission from Elsevier; image credit Duane Raver)

20.1 Disaccharides: Maltose, Sucrose, Trehalose, and Cellobiose

Maltose, sucrose, trehalose, and cellobiose are disaccharides (Fig. 20.3). In maltose, two D-glucose residues are joined by a glycosidic linkage between the α-anomeric form of C-1 on one sugar and the hydroxyl oxygen atom on C-4 of the adjacent sugar (Fig. 20.3). Sucrose is composed of D-glucose and D-fructose (Fig. 20.3). Trehalose consists of two glucose units joined by a 1 → 1 α-bond and cellobiose of two β-1,4-glycosidic-bound glucose molecules, respectively (Fig. 20.3). Nutritional studies with these disaccharides are sparse.

Prior to food detection and intake, certain dietary constituents have the potential to act as attractants. Using the Baltic shrimp (*Palaemon adspersus*), Solari et al. (2017) compared the effectiveness of amino acids (AAs) and carbohydrates in food detection. Besides a number of AAs, carbohydrates (trehalose, maltose, cellobiose, and fructose) are effective in inducing antennular flicking, and maltose being the most potent one.

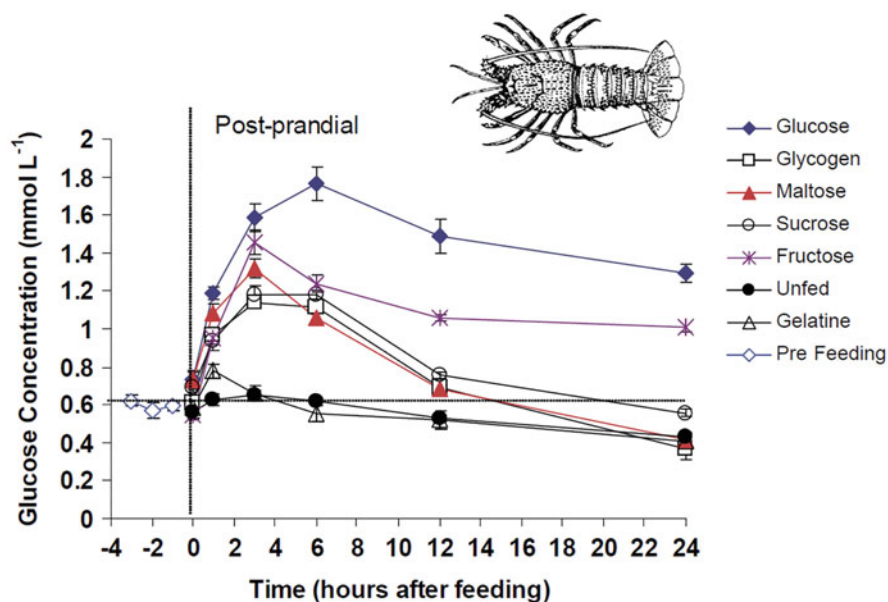


Fig. 20.2 Time course of changes in hemolymph glucose concentration of juvenile red rock lobsters provided with a meal containing glucose, glycogen, maltose, fructose, or sucrose in a gelatin base, gelatin only, and unfed controls. The dotted horizontal line represents mean preprandial level. Lobsters were fasted for 4 days and fed at time 0 h. Mean \pm SEM, $n = 6$. (From Radford et al. (2005), with permission from Elsevier, image credit An Encyclopaedia of New Zealand, edited by A. H. McLintock, originally published in 1966, accessed 26 Jul 2020)

Based on elevated hepatopancreas amylase activity, nutrient digestibility, and weight gain (WG), Niu et al. (2012) identified that *Penaeus monodon* utilizes wheat starch and sucrose as carbohydrate sources better than potato starch, cornstarch, dextrin, maltose, or glucose. Similarly, Pascual et al. (1983) found that sucrose supports the survival of this shrimp best. At a 10% inclusion level, the survival follows the sequence sucrose>sago palm starch>maltose, dextrin>maize starch>cassava starch, molasses (with no survivors). At the 40% inclusion level, the sequence is sucrose>maize starch>dextrin, and no survivors are recorded with maltose, cassava starch, molasses, and sago palm starch.

In crustaceans, there exist specific disaccharide transepithelial transporters (\rightarrow Chap. 16). In their model species *Homarus americanus*, Likely et al. (2015) found that sucrose is transported across the intestine largely intact with no glucose or fructose appearing in the luminal perfusate. In addition, also trehalose is substrate of the sucrose transport system. Simon (2009) reported that the dietary use of sucrose and trehalose in the red rock lobster (*Jasus edwardsii*) as energy source, however, is limited due to poor hydrolysis of these disaccharides.

Trehalose is a blood sugar in invertebrates, particularly crustaceans, nematodes, or rotifers serving as energy-storage compound (Johnston and Davies 1972; Nelson

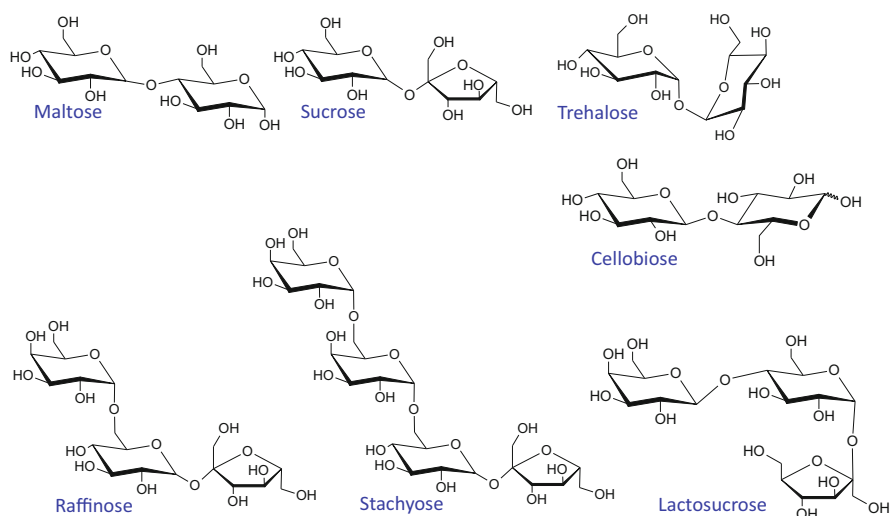


Fig. 20.3 Structures of oligosaccharides: maltose, sucrose, trehalose, cellobiose, and raffinose, stachyose, and lactosucrose

and Cox 2005). Moreover, it plays a major role in anhydrobiosis of invertebrates (Clegg 1965; Tunnacliffe and Lapinski 2003; Hengherr et al. 2011) (for a comprehensive survey, refer to Steinberg (2012)). Furthermore, Schwach (1972) showed that spinycheek crayfish (*Orconectes limosus*) uses trehalose for chitin biosynthesis after conversion to glucose, and Shi and Chung (2014) observed also in a decapod crustacean (blue crab, *Callinectes sapidus*) that trehalose can function as glucose donor for hyperglycemia in addition to glycogen.

In one of the very few dietary trehalose studies, Zhu et al. (2018) identified that this disaccharide stimulates the immunity in *Litopenaeus vannamei*. Trehalose supplements improve not only WG, feed efficiency, and survival rate but also pathogen resistance against *Vibrio alginolyticus*.

Effects of dietary disaccharides in selected aquatic invertebrates and fishes are listed in Table 20.1.

Recently, a beneficial effect was identified with cellobiose (Cao et al. 2020). In mitten crabs, suffering from tremor disease (caused by *Spiroplasma eriocheiris*, a wall less bacterium (Liu et al. 2018)), reduced contents of cellobiose are identified by functional metabolomics. Therefore, crabs are fed-graded supplements of dietary cellobiose prior to *S. eriocheiris* injection. Actually, cellobiose addition reduces the mortality of the crabs (Fig. 20.4) and decreases the copies of the pathogen in the hemocytes.

Table 20.1 Effects of dietary disaccharides on life history traits of selected aquatic invertebrate and fish species

Species	Disaccharide	Trait/effect	Reference
Invertebrates			
<i>Biomphalaria glabrata</i>	Mal	Net uptake by drinking	Thomas et al. (1990)
<i>Litopenaeus vannamei</i>	Suc	Hyposalinity resistance↑	Wang et al. (2016)
	Tre	WG↑, survival↑, pathogen resistance↑	Zhu et al. (2018)
<i>Macrobrachium nipponense</i>	Mal	Growth↔, survival↔	Kong et al. (2019)
<i>Penaeus monodon</i>	Suc	Survival↑, WG↓	Pascual et al. (1983)
	Mal	Survival↓, WG↓	
Fishes			
<i>Acipenser transmontanus</i>	Mal, Suc	WG _{Mal} > WG _{Dex,Str,Suc}	Hung et al. (1989)
<i>Epinephelus lanceolatus</i>	Mal	WG _{Str} > WG _{Mal}	Lu et al. (2018)
<i>Heteropneustes fossilis</i>	Mal, Suc	WG _{Str,Dex,Suc} > WG _{Mal}	Erfanullah (1999)
<i>Ictalurus punctatus</i>	Mal, Suc	GR _{Dex} > GR _{Str} > GR _{Mal, Suc}	Wilson and Poe (1987)
<i>Morone saxatilis</i>	Mal	WG _{Dex} > WG _{Mal}	Rawles and
<i>Morone chrysops</i> ♀ × <i>M. saxatilis</i> ♂	Mal	WG _{Mal} ~ WG _{Dex}	Gatlin III (1998)
<i>Oncorhynchus mykiss</i>	Suc	Protein sparing	Pieper and Pfeffer (1980)
<i>Oncorhynchus tshawytscha</i>	Mal, Suc	WG↓	Buhler and Halver (1961)
<i>Paralichthys olivaceus</i>	Mal	WG _{Mal} > WG _{Glc}	Lee et al. (2003)
<i>Rachycentron canadum</i>	Mal, Suc	Hepatic lipid↓, hepatic glycogen↓	Cui et al. (2010)
<i>Sparus aurata</i>	Mal	Hepatic glycolysis↑, hepatic gluconeogenesis↓, hepatic lipogenesis↓	Enes et al. (2010)

Glc glucose; Mal maltose; Suc sucrose; Tre trehalose; Str starch; Dex dextrin; ↑ support/increase; ↔ no apparent effect; ↓ reduction/inhibition; WG weight gain; GR growth rate

20.2 Raffinose

This trisaccharide is composed of D-galactose, D-glucose, and D-fructose (Fig. 20.3). It is found in beans, cabbage, Brussels sprouts, broccoli, asparagus, other vegetables, and whole grains. In some plants, it is more significant as storage carbohydrate than starch (Navarro et al. 2019).

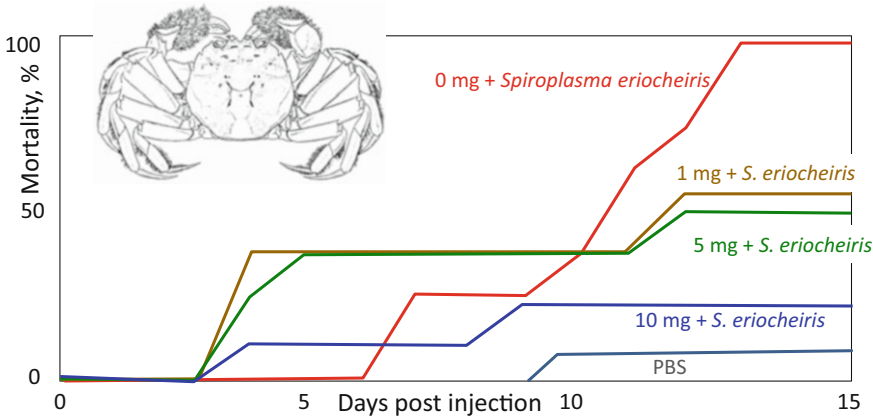


Fig. 20.4 Graded dietary cellobiose (mg g^{-1}) reduces mortality in *Eriocheir sinensis* challenged with *Spiroplasma eriocheiris*. (Redrawn from Cao et al. (2020), with permission from Elsevier; image credit FAO). PBS phosphate-buffered saline solution (negative control)

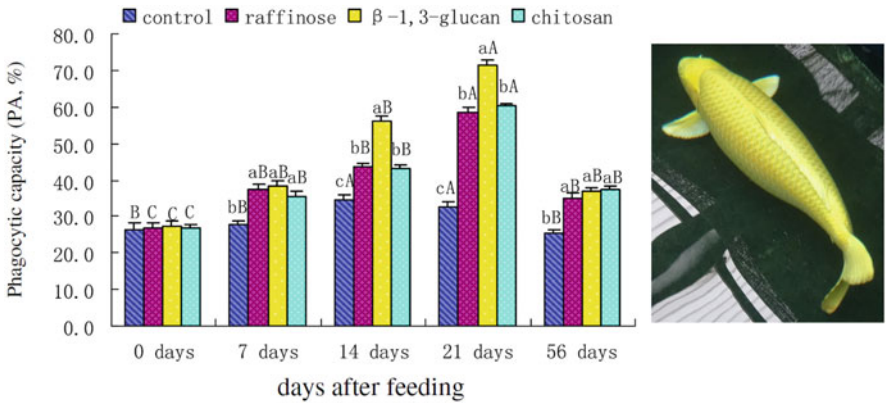


Fig. 20.5 Phagocytic capacity of *Cyprinus carpio* koi-fed different diets. Data are means \pm SEM from 15 individuals with SD. a,b,c: Significant difference ($P < 0.05$) among different groups within the same period. A,B,C: Significant difference ($P < 0.05$) within the same groups among different periods. (From Lin et al. (2011), with permission from Elsevier); image: Nishikigoi (koi) breeding in Niigata, Japan (©CEW Steinberg)

Only a few papers address the effects of dietary raffinose: Lin et al. (2011) describe raffinose as immunostimulant and report that dietary supplementation, as low as 0.2%, enhances the nonspecific immunity of koi carps through increases of total leukocyte counts, respiratory burst, phagocytic (Fig. 20.5), lysozyme, and superoxide dismutase (SOD) activities which result in increased resistance against *Aeromonas veronii*.

In another study in common carp, mixed results are obtained. Whereas dietary application of raffinose does not improve growth performance and feed utilization,

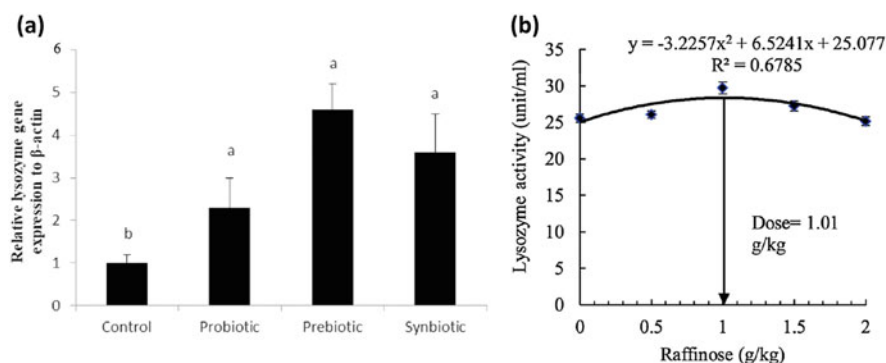


Fig. 20.6 (a) Effects of feeding different experimental diets including control (no supplementation), prebiotic (2 g raffinose, RF, kg^{-1} diet), probiotic (6×10^8 CFU g^{-1} *Pediococcus acidilactici*, PA) and synbiotic (2 g RF kg^{-1} + 6×10^8 CFU g^{-1} PA) on the expression of lysozyme in common carp skin. (From Hoseinifar et al. (2019), with permission from Elsevier). (b) Relationship between serum lysozyme activity of Nile tilapia and dietary levels of raffinose. Values expressed as means \pm SE ($P < 0.05$). (From Abdel-Latif et al. (2020), with permission from Elsevier)

mucosal immune responses increases indicated by upregulation of the lysozyme gene in the skin (Fig. 20.6a) (Hoseinifar et al. 2019). In Nile tilapia, however, not only serum lysozyme activity but also growth performance and feed utilization are beneficially affected (Fig. 20.6b) (Abdel-Latif et al. 2020).

The supplementation of hybrid sturgeon's (*Acipenser baerii* ♀ \times *A. schrenckii* ♂) diet with raffinose improves growth by means of changing intestinal histology and intestinal microbiota (Xu et al. 2018). However, there is not yet a clear explanation for the underlying mechanism.

20.3 Lactosucrose

Lactosucrose is a trisaccharide consisting of galactose, glucose, and fructose (Fig. 20.3) and is occasionally used as prebiotics (Han et al. 2009). Kihara et al. (1995) examined dietary lactosucrose in terms of WG and thickness of the intestinal *tunica muscularis* in farmed red sea bream (*Pagrus major*). Lactosucrose does not affect WG but strengthens the intestinal *tunica muscularis*. In turbot, Mahious et al. (2006) did not find improved growth on lactosucrose diet.

Later, Kihara and Sakata (2001) reported that this trisaccharide improves the microbial *isobutyric acid* (a short-chain fatty acid, SCFA)¹ content in the intestine of rainbow trout, pointing out the prebiotic role of lactosucrose. Chu et al. (2013)

¹Short-chain FAs produced as products of bacterial fermentation are responsible for various beneficial effects in animals feeding prebiotics-supplemented diets (\rightarrow Chap. 26).

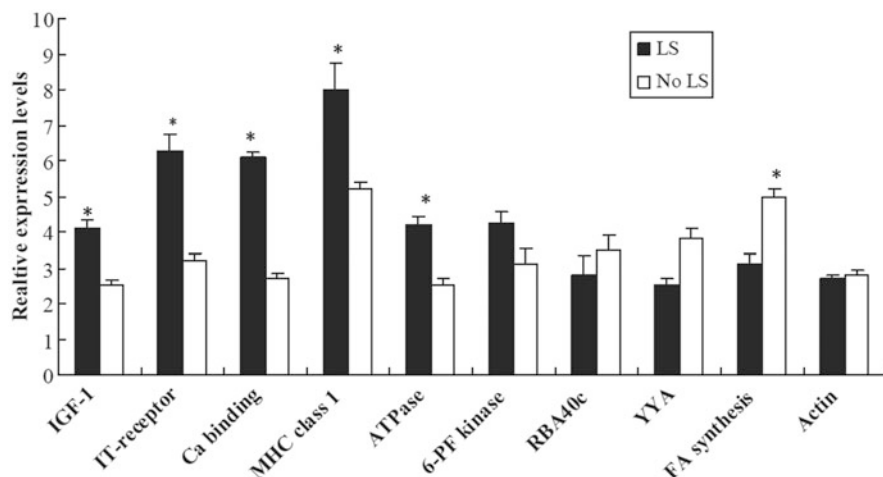


Fig. 20.7 Relative expression of 10 selected genes in grass carp liver from both 15 g kg⁻¹ lactosucrose (LS) supplementation (trial group) and control group. The data are presented as ratios of trial/control. Expression level of each gene >1.5 fold in difference is considered as significant difference marked with *. (From Chu et al. (2013), with permission from Wiley). Selected genes: growth—*insulin-like growth factor igf-1* immunoreaction—*immune-type receptor it-receptor* and *histocompatibility MHC class I mhc class I*, metabolism—*ATPase (Na⁺/K⁺ transporting 1)*, or sugar metabolism—*6-phosphofructo-2-kinase (6-PF kinase)*; *actin* reference gene

choose dietary lactosucrose as dietary antibiotic alternative for grass carp juveniles with an appropriate dose of 15 g kg⁻¹. Fish growth is enhanced as reflected by increased transcription of growth and immune genes; a fatty acid synthesis-related gene shows reduced expression (Fig. 20.7). This indicates that the prebiotic activity should be tested and reconfirmed with different fish species.

20.4 Stachyose

The galactooligosaccharide stachyose is a tetrasaccharide consisting of two α -D-galactose units, one α -D-glucose, and one β -D-fructose unit (Fig. 20.3) and occurs naturally in numerous vegetables, rapeseed, and cottonseed (Navarro et al. 2019). It is one of the so-called anti-nutritional factors in soybean meal (\rightarrow AAN III “Fish Meal Replacement”).

The only available papers on dietary stachyose report that this saccharide alters the intestinal microbiota and improves the intestinal mucosal barrier function in juvenile turbot (Hu et al. 2015; Yang et al. 2018). Contents below 5% dietary stachyose exert promoting effects on intestinal cellulose-degrading bacteria and gene expressions of barrier-forming tight junction proteins and, thus, improve the digestion of stachyose and enhance the mucosal barrier function in the intestine of

juveniles. Even a moderate level of stachyose (1.25%) improves growth, feed utilization, digestive enzyme activities, and distal intestine structures (Hu et al. 2015). However, 5% dietary stachyose reveals ambivalent effects by greatly modifying the composition of the intestinal microbiota with an increase of both beneficial and potential pathogenic bacteria (Yang et al. 2018). This means that stachyose behaves Janus-faced as prebiotic; it elicits hormetic response (Calabrese 2020). Furthermore, this example points out that feeding trials with so-called anti-nutritional factors should consider and test the low-dose range for potential beneficial effects much more carefully.

20.5 Fructooligosaccharides

Fructooligosaccharides (FOS) are oligomeric fructans; they are oligosaccharides with $\beta(2\rightarrow1)$ fructosylfructose glycosidic bonds (Fig. 20.8) (Chen et al. 2016). They are successfully used as prebiotics (Chen et al. 2017; Poolsawat et al. 2020). Corresponding studies will be referenced in AAN III “Prebiotics”; here, only selected examples shall illustrate this potential.

Chen et al. (2017) evaluated the effects of graded FOS additives at 0.1% \rightarrow 2% on growth, superoxide dismutase (SOD) activity, lipid peroxidation, production of intestinal SCFA, and hepatopancreatic histopathology in postlarvae of the giant freshwater prawn (*Macrobrachium rosenbergii*). The optimal FOS level is 0.4%, indicated by optimal WG and intestinal microbial production of SCFA (Fig. 20.9a). However, higher levels induce oxidative stress (Fig. 20.9b) and impair the hepatopancreatic condition.

On the biomolecular level, Zhang et al. (2019) showed alterations in hepatic steroid biosynthesis in zebrafishes on FOS diets. The results indicate that FOS reduce lipid levels but do not alter body weight. The authors confirm the great potential of FOS as dietary prebiotics in improving health of fishes.

20.6 Xylooligosaccharides

Xylooligosaccharides (XOS) are sugar oligomers made up of xylose units (Fig. 20.10a). Their C5 structure is fundamentally different from other prebiotics, which are based upon C6 sugars (Vázquez et al. 2000). As prebiotics, xylooligosaccharides selectively support beneficial gut bacteria, such as *Bifidobacteria* and *Lactobacilli* (Ma et al. 2017). Their metabolism leads to release of SCFAs as products (Fig. 20.11) (\rightarrow Chap. 26).

As new prebiotics candidate, Geraylou et al. (2013a, b) proved the activity of arabinoxylan oligosaccharides (AXOS) in juvenile Siberian sturgeon (*Acipenser baerii*). The analysis of the bacterial 16S rRNA gene clearly demonstrates beneficial shifts in gut microbiota, primarily in the phylum *Firmicutes*, and higher

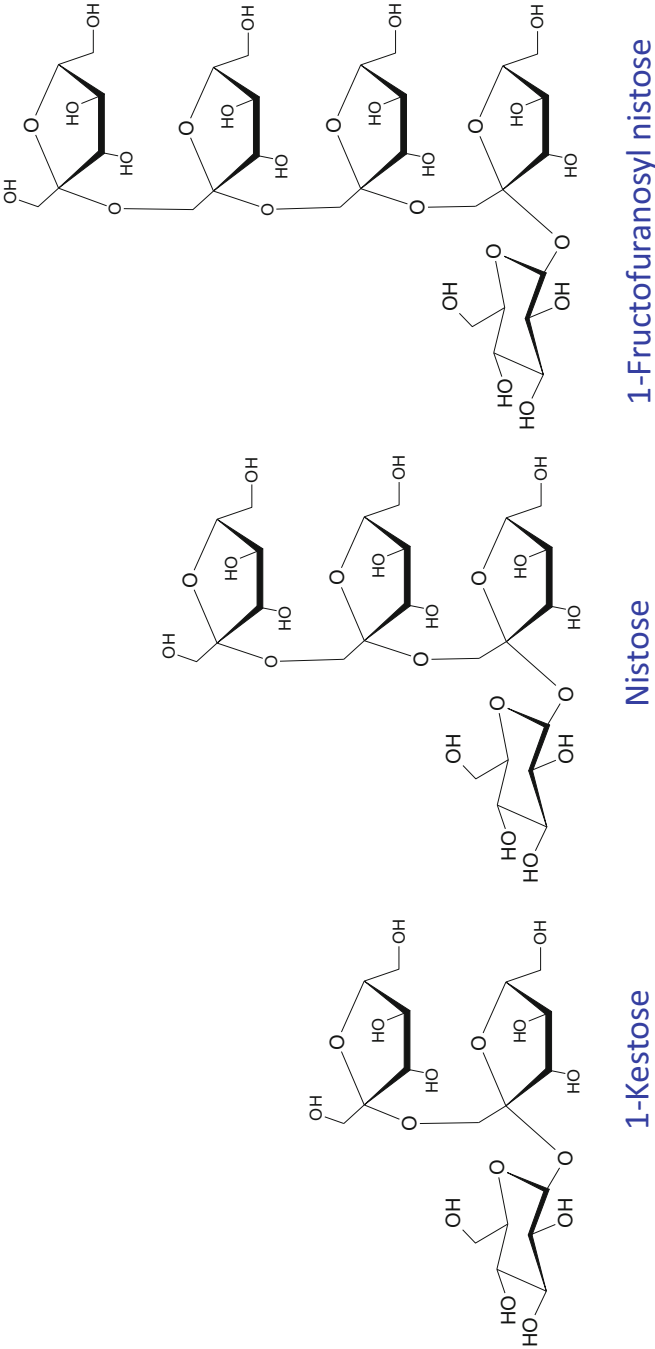


Fig. 20.8 Structure of selected fructooligosaccharides

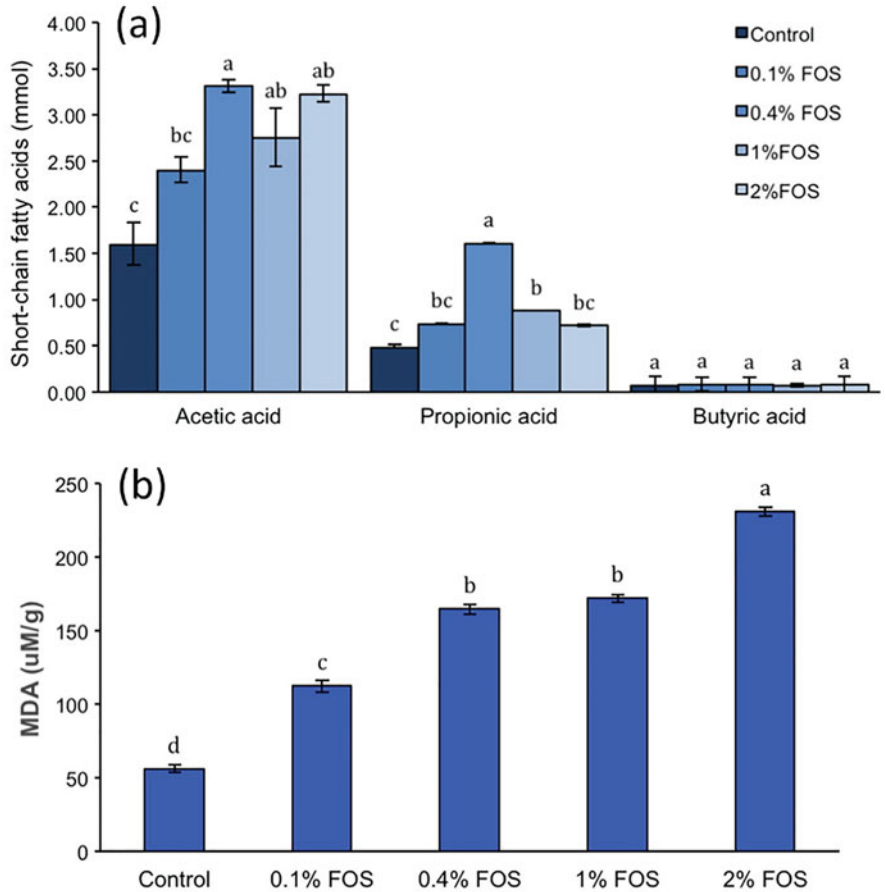


Fig. 20.9 (a) Mean \pm SE acetic acid, propionic acid, and butyric acid from the tail muscle of *Macrobrachium rosenbergii*; (b) mean \pm SE malonaldehyde (MDA) equivalents ($\mu\text{M g}^{-1}$, indicative of lipid peroxidation) within the muscles after 56 days of being fed diets with increasing levels of FOS (From Chen et al. (2017), with permission from Elsevier)

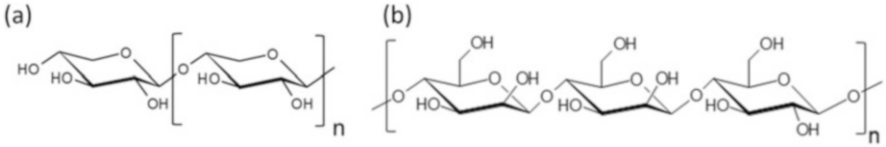


Fig. 20.10 Molecular structures of a hypothetical xylooligosaccharide (a) and konjac mannan (b)

concentrations of SCFAs at 2% dietary AXOS. Moreover, the impact of AXOS follows an optimum curve, as supplementation with 4% of AXOS leads to mainly reduced SCFA release (Fig. 20.11a). AXOS with a lower degree of polymerization increase species richness in hindgut microbiota but has lower effect on SCFA

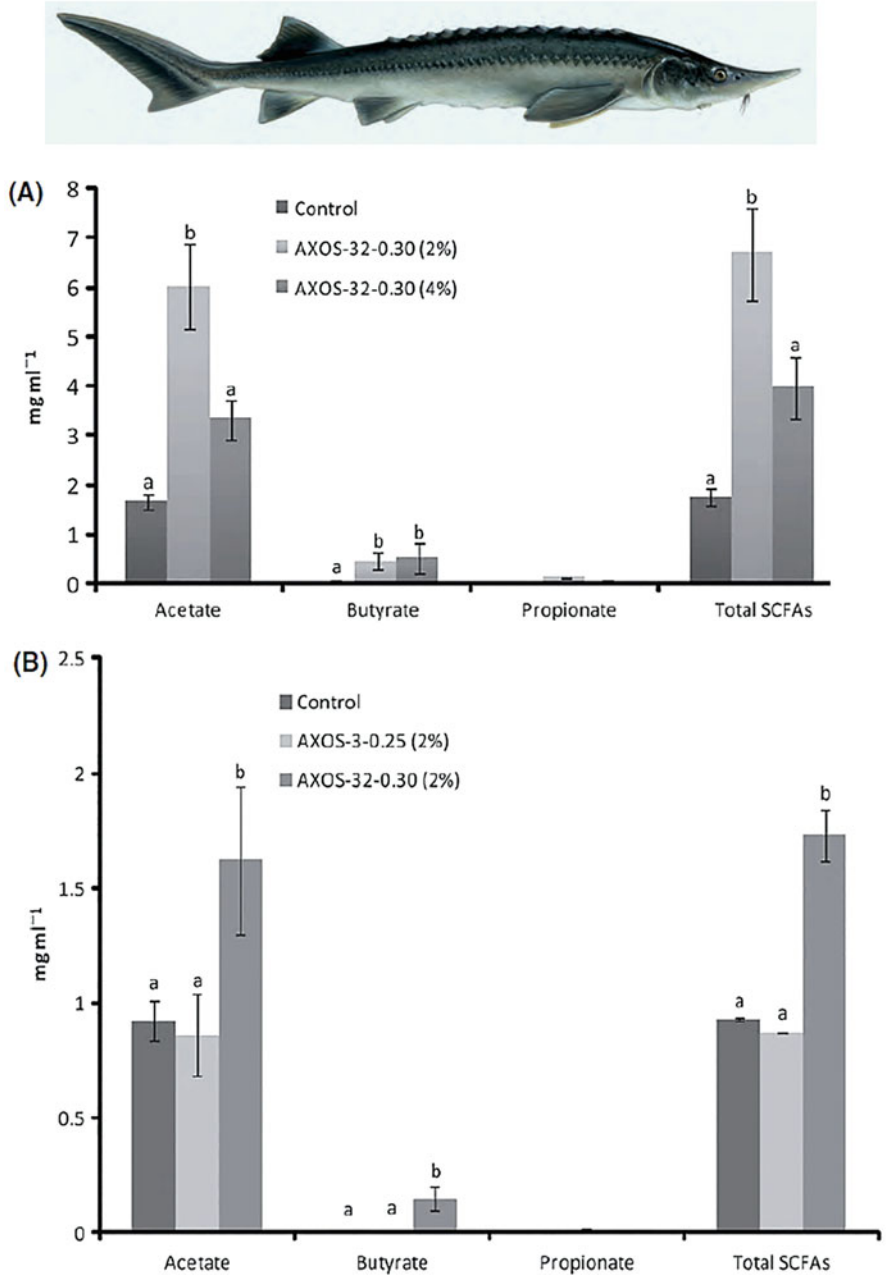


Fig. 20.11 Short-chain fatty acid production of hindgut microbiota in *Acipenser baerii* fed: (a) a control diet or diet supplemented with 2% or 4% AXOS-32-0.30 (trial 1); (b) a control diet or diet supplemented with 2% AXOS-32-0.30 or 2% AXOS-3-0.25 which differ in the degree of polymerization (trial 2). Values are means \pm SE. (From Geraylou et al. (2013b), with permission from Wiley; image credit cc.europa.eu)

concentration and fish growth. Therefore, the prebiotic potential of AXOS appears to depend on the degree of polymerization.

In trials of replacing fish meal with rice protein concentrate in omnivorous Wuchang bream, Abasubong et al. (2018, 2019) found that XOS improve WG, antioxidant capability, digestive functions, TOR signaling, and immune responses against *Aeromonas hydrophila*. The studies reveal that fish meal can entirely be replaced by rice protein concentrate with 1.5% XOS supplementation without adverse effects on WG. However, high doses of XOS (3%) exert detrimental effects on innate immunity and antioxidant defenses.

A comparable prebiotic effect is identified in the carnivorous white sea bream (*Diplodus sargus*): Guerreiro et al. (2016) show that XOS enhance the immune status through increases in alternative complement pathway, lysozyme, and total immunoglobulin. Furthermore, dietary XOS decrease lipogenesis, are independent of the dietary protein source, and improve growth in individuals-fed diets with high shares of plant ingredients (Guerreiro et al. 2015).

20.7 Konjac Oligosaccharides

Konjac mannan is a glucomannan and main component of tubers of konjac, a perennial Araceae species. It is a heteropolysaccharide consisting of β -D-glucose and β -D-mannose (Fig. 20.10b), with a glucose:mannose ratio of 1:1.6 (Takigami 2009). Consequently, the hydrolysis of konjac mannan by mannanase produces oligosaccharides containing both glucose and mannose residues with varying shares.

Mainly oxidized konjac oligosaccharides are applied in feeding trials. Zheng et al. (2015) found that H_2O_2 and HCl effectively reduce the molecular weight of konjac glucomannan; the products increase antioxidant capacity (Zhang et al. 2013), improve lipid metabolism and gut health (Zhang et al. 2017), and show enhancing effects on immunity and gene expression in *Schizothorax prenanti* (Fig. 20.12). The dietary optimum is 8.0 kg^{-1} . The improved immunity translates into increased resistance against *Aer. hydrophila* (Zheng et al. 2016). Additionally, even commercial konjac oligosaccharides are similarly effective as oxidized products (Chen et al. 2019); probably, they contain oxidized konjac oligosaccharides.

20.8 Concluding Remark

Only a few studies on oligosaccharides as dietary supplements do exist with respect to growth, immunity, and modulation of gut histology. Furthermore, several of these studies appear not consistent (e.g., hybrid sturgeon vs. common carp on raffinose diets). This inconsistency may serve as an incentive for studying the response of aquatic organisms applying multi-omics techniques (genomics, epigenomics, proteomics, or metabolomics). Particularly, the interaction of host and microbiome

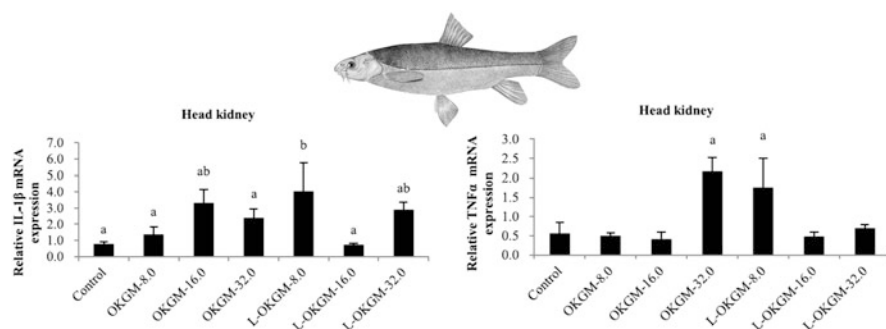


Fig. 20.12 Graded dietary oxidized konjac glucomannan with two different molecular weights affect the transcription of pro-inflammatory *il-1β* and *tnfa* in head kidney of *Schizothorax prenanti*. Data are means \pm SE. Different superscripts indicate significant differences ($P < 0.05$). (From Zheng et al. (2015), with permission from Elsevier; image credit Kessler (1874))

deserves more attention and may contribute to solving apparent contradictions. The gut microbiota contributes to host physiology through the production of a myriad of metabolites, best known are SCFAs. These metabolites exert their effects within the host as signaling molecules and substrates for metabolic reactions (Krautkramer et al. 2020). As a role model for improved understanding of this interaction, Delogu et al. (2020) show that upgrading multi-omic tool kits with traditional absolute measurements unlocks the scaling of core biological questions to dynamic and complex microbiomes, creating a deeper insight into interorganismal relationships that drive the greater community function.

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Chapter 21

Starch—‘*Gluey Promise*’



Abstract This chapter comprises nutritional and regulatory functions of dietary starches of various sources. The limits of dietary starch contents in aquafeeds for carnivores and omnivores are well established. High dietary starch supply can induce hyperglycemia, hepatomegaly, elevated fat deposition, enteritis, and retarded growth. Transcriptomics in selected carnivores reveal that high starch diets lead to increased β -oxidation, depressed gluconeogenesis, inhibited glycolysis, impaired endocrine system, and glucose intolerance. Appropriate dietary starch, however, can beneficially modulate the intestinal microbiota and support microbial production of short-chain fatty acids.

Although the digestive and metabolic systems of aquatic animals are more suitable for using proteins and lipids rather than carbohydrates as energy, starchy feeds are an important source of energy and less problematic than many mono- and disaccharides (Enes et al. 2009). In the aquaculture industry, starches are often intentionally included in the formulations of aquafeeds for ethical and pragmatical reasons: They provide an inexpensive form of energy and can act as binders, which subsequently improve pellet quality, such as durability and water stability (Stone et al. 2003a). This chapter presents selected studies of dietary starch and its degradation products (dextrins) in aquafeeds with particular reference on regulatory activity of these dietary components.

21.1 Starches

Starch is a polymeric carbohydrate consisting of numerous glucose units joined by glycosidic bonds (Fig. 21.1). It is produced by most green plants as energy storage and is contained in large amounts in human staple foods like potatoes, wheat, corn, rice, and cassava. The amount of starch included in aquafeeds varies from ~20% for carnivores and up to 45% for omnivores (Romano and Kumar 2019).

Dextrins are low-molecular-weight carbohydrates produced by the hydrolysis of starch. Consequently, they are mixtures of polymers of D-glucose units linked by

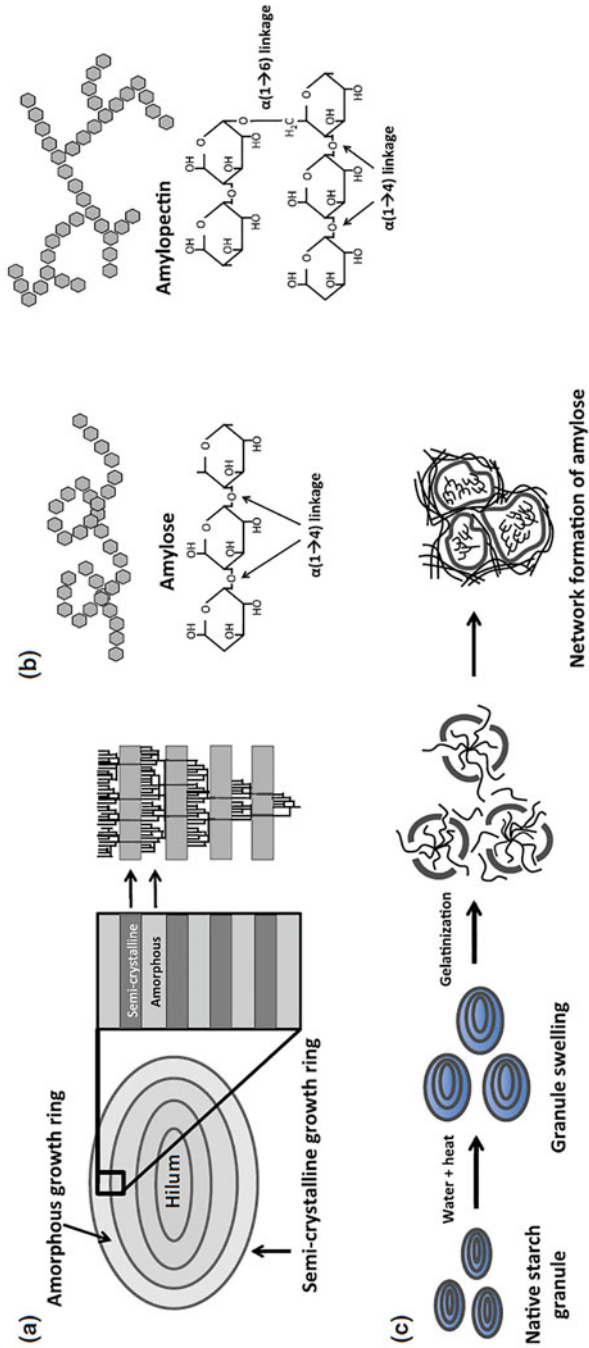


Fig. 21.1 Structure of a starch granule with alternating amorphous and semicrystalline regions (a). Chemical structure of helical amylose and highly branched amylopectin and while both are composed to $\alpha(1\rightarrow4)$ linkages, amylopectin have $\alpha(1\rightarrow6)$ linkages at each branch (b). Structural modifications of starch granules when undergoing gelatinization (c). (From Romano and Kumar (2019), with permission from Wiley)

α -1 \rightarrow 4 or α -1 \rightarrow 6 glycosidic bonds. Starches are digested primarily by the enzyme α -amylase, which cleaves the α -1 \rightarrow 4 bonds, but not the α -1 \rightarrow 6 bonds (Berg et al. 2015). Usually, dietary dextrans are well utilized, much better than monosaccharides, such as glucose or fructose. Their dietary value here is not separately considered.

Starch granules have alternating amorphous and semicrystalline regions due to their amylopectin and amylose structures, respectively (Fig. 21.1a). Amylopectin comprises the majority of the starch granule and is water soluble, whereas amylose is responsible for the crystalline regions due to being tightly packed into glucan chains (Fig. 21.1b). The presence of amylose is largely responsible for the starch granule being insoluble in cold water (Romano and Kumar 2019). Consequently, gelatinization of the native starch granule is required in almost all culinary uses of starch—a process of breaking down the intermolecular bonds. This leads to a change in organization of the granules as a function of temperature and water content and to increased bioavailability (Fig. 21.1c) (Waigh et al. 2000). Starches are defined “gelatinized” (also termed as “precooked,” “cooked,” “pregelatinized,” or “extruded”) when their intermolecular bonds are broken prior to their inclusion in the diets.

21.1.1 Invertebrates

Besides a few studies of echinoderms and mollusks, mainly crustaceans have been studied in terms of dietary starch utilization. In herbivorous and omnivorous species, dietary starch reveals a variety of beneficial effects.

21.1.1.1 Mollusks

Albentosa et al. (1996) figured out that the grooved carpet shell (*Ruditapes decussatus*) has low nutritional requirements in proteins and lipids but high in carbohydrates. Since this mollusk is an aquaculture candidate, attempts have been carried out to find a, at least partial, substitute for live microalgae (Fernández-Reiriz et al. 2015) in the diet of this clam, including cornstarch (Fernández-Reiriz et al. 1999). These authors confirmed that the lipid content of the diet affects growth but does not explain it totally; rather, the starch content explains the differences in growth found when diets with the same composition in lipids are used.

Although the energy demand for gonad development in conditioned fat horse mussels (*Modiolus capax*) appears to be dependent more on temperature than on diet, one effective way of providing sufficient energy and covering most nutritional needs of gametogenesis is through low-cost, carbohydrate-rich products such as cereal flours, especially cornstarch. The combination of high temperature and diet prepared from cornstarch favors gonad development and maturation and reduces conditioning timing (López-Carvallo et al. 2017).

In the same line of evidence, Mazón-Suástegui et al. (2017) reported that a small addition of cornstarch (~0.15% of live weight) to microalgae diets boosts growth

during nursery procedures and performance of the Pacific calico scallop (*Argopecten ventricosus*). At the beginning of the trial, there are more scallops at stages IV and VI, with a high count of degenerated oocytes. After the conditioning period, the scallops on microalgae + cornstarch have an increased frequency of mature-spawning stage V. This study points out the significance of starch in the diets of mollusks of commercial interest; this applies also to *Crassostrea corteziensis*, *Modiolus capax*, *Paphies australis*, *Pinctada mazatlanica*, *Pinna rugosa*, and *Pteria sterna* (Mazón-Suástegui et al. 2017).

In juvenile pink abalone (*Haliotis corrugata*), Montañó-Vargas et al. (2005) showed that dietary carbohydrates (provided as cornstarch) and proteins are the primary and secondary sources to suitable energy requirements. Cornstarch can replace dietary macroalgal powder as energy source up to 75% in juvenile Japanese (disk) abalone (*H. discus hannai*) (Ferreira et al. 2015). The South African abalone (*H. midae*), however, does not benefit from dietary starch supplementation: Different levels of gelatinized cornstarch or α -cellulose do not influence the apparent digestibility of protein, fat, fiber, or starch, pointing out the pure herbivorous dietary habit of this species (Sales and Britz 2002).

“Solar-powered” sea slugs, such as *Elysia timida* (Fig. 21.2), live on stolen chloroplasts (kleptoplasts) and accumulate photosynthate (Laetz et al. 2017). Kleptoplasts function as both, nutritive producer and storage device, holding onto

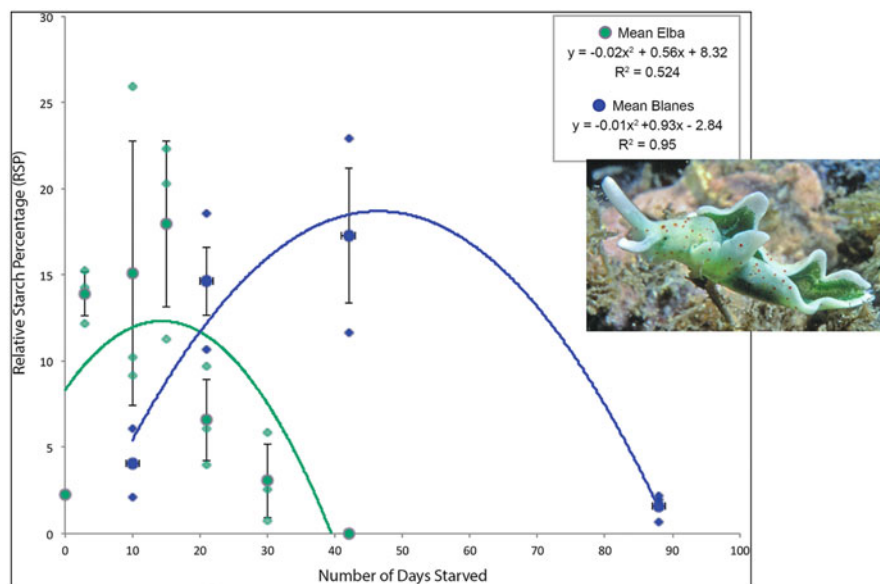


Fig. 21.2 Relative Starch Percentage (RSP) in two populations of *Elysia timida*. The Mean RSP for the Blanes fall population slugs is shown in blue, and the Elba spring population is shown in green (including two specimens which starved for 42 days). Best fit curves show the shift in total days starved between the two populations and show a higher overall RSP in the Blanes fall population. (From Laetz et al. (2017), credit Springer Nature; image credit Parent Géry, Wikimedia)

the starch reserves until they are finally used to withstand extended starvation periods (Fig. 21.2). Consequently, this species shows high physiological plasticity indicated by the different starvation periods in various populations studied.

21.1.1.2 Echinoderms

Dietary carbohydrates reduce glucose tolerance and prolong hyperglycemia in the sea cucumber *Apostichopus japonicus* (Xia et al. 2015), indicated by elevated glucose level in the celomic fluid of individuals fed the highest carbohydrate level, and in the activity decline of metabolic enzymes (pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase). A ~50% dietary carbohydrate levels are optimal for *A. japonicus*. Furthermore, in this herbivorous/detritivorous echinoderm, cornstarch proves to be superior to other starch source, such as potato or tapioca starch (Wen et al. 2017), and can replace dietary *Gracilaria lemaneiformis* (red alga), *Ulva lactuca* (sea lettuce), and *Sargassum muticum* (Japanese wireweed) (Wen et al. 2018).

Also in sea urchins, starch serves as a suitable energy source. Cuesta-Gomez and Sánchez-Saavedra (2017) fed purple sea urchins (*Strongylocentrotus purpuratus*) diets that contained 30→17% protein and 42→58% cornstarch. A diet containing ~20% protein and ~55% carbohydrates benefits this farmed species best. Similar results apply to cultured green sea urchin (*Lytechinus variegatus*) (Heflin et al. 2016). Regardless of diet combination, individuals maintain an average dietary protein intake of ~0.05–0.06 g day⁻¹, whereas dietary carbohydrate intake ranges from 0.04 to 0.14 g day⁻¹. This indicates that large adult *L. variegatus* in aquaculture have a tightly regulated intake target for protein, but not for carbohydrate (Fig. 21.3), confirming the general validity “nutrient-specific foraging” hypothesis (Mayntz et al. 2005) (→Chap. 2) even for omnivores.

21.1.1.3 Crustaceans

Crustaceans exhibit a remarkable plasticity in their feeding habits and food type, but most knowledge on starch digestion and utilization comes from research of only a few species. In his review, Shiao (1998) summarized that practically all studied penaeid shrimps are able to utilize starch from various sources. Later, Wang et al. (2016b) specified the starch requirement for *Fenneropenaeus chinensis* as 26% and *Litopenaeus vannamei* as 20%. Several more farmed crustaceans have been tested in terms of dietary starch requirement and effects—with varying success as listed in Table 21.1. This varying success with apparently identical species is based, to a major extent, to the heterogeneity of applied methods, genetics of animal strains, developmental stages, and dietary (micro)-composition. The comparison of digestibility coefficients of *Artemesia longinaris* and *Pleoticus muelleri* characterizes the first shrimp as herbivorous and the second one as omnivorous (Fig. 21.4).

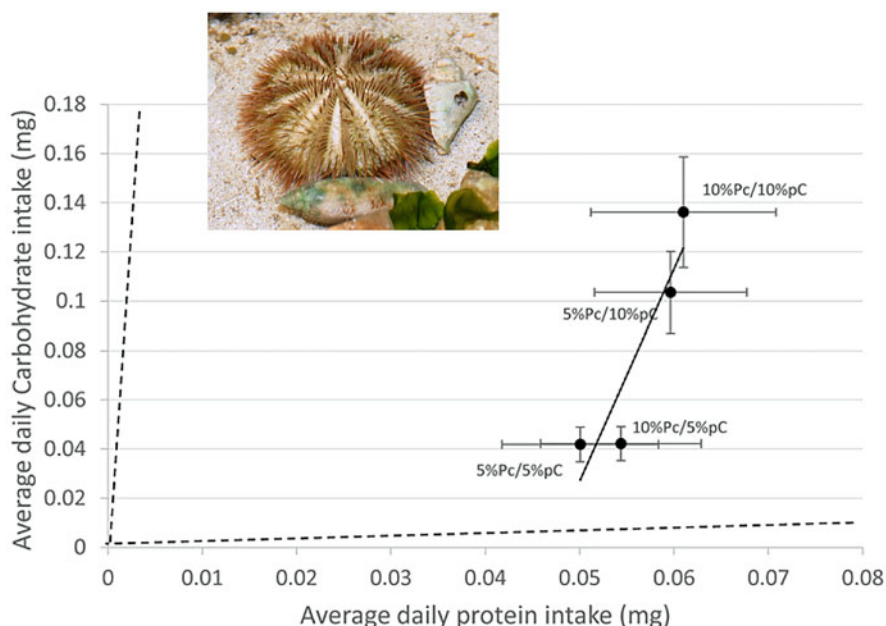


Fig. 21.3 Average daily macronutrient intake by diet combination (closed circles) of *Lytechinus variegatus*. Diet combinations are represented as diet 1/diet 2. As a code, P and C represent the predominant nutrient (protein or carbohydrate, respectively) in the diet, and p and c represent the lesser nutrient. Percentages (5 or 10%) represent the feed concentration (% dry matter) in the diet. Horizontal error bars represent dietary protein (SEM). Vertical error bars represent dietary carbohydrate (SEM). Dashed lines indicate nutrient rails as fed in this study. The solid line indicates data trend line. (From Heflin et al. (2016), with permission from Elsevier; image credit Hans Hillewaert, Wikimedia)

An instructive study was carried out by Johnston and Yellowless (1998). The author determined the digestive capabilities of the commercially important slipper (flathead) lobster *Thenus orientalis* by comparing the range and concentration of digestive enzymes produced with the preferred diet. *T. orientalis* is a specialized predator of bivalve mollusks. Detection of polysaccharase, α -amylase, glycosidases, maltase, and α -glucosidase, indicate, however, that *T. orientalis* is capable of completely hydrolyzing glycogen and starch to glucose. The greater α -glucosidase activity in *T. orientalis* compared with that of *Homarus americanus*, *Cancer borealis*, and *C. irroratus* and higher maltase activity compared with that of *Callinectes sapidus* indicate that the slipper lobster hydrolyzes starch and glycogen more efficiently than the other carnivorous decapods. This is attributed to the greater amount of glycogen ingested by slipper lobster due to high concentrations in adductor muscles of bivalves, unique to the diet of these lobsters.

Table 21.1 Effect of dietary starch in selected crustacean species

Crustacean species, common name	Starch source	Level, %	Affected trait	References
<i>Artemesia longinaris</i> , Argentine stiletto shrimp	Manioc	0→30	ADC 83.7→51.2 (→Fig. 21.4)	Velurtas et al. (2011)
<i>Farfantepenaeus duorarum</i> , Pink shrimp	Unspecified	0, 10, 40	Max WG, survival @ 40	Sick and Andrews (1973)
<i>Fenneropenaeus indicus</i> , Indian white prawn	Unspecified	30	Growth Mal, starch > monosaccharides, protein sparing	Boonyaratpalin (1998)
<i>F. merguensis</i> , Banana shrimp	Unspecified	30	Growth Mal, starch > monosaccharides, protein sparing	Boonyaratpalin (1998)
<i>Jasus edwardsii</i> , Spiny rock lobster	Various	0, 35	ADC Dex 99, CMC; ~94, wheat 91, gelat. corn 84, potato 60, corn 59	Simon (2009)
		0, 7, 17, 27	WG↓, survival↔	Simon and Jeffs (2011)
<i>Jasus lalandii</i> , South African Cape rock lobster	Marine plants	N.A.	Utilization of marine plants, indicated by amylase activity	Barkai et al. (1996)
<i>Litopenaeus setiferus</i> , Atlantic white shrimp	Unspecified	0, 30	WG↑, protein sparing	Andrews et al. (1972)
<i>L. stylirostris</i> , Western blue shrimp	Wheat	1→33	ES protein > carbohydrate	Rosas et al. (2000)
<i>L. vannamei</i> , Pacific white shrimp	Corn	10, 10, 30, 40	WG↔ (protein depending); max survival @ ~20	Bages and Sloane (1981)
	Corn	?	ADC↓	Lawrence et al. (1989)
	Various forms of corn	1, 10, 20	WG↔, amylase↑	Le Moullac et al. (1994)
	Various forms	35	ADC↔	Cousin et al. (1996)
	Wheat, corn, potato	20	Hypoosmotic stress resistance↑, optim. wheat	Wang et al. (2016c)
<i>Macrobrachium nipponense</i> , oriental river prawn	Corn	5→35	WG↑<15	Ding et al. (2017)
	Raw corn Gelat. Corn	18	WG↑, survival↔	Kong et al. (2019)
<i>M. rosenbergii</i> , Giant river prawn	α-potato starch	34→64	WG↑	Gabriel et al. (1988)

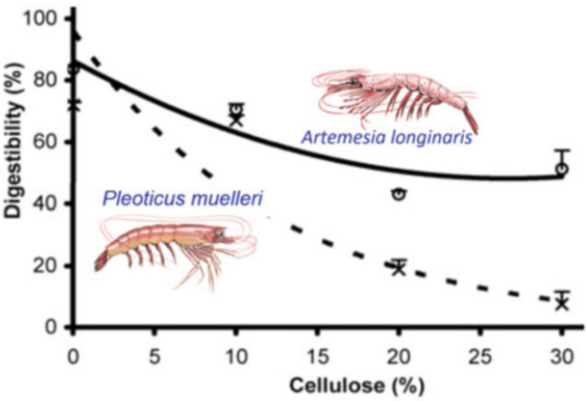
(continued)

Table 21.1 (continued)

Crustacean species, common name	Starch source	Level, %	Affected trait	References
	α-potato starch, soluble starch	40	WG both starches >Dex, Suc > Glc, Glg	Gomez Díaz and Nakagawa (1990)
	Corn	0, 15, 30	WG↔, survival↔	Querijero et al. (1997)
<i>Marsupenaeus japonicus</i> , Kuruma shrimp	Potato	10	FE starch > Glg > Suc > Dex >> Glc	Deshimaru and Yone (1978)
<i>Panulirus argus</i> , Caribbean spiny lobster	Corn, potato, rice	20	ADC rice, potato>corn	Rodríguez-Viera et al. (2014)
<i>Penaeus monodon</i> , black tiger prawn	Wheat, potato, corn	20	ADC wheat > potato, corn	Niu et al. (2012)
<i>Pleoticus muelleri</i> , Argentine red shrimp	Manioc	0→30	ADC 71.9→7.6 (→Fig. 21.4)	Velurtas et al. (2011)
<i>Procambarus clarkii</i> , Red swamp crayfish	Corn	5→35	Max WG @ 20	Xiao et al. (2014)
<i>Scylla serrata</i> , Mud crab	Various	15→60	ADC: Corn > wheat > potato, rice	Truong et al. (2008)

ADC apparent digestibility coefficient, %, *Mal* maltose, *Dex* dextrin, *Glc* glucose, *Glg* glycogen, *Suc* sucrose, *CMC* carboxymethyl cellulose, *WG* weight gain, *ES* energy source, *FE* feed efficiency, ↑ increase/promotion, ↔ no apparent effect, ↓ decrease/reduction

Fig. 21.4 Apparent protein digestibility at four levels of dietary starch (30→0)/cellulose (0→30) in *Artemesia longinaris* and *Pleoticus muelleri*. Values are means of three observations. Error bars indicate SD. (From Velurtas et al. (2011), credit Latin American Journal of Aquatic Research; images credit Biodiversity Heritage Library)



21.1.2 Fishes

Although some fish species have evolved symbioses with microbes that digest structural polysaccharides, many fishes do not rely on microbial endosymbionts to

Table 21.2 Summarized digestive efficiencies (ratio of energy or nutrient assimilated to energy or nutrient ingested) of soluble carbohydrates (starch) in fishes of different trophic habits consuming artificial feeds

Species name, common name	Trophic habit	Digestive efficiency, %
<i>Cyprinus carpio</i> , common carp	Omnivorous/benthivorous	77–96
<i>Dicentrarchus labrax</i> , European sea bass	Carnivorous	66–98
<i>Ictalurus punctatus</i> , channel catfish	Carnivorous/omnivorous	59–78
<i>Oncorhynchus mykiss</i> , rainbow trout	Carnivorous	16–80
<i>Oreochromis niloticus</i> , Nile tilapia	Omnivorous/detritivorous	83–96
<i>Pagrus major</i> , Red Sea bream	Carnivorous	66–82

From German (2011), with permission from Elsevier

do so; the fishes largely ignore the recalcitrant polysaccharides in their diet and digest only the soluble components of their food through the action of endogenous digestive enzymes (German 2011). Fishes consuming different foods will encounter the macronutrients in different concentrations (e.g., carnivores will consume more proteins and lipids, and herbivores more carbohydrates → “Nutrient-Specific Foraging,” Chap. 2) and, thus, will use different nutrients as fuel sources and exhibit differences in digestive efficiencies of these nutrients (Table 21.2). However, this claim does not adequately consider the potential of appropriate probiotic supplementation to improve the utilization of complex carbohydrates and the production of beneficial metabolites, such as short-chain fatty acids (SCFAs) (→Chap. 26).

The issue of prebiotic and probiotic supplementation deserves increased attention, since even prebiotics-like compounds can improve carbohydrate utilization. Chromium (Cr) enhances carbohydrate utilization in terrestrial animals, presumably via insulin production. Cr acts as a cofactor for the interaction of insulin with specific membrane receptor sites and thereby synergistically activates insulin-sensitive metabolic pathways like carbohydrate metabolism and works with insulin to move glucose into cells (Watanabe et al. 1997). In fact, Wang et al. (2019) showed that an appropriate Cr polynicotinate dose improves growth and feed utilization even of carnivorous golden pompano (*Trachinotus ovatus*) juveniles on starch as dietary carbohydrate. Excess dietary Cr, however, turns out to be toxic.

Carnivorous fishes have lower digestive efficiencies of starch than omnivorous, detritivorous, or herbivorous species. This is exemplified by comparing herbivorous grass carp (*Ctenopharyngodon idella*) and carnivorous Chinese longsnout catfish (*Tachysurus dumerili* (*Leiocassis longirostris*)) orally administered with starch (Su et al. 2020). Grass carps exhibit a rapid increase and faster clearance rate of plasma glucose than Chinese longsnout catfish. This effect is closely related to enhanced levels of digestion, glycolysis, glycogen metabolism, and glucose-induced lipogenesis in grass carp, as well as the inhibition of gluconeogenesis (Fig. 21.5) (→Chap. 14).

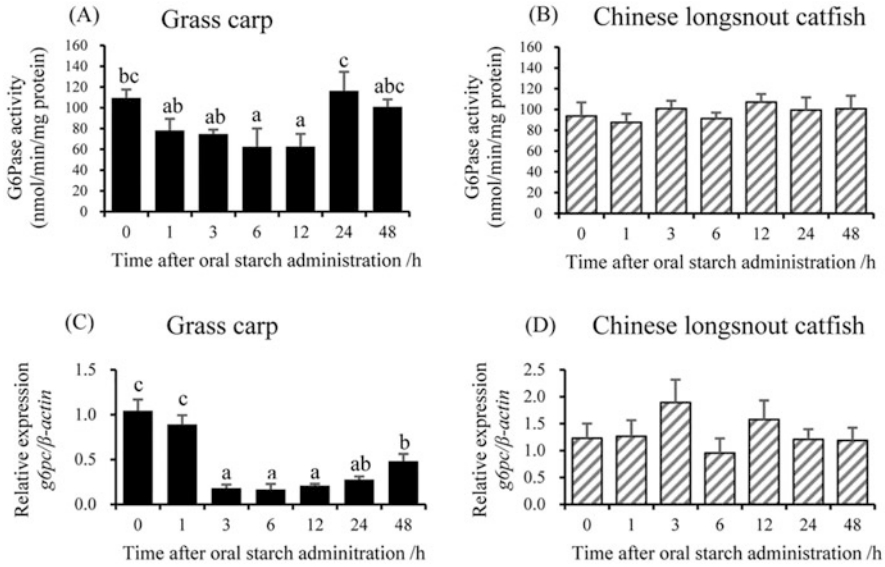


Fig. 21.5 The enzymic activity and expression levels of hepatic gluconeogenesis in grass carp and Chinese longsnout catfish following the oral administration of starch. The enzymic activity of glucose 6-phosphatase (G6Pase) in (a) grass carp and (b) Chinese longsnout catfish; the expression levels of *g6pc* in (c) grass carp and (d) Chinese longsnout catfish. Each column represents the mean of six replicates. Different superscripts indicate significant differences ($P < 0.05$). (From Su et al. (2020), with permission from the Cambridge University Press)

21.1.2.1 Carnivores

To explain variations in estimates of starch digestibility and predict its digestible contents of salmonid fish feeds, Hua and Bureau (2009) developed a mathematical model, based on dietary inclusion levels of different types of starch (raw and gelatinized starch) from studies conducted with salmonids fed near satiety or in excess:

$$\begin{aligned} \text{Digestible starch content (\% of diet)} = & 0.560 \text{ raw starch} - 0.011 \text{ raw starch}^2 \\ & + 0.904 \text{ gelatinized starch} - 0.009 \text{ gelatinized starch}^2 + 0.016 \text{ temperature} \\ & \times \text{raw starch} + 0.009 \text{ temperature} \times \text{gelatinized starch} - 0.270 \text{ fish species} \\ & \times \text{raw starch} - 0.068 \times \text{fish species} \times \text{gelatinized starch} - 0.158 \\ & \times \text{feces collection method} \times \text{gelatinized starch.} \end{aligned}$$

In the equation, fish species = 0 is for rainbow trout, 1 for Atlantic salmon, and feces collection method = 0 for passive collection, 1 for active collection (dissect or stripping). Dietary lipid and water salinity are not identified as significant determining factors. The model provides quantitative estimate of the effect of increasing

starch concentration in diets on digestibility of starch. The model could be validated: A highly significant linear relationship is observed between predicted digestible starch content and observed values for an independent data set conducted with salmonids fishes (Hua and Bureau 2009).

Box: Raw Versus Gelatinized Starch

For many fish species, gelatinized starch is significantly more digestible than raw starch (Hua and Bureau 2009). For example, apparent digestibility coefficients (ADCs) of raw cornstarch fed to rainbow trout range from 30 to 40% while those for gelatinized cornstarch lie between 87 and 90% (Bergot and Breque 1983). The statistical model confirms that the physicochemical state (raw vs. gelatinized) of the starch is a key determinant of starch digestibility in salmonids. The digestibility values estimated by the model are 56% for raw starch (average across starches of different plant origins) and 90% for gelatinized starch at low inclusion levels (Hua and Bureau 2009). Therefore, it appears very reasonable to check whether this model can be translated to other fish groups or even be generalized.

The high nutritional value of gelatinized starch applies also to cyprinids, such as *Labeo rohita* fry (Mohapatra et al. 2003) or juveniles (Kumar et al. 2006) and fingerlings of *Gibelion (Catla) catla* (Yengkokpam et al. 2007). A preference of gelatinized over raw starch is also reported for juvenile striped jack, yellowtail (Takeuchi et al. 1992), or juvenile silver perch (Stone et al. (2003a, b, c)).

Noteworthy, in a later study, however, Kumar et al. (2015) found the opposite in *L. rohita* fingerlings: Dietary gelatinized starch causes stress response leading to decreased antioxidant capacity, depressed immunity, and reduced resistance to *Aeromonas hydrophila* infection. The opposite applies to dietary raw starch. Experimental flaws cannot be excluded. Similarly, Peres and Oliva-Teles (2002) showed that the total replacement of raw by gelatinized starch in feed for European sea bass juveniles reduces growth and feed intake, without affecting protein or energy retentions. The carnivorous *Acanthopagrus latus* behaves similarly (Wu et al. 2007). Also, *Hemibagrus nemurus* prefers raw over cooked potato starch (Hamid et al. 2011). A convincing explanation for the inconsistent and even contrasting observations is lacking.

One critical aspect has to be considered: With increasing dietary starch, often a decrease in protein digestibility goes along as observed in gilthead sea bream (Couto et al. 2012), European sea bass (Enes et al. 2006), white sea bream (Sá et al. 2007), and rainbow trout (Storebakken et al. 1998). Couto et al. (2012) listed potential mechanisms: One explanation for this reduced digestibility is the adsorption of proteolytic enzymes on starch molecules, thus rendering them unavailable for enzymatic action and nutrient provision (Spannhof and Plantikow 1983). In fact,

Storebakken et al. (1998) observed reduced protein digestibility associated with dietary glucose and mention that monosaccharides may inhibit the amino acid (AA) transport in the intestine (Ferraris and Ahearn 1984; Vinardell 1990). Increased glucose release in high-starch diets can be another explanation for the decreased ADC of protein. Multi-omic studies warrant future attention to identify underlying modes of action.

It has been hypothesized that the inclusion of different carbohydrate classes is one key driver of nonadditive effects in digestibility studies and that the different fiber classes are one of the possible factors that causes nutrient interactions resulting in loss of additivity (Glencross et al. 2007). Therefore, Glencross et al. (2012b) compared the effect of increasing inclusion levels of various polysaccharides on dry matter, protein, and energy digestibility of diets fed to rainbow trout (Fig. 21.6). The different polysaccharides comprise gelatinized starch, cellulose, liginosulfonate, pectin, and mannan. There are clear differences among the digestibility parameters: Cellulose addition results in a reduction in both dry matter and energy that is largely commensurate with its inclusion level, but its effect on protein digestibility is marginal. Starch has the least effect on any of the digestibility parameters of all examined polysaccharides. At low inclusion levels, liginosulfonate has the greatest impact on all digestibility parameters, particularly on protein digestibility. This study shows that although this species can digest gelatinized starch, it has almost no ability to digest any of the nonstarch polysaccharides (NSPs) tested (Fig. 21.6) (Glencross et al. 2012b).

Studying high dietary starch (HSD) inclusion as protein- or lipid-sparing approach, García-Meilán et al. (2020) found digestive plasticity in gilthead sea bream. When lipid is replaced with starch in 46% protein diets, compensatory mechanisms involving changes in intestinal pH, pancreatic and intestinal digestive enzymes, and nutrient absorption capacities take place, allowing maintenance of feed utilization and growth and showing that animals are able to modulate the digestive process when protein requirements are met. In contrast, when dietary protein is replaced with starch in 17% lipid diets, the digestive compensatory mechanisms are not sufficient, and increase in food conversion ratio (FCR) and reduction in voluntary feed intake and specific growth rate (SGR) occur.

Further effects of dietary starch, raw or gelatinized, are compiled in Table 21.3. Data of salmonids are excluded from this table since they are the basis of the model by Hua and Bureau (2009) presented above. Again, it becomes obvious that in omnivorous and herbivorous fishes, dietary starch plays a more significant nutritional role than in carnivorous ones. Hence, the latter have been the subject of most dietary starch studies (Kamalam et al. 2017).

Although metabolic pathways are shared among carnivorous fish, results from hepatic enzyme assays, gene expression, and signaling cascades, as well as lipogenic and metabolic flux analysis, support the notion that there is significant diversity in the underlying regulation of dietary nutrient assimilation in different fish species. To overcome limitations of molecular-based studies that select a single post-feeding time point to draw conclusions, Wade et al. (2020) used metabolic tracers to track dietary nutrient assimilation over several days of feeding. The authors demonstrate

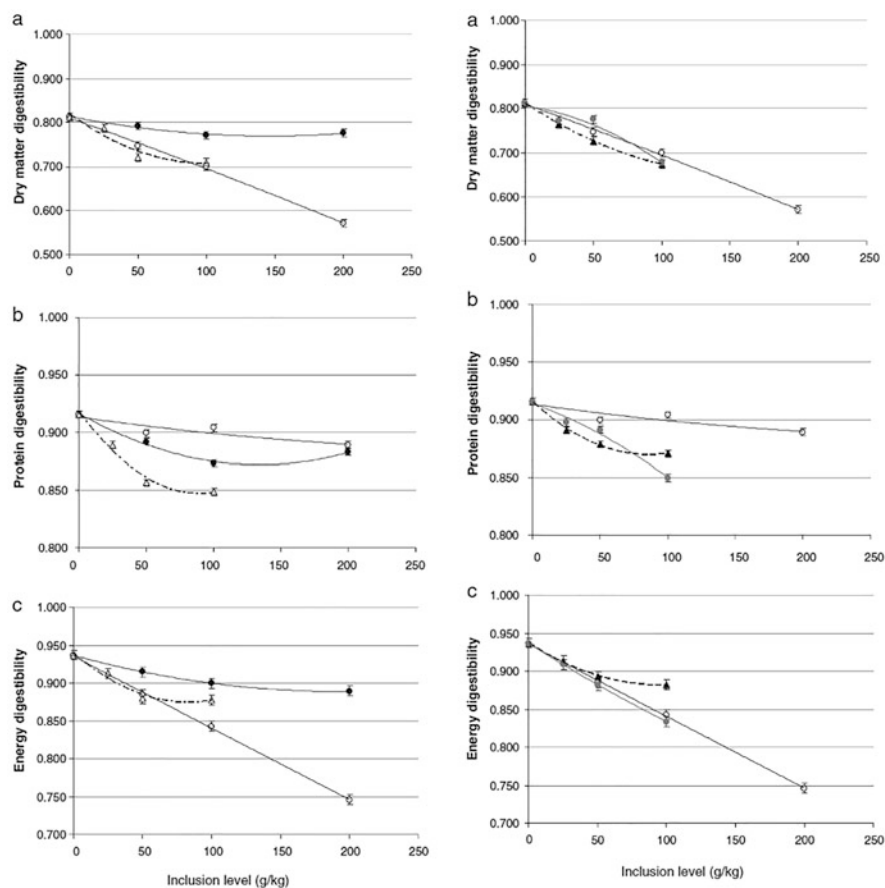


Fig. 21.6 Effect of dietary polysaccharide level (g kg^{-1}) on digestibility coefficient of dry matter (a), nitrogen (b), and energy (c) of each of the experiment diets of rainbow trout. **Left column** cellulose ○; starch ●; lignosulfonate Δ. **Right column** cellulose ○; pectin ▲; mannan ●. (From Glencross et al. (2012b), with permission from Elsevier)

that barramundi on dietary starch utilizes a unique series of specific hepatic regulatory mechanisms to assimilate and store excess dietary carbohydrate energy in the form of lipids to the detriment of protein utilization and growth.

21.1.2.2 Omnivores and Herbivores

The ability of utilizing starch differs between omnivores. One comparative study has been conducted by Gominho-Rosa et al. (2015) (Fig. 21.7a): Nile tilapia is more adapted to utilize and digest starch than jundiá catfish (*Rhamdia quelen*), regardless of the plant source.

Table 21.3 Effect studies of dietary starch in selected fish species of different trophic levels

<i>Species</i> , common name, apparent trophic habit	Starch source and level, %	Effect	References
<i>Acanthopagrus latus</i> , Yellowfin Sea bream, c	Corn, tapioca, potato, 20, raw or gelatin	Raw: WG↔, SGR↔ Gelatin: WG↓, SGR↓	Wu et al. (2007)
<i>Anguilla anguilla</i> , European eel, c	Corn, 20→50, gelatin	Optim WG @ 30	Suárez et al. (2002)
	Wheat meal 20→56	Protein sparing	Degani and Viola (1987)
	N-free extract, 15→42	Protein sparing	Heinsbroek et al. (2007)
<i>Anguilla japonica</i> , Japanese eel, c	Potato, 23	Ovulation problems in farmed females	Higuchi et al. (2019)
<i>Arapaima gigas</i> , arapaima, c	Corn, 2.7→32	ADC protein: Starch>brans ADC dry matter: Starch>brans	dos Santos-Cipriano et al. (2015)
<i>Argyrosomus japonicus</i> , Japanese meagre, c	Corn, 0→30	Optim WG, FCR, PER @ 12–18	Li et al. (2015)
	Gelatin corn, 4→25	WG↑<16.4, protein sparing	Mabasa et al. (2018)
<i>Atractosteus tropicus</i> , tropical gar, c	Corn, 0, 7.5, 15	Growth↑, survival↑, cannibalism↓	Frias-Quintana et al. (2016)
<i>Barbonymus gonionotus</i> ♀ × <i>Hypsibarbus wetmorei</i> ♂, Hybrid lemon fin barb, o	Tapioca, 20, 25, 30, 35, 40	WG↓ @ >35; Optim WG, FE and SCFAs @ 33.5 (→Fig. 21.7b)	Sulaiman et al. (2020)
<i>Bidyanus bidyanus</i> , Silver perch, o	Wheat, 15→60 Raw or increasingly gelatin	Optim growth @ 30 Growth↓ @ ≥45 Gelatinization improved growth α-amylase addition↔ Protein sparing	Stone et al. (2003a, b, c)
<i>Caranx vinctus</i> , Cocinero, c	Potato, 10 α- or β-starch 5 + 5 mixture	Growth: α-starch> > β-starch Growth↑ @ gelatinization↑	Takeuchi et al. (1992)
<i>Carassius gibelio</i> , Prussian (gibel) carp, o	Corn, 6→38	≤30: SGR↑, FE↑, PRE↑ >30: SGR↓, FE↓, PRE↓	Li et al. (2016b)
	Corn, 24→40	Growth↓, FE↓ @ ≥32	Tan et al. (2009)
<i>Cirrhinus mrigala</i> , Mrigal carp, o	Unspecified, 30, 40, 50	WG↔, SGR↔, FCR↔	Aderolu and Sahu (2015)
<i>Clarias batrachus</i> , Philippine catfish, o	Unspecified, 15–45	<35: Growth↑, myogenesis↑; >35: Growth↓, lipogenesis↑	Talukdar et al. (2019)
<i>C. gariepinus</i> , North African catfish, c	Corn, 6→30	Optim WG, PER, FCR @ ~2.5	Ali and Jauncey (2004)

(continued)

Table 21.3 (continued)

<i>Species</i> , common name, apparent trophic habit	Starch source and level, %	Effect	References
	Corn, 0, 25, gelatin	WG↔	Romano et al. (2018)
<i>Cromileptes altivelis</i> , Hump-back grouper, c	Corn, tapioca, 10, 20	WG (↑) @20, protein sparing	Shapawi et al. (2011)
<i>Ctenopharyngodon idella</i> , Grass carp, h (o)	Wheat, 20–47	Optim growth @ 33 >33→hepatic fat deposition	Tian et al. (2012)
	Corn, 6–38	SGR↑, FE↑, PRE↑	Li et al. (2016b)
	Wheat, 17–26	Optim growth @ P34, C17 >17→hepatic fat deposition	Chen et al. (2012)
<i>Cyprinus carpio</i> , Common carp, o	Unspecified, 25, 50	PER↑, liver damage @ 50	Li et al. (2016a)
	Corn, 0→38	Optim WG, PER, FCR @ 38	In Kamalam et al. (2017)
<i>Dentex dentex</i> , Common dentex, c	Unspecified, gelatin, 12, 18	Protein sparing <24, optim FE < 18	Pérez-Jiménez et al. (2015)
<i>Dicentrarchus labrax</i> , European sea bass, c	Unspecified, 14→31, precooked	Optim WG, FCR @ 25	Pérez et al. (1997)
	Corn, 0→25	Hepatic glycogen↑	Russell et al. (2001)
	Unspecified, 25 raw or gelatin; 12.5 raw + 12.5 gelatin	Replacement of raw by gelatinized: growth↓, feed intake↓ Protein, energy retentions↔	Peres and Oliva-Teles (2002)
	Pea, 0.2, 17.8	Gluconeogenesis↓, de novo lipogenesis in muscle↑	Viegas et al. (2015)
	Pea, 33 digestible or raw	Hepatic adiposity↑	Viegas et al. (2016)
	Wheat, corn, rye, barley, 30	Younger individuals: growth↔ Older individuals: rye PD↓	Couto et al. (2017)
<i>Diplodus cervinus</i> , Zebra Sea bream, c	Corn, 9→44	Energy utilization P > C Optim growth P:C 45:21	Coutinho et al. (2016)
<i>D. sargus</i> , White Sea bream, o	Corn, 35 gelatin	Growth↔ independent of feeding frequency	Enes et al. (2015)
	Normal or waxy corn, 26, 42	WG↔ ≥42, normal>waxy	Sá et al. (2008)
<i>Epinephelus akaara</i> , Hong Kong grouper, c	Corn, 0→30	Max WG @ 7.6, no protein sparing	Wang et al. (2016a)
<i>E. lanceolatus</i> ♂ × <i>E. fuscoguttatus</i> ♀, Hybrid grouper, c	Corn, 0, 7, 14, 21, 28	Hepatic glycogen↑, protein sparing @ 7–28	Luo et al. (2016)

(continued)

Table 21.3 (continued)

Species, common name, apparent trophic habit	Starch source and level, %	Effect	References
<i>Epinephelus malabaricus</i> , Malabar grouper, c	Corn, 14, 20, 25	WG↔, protein sparing	Shiau and Lin (2001)
<i>Gadus morhua</i> , Atlantic cod, c	Potato, 0–30	Growth↔, protein retention↔, fat retention↔	Hemre et al. (1989)
<i>Gibelion (Catla) catla</i> , Catla, o	Corn, rice, tapioca, 40, 50 gelatin	Mortality↔, hepatocyte hypertrophy↑, body lipid accumulation↑; nutrient utilization @ 50: Corn>tapioca>rice	Yengkokpam et al. (2005, 2007)
<i>Hemibagrus nemurus</i> , Asian redbtail catfish, c	Corn, 14 raw or gelatin	WG: raw>gelatin	Hamid et al. (2011)
<i>Labeo rohita</i> , Roho labeo, o	Unspecified, 26→47 gelatin	WG↑, PER↑ @ ≤45	Mohapatra et al. (2003)
	Corn, 0→100, raw or gelatin	Raw: amino acid catabolism↑, gluconeogenesis↑, TCA cycle↑ Gelat.: Glycolysis	Kumar et al. (2010)
	Unspecified, 50 raw or gelatin	Raw: immunity↑ Gelatinized: immunity↓ temperature dependent Increased utilization of raw starch @ temperature↑	Kumar et al. (2015, 2016)
	Corn, 40→58 gelatin	n-3 PUFA↓	Ciji et al. (2013)
	Unspecified, 30→45, gelatin	Growth↔, immunity↑	Anand et al. (2018)
<i>Lophiosilurus alexandri</i> , Pacman catfish, c	Corn bran, 5→25	Best WG @ 10	Oliveira et al. (2020)
<i>Larimichthys crocea</i> , large yellow croaker, c	Corn, 2→32	Optim WG @ 19	Cheng et al. (2013)
<i>Lateolabrax japonicus</i> , Japanese sea bass, c	Corn, 0, 6, 12, 18, 24, 30	WG↑ @ 18–27	Zheng et al. (2015)
<i>Lates calcarifer</i> , Barramundi, c	Various, 49.1→93.9	Amylopectin↑→starch digestibility↓	Glencross et al. (2012a)
	Unspecified, 1.6, 32.5	De novo lipogenesis in visceral adipose tissue↑	Viegas et al. (2019)
	Pregelatinized wheat starch, 20	Hepatic lipogenesis↑	Palma et al. (2020); Wade et al. (2020)
<i>Megalobrama amblycephala</i> , Wuchang (blunt snout) bream, o	Cassava, 0→45	Immunity↑ ≤31	Zhou et al. (2013)
	Wheat, 100 raw, 100 gelatin, or 75 raw+25 gelat	Glucose transportation↑, glycolysis↑, lipogenesis↑, lipid deposition↑, glucose tolerance↑, gluconeogenesis↓, FA oxidation↓	Zhang et al. (2020a)

(continued)

Table 21.3 (continued)

<i>Species</i> , common name, apparent trophic habit	Starch source and level, %	Effect	References
<i>Micropterus salmoides</i> , largemouth black bass, c	Wheat, cassava, pea, high-amylose corn, 10	Optim growth with pea and corn	Song et al. (2018)
	Corn, 0→25	SGR↓, PER↓, no protein sparing	Ma et al. (2019)
<i>Morone chrysops</i> ♀ × <i>M. saxatilis</i> ♂, Sunshine bass, c (p)	Corn, 0→25	Growth: amylose>amyopectin	Rawles and Lochmann (2003)
<i>Mylopharyngodon piceus</i> , Black carp, c	α-starch, 20→40	Growth↓, FCR↔, PER↓ 40: Damage of hepatic structures	Miao et al. (2016)
<i>Oreochromis niloticus</i> , Nile tilapia, o	Unspecified, 16.6, 26.8: protein replacement by starch	Hepatic fat deposition↑	Xiong et al. (2014)
	Corn, 16→40	25% protein sparing possible	Azaza et al. (2015)
	Corn, tapioca, 25 raw or gelatin	SCFA↓	Kanmani et al. (2018)
	Unspecified, 10–40, gelatin	FI (↑), intake of digestible energy↓	Tran-Duy et al. (2008)
	Wheat bran, cassava residue, ground corn, broken rice	Amylase (↑), maltase (↑) (also see Fig. 21.7)	Gominho-Rosa et al. (2015)
<i>O. niloticus</i> GIFT, o	Corn, 2→32, gelatin	WG, SGR, FE, PER ↑<19	Chen et al. (2020)
	Lipid 5.6→12 Starch 22.5→13.8	Glucose homeostasis↓	Du et al. (2020)
<i>O. mossambicus</i> × <i>O. niloticus</i> , Hybrid red tilapia, o	<i>Sorghum</i> , 15→35	WG, SGR, PER, NPU↑ <30	Yones and Metwalli (2016)
<i>O. niloticus</i> × <i>O. aureus</i> , Hybrid tilapia, o	Corn, 6→46	Optim WG, SGR, FE, PER @ ≥22	Wang et al. (2005)
	Corn, 33, 37, 41	WG↔, protein sparing	Shiau and Peng (1993)
<i>Pangasianodon hypophthalmus</i> , Striped catfish o	Cassava, 0→63	Optim protein sparing @ 10	Hung et al. (2003)
<i>Pangasius bocourti</i> , Basa catfish, h		Optim protein sparing @ 20	
<i>Rachycentron canadum</i> , Cobia, c	Corn, 0→30, gelatin	Optim SGR @ ~20	Ren et al. (2011)

(continued)

Table 21.3 (continued)

<i>Species</i> , common name, apparent trophic habit	Starch source and level, %	Effect	References
<i>Rhamdia quelen</i> , South American catfish, o	Wheat bran, cassava residue, ground corn, broken rice	Amylase↑, maltase↑ (also see Fig. 21.7)	Gominho-Rosa et al. (2015)
<i>Salminus brasiliensis</i> , Dorado, c	Corn, rice, 0→30	WG↑ <14.5	Moro et al. (2016)
<i>Sebastes schlegelii</i> , Korean rockfish, c	α-, β-starch, 6→30	Optim WG @ 18	Lee and Kim (2009)
<i>Seriola lalandii</i> , Yellowtail amberjack, c	Gelatin wheat starch, 10→40	WG↔, protein sparing @ < 10	Booth et al. (2013)
<i>S. quinqueriata</i> , Japanese amberjack, c	Potato, gelatin, or raw, 10; mixture 5 + 5	Growth: α-starch > β-starch Growth↑ @ gelatinization↑	Takeuchi et al. (1992)
<i>Silurus meridionalis</i> , Chinese largemouth catfish, c	Corn, 15, 30, precooked	WG ↔ ≤15	Fu (2005)
<i>Solea senegalensis</i> , Senegalese sole, c	Pea, 18–27	WG↔, FCR↓	Conde-Sieira et al. (2016)
<i>Sparus aurata</i> , Gilthead Sea bream, c	Corn, 5→26, gelatin	Protein sparing ≤20	Fernández et al. (2007)
	Wheat, 20, 40	PRE↑↔, depending on feeding time	Felip et al. (2015)
	Pea, 13.5, 32	Growth↔, apparent digestibility↔, FE↔	Rocha et al. (2016)
	Wheat, 5.4→23.6	Glycogenesis↑	Ekmann et al. (2013)
<i>Tachysurus dumerili</i> (formerly <i>Leiocassis longirostris</i>), Chinese longsnout catfish, c	α-starch, 6	WG Dex > C	Tan et al. (2006)
	Corn, α-starch, 6→27	Optim WG @ 17	Tan et al. (2007)
<i>Takifugu obscurus</i> , Obscure pufferfish, o	Corn, 10→30, gelatin	Optim WG, PER, FCR @ ~23	Liu et al. (2015)
<i>Trachinotus ovatus</i> , Pompano, c	Corn, 0→28 raw	Optim WG, PER, FCR @ 12–17	Zhou et al. (2015)
	Corn, 0→25 raw	Optim WG, health @ 25	Zhao et al. (2020)

c carnivorous, o omnivorous, h herbivorous, p piscivorous, *gelatin* gelatinized, *optim* optimum, ↑ increase, ↓ reduction/impairment, ↔ no apparent effect, *gelat* gelatinized, *ADC* apparent digestibility coefficient, *WG* weight gain, *SGR* specific growth rate, *FA* fatty acid, *FE* feed efficiency, *FI* feed intake, *FCR* food conversion ratio, *Dex* dextrin, *P* protein, *C* starch, *Glc* glucose, *PER* protein efficiency ratio, *NPU* net protein utilization, *PD* protein digestibility, *PRE* protein retention efficiency, *SCFAs* short-chain fatty acids

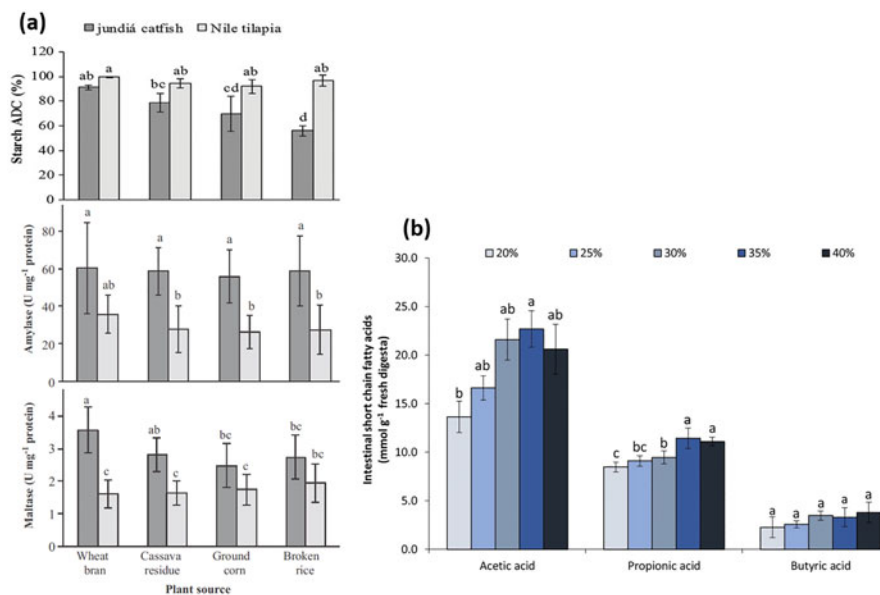


Fig. 21.7 (a) Apparent digestibility coefficients (ADC) of starch (mean \pm SD, $n = 3$) from different plant sources in jundiá catfish and Nile tilapia juveniles and activities of amylase and maltase (mean \pm SD, $n = 10$) in the gut of juveniles. Different letters indicate significant differences (Tukey test, $P < 0.05$). (From Gominho-Rosa et al. (2015), with permission from Elsevier). **(b)** Intestinal short-chain fatty acids in hybrid lemon fin barb fed increasing level of tapioca starch. Different letters within each SCFA group indicate significant differences ($P < 0.05$) among the dietary treatments. (From Sulaiman et al. (2020), credit Elsevier)

Dietary starch can support the microbial production of SCFAs (Titus and Ahearn 1988; Smith et al. 1996). These by-products from microbial fermentation within the intestine are beneficial not only as energy source but also as biological control of pathogenic microbes (Leenhouders et al. 2008) (\rightarrow Chap. 26). In hybrid lemon fin barb, a tropical carp hybrid produced by crossing female silver barb (*Barbonymus gonionotus*) with male lemon fin barb (*Hypsibarbus wetmorei*), the most abundant intestinal SCFA is acetic acid, followed by propionic acid and butyric acid (Fig. 21.7b) (Sulaiman et al. 2020). The maximum amount of intestinal acetic acid is observed in fishes fed 35% carbohydrate. However, increasing carbohydrate contents lead also to elevated propionic acid contents and reduced growth. Whether there is a mechanistic connection remains obscure.

Reporting starch effects on the structure of the intestinal microbiota, Zhao et al. (2020) showed in juvenile *Trachinotus ovatus* that 5% and 20% starch levels increase the abundance of *Photobacterium*, 10% and 20% starch levels elevate *Mycoplasma* numbers, and 15% starch level promotes *Vibrio*. It is well known that many species and strains of *Vibrio* and *Photobacterium* are pathogenic (Holben et al. 2002; Liu et al. 2016). These pathogens are present in the intestines, and 5%–20% starch levels may favor the development of these pathogens and increase the

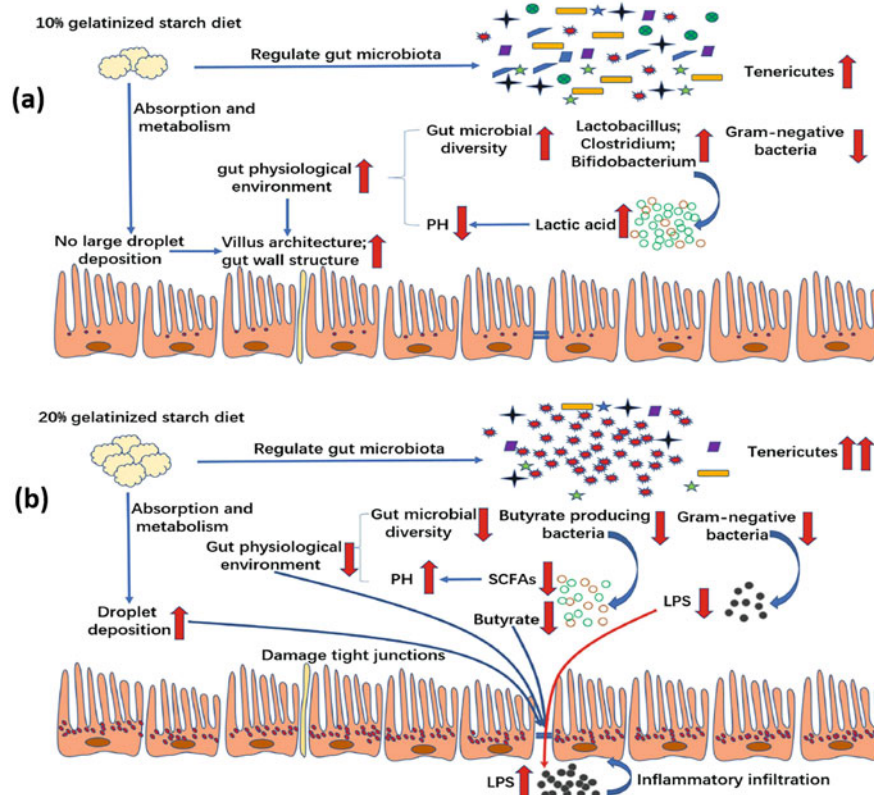


Fig. 21.8 Mechanism by which different contents of gelatinized starch diet influence the gut health in Chinese perch. **(a)** 10% gelatinized starch is beneficial to the structure of gut by changing gut microbiota diversity, beneficial bacteria quantity, and lactic acid content. **(b)** 20% gelatinized starch diets affect gut health by altering microbial composition and gut lipid metabolism in non-mammals with butyric acid playing a key role in that process. (From Zhang et al. (2020b), credit Frontiers Media)

risk of diseases in *T. ovatus*. However, the regulatory mechanism of starch on intestinal microflora composition remains to be elucidated in detail. Particularly, the fact that medium levels of starch appear to be more risky than the highest level tested needs verification and mechanistic explanation.

Simultaneously, Zhang et al. (2020b) reported that gelatinized dietary starch alters gut microbiota composition in Chinese perch. The addition of 10% starch is beneficial to gut microbiota diversity and quantity, lactic acid content, and microbial functionality (Fig. 21.8a). In contrast, the addition of 20% starch lowers the level of gut microbial diversity, and *Mycoplasma* becomes the dominant species. Butyric acid-producing bacteria and butyric acid level (→ Chap. 26) are significantly reduced (Fig. 21.8b). Furthermore, the gut permeability in this group is also increased due to the decreased mRNA expression levels of tight junction proteins caused by the

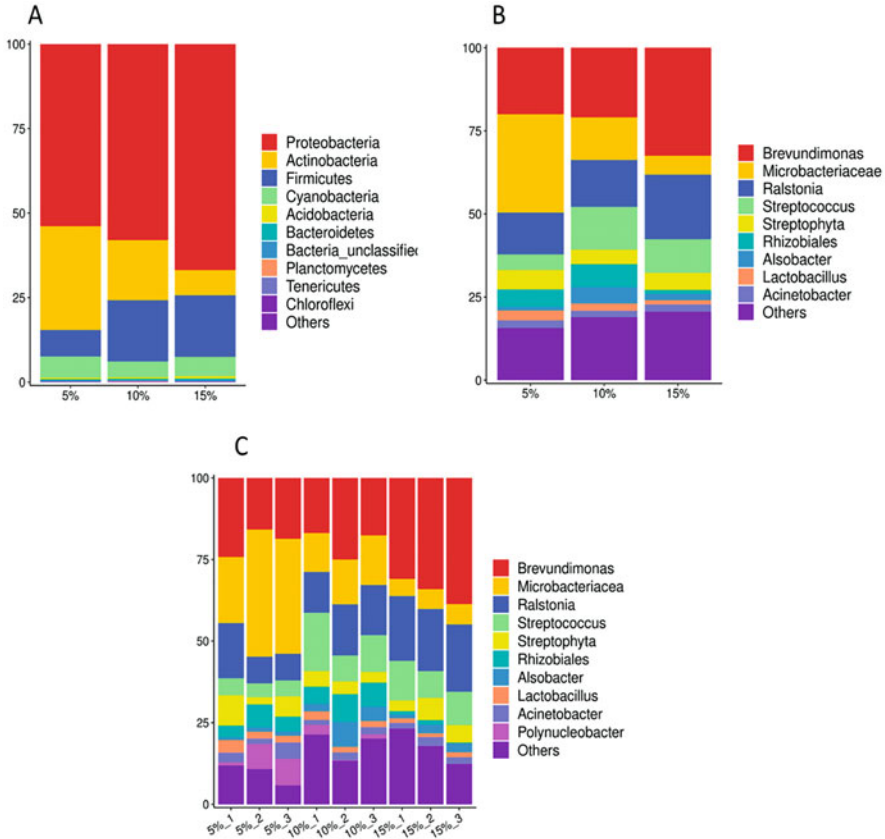


Fig. 21.9 Intestinal bacterial composition in three dietary groups at the phylum (a) and genus (b and c) levels. Only the top 10 most abundant (based on relative abundance) bacterial phyla and genera are shown. Other phyla genera are all assigned as “Others.” (From Zhou et al. (2021), with permission from Elsevier)

butyric acid deficiency and gut lipid droplets accumulation (→Chap. 24). Subsequently, lipopolysaccharides penetrate into the plasma and cause inflammation.

This finding is supported by a study in largemouth bass (*Micropterus salmoides*). Zhou et al. (2021) showed that high starch diets increase lipopolysaccharide and diamine oxidase indicating intestinal mucosal injury (O’Dwyer et al. 1988). Diamine oxidase is involved in the metabolism, oxidation, and inactivation of histamine and other polyamines (Seiler 2004). Furthermore, growth is closely related to intestinal health. HSD can increase intestinal permeability, which may lead to increased susceptibility to inflammation and decreased antioxidant capacity. Additionally, the HSD exacerbates the imbalance of intestinal microbes (Fig. 21.9). At the phylum level, *Proteobacteria* and *Actinobacteria* are dominant, followed by *Firmicutes* and *Cyanobacteria*. On 15% dietary starch, the relative abundance of beneficial

Proteobacteria and *Firmicutes* increases and that of *Actinobacteria* decreases. Consequently, the concentrations of beneficial SCFAs change.

21.1.3 Nutrigenomics

Applying RNA-seq technique, Xiong et al. (2014) evaluated the effects of different dietary protein to starch ratios on the hepatic gene expressions of Nile tilapia. Key pathways modulated by low ratios include glycolysis, gluconeogenesis, AA biosynthesis, fatty acid (FA) biosynthesis, and FA metabolism, resulting in increased plasma cholesterol, plasma triacylglycerol, liver lipid, and muscle lipid content. This profile indicates elevated fat deposition.

Also using next-generation transcriptomics, Zhang et al. (2019) tested the effects of dietary cornstarch on glycometabolism in carnivorous largemouth bass. Ca. 51,000 unigenes are obtained from the liver. High level of dietary cornstarch exhibits a strong inhibitory effect on the genes. The transcripts are categorized into 84 subcategories of three major categories: biological process, cellular component, and molecular function. Gene Ontology (GO) annotation shows that “biological process,” “cellular process,” “single-organism process,” and “metabolism process” are frequently represented. These results point out that liver and pancreas intensively participate in glucose and lipid metabolism. In “cellular component,” high frequencies are cell, cell part, and organelles; in “molecular function,” the binding, metabolism process, and catalytic activity appear frequently. The increase of dietary cornstarch is combined with severe alterations in liver histology and lipid accumulation.

Another Guangzhou laboratory, Zhang et al. (2020c) studied selected genes in juvenile largemouth bass fed graded starch diets. After 20 days, *hexokinase* (*hk*) transcription decreases in fishes fed dietary starch $<150 \text{ g kg}^{-1}$ (Fig. 21.10). *Insulin receptor 1* (*irs1*), *glucagon-like peptide-1 receptor* (not shown), and *glucose transporter2* (*glut2*) transcripts decrease with increasing dietary starch after 10 days of feeding (Fig. 21.10). Dietary starch levels are negatively correlated with the expression of key gluconeogenesis-related enzymes, including *glucose-6-phosphatase* (*g6pase*)—this trend becomes more evident with increasing time (Fig. 21.10). The major trend of energy-related genes, such as *hexokinase* (*hk*), *phosphoenolpyruvate carboxykinase* (*pepck*), and *citrate synthase* (*cs*), is also decreased transcription with increasing dietary starch content, whereas *carnitine ester acyl transferase* (*cpt-1*) transcription increases with increase dietary starch (Fig. 21.10). CPT-1 is located in outer membranes of mitochondria and catalyzes the conversion of FA CoAs into FA carnitine for entry into the mitochondrial matrix as substrate of β -oxidation (Kerner and Hoppel 2000). The enhanced β -oxidation in fishes on HSD can be considered as self-protection measure to decrease the lipid content in the liver. These results also indicate that glycolysis and gluconeogenesis are depressed, and the endocrine system is impaired in response to HSD. This indicates that glucose homeostasis is disturbed, eventually leading to glucose intolerance.

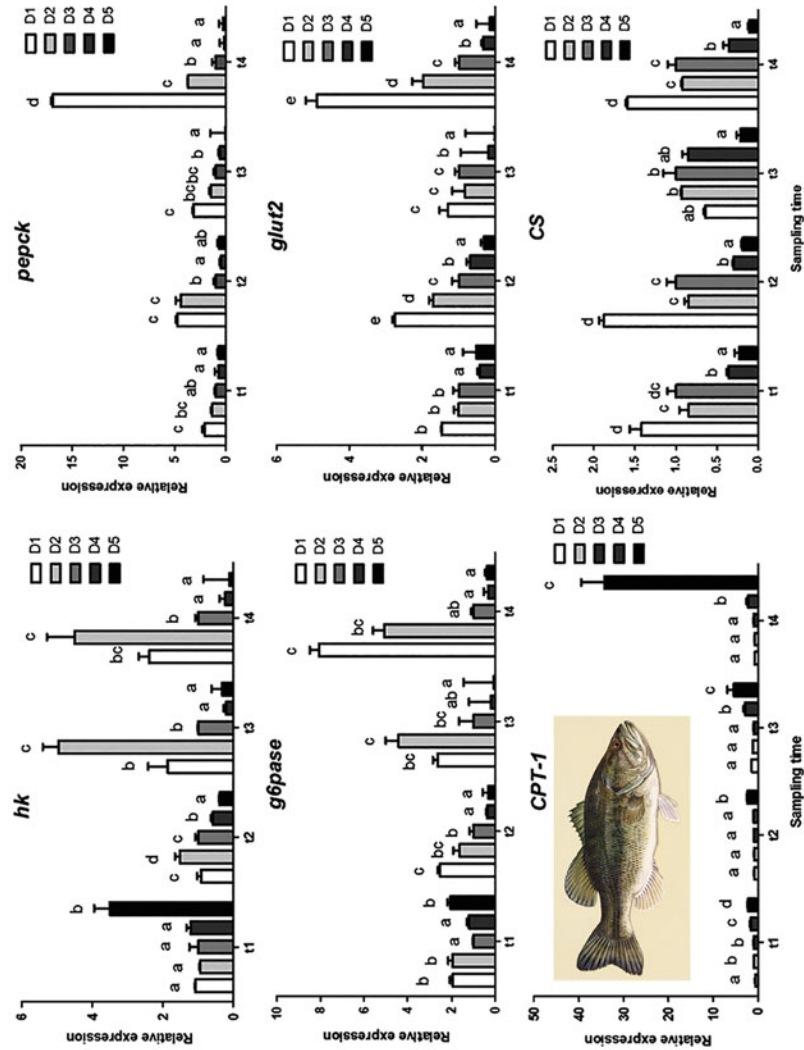


Fig. 21.10 Effect of dietary starch level on the transcription of hepatic energy metabolism-related genes in largemouth bass. Values are means \pm SEM of three replicates. The sampling times t1, t2, t3, and t4 mean 10, 20, 40, and 60 days, respectively. Different superscripts indicate significant differences ($P < 0.05$). *cpt-1*

21.2 Concluding Remarks

Briefly recalling the concern of lacking experimental standardization from previous chapters, different results of given species are due to strain specificity, different stress situations, various sizes of specimens, daily and seasonal variations in hematological and blood biochemical features, feeding history, ontogenetic developmental stage, neglected circadian rhythmicity, different stocking density, or differences in aquafeed composition, other than commonly analyzed organic macro- and micronutrients. As long as a common test protocol is missing, “apples may be compared with pine-apples” (→Chap. 40), and future studies risk not to reach levels above the esoteric level in terms of Kuhn (2012).

HSDs reveal that even in herbivorous and omnivorous fishes, a variety of adverse effects might occur. Above dietary starch contents of 30 to 45%, growth reduction, reduced immunity and tight junction, diminished release of SCFA, increased lipogenesis and lipid accumulation, or liver damage can be observed. Underlying mechanisms should be unraveled in detail with sophisticated “omics” techniques in order to provide a platform for the assessment of potential improvement. Can the addition of probiotics, for instance, increase crowding stress resistance, strengthen immunity, and improve growth performance by the release of SCFAs in the intestine or by interference in signaling pathways?

In addition and more general, Panserat’s (2009) recommendations of how to improve the use of dietary carbohydrates by farmed fish still apply through:

- Genetic selection (→Chap. 40).
- Transgenesis (for techniques, →AAN III “Fish Oil Replacement”).
- Nutritional programming, →AAN III “Nutritional Programming”.
- Feeding appropriate pre- and/or probiotics (→corresponding chapters in AAN III).

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Fig. 21.10 (continued) *1* carnitine ester acyl transferase; *cs* citrate synthase; *g6pase* glucose-6-phosphatase; *glp-1r* glucagon-like peptide-1 receptor; *glut2* glucose transporter2; *hk* hexokinase; *igf-1r* insulin-like growth factor i receptor; *irs1* insulin receptor substrate; *pepck* phosphoenolpyruvate carboxykinase. Five diets were formulated with graded levels of dietary cornstarch (0, 50, 100, 150, 200 g kg⁻¹; D1...D5. (From Zhang et al. (2020c) with permission from Wiley, image credit Duane Raver & U.S. Fish and Wildlife Service)

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Chapter 22

Nonstarch Polysaccharides—‘Neither Sweet Nor Gluey—Adverse?’



Abstract This chapter comprises nonstarch polysaccharides (NSPs), such as cellulose, hemicellulose, β -glucans, pectins, and gums, creating a fluid transition from NSPs to fibers. They are not directly digested, but several of them might serve as prebiotics, if appropriate gut microbes can be established by exogenous probiotics or dietary gut microbiota modulation. Promising tools are also pretreatment or addition of exoenzymes. In addition to nutritional purposes, NSPs have the potential to stimulation of nonspecific immune response and disease resistance. Again, the immune response can even be boosted, if probiotic bacteria are added. In several instances, the “anti-nutritional” NSP activity appears to a function of dosage (\rightarrow hormesis effect) rather than intrinsic properties themselves; often, the low-dose range is not well covered.

Polysaccharides, other than glycogen and starch, are not degraded by endogenous digestive enzymes (Krogdahl et al. 2011). Nonstarch polysaccharides (NSPs) comprise cellulose,¹ hemicellulose, β -glucans, pectins, and gums, creating a fluid transition from NSP to fibers (Fig. 22.1). Several NSPs are used as binders in aquafeed production (Pearce et al. 2002). Furthermore, many of the NSPs can function as prebiotics (\rightarrow AAN III “Prebiotics”).

NSP substrates belong to the natural diet of herbivores and omnivores but not of carnivores. However, NSPs often occur in diets for farmed carnivores as constituents of grains, as soybean meal, or as specific binders. In Atlantic salmon, Kraugerud et al. (2007) showed that NSPs from soybean are inert in terms of nutrient digestibilities and intestinal pathologies compared to the fish meal reference, but it affects fecal mineral (Mn, K) excretion.

¹Recently, Fischer et al. (2020) demonstrated in mammals the potential of dietary cellulose to induce cellular and molecular anti-inflammatory mechanisms via maturation of the intestinal microbiota and thus providing an immunological and molecular rationale for the health benefits of cellulose. Supportingly, Kim et al. (2020) found that high-cellulose feeding enhances mucus production by goblet cells. And vice versa, a dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility in mammals (Desai et al. 2016).

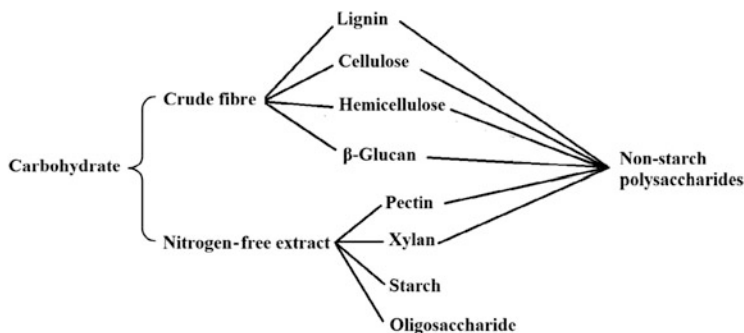


Fig. 22.1 The major components of dietary carbohydrates for farmed animals. (From Zheng et al. (2020), with permission from Wiley)

The composition of the intestinal microbial communities can adapt, when the host is fed different ingredients. Therefore, Serra et al. (2019) tested the hypothesis that a selective pressure of plant-based diets on fish gut microbiota is a beneficial strategy for an enrichment of bacteria with a secretome able to mobilize dietary NSPs. In European sea bass, the authors identified two promising probiotic candidates, *Bacillus subtilis* and *B. licheniformis*, and showed that one of them improves growth and feed utilization in individuals on plant-based diets supplemented with probiotics.

22.1 Supplementation of Exoenzymes

Several attempts tried to increase the digestibility of NSPs by exoenzymes (→also Chap. 39). Therefore, a brief characterization of corresponding enzymes in invertebrates will follow (Fig. 22.2). Cellulases hydrolyze the 1,4-β-D-glucosidic linkages in cellulose, lichenin, and cereal β-D-glucans. Three classes of cellulases are recognized on the basis of the mode of enzymatic actions and the substrate specificities: endoglucanases, exoglucanases, and β-glucosidases (Watanabe and Tokuda 2010). In aquatic arthropods, besides endo-1,4-β-glucanases (Byrne et al. 1999), also β-1,3-glucanase (laminarinase)-related proteins have been found (Linton 2020). The presence of endo-1,4-β-glucanases does not necessarily indicate the ability to digest cellulose (Linton 2020). Furthermore, one major function of β-1,3-glucanase is pattern recognition in antibacterial response based on microbial cell wall hemicelluloses (Cerenius and Söderhäll 2018). β-1,3-glucanase hydrolyses 1.3-β-glucans. These polysaccharides are present in cell walls of algae, protozoans, and yeasts; they also occur as storage polysaccharides in protozoans and algae (Linton 2020) and are often applied as prebiotics in functional aquafeed.

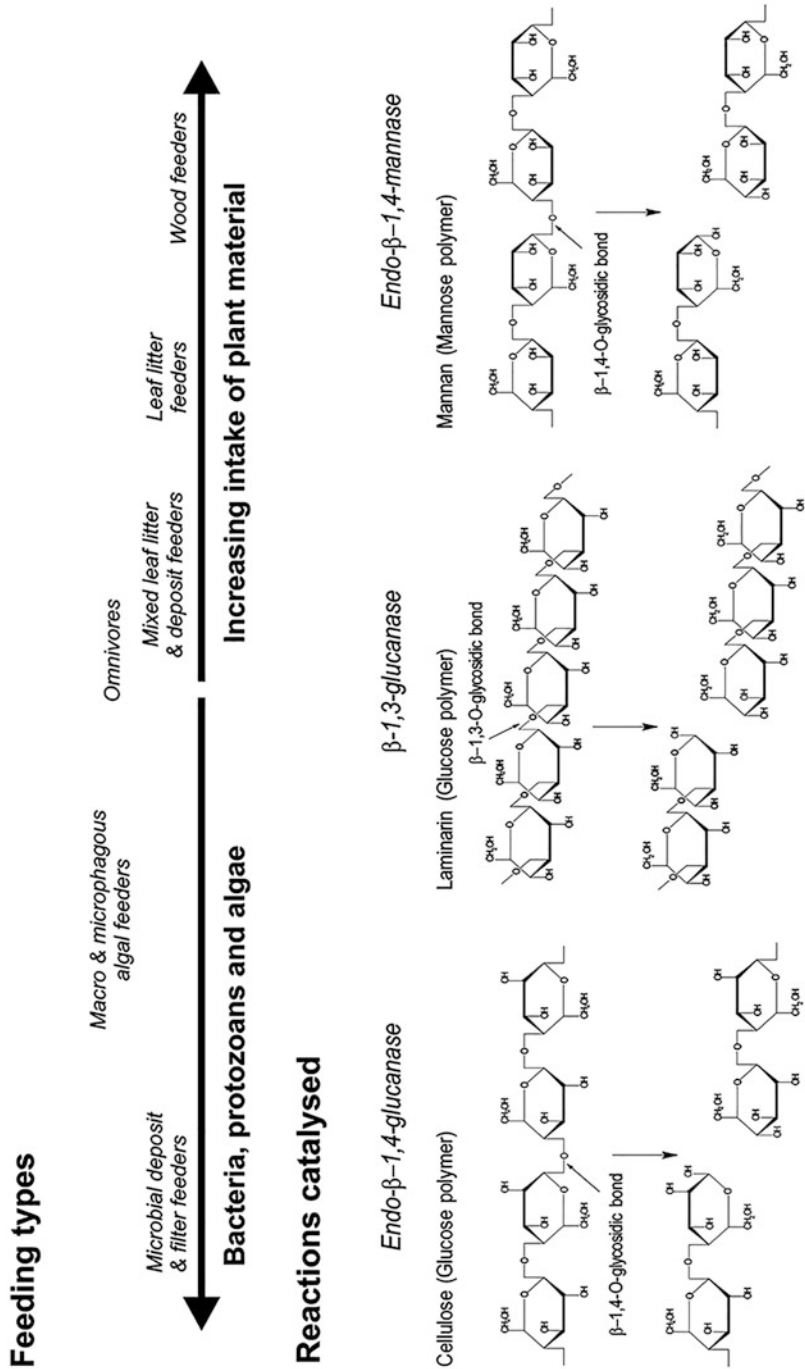


Fig. 22.2 Invertebrate feeding types that display cellulase (endo-1,4- β -glucanase), and hemicellulose (β -1,3- β -glucanase and endo-1,4- β -mannase) activities. (From Linton (2020), with permission from Elsevier)

Linton (2020) adds endo-1,4- β -mannase to the catalog of hemicellulases, which are characterized primarily from mollusks [*Aplysia kurodai* (sea hare), *Haliotis discus hannai* (abalone), *Mytilus edulis* (blue mussel), *Pomacea insularis* (mud snail), *Littorina brevicula* (periwinkle)]. In these animals, β -1,3-glucanase and endo-1,4- β -mannase are endogenously produced and not exclusively synthesized by symbiotic bacteria or protozoans. Endo-1,4- β -mannase hydrolyzes 1,4- β -glycosidic bonds within hemicellulose, mannan, glucomannan, and galactomannan (Fig. 22.2). Mannan is a polymer of mannose units, which are joined by 1,4- β -glycosidic bonds (Fig. 22.2) (Moreira and Filho 2008). The endo-1,4- β -mannase is expressed within digestive tissues and thus functions as digestive enzyme in aquatic mollusks. Its high expression in some species indicates that it is an important enzyme in cellulose and hemicellulose digestion (Linton 2020).

Dalsgaard et al. (2016) showed that supplementing β -glucanase and protease to a diet containing solvent-extracted soybean meal (SBM) causes a breakdown of NSPs in juvenile rainbow trouts, followed by an increase of apparent digestibility of some dietary nutrients. This indicates that exogenous enzymes other than phytase can reduce anti-nutritive effects of SBM and thereby improve nutrient digestibility and utilization.

Less promising, Denstadli et al. (2011) found also in rainbow trouts that although the treatment with NSPs degrading enzymes has significant effects on reductions in total NSPs in vitro, the released substrates do not contribute to improved fish performance.

Recently, Staessen et al. (2020) provided information about one underlying mechanism of the reduced apparent digestibility coefficient (ADC) of lipids in rainbow trout fed high-NSP diets. High feed intake and high dietary NSPs level result in an increase of fecal bile acid loss that is related to a lack of bile acid availability in the small intestine. Moreover, a negative bile acid balance correlates with strong decrease in fat ADC. Consequently, dietary bile acid supplementation remedies this decrease. The ADC of protein and starch are independent from bile acid supplementation. The study indicates that reduced fat ADC is combined with enhanced fecal bile acid loss.

In an interesting meta-analysis of dietary NSPs in omnivorous tilapia, Maas et al. (2020) though confirming a general adverse effect of NSPs on growth performance and nutrient digestibility (crude protein, fat, energy), an average NSP digestibility of ~24% can be calculated in 95 cases. Out of these 95 cases, 88% of them show positive NSP digestibility contributing to energy digestibility. Besides being an energy source, specific types of NSPs have immunomodulating activity by production of short-chain fatty acids (SCFA) via gut microbiota. Nevertheless, NSPs are potentially digested by a wide range of fishes, especially by warmwater species with long gut adapted to feeding on plant matter, as these factors favor gut fermentation (Maas et al. 2020).

22.2 Selected Nonstarch Polysaccharides

22.2.1 Guar Gum

The assumption of adverse NSP effects applies to gibel carp (*Carassius gibelio*). Gao et al. (2019) tested guar gum (guaran), a galactomannan polysaccharide (Fig. 22.3) from powdered endosperm of *Cyamopsis tetragonoloba* seeds. It has thickening and stabilizing properties useful as dietary fibers and binder in feed applications (Thombare et al. 2016). Gao et al. (2019) supplemented diets with 0, 1, 3, and 5% guar gum. 1% guar gum has no effect on growth, feed efficiency, or protein retention. However, this level of supplementation increases *microvilli* length in the distal intestine (Fig. 22.3) as well as fecal viscosity. In contrast, 5% guar gum supplementation decreases feed intake and induces distal intestine damage, as indicated by shortened *microvilli* length. This supplementation also reduces the activity of intestinal digestive enzymes, decreases plasma cholesterol and triglycerides, and finally reduces fish growth. Hence, the optimal level of guar gum dietary supplementation for gibel carp is 1%. The curve in Fig. 22.3, as many others as well, clearly shows a hormetic response, which is seldom discussed in aquaculture papers and shall be revisited in detail in AAN III “Modes of Action.”

In African catfish (*Clarias gariepinus*), Leenhouwers et al. (2006) showed that dietary guar gum inclusion (0, 4, 8%) increases digesta viscosity, which may explain the often observed reduced nutrient digestibilities and increases in digestive organ weights. Similarly, Ramos et al. (2015) found that dietary guar gum (4, 8, 12%) causes an anti-nutritional effect, reducing growth and feed intake in mullet (*Mugil liza*) juveniles. Furthermore, the supplementation alters the body composition and increases hepatic cholesterol and glycogen levels. Guar gum modulates bacterial profiles and densities in different tract sections. Comparably adverse effects (reduced ADCs) appear also in Nile tilapia (gradation 0, 8%) and in European sea bass (gradation 0, 4%) (data in Glencross et al. (2012)).

In contrast to these studies, Enes et al. (2013) found that guar gum had no effect on glucose utilization and lowering plasma cholesterol and triacylglyceride levels in carnivorous white sea bream (*Diplodus sargus*). Dietary inclusion of guar gum of

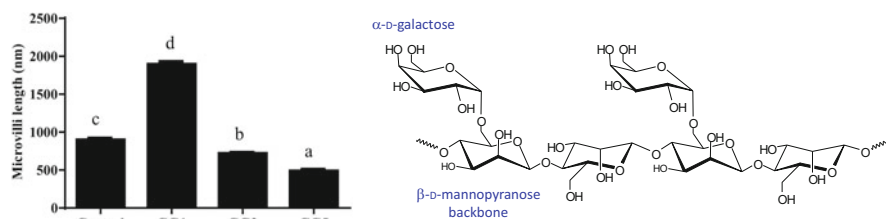


Fig. 22.3 Effects of different levels of guar gum supplementation on the length of *microvilli* in gibel carp, which were fed the control diet and diets supplemented with 1, 3, or 5% guar gum (GG1, GG3, GG5), respectively. Different superscripts indicate significant differences ($P < 0.05$). (From Gao et al. (2019), with permission from Elsevier). Right: Structure of guar gum

even 12% does not impair growth and feed utilization. It does not promote the decrease in plasma glucose and cholesterol levels but seems to reduce gluconeogenesis.

Guar gum and similar other NSPs have often been applied as binder in aquafeeds, and the experiences are collected in Table 22.1. It becomes obvious that NSP contents above a dose of a few percent usually reduce nutrient utilization and growth of farmed animals; however, below these doses, beneficial effects of dietary NSPs can be found (Fig. 22.3). Since the record of beneficial outcomes is sparse, it can be assumed that the gradation of guar gum contents usually is too coarse and the beneficial effects of low doses have been overlooked. Moreover, even a nutritional paradigm has been constructed on this fragile basis; therefore, this concern may serve as an incentive for studying the response of organisms to supplemented feed more carefully and on the basis of optimized graduated supplementation.

22.2.2 Xylan

“Xylan” is a group of hemicelluloses that represents the third most abundant biopolymer on Earth. It is found in the secondary cell walls of dicot plants and in all cell walls of grasses (Deutschmann and Dekker 2012). Its major structural unit is β -1,4-linked D-xylan (Fig. 22.4).

Traditionally, xylan is considered anti-nutritional in plant-derived feeds (Sinha et al. 2011). Uncritically, Ren et al. (2019a) accepted this notion, although they do not find any reduction in survival, growth rate, serum contents of glucose, high-density and low-density lipoproteins, total cholesterol, digestive enzyme activities, or histomorphology of the hindgut in Chinese mitten crab after feeding as much as 5.3% dietary xylan. The authors observed only a slight reduction in the apparent digestibility of dry matter but not of protein. Unfortunately, they tested only this high single dose and most likely overlooked potentially beneficial effects of lower doses (hormesis effect).

Phenotypically, Hu et al. (2015) found that dietary xylan affects growth and feed utilization of juvenile turbot, with beneficial effects at an intermediate supplemental level of 1.25% but with adverse effects at higher levels (5%). More sophisticatedly, Yang et al. (2019) showed also in juvenile turbot that 1.25%, but not 5%, dietary xylan beneficially regulates immune responses, increases gene expression of barrier-forming tight junction proteins (Fig. 22.5), increases the abundance of protease-producing bacteria *Halomonas*, and decreases that of *Bacteroides* and *Bifidobacterium*. This fact confirms a prebiotic character of 1.25% dietary xylan by enhancing intestinal mucosal barrier function and regulating microbiota community. However, 5% dietary xylan exerts adverse effects on intestinal health, characterized by disrupted cell-cell tight junctions, increased gene expression of *muc-2* and pro-inflammatory *il-1 β* , as well as alterations in the abundance of *Clostridium* sp., *Escherichia coli*, and *Prevotella copri*, and bacteria are associated with intestinal diseases.

Table 22.1 Nonstarch polysaccharide (gums) affecting traits of selected farmed animals

Species	Nonstarch polysaccharide, level %	Affected trait	References
<i>Invertebrates</i>			
<i>Apostichopus japonicus</i>	GG 10	WG↑, SGR↑	Won et al. (2018)
<i>Octopus maya</i>	Alginate 1 Gelatin 2	ADC↓ ADC↔	Rosas et al. (2008)
<i>Pseudocentrotus depressus</i>	Alginate 30 Polysaccharide from <i>Alcaligenes faecalis</i> 15	SGR _{Alginate} > SGR _{Af} FER _{Alginate} < FER _{Af}	Akiyama et al. (1997)
<i>Strongylocentrotus droebachiensis</i>	GG, gelatin, or alginate 3, 5	Gonad yield best @ gelatin	Pearce et al. (2002)
<i>Fishes</i>			
<i>Carassius gibelio</i>	GG 0→5	Microvilli growth↑ @ 1; growth↓ @ ≥3; (→Fig. 22.3)	Gao et al. (2019)
	Gelatin 1→5 or carboxymethyl cellulose 1, 3		Gao et al. (2020)
<i>Clarias gariepinus</i>	GG 0, 4, 8	WG↔, FCR↔, protein content↓, energy content↓	Leenhouwers et al. (2006)
<i>Cyprinus carpio</i>	Galactomannan, <i>Sesbania bispinosa</i> endosperm 7→14	Growth↓ @ ≥7 due to increase in chyme viscosity	Hossain et al. (2001)
<i>Diplodus sargus</i>	GG 4, 8, 12	Growth↔, FER↔, gluconeogenesis↓	Enes et al. (2013)
<i>Mugil liza</i>	GG 0, 4, 8, 12	Growth↓, feed intake↓	Ramos et al. (2015)
<i>Oncorhynchus mykiss</i>	Alginate 2.5, 5, 10 GG 2.5, 5, 10	ADCs↓ ADCs↓	Storebakken (1985)
	Six alginate 5	ADCs↓	Storebakken and Austræng (1987)
	Mid viscosity GG 0→0.4 High viscosity GG 0→0.3	Growth↔ Growth↔	Brinker (2007)
	100% plant protein + Lys + Met + GG 0.3	Growth↔	Brinker and Reiter (2011)
<i>Oreochromis niloticus</i>	GG 3	ADCs↓	Fagbenro and Jauncey (1995)
	Galactomannan, <i>Sesbania bispinosa</i> endosperm 3→12	Growth↓ ≥5.8 due to increase in chyme viscosity	Hossain et al. (2003)
	GG 0, 8	ADCs↓	Amirkolaie et al. (2005)
<i>Sparus aurata</i>	GG 0, 4	ADCs↓	Leenhouwers et al. (2004)

↑support/increase, ↓reduction, ↔ no apparent difference to control, GG guar gum, ADC apparent digestibility coefficient, ↓FCR feed conversion ratio, FER feed efficiency ratio, SGR specific growth rate, WG weight gain

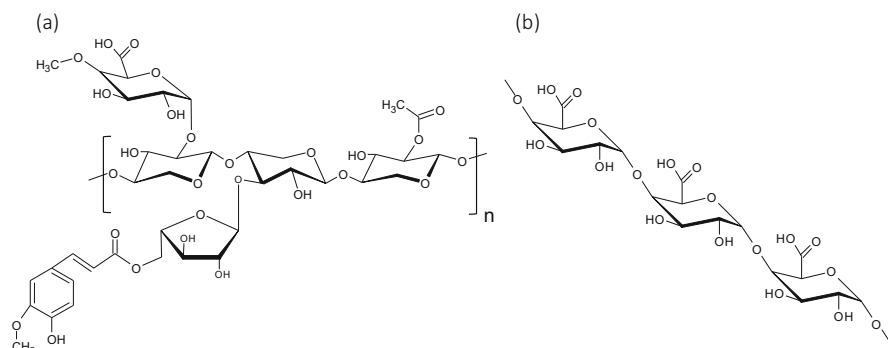


Fig. 22.4 Structural units of xylan (a) and pectin (b)

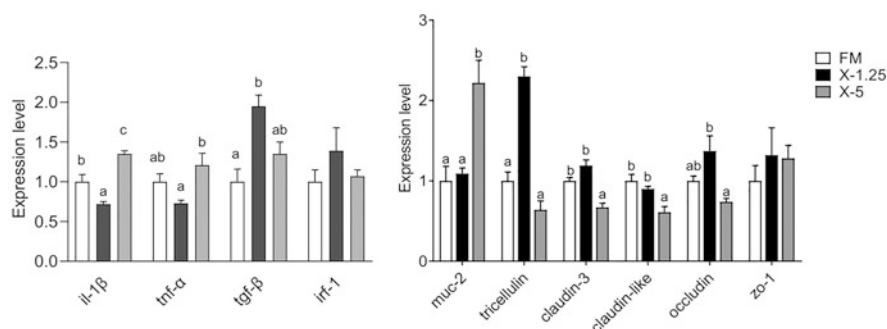


Fig. 22.5 Relative mRNA expression of immune-relevant genes (pro-inflammatory: *il-1β*, *tnf-α*; anti-inflammatory: *tgf-β*), *muc-2*, and tight junction protein genes (*Muc-2* is the main gel-forming and most abundant mucin in the intestine. Tight junction proteins include members of the claudin family, tricellulin, and zonula occludens 1 (*zo-1*)) in the intestine of juvenile turbot on graded dietary xylan (1.25 or 5%). Error bars denote SEM ($n = 6$) and different letters significant differences among groups ($P < 0.05$). (From Yang et al. (2019), with permission from Elsevier)

22.2.3 Pectin

Pectin is a structural heteropolysaccharide, a heterogeneous grouping of acidic structural polysaccharides (Fig. 22.4), contained in primary cell walls of terrestrial plants. It is considered an anti-nutritional factor (Francis et al. 2001).

22.2.3.1 Crustaceans

In Chinese mitten crabs, Ren et al. (2019b) identified a slight anti-nutritional effect when fed 2.7% dietary pectin in a rapeseed meal diet. Specific growth rate and protein efficiency, however, do not differ from the control. Only digestibility of dry

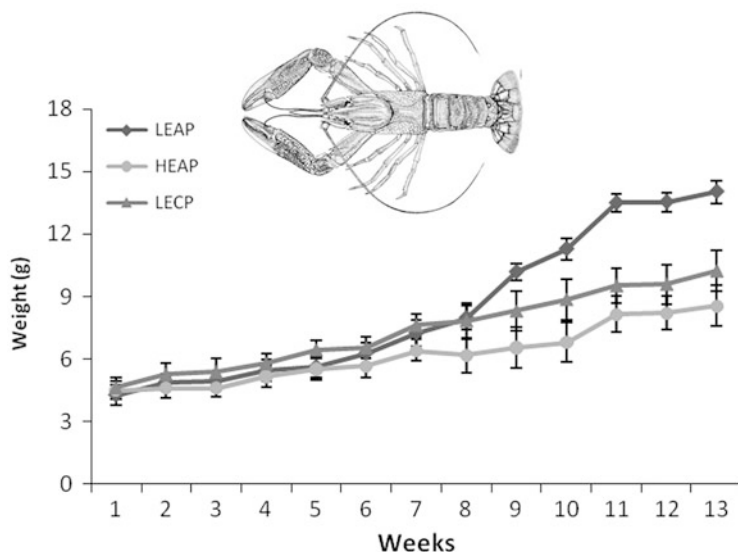


Fig. 22.6 Growth of juvenile *Cherax quadricarinatus* fed low ester pectin from apple (LEAP) pellets, high ester pectin from apple (HEAP) pellets, and low ester pectin from citrus (LECP) pellets. Values are reported as means \pm SEM. (From Volpe et al. (2015), with permission from Wiley; image credit FAO)

matter, activity of aspartate transaminase (AST), and content of high-density lipoprotein (HDL) decrease, whereas the transcription of one of two peritrophin genes increases. This indicates slightly increased digestive efforts, in particular intensified function of the peritrophic matrix² in order to prevent nutrient loss. A real anti-nutritional factor, however, should have eventually resulted in reduced growth or diminished survival of larvae or juveniles.

Further invertebrate studies compared feed pellet qualities for two *Cherax* species. In *C. albidus* (*C. destructor albidus*, yabby complex), Volpe et al. (2008) showed that fresh diet (frozen fish plus fresh potatoes) renders 24% weight gain (WG), alginate and agar diets 36%, and pectin diet even 80%, respectively. Furthermore, pectin stimulates amylase activity best (Volpe et al. 2012). In the sister species, *C. quadricarinatus* (Australian red claw crayfish), Volpe et al. (2015) compared growth stimulation of two low-molecular pectins and found that from week seven onward, WG in the group on low apple pectin increases faster than in the groups on citrus pectin and high apple pectin (Fig. 22.6). The authors assume that the applied apple pectin pellets promote growth of pectinase-producing bacteria best. Taken together, in these crustaceans, dietary pectin appears not to show any anti-

²The peritrophic membrane is a semipermeable, noncellular structure which surrounds the food bolus in an organism's midgut.

nutritional activity; rather, it could emerge as a prebiotic candidate, if they authors would have fed also a control diet for comparison.

22.2.3.2 Fishes

Applying (too) high doses of pectin (30%) in the diet of juvenile yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*), Ren et al. (2020) confirmed—not astonishing—the anti-nutritional effect via growth retardation and structural damage of liver and intestines. Also, Irvin et al. (2016) found reduced digestibility in barramundi on diets with 5 to 20% pectin content. One cannot avoid the impression that the experimental approaches of these tests are biased.

In contrast to the above studies with rather high pectin doses, Van Doan et al. (2018, 2019) fed pectin from orange peels only in the low percent range (0.5–2.0) to Nile tilapia and detected a clear stimulation of the nonspecific immune response, disease resistance, and growth. The immune response can even be boosted, if the prebiotic pectin is combined with a probiotic bacterium (*Lactobacillus plantarum*) resulting in an improved resistance against *Streptococcus* infection (Van Doan et al. 2019). The prebiotic feature of pectin is confirmed also in a South American catfish (*Rhamdia quelen*) on 5% pectin diet (Goulart et al. 2017).

Together, these studies lead to the assumption that the anti-nutritional activity of pectin is a function of its dosage and bias rather than an intrinsic property of the pectins themselves. If a well-graded dosage is applied, the pectin effects will surely follow a hormetic dose-response curve (Calabrese 2020). This means that low pectin doses emerge as prebiotics and enable health promotion (Naqash et al. 2017). Furthermore, the role of gut microbiota in health modulation has to be considered in depth, since beneficial microorganisms and their metabolites (SCFA) improve animal growth and health. In addition to the gut microbiota-mediated effects, various reports in livestock nutrition demonstrate antiviral and antibacterial effects of pectin (Wiese 2019); these studies do not yet have aquaculture equivalents.

22.3 Fibers

22.3.1 Crustaceans

A study of the crayfish *Paranephrops zealandicus* (southern koura) supports the notion of fibers as major constituent in the natural diets. In stomachs of this species from New Zealand, streams in native bush (mainly tree leaves and dicotyledonous seeds) and exotic pasture settings (mainly grass stems and monocotyledonous seeds) are dominated by allochthonous fibrous materials (Hollows et al. 2002). Therefore, it is now central to consider specific effects of dietary fibers.

D'Abramo and New (2009)³ reviewed these effects in *Macrobrachium rosenbergii*. In experiments with dietary fibers up to 30% (DW), growth of *M. rosenbergii* is unaffected (Fair et al. 1980). González-Peña et al. (2002) fed diets that contained 0, 5, 10, and 15% α -cellulose to large (37 g) and small (13.5 g) *M. rosenbergii* individuals. For large prawns, the mean rate gastric evacuation increases at the 10 and 15% levels. WG, food conversion ratio (FCR), and protein efficiency ratio (PER) also improve. For smaller prawns, improvement of WG, FCR, and PER only occurs at the 10% inclusion level. In a companion study, inclusion of dietary α -cellulose at levels of 0, 5, 10, and 15% in semi-purified diets fed to juvenile *M. rosenbergii* is associated with increases in specific dynamic action (SDA) (González-Peña and Moreira 2003). SDA is manifested by an increase in oxygen consumption and heat production associated with metabolism of consumed food. The SDA for the 15% cellulose diet is higher than that of any other diet. Based on mean excretion rates of ammonia and oxygen:nitrogen ratios, protein is catabolized at a slower rate in the diet containing 15% cellulose, thereby enhancing its assimilation. In both studies, however, cellulose levels higher than 15% are not tested.

In contrast, in juvenile *Cherax quadricarinatus*, despite the presence of cellulase activity in the digestive system, the presence of α -cellulose in the diet does not lead to increased growth. Furthermore, dietary inclusion levels above 12% adversely impact survival, growth, and FCR (Pavasovic et al. 2006).

22.3.2 Fishes

In search of dietary replacements of marine proteins, several attempts have been carried out to include materials with high-fiber contents. The phenotypic results are rather mixed; evaluations of underlying biomolecular mechanisms are essential but often lacking. Nevertheless, the digestibility of feed generally decreases with increasing fiber content as shown—one of many examples—with increasing inclusion levels of soluble and insoluble lupin (*Lupinus angustifolius*) fibers and purified cellulose (Fig. 22.7) fed to rainbow trout (Glencross 2009).

Testing juvenile silver catfish (*Rhamdia quelen*) diets, Adorian et al. (2015, 2016) answered the question of whether dietary fiber in the nutrition is a prebiotic nutrient or an antinutrient. The authors compared commercial prebiotic Actigen® (\rightarrow AAN III “Prebiotics”), citrus pulp, brewery yeast biomass, yeast autolysate, and linseed fiber. Fishes on yeast autolysate or linseed fiber diets show superior performance (Fig. 22.8), as well as high values of crude protein and fat deposited in the body. Linseed fiber does not affect the digestive ability, indicating that the fiber has no anti-nutritional activity; rather, this paper demonstrates the potential use of fibers as prebiotics to promote growth—at least in juvenile silver catfish.

³Extract taken with permission from Wiley-Blackwell, appropriate references added.

Fig. 22.7 Effect of diet carbohydrate level (%) on energy digestibility (%) of each of the experimental diets in rainbow trout. Each of the different NSP types is represented by a different shaded circle (cellulose black; soluble NSPs white, and insoluble NSPs gray). + indicates the reference diet. (From Glencross (2009), with permission from Elsevier)

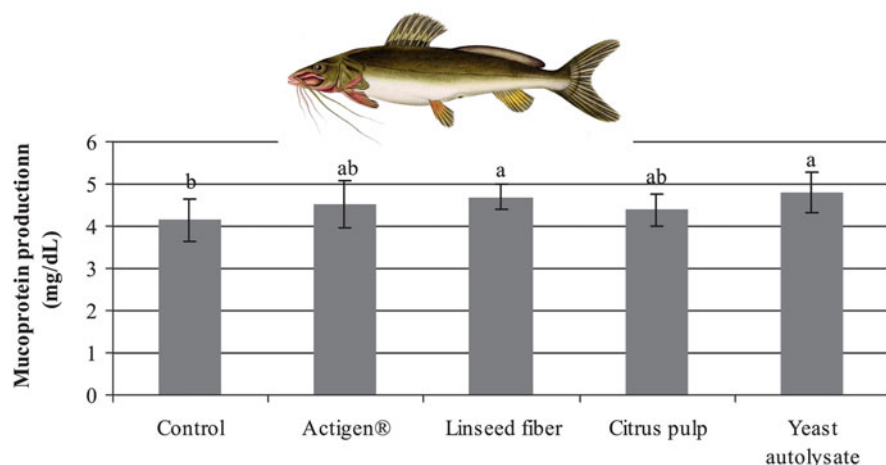
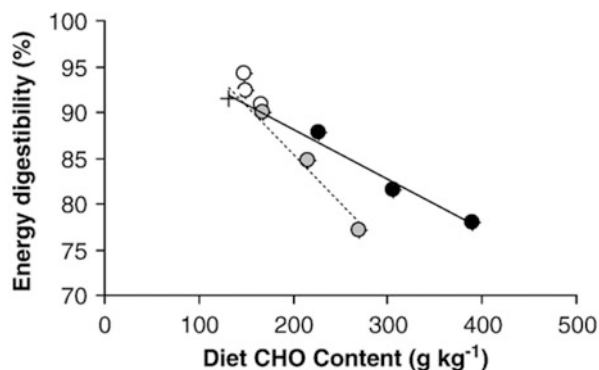


Fig. 22.8 Effect of dietary fiber concentrates on mucoprotein production (mg dL⁻¹) in silver catfish. Values are means \pm SE. Different letters represent significant differences ($P < 0.05$). (From Adorian et al. (2016), with permission from Elsevier; image credit Paul Louis Oudart “Voyage dans l’Amérique méridionale”)

Consequently, this laboratory continues the studies only with linseed fiber and shows that this dietary material has stress-reducing, immunostimulatory, and growth-promoting effects (Adorian et al. 2019). Furthermore, an increased presence of insoluble fiber results in improved immune parameters of the plasma and skin mucus, as well as elevated final weight—a clear indication of the prebiotic potential of these fibers. This indication is reinforced: Linseed fibers (1:2 and 1:4 soluble fiber/insoluble linseed) modulate the production of SCFAs and improve growth as well as plasma and skin mucus parameters (Adorian et al. 2020).

In the same line of evidence, Yossa et al. (2018) reported that inclusions of up to 0.25% of dietary purified lignin alone and 0.25–0.50% of dietary hemicellulose

alone or in combination with dietary lignin can be considered as candidate prebiotic in Atlantic salmon nutrition.

Also looking specifically for beneficial effects of dietary fibers, Bonvini et al. (2018) showed that different fiber levels derived from sunflower and soybean hulls have no effects on overall performances and feed efficiency in carnivorous European sea bass (*Dicentrarchus labrax*). Blood biochemistry and gut histology confirm a good nutritional and health status under all feeding treatments. Moreover, the inclusion of fiber derived from these hulls has no influence on digesta transit time and digesta moisture. In contrast, higher dietary fiber levels seem to increase the time required emptying the stomach. The authors conclude that it is feasible to include insoluble fiber up to ~16% in feed formulations for on-growing of European sea bass without adversely affecting growth and health. Particular beneficial effects of fiber inclusion, however, are not reported.

The *Dicentrarchus* example has to be considered as extreme because most studies recommend fiber contents below 7–8% (NRC 2011), independent of the feeding habit of the species under consideration. Confirming this figure, Adamidou et al. (2011) showed that the inclusion of fiber up to 5% does not affect growth or nutrient digestibility in omnivorous sharpsnout sea bream (*Diplodus puntazzo*). McGoogan and Reigh (1996) detected higher digestibility of protein by red drum (*Sciaenops ocellatus*) on feeds with <2% fiber; above this level, no relationship can be established between fiber contents and protein digestibility of feeds. In addition, Altan and Korkut (2011) found a nonlinear relationship between fiber content and effects: Low dietary concentrations of dietary fiber (3–5%) may be beneficial for growth, like (other) prebiotics, but elevated dietary fibers (>8%) decrease dry matter digestibility and reduce the availability of other nutrients. Recently, Zhong et al. (2020) showed that the optimum level (4%) of dietary soybean fiber improves growth, plasma biochemical indices, and liver function in *Micropterus salmoides*. This information is intriguing since this species is strictly carnivorous in the wild.

In a companion paper, Lin et al. (2020) show that dietary soybean fibers induce health benefits in the intestine. Supplemental soybean fibers improve the intestinal antioxidant ability and alleviate potential intestinal disruption. Moreover, dietary fibers enhance the immune response by upregulation of anti-inflammatory and downregulation of pro-inflammatory genes (Fig. 22.9).

In contrast to the cellulose studies, Yarahmadi et al. (2014, 2016) devoted their papers to fermentable fibers in form of a commercial product (Vitacel[®], consisting mainly of cellulose and hemicellulose) and found that these diets upregulate immune-related genes (*lysozyme*, *tnf- α*) and corresponding enzyme activity. Furthermore, these diets downregulate the stress-responsive *hsp70* and elevate innate immune response and pathogen resistance of rainbow trout.

Recently, Leigh et al. (2018) have put forward an interesting hypothesis. The authors fed zebrafish diets ranging from high to low quality (i.e., ranging from high-protein to low-fiber to low-protein to high-fiber diets) and monitored how gut length and surface area (\rightarrow AAN I Chap. 2 (Steinberg 2018)), as well as the activity of digestive enzymes (amylase, maltase, trypsin, aminopeptidase, and lipase), shift in response to the dietary changes. Most intriguing, zebrafishes fed low-protein to high-

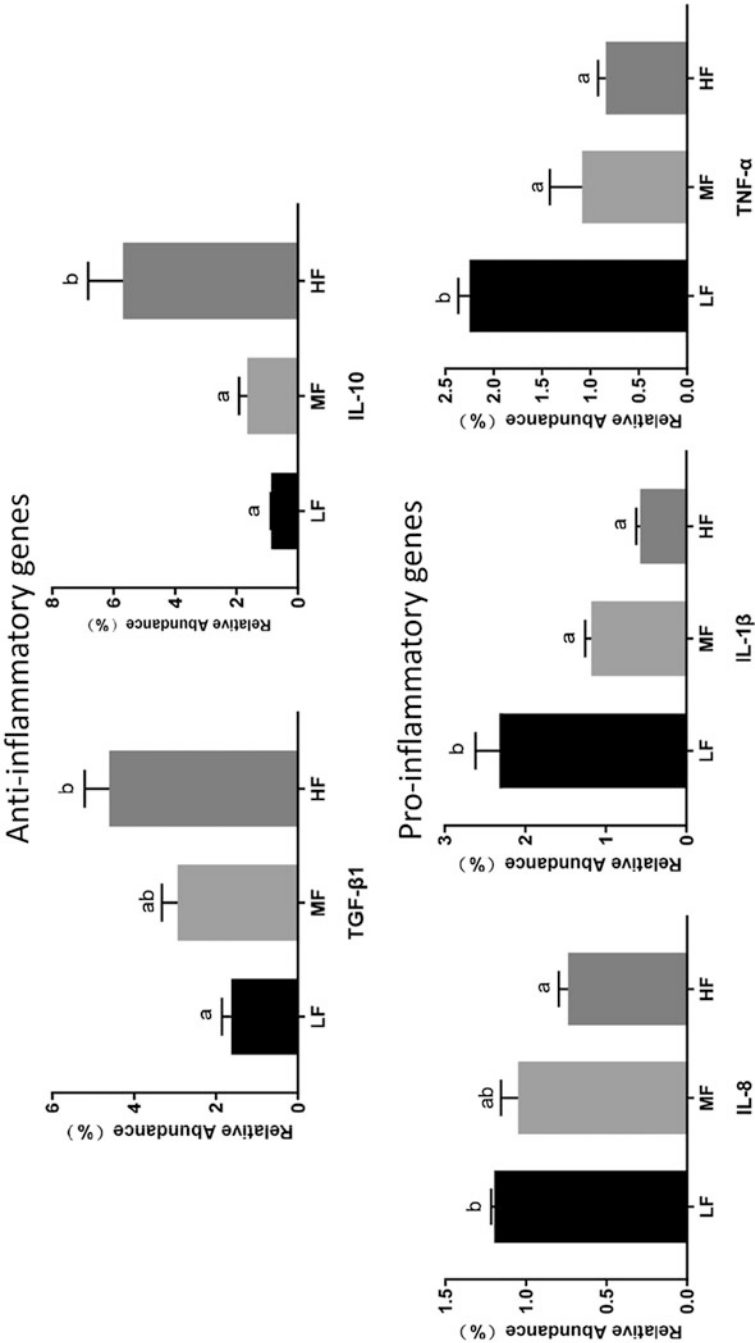


Fig. 22.9 Intestinal expression levels of anti-inflammatory and pro-inflammatory cytokines genes in largemouth bass fed graded levels of fibers. Data are expressed as mean (SEM). Values in each column with different superscripts have significant differences ($P < 0.05$). *LF* 0% soybean fiber; *MF* 4% soybean fiber; *HF* 8% soybean fiber. (From Lin et al. (2020), with permission from Elsevier)

fiber diet show elevated lipase activity. The authors interpret this phenomenon as lipid-scavenging mechanism when individuals consume high-fiber foods. This hypothesis is worth being challenged in a broader context with more species.

Studies on dietary effects of fibers in fishes are often restricted to purified fiber, such as cellulose, pectin, and starch. To feed more realistic fiber diets, Rodrigues et al. (2012) included plant ingredients with different levels of broken rice (BR), ground corn (GC), wheat bran (WB), citrus pulp (CP), and soybean hulls (SH) into feeds for the two omnivores, jundiá catfish, and Nile tilapia. The species differ in intestine morphology: Tilapia is typical omnivorous with long intestines, whereas the opportunistic omnivorous jundiá has short intestines and no pyloric *ceca*. Consequently, it can be hypothesized that the first species benefits more from nutritionally concentrated feed ingredients than the second one. Fibrous diets (CP, WB, and SH) are less digestible for both species than starchy ones (BR and GC). As expected, jundiá is less able to utilize starch and protein from plant sources than tilapia does. Growth of tilapia follows plant sources digestibility, but no significant differences are detected in jundiá, probably because of its slower growth rate. Muscle layer is thicker in the distal intestine of jundiá fed CP diet, which possibly indicates an adaptation to propel the large volume of viscous digesta along the intestine. Therefore, despite its lower ability to utilize starchy plant sources, jundiá shows an adaptive capacity to utilize fibrous diets, which is in agreement with its opportunistic omnivorous feeding habit tending toward carnivorous. However, fibrous sources in general are not advantageous to both species (Rodrigues et al. 2012).

22.4 Concluding Remarks

A short chapter only, however, several remarks remain to be made:

1. Animals are no engines with a constant performance throughout the day. Almost no nutritional studies consider the circadian rhythmicity of the nourished animals. This topic has been dealt with in depth in AAN I “Chrononutrition” (Steinberg 2018). Usually, it takes some time to accept new ideas from other disciplines and set them into scientific practice. Satisfactorily, a few emerging studies consider this issue (Basili et al. 2020).
2. Many studies used a too coarse gradation of dietary NSPs. Therefore, it is not astonishing that the classification of several NSPs as anti-nutritional principles emerged. This classification continues to be applied. However, a finer gradation, as shown with guar gum (Fig. 22.3), gelatin, or carboxymethyl cellulose supplementation as dietary supplementations, reveals that there are not linear but biphasic U-shaped or inverse U-shaped dose-response relationships. This phenomenon is termed hormesis and implies that a low dose of an effective principle may trigger from an organism the opposite response to a very high dose. According to Calabrese (2020), “the hormetic model is not an exception to the

rule—it is the rule.” Applied to aquatic animal nutrition, this calls for rethinking of one of its central beliefs. Pragmatically, this means that the low-dose region has to be studied much more thoroughly and in dependence of the circadian rhythm (Jagota et al. 2019).

3. Besides the necessary application of actual biomolecular techniques, it becomes obvious from the comparison with livestock nutrition that future studies should cover aspects of direct and indirect effects of NSPs on gut microbiota as here depicted with pectin. Simply, an old question has to be studied with new tools (Vestrum et al. 2018): Do NSPs display antiviral and antibacterial effects also in aquatic animals? Do NSPs strengthen the gut barrier?

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Chapter 23

Lipids—‘*The Greasy, Unhealthy Stuff*’



Abstract Lipids are vital. This chapter focuses on recent biomolecular progress in dietary lipid with farmed invertebrates and fishes as well as ecological model organisms. Deficient or excess levels of lipids aggravate disease/pathogen susceptibility; modes of action are sketched and discussed in depth in Chap. 24. First indications point out that different lipid doses trigger different pathways; whether this notion is generalizable remains to be elucidated. The role of lipids as elicitor of biomolecular regulatory pathways of digestion, immunity, and disease resistance is beginning to be understood. First inventories of intestinal microbiota in fishes on different lipidic diets show there might be a potential of probiotic modulation. High-fat diets (HFD) can compromise the immune response by influencing the physical properties of immune cell membranes, membrane-associated signaling molecules, and receptor sites. HFDs combined with high dietary carbohydrates emerge as risky diet, since it upregulates disproportionately large numbers of genes associated with mitochondrial metabolism, neurodegenerative diseases, and liver dysfunction. The involvement of epigenetic pathways in lipid metabolism is beginning to be detected: Dietary fat does not influence growth in a given species but alters hepatic expression of miRNAs and genes related to lipid metabolism causing severe lipid deposition. HFDs induce a fatty liver, which blocks the TCA cycle, disrupts protein and carbohydrate metabolism, and results in reduced growth. Moreover, with “enteroendocrine cells silencing” by HFD via gut microbiota modulation, a new mechanism of nutrient sensing and signaling is detected that might serve as basis for new probiotic HFD treatments.

Based on belief and passed on as “accepted wisdom,” lipids have rather a bad reputation in human nutrition; the greasy, ugly stuff has to be avoided. Consequently, a plethora of “science-based” guidebooks and recommendation brochures about low-fat diets do exist in order to restrict fat, saturated fat, as well as cholesterol. Therefore, adipocytes (fat-storing cells) are perhaps the most vilified nonmalignant cell type in a body. Given that context, it has been easy to overlook the benefits provided by healthy adipose tissues (Rosen and Spiegelman 2014). Instead, food and pharmaceutical industries offer seemingly healthy food and expensive supplemental drugs to reduce putative symptoms of fat consumption. Low-fat diets are intended to

reduce diseases such as heart disease and obesity (Fogelholm et al. 2012). However, research has not supported the efficacy of low-fat diets in lowering plasma cholesterol, weight, and heart diseases (Corr and Oliver 1997). Rather, even gross lipids are vital and adipocytes take part in regulatory pathways. Therefore, this chapter will focus on this aspect in aquatic animal nutrition.

Particularly the cholesterol issue appears questionable, since the daily demand of central organs, such as brain, often exceeds the daily dietary uptake: There is a distinct endogenous cholesterol production in animals, including most aquatic ones. Rather, evidence is accumulating that they benefit from dietary cholesterol in healthy development (Kanazawa et al. 1975; Yun et al. 2012; Norambuena et al. 2013). Arthropods (such as crustaceans) and mollusks are incapable of *de novo* synthesis of cholesterol (Zande 1967); therefore, dietary cholesterol is essential for their growth and survival (van den Oord 1964; Zandee 1966; von Elert et al. 2003; Kumar et al. 2018). The cholesterol issue will be revisited in Chap. 31.

Dietary lipids play important roles in the energy production of animal tissues and as sources of essential fatty acids (EFAs) and, thereby, indirectly take part in controlling pathways. Lipids are the generic names assigned to fat-soluble compounds. They are classified as:

- Lipids: Fatty acid (FA) esters of glycerol (Fig. 23.1)
- Sterols: Polycyclic, long-chain alcohols (Fig. 23.1)
- Phospholipids: Esters of FAs and a hydrophilic “head” containing a phosphate group (Fig. 23.1)
- Waxes: FA esters of long-chain alcohols, which serve as dietary source of marine salmonids (Fig. 23.1)

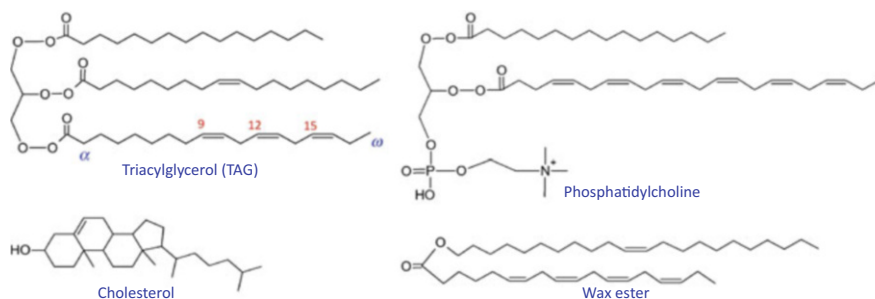


Fig. 23.1 Example of an unsaturated fat triacylglycerol (TAG, $C_{55}H_{98}O_6$). Left vertical part: glycerol; right horizontal parts, from top to bottom: palmitic acid (PA), oleic acid (OA or OL), and α -linolenic acid (ALA). Cholesterol. Phosphatidylcholine, the first phospholipid identified as such in the egg yolk of chickens. Wax ester (22:1 alcohol and 18:4 fatty acid)

23.1 Lipid Requirement

Lipids are concentrated source of energy (9.44 kcal g^{-1} or 39.5 kJ g^{-1}), containing ~ 2.25 -fold more energy than carbohydrates (Molina-Poveda 2016). Fishes consuming different foods will encounter the macronutrients in different concentrations (e.g., carnivores will consume more proteins and lipids, and herbivores more carbohydrates) and, thus, will use different nutrients as fuel sources and will exhibit differences in digestive efficiencies of these nutrients (Table 23.1). Many fishes consuming lipid-rich prey (e.g., carnivores and diatom consumers) use lipids as primary energy source, allowing protein to be utilized primarily for tissue maintenance and growth (German 2011). In nature, animals have evolved strategies to select and ingest foods in order to balance the gain of several nutrients. They balance protein and lipid intake and to meet the specific requirement (Mayntz et al. 2005; Machovsky-Capuska et al. 2016) (\rightarrow “Nutrient Specific for Aging,” Chap. 2). With exogenously provided feeds in aquaculture, the options of selection are rather limited for farmed animals.

Unlike proteins, lipids are a diverse range of very different compounds that are grouped together simply based on their solubility in organic solvents. Most lipids are “complex,” meaning that they contain FAs usually esterified to alcohol groups in the case of acylglycerols (glycerides) and to amino groups in the case of sphingolipids (Sanchez et al. 2018). In terms of function, lipids can be divided into two groups: (1) polar lipids, including phospholipids that play predominantly structural roles, and (2) neutral lipids, including triacylglycerols (TAG or triglycerides, Fig. 23.1), whose role is storage, primarily of energy, but also of cellular components. Simple lipids do not contain FAs, with the most important one in animals being cholesterol that can be unesterified as a functional component of cell membranes or in a storage form esterified to an FA (NRC 2011).

Other functional lipids are several hormones and fat-soluble vitamins, such as vitamins A, D, E, and K (\rightarrow Chapters on Vitamins). For more information about function, biochemistry, and nutrigenomics of lipids, the audience is referred to reviews by Sargent et al. (2002), Glencross (2009), Monroig et al. (2013), or

Table 23.1 Summarized digestive efficiencies of lipids in selected fishes of different trophic levels consuming artificial feeds

Species name, common name	Trophic habit	Digestive efficiency, %
<i>Cyprinus carpio</i> , Common carp	Omnivorous/benthivorous	92–96
<i>Dicentrarchus labrax</i> , European sea bass	Carnivorous	80–99
<i>Ictalurus punctatus</i> , Channel catfish	Carnivorous/omnivorous	76–97
<i>Oncorhynchus mykiss</i> , Rainbow trout	Carnivorous	71–97
<i>Oreochromis niloticus</i> , Nile tilapia	Omnivorous/detritivorous	72–98
<i>Pagrus major</i> , Red sea bream	Carnivorous	90–96

From German (2011), with permission from Elsevier

Castro et al. (2016). The following chapter will focus on recent biomolecular progress in farmed invertebrates and fishes as well as ecological model organisms.

23.1.1 *Invertebrates*

Several recent field studies disclose conspicuous plasticity of lipid metabolism and different strategies of invertebrates in response to changing or even deteriorating food qualities.

23.1.1.1 *Fresh Water*

Koussoroplis et al. (2017) evaluated the short- and long-term response of *Daphnia pulex* lipases to quality shifts in diets consisting of graded mixtures of *Scenedesmus* (green alga) and *Synechococcus* (cyanobacterium), two genera with contrasting lipid compositions. Five lipases respond to small changes in food quality. Furthermore, the gene expression of one lipase is strongly correlated to gut lipase/esterase activity, pointing out its major role in digestion: buffering the lipid variability in the diets. Most importantly, *D. pulex* not only responds to the shift in diet quality but also to the amount of change. These findings demonstrate that the lipid-related enzyme machinery of *D. pulex* is finely tuned to diets (Koussoroplis et al. 2017). In a companion paper, Schwarzenberger and Fink (2018) showed that such tuning is combined with costs caused by upregulation of digestive enzyme gene expression and activity in order to adjust to low food quality provided by cyanobacteria.

Another well-studied freshwater crustacean, the giant river prawn (*Macrobrachium rosenbergii*) has no fixed dietary lipid demand (D’Abramo and New 2010). The required amount is essentially based upon satisfaction of the needs for EFAs, in combination with its supply as energy source to spare dietary proteins. With a good source of dietary protein, levels of dietary lipid which contain the appropriate levels of EFAs can be as low as 2%. Dietary lipid levels which exceed 10% are generally associated with reduced growth, likely due to oxidative stress that the processing of excess lipids causes.

23.1.1.2 *Marine*

Crustaceans

Brown shrimp (*Crangon crangon*) reflects the seasonal food availability and feeding activity by gene expression of digestive enzymes. Elevated expression of phospholipase indicates utilization of intracellular lipid reserves, in this case polar lipids of membranes, which may fulfill an extra function as energy storage compounds besides their structural function (Martínez-Alarcón et al. 2020). Gene expression

of digestive enzymes involved in lipid metabolism is modulated by the lipid content in the midgut gland and is related to food availability. In spring, expression of phospholipase (involved in the catabolism of phospholipids) is twice as high as the expression of TAG lipase (involved in the catabolism of TAG). In summer, the ratio changes and the expression of TAG lipase increases. This tendency continues in autumn, showing twice as high expression of TAG lipase as phospholipase. Toward winter, the ratio changes again, showing a five times higher expression of phospholipase than TAG lipase. During the productive seasons, the relative expression of TAG lipases is higher than that of phospholipases, indicating the primary utilization of dietary TAGs. In early spring and particularly in winter, when food is scarce, the expression of phospholipases favors the utilization of intracellular phospholipids (Martínez-Alarcón et al. 2020).

In order to reduce the overlap of ecological niches, coexisting shrimp species use energy resources differently (Martínez-Alarcón et al. 2019). The authors compared the omnivorous brown shrimp and pink shrimp (*Pandalus montagui*) in the southern North Sea. In *P. montagui*, the variable but usually high lipid levels of the midgut gland are linked to the reproductive cycle. Feeding preference in this species varies with seasons. In *C. crangon*, the low lipid content indicates that the midgut gland does not function primarily as an energy depot to support reproductive processes; rather, the midgut gland apparently serves as a dynamic metabolic center with high turnover rates. Hence, in spite of the limited lipid storage capacity, the brown shrimp is well adapted to cope with a highly variable environment with periods of food paucity and has successfully established large stocks in the North Sea and surrounding waters. The strategies of both shrimps are depicted in Fig. 23.2.

The same hypothesis underlies the study of Jungblut et al. (2018): Does a smart energy-storing strategy enable an invasive crab species to capture new areas? The invasive Asian shore crab (*Hemigrapsus sanguineus*, Fig. 23.3) and the native European green crab (*Carcinus maenas*) share intertidal habitats along European North Atlantic shores and may compete for food. The authors demonstrate that *H. sanguineus* stores much higher amounts of lipids as energy reserves than *C. maenas* (Fig. 23.3). In contrast to *C. maenas*, *H. sanguineus* shows a pronounced seasonality in lipid deposition and depletion. These differences may be due to the higher lipid turnover in *H. sanguineus*. FA compositions of *C. maenas* and *H. sanguineus* differ distinctly from each other. *H. sanguineus* is able to accumulate high lipid quantities in relatively short periods, apparently exploiting macroalgae as primary resource. Therefore, *H. sanguineus* is a stronger herbivore than *C. maenas*, apparently occupying quite a different trophic niche. Even though it consumes energy-poor macroalgae, *H. sanguineus* is able to accumulate large lipid reserves. These deposits buffer periods of food paucity, facilitate reproductive output, and thus contribute to the success of this invasive crab in new areas (Jungblut et al. 2018).

Most studies of dietary lipids in invertebrates, however, focus on farmed crustaceans. In the economically important *Litopenaeus vannamei*, Xie et al. (2019) reported that a proper level of dietary lipid improves survival, stress tolerance, and immunity of postlarval, whereas excess dietary lipid leads to oxidative damage. The

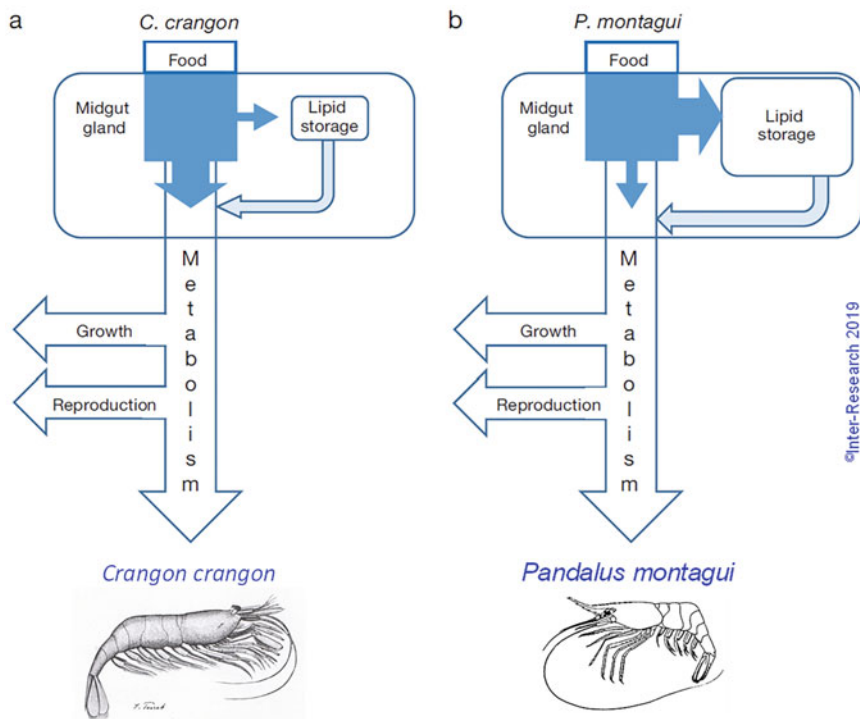


Fig. 23.2 Conceptual scheme emphasizing the metabolic function of the midgut gland in terms of lipid utilization of (a) *Crangon crangon* and (b) *Pandalus montagui*. The differences in arrow widths and lipid storage size indicate relative differences in matter and energy flows. (From Martínez-Alarcón et al. (2019), with permission from Inter-Research; images credit Julius Fürst ~1895 & FAO)

optimal dietary lipid for postlarvae is determined as ~12%. This value lies significantly above the range representative of farmed shrimps (NRC 2011): The highest weight gains (WG) are generally achieved at dietary levels of 5–6% inclusion. Higher levels (>10%) often retard growth (Kanazawa et al. 1977; Davis and Robinson 1986; Sheen and D’Abramo 1991), due to oxidative stress. Chapter 24 will revisit this issue.

The general NRC figure of lipid demand applies to mud crab *Scylla paramamosain*, in which the dietary lipid level of 8.5–11.6% (optimum 9.5%) maintains solid growth and antioxidant capacity of juveniles (Zhao et al. 2015), or to oriental river prawn (*Macrobrachium nipponense*), in which dietary lipid levels above 7% reduce fecundity (Li et al. 2020). In the same line of evidence, dietary lipid level of 15% retards growth and reduces feed utilization in juvenile swimming crab (*Portunus trituberculatus*) (Sun et al. 2020). Furthermore, it decreases lipoprotein clearance, reduces lipid digestion and lipolysis, inhibits mitochondrial and peroxisomal β -oxidation, and results in increased hepatopancreatic lipid deposition.

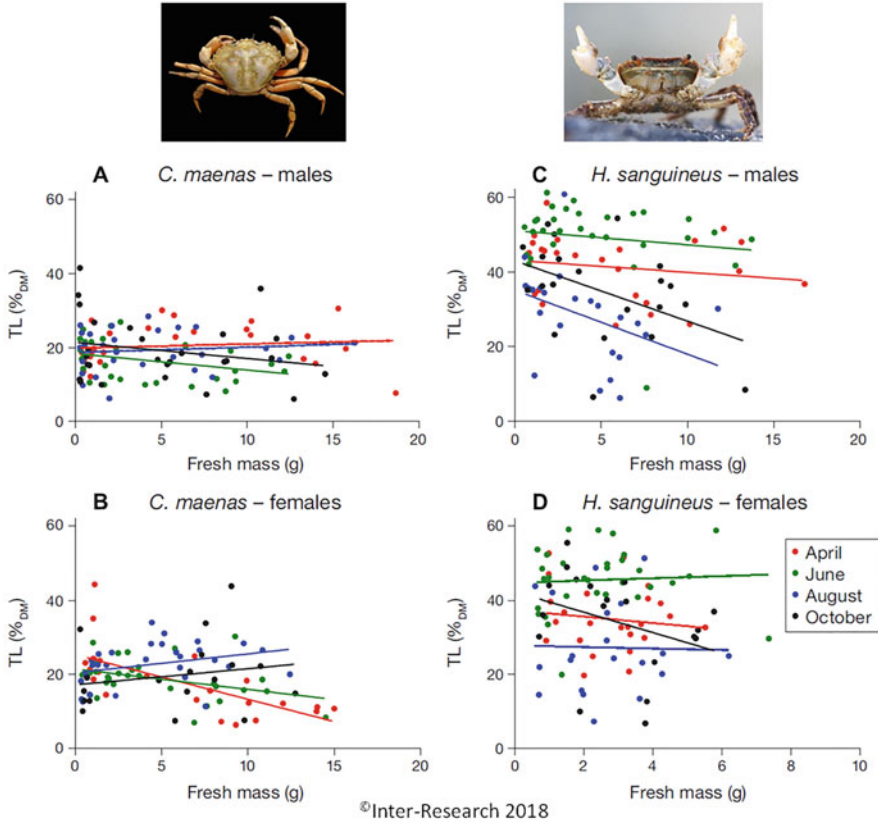


Fig. 23.3 Total lipid content (TL; % of dry mass or %_{DM}) of *Carcinus maenas* (a) males and (b) females and *Hemigrapsus sanguineus* (c) males and (d) females sampled at Helgoland, Germany, between April and October 2015. Note the differing x-axis of (d). (From Jungblut et al. (2018) with permission from Inter-Research; images ©Hans Hillewaert & ©Jonas Mortelmans, Wikimedia)

Glencross (2009) summarized that lipid nutrition in crustaceans presents as a slightly different scenario to that of fish, because crustaceans have a comparatively poor ability to use large amounts of dietary lipid in their diets. It is possible that this relates to the efficacy of lipid emulsion in the digestive systems of the animals, with crustacean digestive systems showing a greater degree of similarity with those of fish larvae, which also have a poorer developed digestive system than adults.

One of the rare non-crustacean, non-echinoderm studies reports that optimal dietary lipid content for juvenile kisslip cuttlefish (*Sepia lycidas*) lies in the range of mud crabs (9.6%) (Han et al. 2017).

Echinoderms

In marine invertebrates with larval planktotrophy as ancestral life history, the evolutionary switch to lecithotrophy¹ depends on modifications of oogenesis to produce energy-rich eggs that support development to the juvenile stage. In this sense, Byrne and Sewell (2019) characterized lipid provision in the large eggs of the echinometrid sea urchin *Heliocidaris erythrogramma* (purple sea urchin, endemic to Australia, is an aquaculture candidate (Keesing 2007)), which has lecithotrophic larvae. In the planktotrophs, TAG is the major energetic lipid. Egg energetic lipids in the lecithotrophs are largely diacylglycerol ether (DAGE) with TAG and wax ester present. Larval development does not significantly deplete energetic lipids, with 70% of the DAGE remaining for the juvenile. TAG supports larval development with a 20–30% decrease by day 3 with no further depletion to day 14. DAGE levels decrease around metamorphosis, followed by a gradual depletion, but 49% of these reserves remain on day 14 (Fig. 23.4). Thus, DAGEs provide a significant nutritive buffer for a considerable time post settlement (Byrne and Sewell 2019).

23.1.2 Fishes

Dietary lipids are a major source of energy in all fishes, especially carnivores. There are metabolic interactions between proteins, lipids, and carbohydrates; it is well understood that lipids in the range of 10–20% of the dry weight of the diet are sufficient to allow protein to be effectively utilized for growth without depositing excessive lipid in the tissues. Under experimental laboratory conditions, high dietary EFA concentrations have an adverse effect on growth and feed efficiency in salmonids (Tacon 1996). The precise amount of lipid required depends on the dietary protein level and, in the cases of omnivores, on the dietary carbohydrate level (Sargent et al. 2002; Machovsky-Capuska et al. 2016).

Furthermore, growth depressions of high dietary neutral lipid levels in freshwater and marine fish larvae have often been reported: in hybrid tilapia, tongue sole, Malabar grouper, Atlantic herring, Senegalese sole, European seabass, gilthead sea bream, or lined seahorse. This may be due to decrease in the efficiency or activity of digestive enzymes, reduction in absorption efficiency, decrease in food intake, increase of internal oxidative stress, or combination of several of these factors. The results collectively indicate that lipid transport from the enterocytes into the body may be more problematic in larval stages dealing with high neutral lipid diets than lipolytic enzymatic capacity, although both factors are likely to intervene (Morais et al. (2007) and references therein).

¹Lecithotrophy is nourishment and development of the embryo only via the yolk originally contained within its egg; the opposite is matrotrophy.

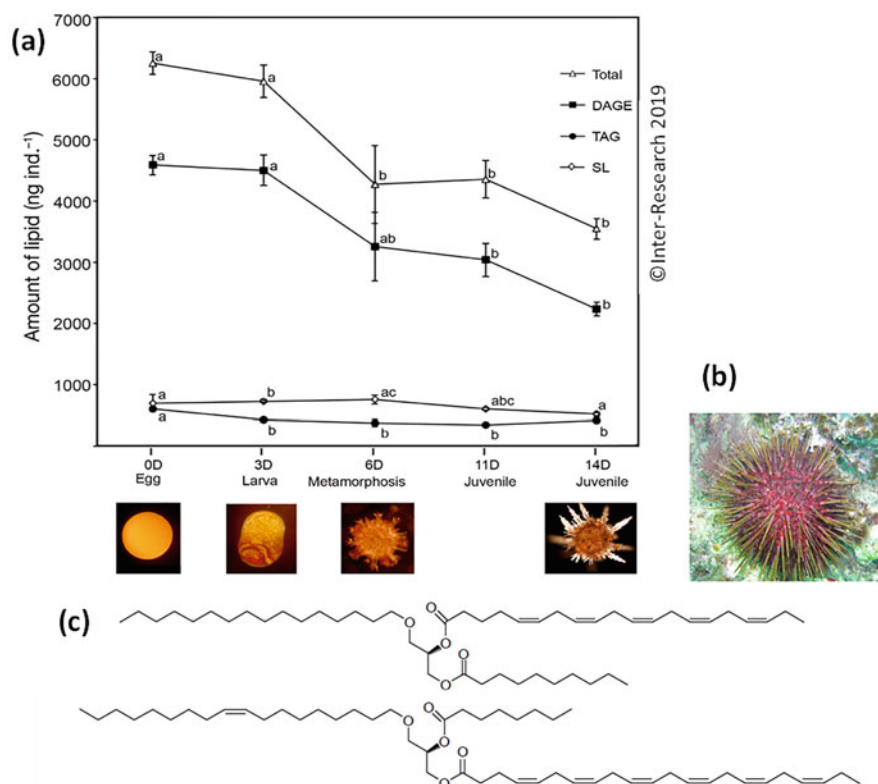


Fig. 23.4 (a) Lipid depletion (mean \pm SE) during the development of *Heliocidaris erythrogramma* from day 0 (OD, egg) to the larva (day 3), metamorphosis (day 6), and the juvenile (days 11 and 14). *Total* total lipid; *DAGE* diacylglycerol ether; *SL* total structural lipid (sterol, acetone-mobile polar lipids, and phospholipid); *TAG* triacylglycerol. Letters indicate results of Tukey's post hoc test; time points with the same letter do not differ. (From Byrne and Sewell (2019), with permission from Inter-Research). (b) Adult purple sea urchin (credit Parks Victoria). (c) DAGE examples: Chemical structures of a normal structured alkyl-long-medium type DAGE of chimyl alcohol possessing eicosapentaenoic acid (EPA) at the *sn*-2 position and capric acid (C10:0) at the *sn*-3 position (top) and a reversed structured alkyl-long-medium type DAGE of selachyl alcohol possessing docosahexaenoic acid (DHA) at the *sn*-3 position and caprylic acid (C8:0) at the *sn*-2 position (bottom). (From Magnusson et al. (2015), credit MDPI, Basel)

Even disease/pathogen resistance might depend on optimum lipid supply: In young grass carp, the enteritis resistance is optimal at 50–55 g lipid kg⁻¹ diets (Feng et al. 2017) (Fig. 23.5a); higher shares increase the morbidity. However, already dietary lipid levels above 40 g kg⁻¹ increase the risk of growth reduction in this herbivore (Du et al. 2005).

Another intriguing example of adverse effects of dietary lipid excess was reported for Japanese eel: Decreasing dietary lipid contents improves larval survival and growth (Furuita et al. 2014). The authors used shark eggs (SE) and hen egg yolk

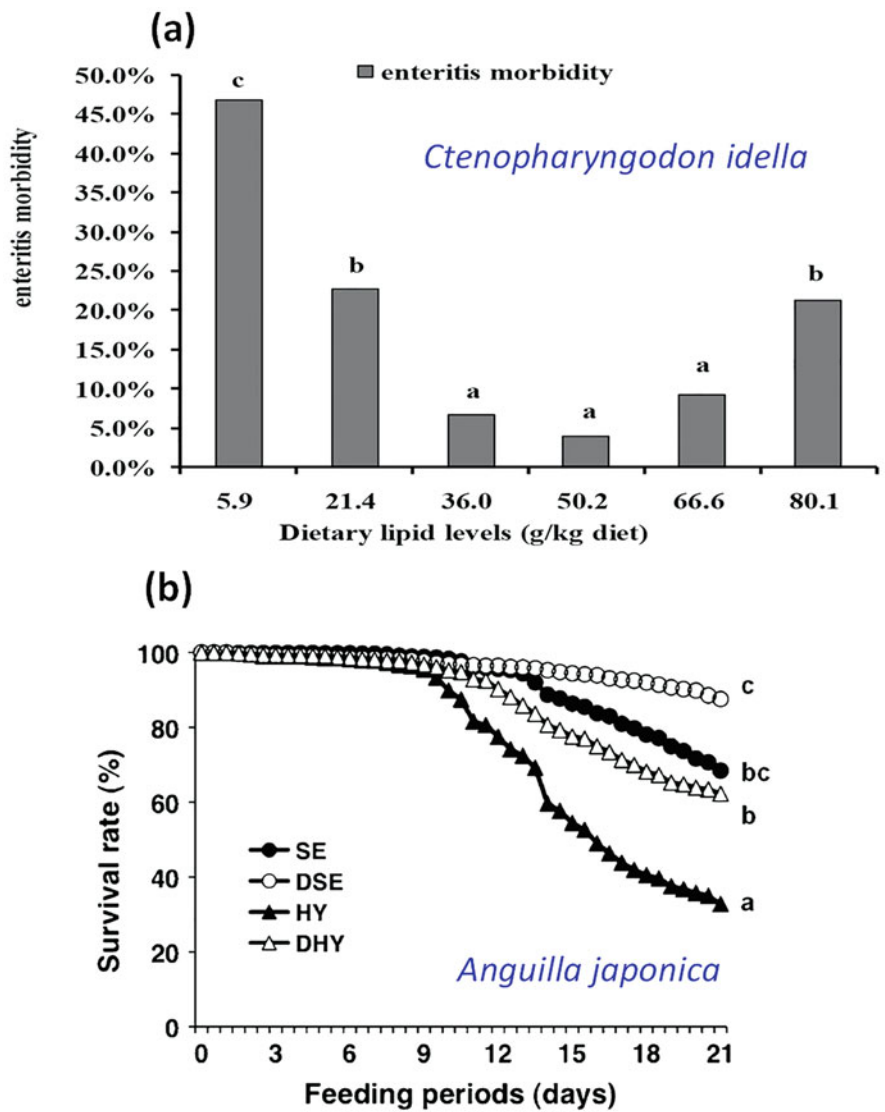


Fig. 23.5 (a) Deficient or excess levels of lipids aggravate enteritis under the infection of *Aeromonas hydrophila* in young grass carp (*Ctenopharyngodon idella*). Different letters indicate significant differences ($P < 0.05$). (From Feng et al. (2017), with permission from Elsevier). (b) Survival rate of Japanese eel larvae fed shark egg and hen egg yolk-based diets. Data are expressed as mean \pm SD. SE shark eggs, DSE defatted shark eggs, HY hen egg yolk, DHY defatted hen egg yolk. Different letters indicate significant differences ($P < 0.05$). (From Furuita et al. (2014), with permission from The Japanese Society of Fisheries Science)

(HY), untreated or defatted with *n*-hexane (DSE, DHY). The highest survival rate occurs in larvae fed DSE, and larvae fed HY show the lowest survival rate (Fig. 23.5b). The best growth is found in larvae fed DSE, followed by SE and DHY, and the worst growth in HY-fed larvae. Detailed mechanisms of lipid toxicity are going to be discussed in Chap. 24.

Dietary lipid provision, however, is not only a matter of low, appropriate, or high-fat (HF) diets resulting in low, appropriate, or reduced growth; rather, different lipid doses trigger different pathways. This intriguing finding is demonstrated in Chinese sturgeon (*Acipenser sinensis*) (Fig. 23.6). In captivity, females rarely successfully develop their gonads. Leng et al. (2019) figured out the effects of dietary lipid level on the ovarian development and the related regulatory mechanism. With three experimental diets containing 10, 14, and 18% lipids, a 24-month feeding trial is conducted in 10-year-old Chinese sturgeons with ovaries at the critical developmental stage. Only the 18% lipid diet promotes ovary development. Metabolomics and transcriptomics indicate that 14% of dietary lipids benefits steroid hormone synthesis, while 18% lipid facilitates the arachidonic acid metabolism, cholesterol biosynthesis, and vitellogenesis (Fig. 23.6) (Leng et al. 2019).

23.1.2.1 Lipid Preference

With increasing demand for alternative lipid sources, the question arises, if fishes would voluntarily select plant lipids, if they have the choice. Luz et al. (2018) provided evidence that *Dicentrarchus labrax* is able to discriminate between diets in which the only difference is the lipid source. Noteworthy, adults and juveniles have different preferences. Adults select diets made from soybean and rapeseed in proportions similar to fish oil (FO) (Fig. 23.7a–e). Demand levels for the other two diets (sunflower, linseed oils) are lower than for FO, while juveniles prefer soybean and FO (Fig. 23.7f–j). The lowest preference levels are associated with fat-free diets.

In a major South American farmed freshwater fish (*Colossoma macropomum*), Pereira et al. (2018) showed that also a frugivorous/omnivorous species is able to discriminate between diets made with distinct oil sources through postabsorptive signals when provided with a nutritional stress challenge (fasting >10 days). This behavior indicates the capacity of this fish to use alternative metabolic pathways to generate energy and maintain homeostasis. Under these conditions, even this fish prefers FO (Fig. 23.8). Lipid reserves have to be replenished, since long-term fasting induces lipid mobilization and digestion (Palmeri et al. 2009; Steinberg 2018).

Remarkably, dietary lipid preferences/supplies, however, do not necessarily translate into differences in growth or feed intake. For instance, replacing up to 60% of FO with mixtures of vegetable oils (VOs) produces no marked effect in growth, lipogenesis, and tissue lipid contents of *D. labrax* fingerlings (Richard et al. 2006). Supportingly, Yildiz and Şener (2004) did not observe any differences in the growth of juvenile *D. labrax* after supplementing diets with oils from different sources (fish, soybean, sunflower).

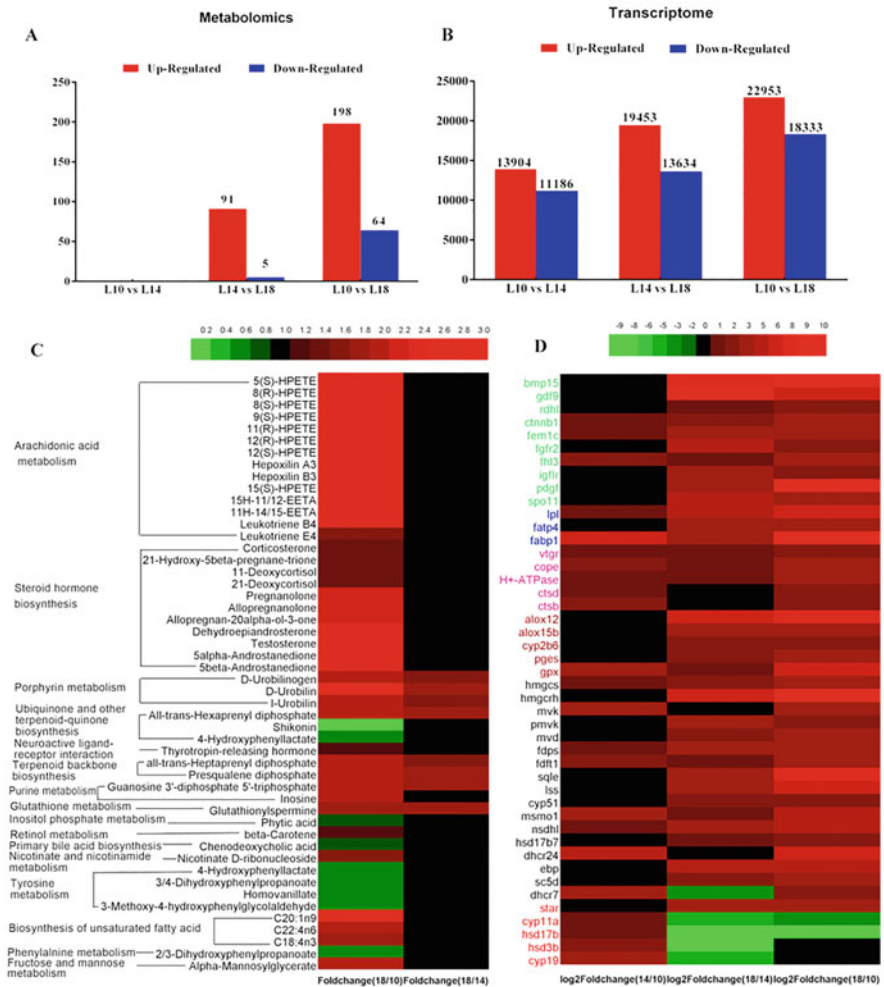


Fig. 23.6 Screening of metabolites and unigenes differentially expressed among three groups of female Chinese sturgeon. **(a)** Differentially expressed metabolites. **(b)** Differentially expressed unigenes. **(c)** Heat map of differentially expressed metabolites with KEGG pathway annotation. **(d)** Heat map of differentially expressed unigenes involved in key pathways for gonad development. Colors in panel **(d)** correspond to different gene functions: Genes with green, blue, and pink color are involved in oocyte growth, lipid deposition, and vitellogenesis, respectively. Genes with brown, black, and red color are involved in arachidonic acid metabolism, terpenoid backbone biosynthesis, and steroid biosynthesis and ovarian steroidogenesis pathways, respectively. (From Leng et al. (2019), with permission from the Cambridge University Press)

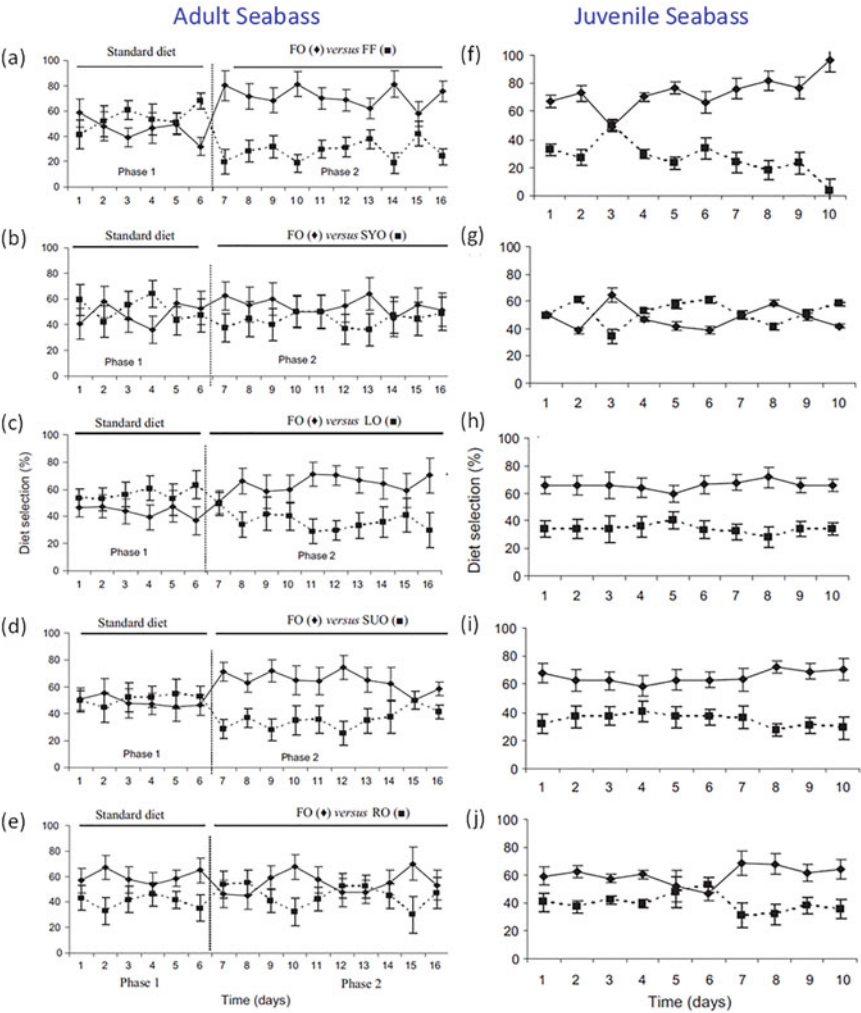


Fig. 23.7 Selection by *Dicentrarchus labrax* of diets with different oil sources provided by self-feeders. (a–e) Self-selection by adults. During phase 1, both dispensers delivered the same standard diet (SD), whereas during phase 2, dispenser F1 provided fish oil (FO) diet and dispenser F2 provided one of the following experimental diets: (a) fat-free (FF); (b) soybean oil (SYO); (c) linseed oil (LO); (d) sunflower oil (SUO); and (e) rapeseed oil (RO) ($n = 12$, mean \pm SEM). (f–j) Selection by juveniles of diets with different oil sources provided by hand: (f) FO versus FF; (g) FO versus SYO; (h) FO versus LO; (i) FO versus SUO; and (j) FO versus RO ($n = 8$, mean \pm SEM) (From Luz et al. (2018), with permission from Wiley)

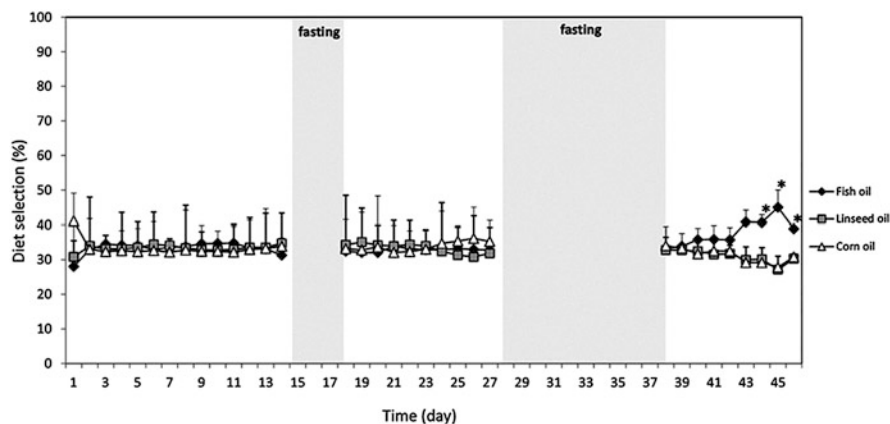


Fig. 23.8 Daily evolution of the oil source selected in the absence of diet organoleptic properties by tambaqui. Values (SD) represent the percentage of total diets selected as 100%. Gray columns indicate fasting periods. Asterisks denote significant differences, $n = 6$, ANOVA, Tukey's test ($P < 0.05$). (From Pereira et al. (2018), with permission from Elsevier)

Tributylin

Tributylin (TB, Fig. 23.9) is a specific triglyceride present in butter. It is an ester composed of three butyric acid and glycerol (Brody 1999). In a variety of freshwater (*Macrobrachium tenellum*, *M. americanum*) and marine shrimps (*Litopenaeus vannamei*, *L. schmitti*, *Farfantepenaeus notialis*, *F. californiensis*), TB shows a much better digestibility than any tested oil as lipid source. Doubtless, these species possess tributyrinase (Espinosa-Chaurand and Nolasco-Soria 2019). Moreover, unsupplemented high soybean meal diets has detrimental effects on *L. vannamei*; however, the addition of 10 g kg^{-1} TB improves growth, innate immunity, and intestinal structure (Liu et al. 2020).

A few papers show that the addition of TB to plant-based diets for carnivores attenuates damages. In juvenile black sea bream, appropriate addition of TB (2.2 g kg^{-1}) in a high soybean meal (SBM)-based diet improves growth, reduces oxidative stress and damage, and attenuates intestinal injuries (Volatiana et al. 2020a, b). Although Hou et al. (2019) showed that the same amount of dietary TB in snakehead (*Channa argus*) enhances intestinal trypsin and pepsin activities, the regulatory pathway remains widely obscure. Some mechanistic hints can be derived from a study in juvenile yellow drum (*Nibea albiflora*). Supplementing a high SBM diet with 1 g kg^{-1} TB, Tan et al. (2020) figured out that mucosal fold height, microvilli length, and microvilli density increase. Moreover, this supplementation suppresses the transcription of pro-inflammatory genes, likely due to modification of intestine microbial community.

Appropriate inclusion of tributyrin ($2.0\text{--}4.0 \text{ g kg}^{-1}$) in plant-based diets is beneficial also to common carp (Xie et al. 2020). It directly or indirectly reduces adverse influences on growth and intestinal barrier function. Direct effects take place mainly by facilitating the development and integrity of intestinal villi and increasing

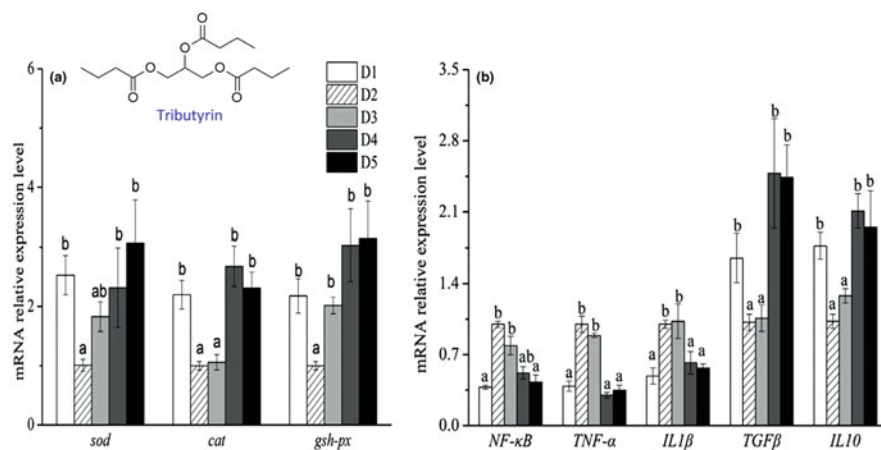


Fig. 23.9 Structure of tributyrin (TB). mRNA relative expression of antioxidant (a) and inflammatory (b) indicators in the intestine of common carp. Values are means \pm SEM ($n = 3$). Different letters indicate significant differences ($P < 0.05$). (From Xie et al. (2020), with permission from Wiley). The control diet (D1) contained 120 g kg⁻¹ fish meal and 500 g kg⁻¹ plant protein blend (soybean, rapeseed, and cottonseed meal), while the other four diets contained plant protein blend with TB at 0.5 g kg⁻¹ (D2), 1.0 g kg⁻¹ (D3), 2.0 g kg⁻¹ (D4), and 4.0 g kg⁻¹ (D5)

digestive enzymatic activities, and subsequently improving growth. In detail, optimal TB diet represses antioxidative genes (*sod*, *cat*, *gsh-px*, indicative of reduced ROS production; Fig. 23.9a), improves the gut microbiota (not shown here), and suppresses the transcription of pro-inflammatory (*nf-κb*, *tgfb*, *il-10*, Fig. 23.9b) and supports that of anti-inflammatory genes (*tnfa*, *il1β*, Fig. 23.9b), likely due to modification of intestine microbial community (Xie et al. 2020).

Together, these studies contribute to the establishment of TB as functional feed additive in plant-based diets for fishes and invertebrates.

23.1.2.2 Lipids and Immunity

Past fish nutrition research mainly focused on establishing minimum nutrient requirements for normal or optimal growth. The role of nutrition, in general, and lipids, in particular, as elicitor of biomolecular regulatory pathways of digestion as well as immunity and disease resistance is going to be understood only recently. Qiang et al. (2017) reported that the optimal dietary lipid level for larval GIFT tilapia (8–11.5%) increase *trypsin* and *lipase* transcription in liver and intestine (Fig. 23.10) and act as immunostimulant. Based on experiences above (Fig. 23.5), it can be expected that excess dietary lipid levels reduce growth and survival, cause stress, and consequently leading to reduced antioxidant defense, impaired liver function, depressed immunity, and reduced resistance to *Streptococcus iniae* infection. This study confirms several older phenotypic ones reporting that appropriate dietary fish lipid supplementation enhances immune response (Table 23.2). To contrast

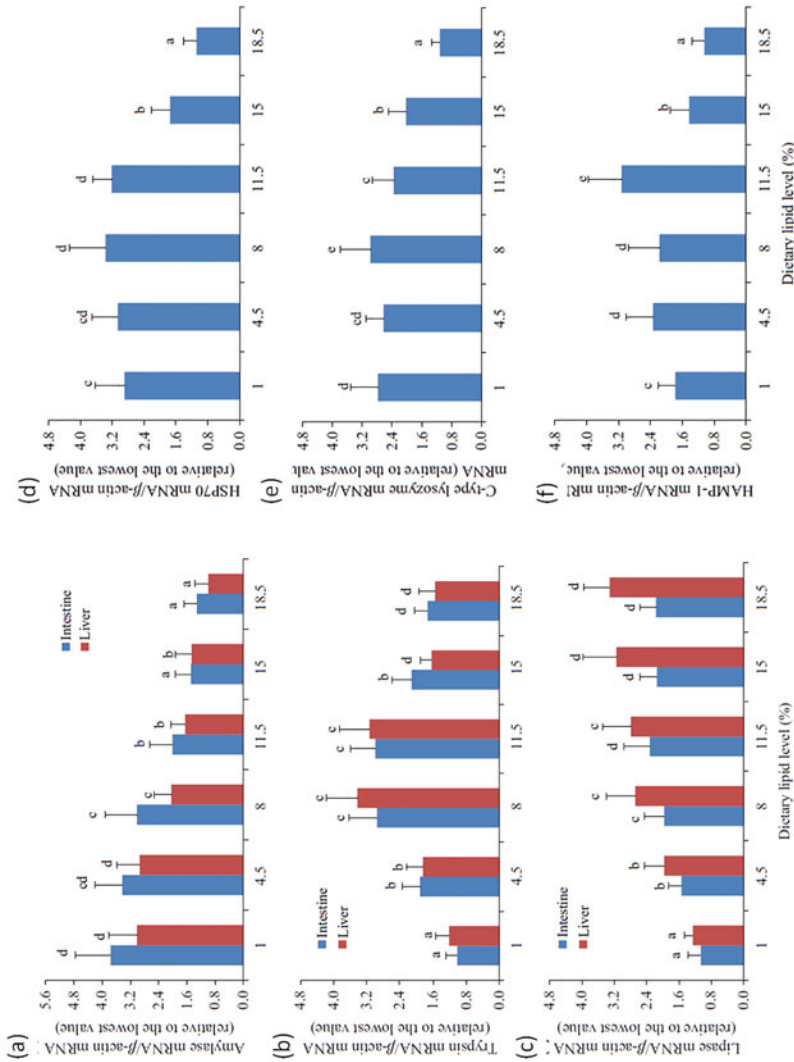


Fig. 23.10 Larval GIFT tilapia fed different dietary lipid levels for 35 days. (a–c) Levels of *amylase* mRNA (a), *trypsin* mRNA (b), and *lipase* mRNA (c) in intestine and liver. (d–e) Levels of hepatic *hsp70* mRNA (d), *C-type lysozyme* mRNA (e), and *hepcidin antimicrobial peptides (hamp-1)* mRNA (f). Data are means \pm SD; different superscripts indicate significant differences ($P < 0.05$). (From Qiang et al. (2017), with permission from Wiley)

Table 23.2 Dietary lipids and oils modulate immunity in aquatic animals—typical studies

Species Common name	Lipid/oil		Immune trait	References
	Source	Dose, %		
Invertebrates				
<i>Eriocheir sinensis</i> juvenile Chinese mitten crab	FO + soy lecithin	6, 10	LYZ↔, LPO↔	Wang et al. (2015)
	FO, LO, SO	6	LYZ↔, LPO↔, phenoloxidase↔, resistance against <i>Aeromonas hydrophila</i> ↔	Chen et al. (2018)
<i>Litopenaeus vannamei</i> juvenile Pacific white shrimp	Oil mixture	6, 8, 10, 12, 14	Hemocytes↑, respiratory burst↑	Zhang et al. (2013)
	FO, SO, lecithin, cholesterol	6, 9, 12	LPO↑ @ 12 immunity↑ @ hyposalinity	Xu et al. (2018)
Fishes				
<i>Ctenopharyngodon idella</i> juvenile, grass carp	1:1 FO+CO	0, 2.5, 5, 7.5, 10	PHA↑, LYZ↑, PER↑, max pathogen resistance @ 7.5; LPO↑@10	Jin et al. (2013)
<i>Dicentrarchus labrax</i> juvenile European sea bass	Cod liver oil	10 fresh or oxidized	HC↔, red blood cells↔, mortality↔	Obach et al. (1993)
<i>Dicentrarchus labrax</i> 90g	RO, LO, or OO	60 FO replacement	LEUK↓, PHA↓ RO: Liver inflammation↑, midgut MA↑	Mourente et al. (2005)
<i>Epinephelus malabaricus</i> juvenile Malabar grouper	1:1 FO+CO	0, 4, 8, 12, 16	LEUK↑, innate immunity↑	Lin and Shiau (2003, 2007)
<i>E. malabaricus</i> juvenile		4, 9	LPO↑ @ 9, LYZ↔, ACH ₅₀ ↔	Lin and Shiau (2005)
<i>Heterobranchius longifilis</i> fingerling Sampa, African catfish	Pork lard, poultry fat, PO, shea butter oil, SfO	Total replacement of FO	Lymphocytes↑, neutrophils↓, ALT↑, AST↑	Babalola et al. (2009)
	PO, SO, or PO+SO		ALT↑, AST↑, ALP↑ @ palm oil ALP↓ @ soybean oil	Babalola et al. (2016)
<i>Hippoglossus hippoglossus</i> juvenile Atlantic halibut	South American marine FO	Different oxidation states	LPO↑, ALP↔, ACP↔	Lewis-McCrea and Lall (2007)
<i>Ictalurus punctatus</i> 62.5 g Channel catfish	Catfish oil, FO ^a	Identical shares	Phagocytosis↑ @ 28 °C Phagocytosis↑ @ 18 °C	Lingenfelser et al. (1995)

(continued)

Table 23.2 (continued)

<i>Species</i> Common name	Lipid/oil		Immune trait	References
	Source	Dose, %		
<i>I. punctatus</i> juvenile	FO	0, 3, 6, 9	LYZ↑, COM↑	Yildirim-Aksoy et al. (2009)
	Mixed oil BT CO LO FO	7	PR↑ PR↑ PR↑ PR↓ PR↓	Fracalossi and Lovell (1994)
	FO SO BT Mixture	7	HC↓, LEUK↑, THR↑ HC↑, LEUK↓, THR↓ HC↑, LEUK↓, THR↓ HC↑, LEUK↓, THR↓	Klinger et al. (1996)
	SO, SO+LIN Algal DHA FO	2	LYZ↔, ACH ₅₀ ↔, HC↔	Faukner et al. (2015)
	FO Virgin coco- nut oil CO Mixture (coconut + CO)	6	ALP↔, ALT↔, AST↔, ACH ₅₀ ↔, <i>tgf-β</i> ↔, <i>c-lyz</i> ↔ <i>il-1 β</i> ↑@ mixture	Apraku et al. (2019)
<i>I. punctatus</i> sub- adult 105g	FO, SO, LO	2	Survival after <i>Edwardsiella ictaluri</i> challenge↔	Suja et al. (2012)
<i>I. punctatus</i> fingerling	Catfish offal oil BT FO	2	PR↑ PR↑ PR↓	Li et al. (1994)
<i>Larimichthys</i> <i>crocea</i> juvenile, 9 g Large yellow croaker	VO	0–100 replacement of FO	Resp. burst↓, ACH ₅₀ ↓ Inflammation↑ (Fig. 23.11)	Tan et al. (2016)
<i>Lateolabrax</i> <i>japonicus</i> juvenile Japanese sea bass	LO, SO	Total replace- ment of FO	PHA↓, LYZ, ↓, ACH ₅₀ ↓	Xu et al. (2015)
<i>Micropterus</i> <i>salmoides</i> juvenile Largemouth bass	Oxidized FO		LPO↑, apoptosis↑	Yin et al. (2019)
	FO, SO	5→20	>10: iNOS ↓, LYZ↓	Zhou et al. (2020)
<i>Mylopharyngodon</i> <i>piceus</i> fingerling Black carp	RO	0–100 replacement of FO	ACH ₅₀ ↔, LYZ↔	Sun et al. (2011)
<i>Nibea coibor</i> juve- nile Chu’s croaker	FO	6, 9, 12, 15	ALT↑, AST↑, LPO↑	Huang et al. (2016)

(continued)

Table 23.2 (continued)

Species Common name	Lipid/oil		Immune trait	References
	Source	Dose, %		
<i>Oncorhynchus mykiss</i> fry Rainbow trout	LO, SaO	100	ACH ₅₀ ↔, LYZ↔, PHA↔	Kiron et al. (2011)
<i>Oncorhynchus mykiss</i> juvenile	BT	50 FO replaced	LYZ↔	Bureau et al. (2008)
<i>Oreochromis mossambicus</i> juvenile Mozambique tilapia	Unrefined peanut oil	50, 100 FO replacement	HC↔, LYZ↔	Demir et al. (2014)
<i>Oreochromis niloticus</i> fry Nile tilapia	CO, BT, FO, LO FO+CO+BT; LO+CO+BT equal levels	7	Only BT: LYZ↓, ACH ₅₀ ↓, but pathogen resistance↑	Yildirim-Aksoy et al. (2007)
<i>O. niloticus</i> juvenile, male	CO, LO, FO, OO, SO	5	Only SO: Immunity↑, resistance against <i>Streptococcus agalactiae</i> ↑	Ferreira et al. (2015)
<i>O. niloticus</i> juvenile	PO	0, 25, 50, 75, 100	AST↑, ALP↑, LYZ↓, resistance against <i>Str. iniae</i> ↓	Larbi Ayisi et al. (2018)
<i>O. niloticus</i> larvae GIFT tilapia	FO+SO 1:1	4–18.5	Max lyz, <i>hamp-1</i> @ 4.5–11.5 (Fig. 23.10) Max survival after <i>Str. iniae</i> challenge @ 11.5	Qiang et al. (2017)
<i>O. niloticus</i> × <i>O. aureus</i> juvenile, 8 g, Hybrid tilapia	SO	4, 6, 8	ALT↓, AST↑, ALP↔	Ma et al. (2016)
<i>Oxyeleotris marmorata</i> juvenile Marble goby	FO, CaO, SO, CaO+SO	12–13	LYZ↔	Ti et al. (2019)
<i>Polyodon spathula</i> ~80 g, American paddlefish	Not specified	3–12	ALT↔, AST↔, LPO↔ SOD optim. @ 9.7	Liu et al. (2018)
<i>Salmo salar</i> fry, Atlantic salmon	FO; VO; phospholipid-rich oil	Total replacement of FO	Immunomodulation only in the pyloric caeca of the intestine but not in the liver	Jalili et al. (2019)
<i>S. salar</i> smolt	FO; RO	Partly replacement; replacement of protein source	Immune genes: <i>igd</i> ↓, <i>mx</i> ↑, <i>ifit5</i> ↑, <i>mhcI</i> (↑)	Caballero-Solares et al. (2018)
<i>S. salar</i> smolt 3 strains: Fat Lean Commercial	VO	Total replacement of FO	HC↑, LYZ↔, LEUK↓ HC↓, LYZ↔, LEUK↔ HC↔, LYZ↓, LEUK↔	Petropoulos et al. (2009)

(continued)

Table 23.2 (continued)

Species Common name	Lipid/oil		Immune trait	References
	Source	Dose, %		
<i>S. salar</i> post-smolt	FO, SfO, LO	16	HC↔, LYZ↔, ACH ₅₀ ↔, PHA↔	Bell et al. (1996)
<i>S. salar</i> juvenile 400g	1:1 CaO + poultry fat	30, 60 anchovy oil replaced	HC↔, ERY↔, LEUK↔, LYM↔, THR↓	Balfry et al. (2006)
<i>Salvelinus alpinus</i> subadult, Arctic charr	SO LO Marine oil ^b	18	PR↑↑ PR↑ PR↔	Lødemel et al. (2001)
<i>Scophthalmus maximus</i> 85 g Turbot	Oxidized FO	7	Phagocytosis↓, LPO↑, mortality after <i>Vibrio anguillarum</i> infection↑	Obach and Laurencin (1992)
<i>Sebastes schlegelii</i> juvenile, Korean rockfish	FO; SO, LO SO+LO	8	Antioxidant enzymes↔	Aminikhoei et al. (2013)
<i>Sillago ciliata</i> fingerling, Sand whiting	FO	0, 10	Resistance against <i>Aphanomyces</i> ↔	Catap and Munday (1998)
<i>Solea senegalensis</i> juvenile Senegalese sole	LO, SO	100 replacement	Complement and cytokine genes↑	Montero et al. (2015)
<i>Sparus aurata</i> juvenile Gilthead sea bream		60 replacement	PHA↓ ACH ₅₀ ↓	Montero et al. (2003)
	LO + SO 1:1	Total replacement of FO	LYZ↔, ACH ₅₀ ↓, PHA↓, mx↑	Montero et al. (2008)
	FO, SO, LO	70→100 FO replacement	Pro-inflammatory genes↑	Montero et al. (2010)
<i>Trachinotus ovatus</i> juvenile Golden pompano	SO; LO	100	zo-1↓, <i>Mycoplasma</i> ↑, <i>Vibrio</i> ↑, <i>Bacillus</i> ↓, <i>Lactococcus</i> ↓ (also refer to Volume III)	You et al. (2019)

BT beef tallow, CaO canola oil, CO corn oil, FO fish oil, LO linseed oil, OO olive oil, PO palm oil, RO rapeseed oil, SaO safflower oil, SfO sunflower oil, SO soybean oil, VO vegetable oil, LIN linoleic acids, DHA docosahexaenoic acid

↑ support/increase, ↔ independent of treatment, ↓ decrease/reduction, PR pathogen resistance, HC hematocrit, iNOS inducible nitric oxide synthase, ERY erythrocytes, LEUK leukocytes, THR thrombocytes, LPO lipid peroxidation, LYM lymphocytes, LYZ lysozyme, COM alternative complement, PER plasma peroxidase, PHA phagocytosis, MA mucus activity, ACH₅₀ alternative complement, AST aspartate aminotransferase, ALT alanine aminotransferase, ALP alkaline phosphatase, ACP acid phosphatase, SOD superoxide dismutase. Enzyme names refer not only to the protein itself but also to its activity

^aCatfish oil has a higher percentage of monounsaturated FAs and slightly higher of ω6 FAs, whereas the menhaden oil diet has a higher percentage of ω3 FAs (Lingenfelser et al. 1995)

^bCommercial product. Genes: *igd* immunoglobulin delta heavy-chain constant region, *mx* interferon-induced GTP-binding protein, *ift5* interferon-induced protein with tetratricopeptide repeats 5, *mhcI* major histocompatibility complex class I heavy chain, *zo-1* zonula occludens-1

beneficial effects of appropriate lipid diets, examples of replacement lipids by various oils are also listed in this table; replacement issues will be revisited in detail in AAN III.

In brief, changes in the sources of dietary lipids by partial or even complete replacement of FO by terrestrial animal or plant lipid sources can adversely affect fish health (e.g., indicated by lipid peroxidation (LPO), inflammation, increase of hepatic enzyme activity) and reduced disease resistance. Lipids modulate or even compromise the immune response by influencing the physical properties of immune cell membranes (phospholipids), membrane-associated signaling molecules (eicosanoids), and receptor sites (i.e., protein kinase C) (Montero et al. 2004).

Alterations on the dietary ratio of $\omega 6/\omega 3$ FAs (feeding certain VO instead of FO) bear the risk of exerting detrimental effects on fish immunity and disease resistance in most marine fish species. Montero et al. (2010) reported that the replacement of dietary FO by VOs adversely affects humoral immunity in gilthead sea bream. Anchovy oil is substituted by linseed (rich in $\omega 3$ FAs) or soybean (rich in $\omega 6$ FAs). Complete FO replacement causes an overexpression of pro-inflammatory cytokines, *tumor necrosis factor- α* (*tnf- α*), and *interleukin 1 β* (*il-1 β*) in the intestine (Fig. 23.11).

Consistently, Tan et al. (2016) identified this phenomenon in large yellow croaker (*Larimichthys crocea*). The addition of dietary VO increases the expression of the two pro-inflammatory cytokines, also studied in gilthead sea bream, and reduces those of anti-inflammatory cytokines in the adipose tissue (Fig. 23.11). This culminates in the activation of the transcription factor nuclear factor- κ B, which controls the expression of an array of inflammatory cytokine genes (TLR-NF- κ B pathway) (Kawai and Akira 2007).

Montero et al. (2015) documented the inflammatory response by excess of $\omega 6$ FAs also in Senegalese sole. The authors fed juveniles with 12% crude lipid diets containing linseed (100LO), soybean (100SO), or fish (100FO) oils and monitored expression profiles of 53 immune-related genes. The use of SO induces an overexpression of genes related to complement pathway, recognizing pathogen associated to molecular patterns, defensive response against bacteria, defensive response against viruses, antigen differentiation, and cytokines and their receptors (Fig. 23.12). This general overexpression indicates an activation of inflammatory processes in the gut.

In addition, dietary VOs alter also the *mx* mRNA expression. Mx proteins are resistance proteins against myxovirus. As studied again in gilthead sea bream, *mx* transcripts are not expressed in healthy individuals on FO. However, dietary inclusion of VOs markedly increases the basal expression of these transcripts indicating a challenge of the immune system (Montero et al. 2008).

Noteworthy, even an increase in immunity by inclusion of non-FOs can take place: In channel catfish, such an inclusion increases the resistance against *Edwardsiella ictaluri* (Fracalossi and Lovell 1994). Li et al. (1994) showed that channel catfish on diets containing 2% menhaden oil are more susceptible to *E. ictaluri* than individuals fed the same amount of catfish offal oil or beef tallow. Therefore, the authors recommend a mixture of menhaden oil and other animal and/or plant lipids in commercial catfish feeds.

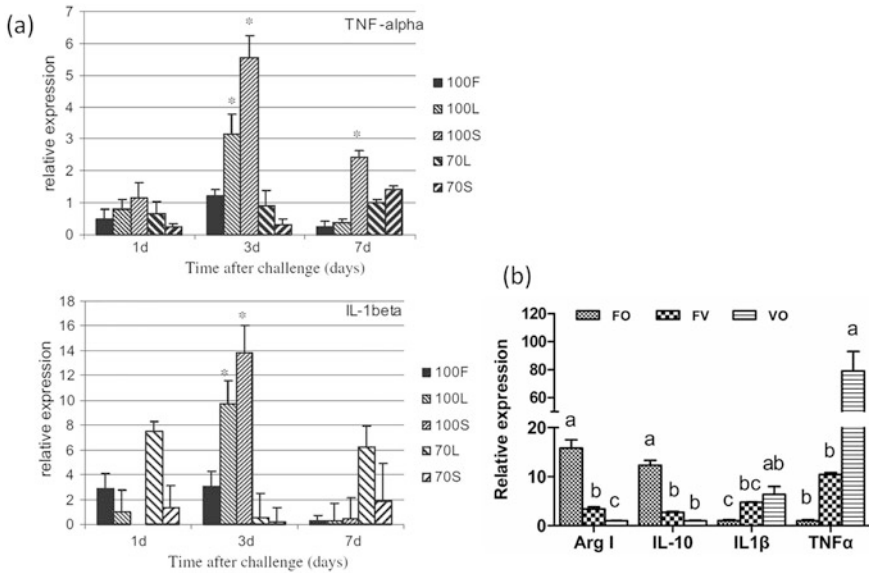


Fig. 23.11 (a) Pro-inflammatory cytokine gene expression in intestine of gilthead sea bream fed experimental diets. Values are expressed as relative expression (*Photobacterium damsela* sp. *piscicida*-infected against noninfected). Samples corresponding to 100 S after 1 day of pathogen exposition were missed. Asterisks denotes significant differences ($P < 0.05$) with fish fed fish oil diet. (From Montero et al. (2010), with permission from Elsevier). Abbreviations: Control diet (100 F) (100 L, 70 L = 100 and 70% linseed oil, respectively; 100 S, 70 S = 100, 70% soybean oil, respectively). (b) Upregulation of pro-inflammatory cytokine genes (*mfx*, *il-1β*) and downregulation of anti-inflammatory cytokine genes (*arg I*, *il-10*) in macrophages in the adipose tissue of large yellow croaker on FO, fish oil:vegetable oil 1:1 (FV), and vegetable oil (VO) diets. Values are means \pm SEM ($n = 3$). Different letters denote significant differences ($P < 0.05$). Abbreviations: *il-1β* interleukin 1β, *mfx* tumor necrosis factor α, *arg I* arginase I, *il-10* interleukin 10. (From Tan et al. (2016), with permission from Elsevier)

23.1.2.3 Lipids and Intestinal Microbiota

The immunity studies in channel catfish suggest that the optimization of the intestinal microflora may beneficially modulate host's resistance against pathogens, if the fishes are fed appropriate dietary lipids. Furthermore, also the stage of ontogenetic development may be important. A first inventory evaluates the effect of total fish oil replacement by a blend of terrestrial vegetable oils (rapeseed, linseed, and palm oils) in the feed on the colonization and the bacterial succession in first feeding of Atlantic salmon, up to 93 days post first feeding (Nikouli et al. 2021). Feeding on either fish oil or terrestrial vegetable oil diets does not result in significant differences in the intestinal gut microbiota (Fig. 23.13) and growth performance parameters. On the contrary, the composition of gut microbiota changes with age, and each stage is characterized by different dominant bacteria. Important to note, operational taxonomic units (OTUs) are associated with probiotic bacteria (detected in all time points

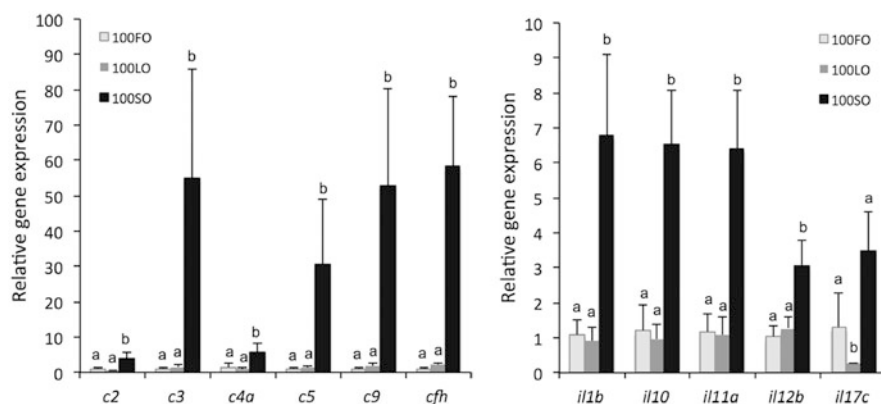


Fig. 23.12 Transcript abundance of complement-related genes (*c2*, *c3*, *c4a*, *c5*, *c9*, *cfh*) and interleukin-related genes (*il-1 β* pro, *il-10* anti, *il-11 α* anti/pro, *il-12 β* anti, *il-17c* pro) in Senegal soles fed 100FO (fish oil), 100LO (linseed oil), and 100SO (soybean oil) diets. Normalization to *gapdh2*. Data are expressed as the mean fold change (mean \pm SEM, $n = 3$) from the calibrator group (100FO). Different letters denote significant differences between diets ($P < 0.05$). (From Montero et al. (2015), with permission from Elsevier)

studied and independently of dietary treatments). One dominant OTU is affiliated with *Weissella cibaria* (Firmicutes), which belongs to the lactic acid bacteria and has antimicrobial activity. Further, OTUs seem to be related to *Delftia acidovorans* and *Pseudomonas viridiflava* (β -proteobacteria) with yet unidentified, probably probiotic, function.

Finally, this study reveals the occurrence of a core microbiota independent of the studied life stages and diet (Fig. 23.13). These findings indicate that total fish oil replacement by terrestrial vegetable oils is feasible. Future work should aim on understanding the functional role of the detected core community which could lead in further feed, growth performance, and host health optimization (Nikouli et al. 2021).

23.2 Metabolism

Lipid metabolism includes catabolic (energy-providing) and anabolic (biosynthetic) processes involving biochemical reactions catalyzed by key enzymes and regulated by, among others, transcription factors. We shall highlight some basics of lipid and FA metabolism.

Acetyl-CoA carboxylase alpha (ACC α) is a cytosolic enzyme that controls the production of malonyl-CoA and plays an important role in the biosynthesis of long-chain FAs (LCFAs). Both 6-phosphogluconate dehydrogenase (6PGD) and glucose 6-phosphate dehydrogenase (G6PD) are key regulatory enzymes involved in NADPH production, essential for FA biosynthesis. Fatty acid synthase (FAS)

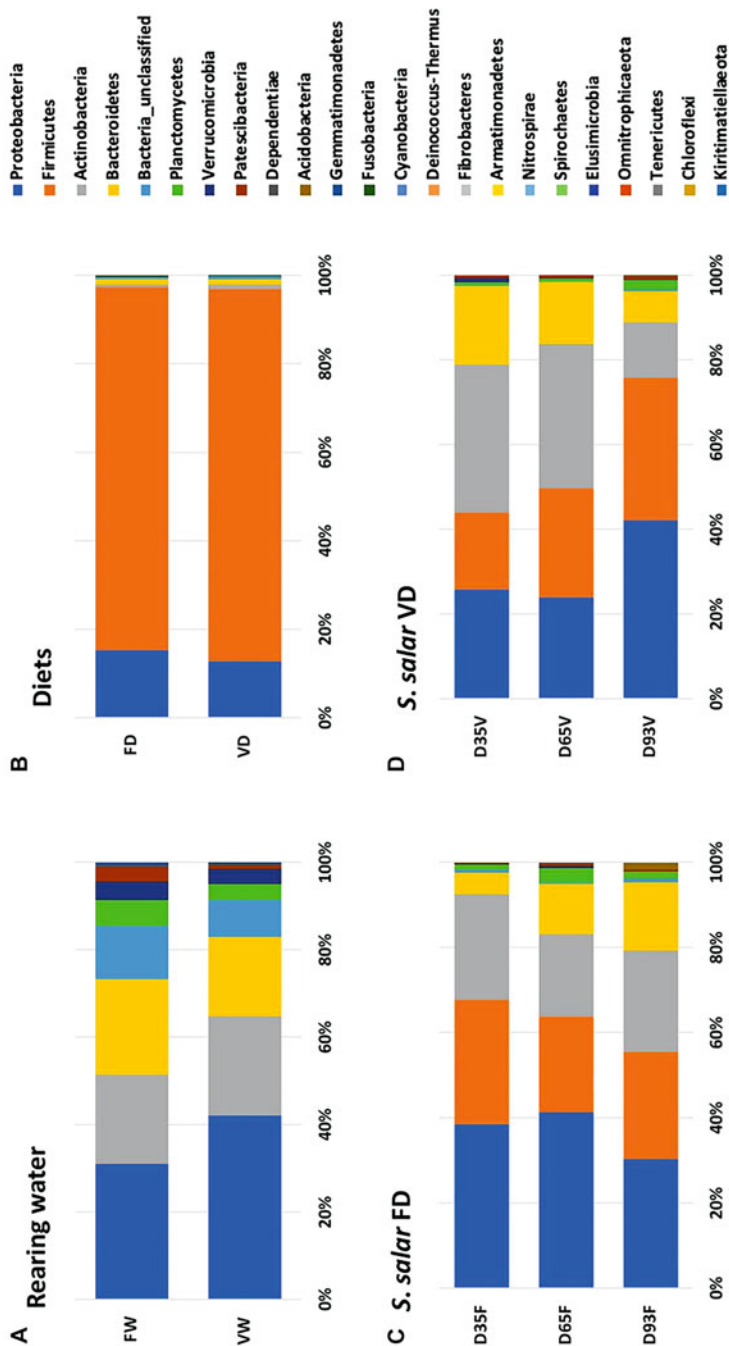


Fig. 23.13 Phylum composition of microbiota from rearing water (a), diets (b), and hindgut of *Salmo salar* samples in fish oil “FD” (c) and vegetable oil “VD” (d) dietary treatments, at D35 (day35), D65, and D93 post first feeding. (From Nikouli et al. (2021), credit Frontiers Media)

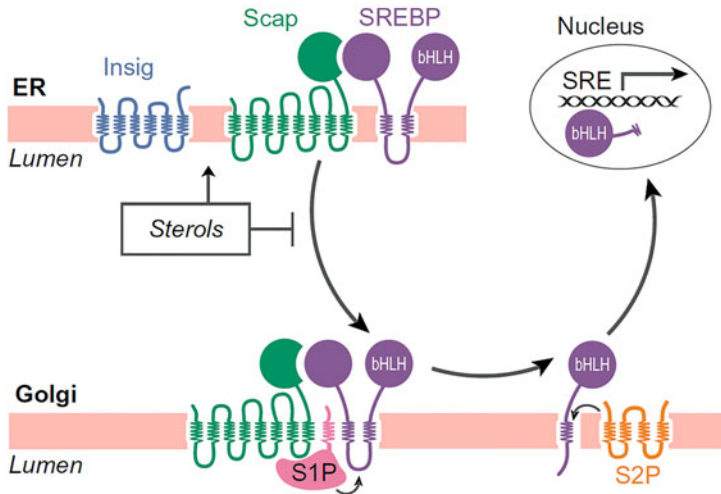


Fig. 23.14 When mammalian cells are deprived of sterols, Scap escorts SREBPs from ER to Golgi. Two Golgi proteases (S1P and S2P) then sequentially cleave SREBP, releasing the active NH₂-terminal transcription factor domain, which travels to the nucleus and activates genes involved in cholesterol synthesis and uptake. High sterol levels trigger the binding of Scap to an ER retention protein, Insig. Transport of SREBP to Golgi and subsequent transcriptional activation are then blocked. (From Radhakrishnan et al. (2010), with permission from Elsevier)

catalyzes *de novo* FA synthesis, whereas sterol regulatory element-binding protein-1 (SREBP1, a transcription factor) is a major regulator of FA and lipid biosynthesis (Spiegelman and Flier 2001).

The SREBP pathway in mammals is presented in Fig. 23.14. When cells are depleted of sterols, a sterol-sensing escort protein (Scap) transports SREBPs from the endoplasmic reticulum (ER) to the Golgi apparatus. Release of SREBPs from the membrane is initiated by Site-1 protease (S1P), a Golgi-located protease that cleaves SREBPs in the luminal loop between the two membrane-spanning sequences. Once the two halves of the SREBP are separated, a second Golgi protease, Site-2 protease (S2P), cleaves the NH₂-terminal basic-helix-loop-helix-leucine zipper (bHLH-Zip, a transcription factor) domain of SREBP at a site located within the membrane-spanning region. After the second cleavage, the NH₂-terminal bHLH-Zip domain leaves the membrane, carrying three hydrophobic residues at its COOH-terminus. The cleaved SREBP enters the nucleus, where it activates genes controlling lipid synthesis and uptake. If sterols are abundant, this pathway is blocked (—|) (Brown and Goldstein 2009).

Among catabolic enzymes, lipoprotein lipase (LPL) hydrolyzes TAGs in plasma lipoproteins and provides free FAs for either storage in adipose tissue or oxidation in other tissues. LPL plays a crucial role in regulating the content of body lipids. Carnitine palmitoyltransferase (CPT1) is regarded as the main regulatory enzyme in FA oxidation catalyzing the conversion of cytosolic fatty acyl-CoA to fatty acyl-

carnitine for entry into mitochondria. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are important enzymes involved in lipogenesis and lipolysis, respectively. Peroxisome proliferator-activated receptor alpha (PPAR α) can modulate expression of genes encoding several mitochondrial FA-catabolizing enzymes in addition to mediating inducible mitochondrial and peroxisomal FA β -oxidation (Jin et al. (2017) and references therein).

23.2.1 Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) have emerged as central factors in sensing FA levels and in regulating FA metabolism. PPARs are ligand-induced transcription factors belonging to the nuclear hormone receptor superfamily with eicosanoids being usually the ligands. PPARs activate the transcription of target genes. In sea bass, PPARs are structural homologs of the mammalian PPAR α , β/δ , and γ isotypes (Boukouvala et al. 2004). *Ppara* is mainly expressed in the liver, *ppary* in adipose tissue, and *ppar β* in all tissues tested, with highest levels in the liver.

The sea bass results are generalizable, since various forms of *ppars* have been identified in fishes: Atlantic salmon (Andersen et al. 2000), zebrafish (Ibabe et al. 2005), rainbow trout, red sea bream (Oku and Umino 2008), Japanese sea bass, large yellow croaker (Dong et al. 2017), Wuchang bream (Zhang et al. 2017), Nile tilapia (Huang et al. 2018), rabbitfish (You et al. 2017), or redlip mullet (Yang et al. 2017). PPARs are involved in the regulation of lipid metabolism in a tissue-specific manner: In turbot, *ppara1* and *ppara2* prevail in the heart, *ppar β* in various tissues, and *ppary* in gills (Zhao et al. 2020).

In addition to tissue-specific distribution of *ppara*, Yang et al. (2017) evaluated the dietary lipid-dependent transcription of this gene in redlip mullets (*Planiliza (Liza) haematocheila*) inhabiting brackish waters. mRNA of *ppara* occurs in all tested tissues with highest expression in liver, followed by brain, stomach, skin, spleen, and visceral fat. The expression is weak in heart and muscles (Fig. 23.15a). Feeding graded lipid levels increases the hepatic *ppara* transcription (Fig. 23.15b). These results indicate that the *ppara* transcription is tissue-specific and that HF diets (HFD) stimulate fat oxidation via activating *ppara* in order to balance the fat deposition.

The third example of *ppara* distribution shows another slight difference (Fig. 23.16). In tiger puffer (*Takifugu rubripes*), highest *ppara1* expression is found in the intestine, whereas the highest level of *ppara2* occurs in heart, liver, and muscle. *ppar β* has highest contents in brain, eyes, and heart (Xu et al. 2020).

Grass carp, too, deserves a closer look: Increases in dietary lipid level do obviously not result in protein sparing (Gao et al. 2011). Young individuals adapt to high lipid intake through increased transcription of *ppary* and *carnitine palmitoyltransferase 1 (cpt1)* in adipose tissues to elevate adipocyte lipid cell differentiation and lipolysis (Fig. 23.17) (Yuan et al. 2016). Moreover, fishes elevate

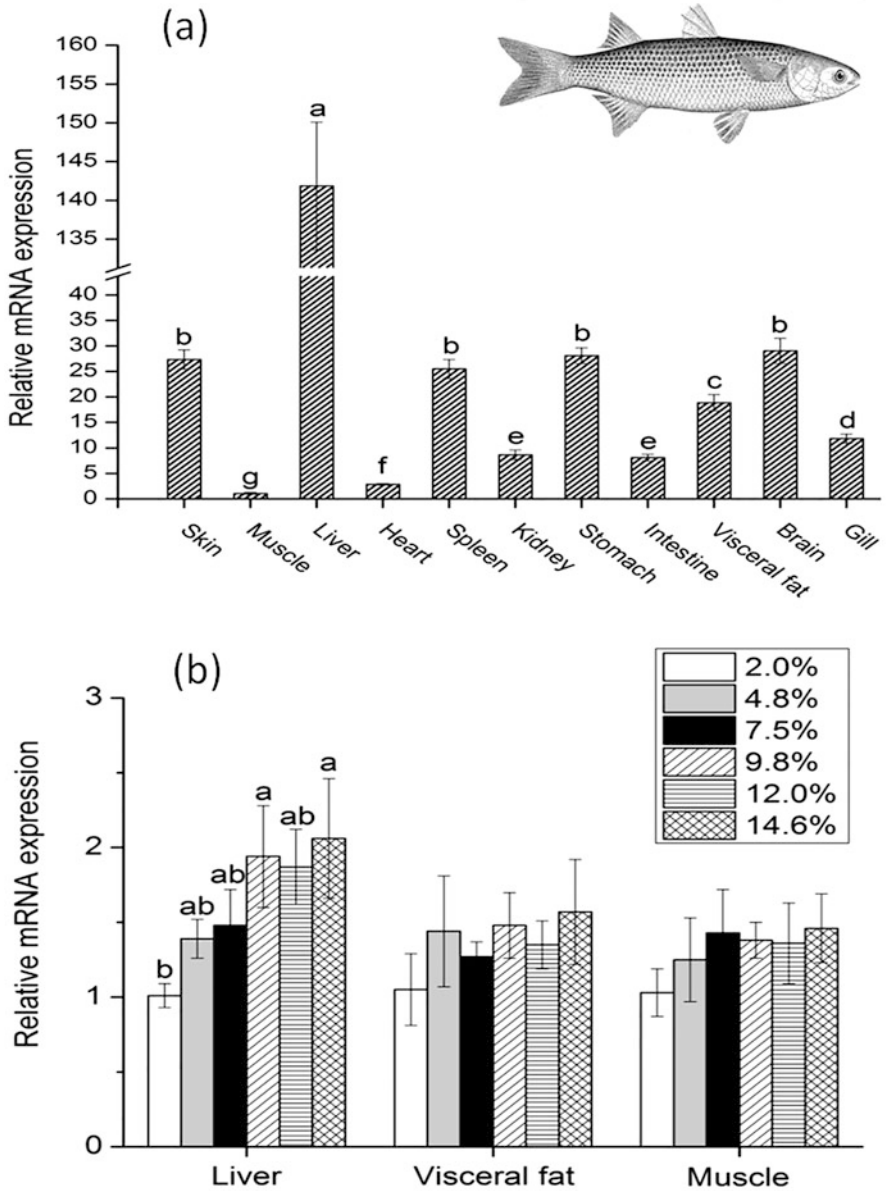


Fig. 23.15 Relative mRNA expression of *ppara* in various tissues of redlip mullet (a). Relative mRNA expression of *ppara* in the liver, visceral fat, and muscle of juvenile redlip mullet fed graded dietary lipid concentrations (b). The *ppara* expression was quantified by qRT-PCR and normalized to β -actin and calculated as the fold increase relative to an arbitrary fold increase of 1 for the expression level of *ppara* in the muscle, which had the lowest value. The values are the means \pm SE. Different letters indicate significant differences ($P < 0.05$). (From Yang et al. (2017), credit Central Fisheries Research Institute (CFRI) Trabzon, Turkey, in cooperation with Japan International Cooperation Agency (JICA), Japan; image credit Turan et al. (2011))

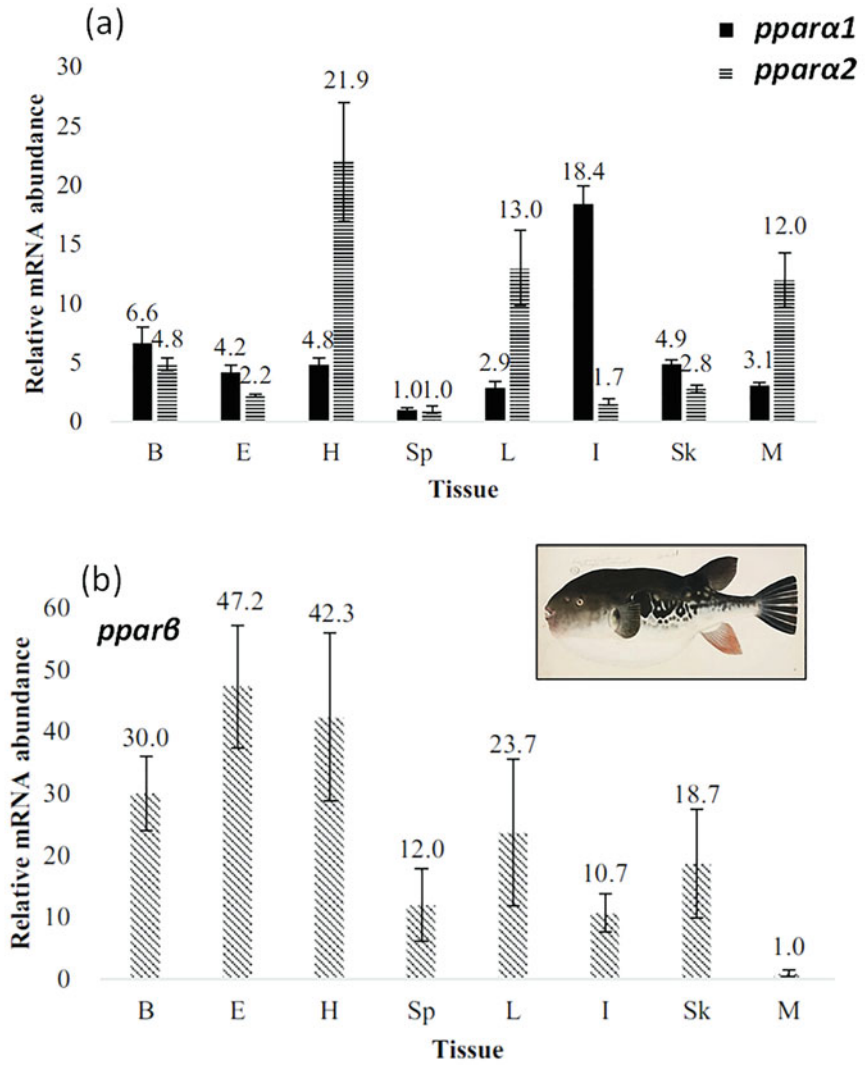


Fig. 23.16 Relative mRNA abundance of *ppara1*, *ppara2* (a), and *pparβ* (b) in different tissues of tiger puffer. Values are means of three replicates ± SE. For each gene, values are expressed as relative to the tissue with the lowest expression. *B* brain, *E* eye, *H* heart, *Sp* spleen, *L* liver, *I* intestine, *Sk* skin, *M* muscle. (From Xu et al. (2020), with permission from Springer Nature; image credit Kawahara Keiga, Naturalis Biodiversity Center, Leiden)

the hepatic lipid uptake but depress the biosynthesis of hepatic FAs (Fig. 23.17) resulting in unchanged hepatopancreasomatic index and liver lipid content. Only in muscles, fishes deposit fat when lipid intake increases above 40 g kg⁻¹.

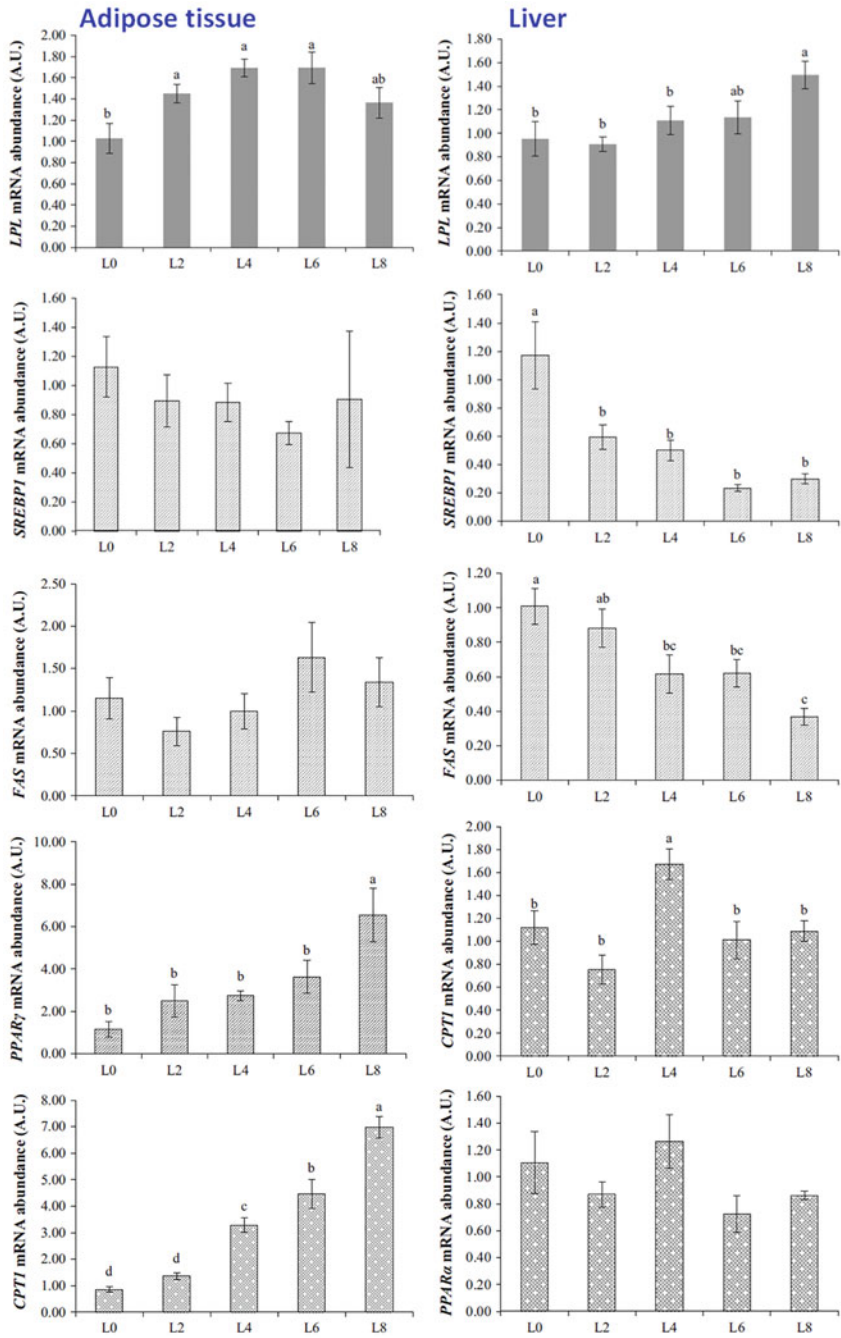


Fig. 23.17 The relative expressions of genes involved in lipid metabolism in adipose tissue and liver of grass carp fed different dietary lipid levels. Normalization to β -actin and *rpl13a*. Values are means \pm SEM of four replicates, and different letters indicate significant differences ($P < 0.05$). (From Yuan et al. (2016), with permission from Elsevier). L0...L8 lipid levels 0...0.80 g kg⁻¹, *lpl*

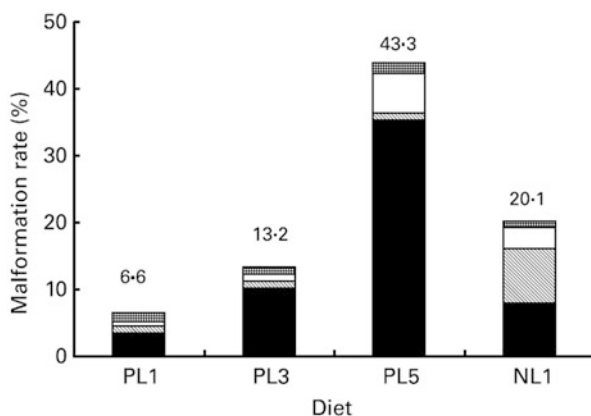


Fig. 23.18 Incidence of skeletal malformations (■ neurocranium; □ maxilla; ▨ operculum; ■ vertebral column) in European sea bass juveniles (age 71 dph) fed isolipidic diets containing different levels of EPA and DHA in different chemical specifications. For details of experimental diets, see text. As fishes fed the NL3 diet died before 37 dph, their incidence of skeletal deformities could not be determined. (From Villeneuve et al. (2005), with permission from the Cambridge University Press)

23.2.2 Retinoid Receptors (RXRs)

Another group of receptors is crucial in lipid metabolism: retinoid receptors (RXRs). They are nuclear receptors that bind to retinoids and then act as transcription factors (Repa et al. (2000); for more information, →AAN I “Transgenerational Effects” (Steinberg 2018)).

Obviously, no studies are available of how dietary gross lipids affect growth and development of fishes via RXR regulation. However, exemplary studies on larval development controlled by EFAs in different lipid fractions have been carried out in European sea bass (*Dicentrarchus labrax*) by identifying several genes involved in morphogenesis (Villeneuve et al. 2005). The authors fed fishes isolipidic diets incorporating different levels of EPA and DHA provided by phospholipid or neutral lipid. Phospholipid fractions containing 1.1% (PL1 diet) to 2.3% (PL3 diet) of EPA and DHA sustain good larval growth and survival, with low vertebral and cephalic deformities. Comparable levels of EPA and DHA provided by the neutral lipid fraction, however, are teratogenic and lethal. Dietary phospholipids containing high levels of DHA and EPA (PL5 diet) induce cephalic (~9%) and vertebral column deformities (35 %) (Fig. 23.18) and adversely affect growth and survival. Moreover, downregulation of *retinoid X receptor α* (*rxra*), *retinoic acid receptor α*, *retinoic*

Fig. 23.17 (continued) lipoprotein lipase, *srebp1* sterol regulatory element binding protein 1, *fas* fatty acid synthetase, *ppar*. . . *γ* peroxisome proliferator-activated receptor type α . . . γ , *cpt1* carnitine palmitoyltransferase 1

acid receptor γ , and *bone morphogenetic protein-4* genes is also noted on PL5 diets at day 16. High levels of dietary PUFA in neutral lipid (NL3 diet) first upregulate the expression of *rxra* at day 16 and then downregulate most of the studied genes at day 23, leading to skeletal abnormalities (Fig. 23.18) and larval death. Moderate PUFA levels in neutral lipids upregulate genes only at day 16, inducing less adverse effects on growth, survival, and malformation than the NL3 group. These results show that retinoid pathways can be influenced by dietary lipids leading to skeletal malformation during larval development; this issue, however, deserves more attention.

23.2.3 MicroRNAs

Since a few years ago, miRNAs are understood to be crucial regulators involved, among other processes, in lipid metabolism of fishes. Pioneering studies have been carried out in rainbow trout. In its liver, postprandial induction of the highly abundant and liver-specific *miR-122*² (Mennigen et al. 2012) is shown to depend on insulin and/or macronutrient composition (Mennigen et al. 2014b) and to be functionally involved in the regulation of postprandial glucose and TAG homeostasis, likely by regulating fatty acid synthase protein abundance (Mennigen et al. 2014a) and adenosine monophosphate-activated protein kinase (AMPK)³ (Lu et al. 2020). In glucose-tolerant Wuchang bream, an HFD affects the hepatic miRNA profile and is predicted to target key genes in lipid metabolism (Zhang et al. 2014).

To identify miRNAs involved in GIFT lipid metabolism, two hepatic small RNA libraries from HFD-fed and normal-fat diet (NFD)-fed GIFT were constructed (Tao et al. 2017). The authors found 204 known and 56 novel miRNAs. Moreover, they examined levels of six known miRNAs differentially expressed: *miR-30a-5p*, *miR-34a*, *miR-145-5p*, *miR-29a*, *miR-205-5p*, and *miR-23a-3p* and their potential target genes (Fig. 23.19). The expression levels of the mRNAs encoding elongation of the very long-chain fatty acid protein 6 (*elovl6*), stearoyl-coenzyme A desaturase (*scd*), acetyl-CoA carboxylase beta (*acac β*), and superoxide dismutase 1, soluble (*sod1*) are lower in the HFD group than in the NFD group. The expression levels of the mRNAs encoding serum response factor (*srf*) and steroid 5 α -reductase 2 (*srd5a2*) are higher in the HFD group than in the NFD group (Tao et al. 2017).

Subsequently, Tao et al. (2018b) functionally characterized *miR-205-5p*, which shows the strongest change between HFD and NFD fishes (Fig. 23.19). HFD inhibits growth of juvenile GIFT and *miR-205-5p* is upregulated in the liver. Acetyl-CoA carboxylase β (*acac β*) mRNA is identified as target of *miR-205-5p*. Consequently,

²Noteworthy, the comparison of *miR-122* from genomic sequences reveals strong conservation of *miR-122* across vertebrate classes; however, its function shows species-specific actions in fishes (here grass carp) and mammalian models (Lu et al. 2020).

³The AMPK and TOR pathways are interlinked, opposing signaling pathways involved in sensing availability of nutrients and energy and regulation of cell growth (\rightarrow Box “Yin and Yang of Energy Regulation” in Chap. 5).

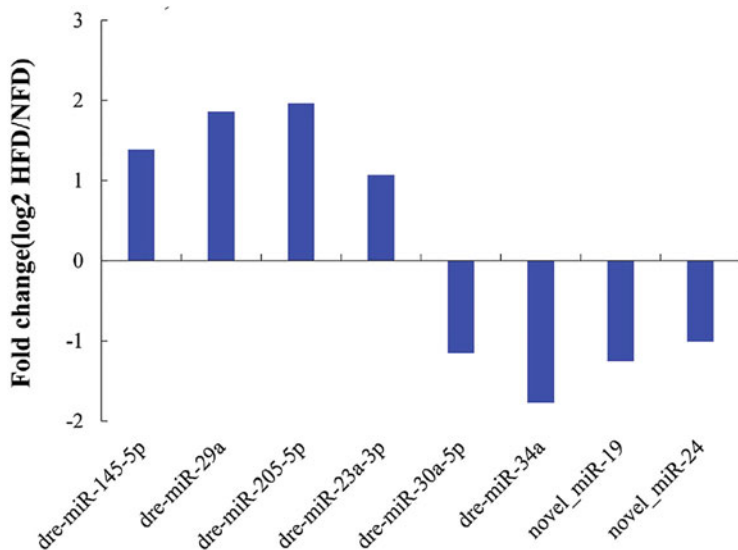


Fig. 23.19 Fold changes of several differentially expressed miRNAs between the high-fat diet (HFD) and normal-fat diet (NFD) GIFT tilapia groups. Fold changes were calculated as \log_2 (HFD/NFD). (From Tao et al. (2017), with permission from Elsevier)

miR-205-5p silencing increases *acacβ* mRNA expression and affects FA synthesis. With *miR-122*, Qiang et al. (2018) identified another miRNA attacking central lipid metabolism genes highly abundant in the liver. The inhibition of *miR-122* upregulates *scd* transcription. Under HFD stress, upregulation of *scd* promotes the expression of genes related to fat synthesis (*srebp-1*, *lpl*) and increases hepatic TAG and cholesterol contents. Furthermore, *miR-122* inhibition results in reduced plasma cholesterol and alterations in genes of the cholesterol metabolism (*srebp-2*, *lxr*, *abca5*, *ugt1a3*) (Mennigen et al. 2014a).

Tao et al. (2018a) enlarged the catalog of miRNAs involved in lipid metabolism of HFD-fed GIFT: *miR-122*, *miR-29a*, and *miR-145-5p* are upregulated, whereas *miR-34a* is downregulated. This study confirms the link between miRNAs and fatty liver induced by HFD and indicates that HFD can lead to excess fat deposition in the liver, which disrupts hepatic lipid metabolism and reduces the antioxidant defense.

miR-122 appears to a major key in hepatic lipid metabolism. In grass carp, Tang et al. (2019) found that 2 g kg⁻¹ dietary fat does not influence growth; however, it alters hepatic expression of miRNAs and genes related to lipid metabolism causing severe lipid deposition. *Pparγ* and *srebp-1* are upregulated in addition to *lxra* (*liver X receptor alpha*) and *abca1*. In hepatic lipid metabolism, *miR-16*, *miR-30*, *miR-33a*, and *miR-122* are downregulated. In addition, *miR-33* plays an important role in cholesterol homeostasis and high-density lipoprotein biogenesis (Tang et al. 2019). In Wuchang bream, *miR-30c* is related to *pparγ* (Zhang et al. 2014), which promotes cellular cholesterol efflux by inducing the expression of *lxra* and *abca1*. In addition,

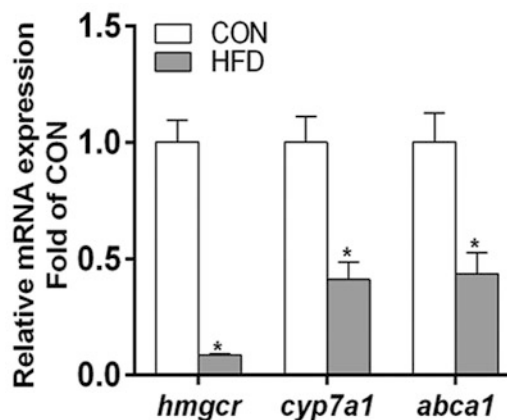


Fig. 23.20 Downregulation of genes involved in cholesterol metabolism of yellow catfish. Values are means \pm SEMs ($n = 3$ replicates of 3 fishes). *Different from CON (control), $P < 0.05$ (Student's t -test). *abca1* adenosine triphosphate binding cassette transporter A1, *cyp7a1* cholesterol 7 α -monooxygenase A1, HFD high-fat diet, *hmgcr* hydroxymethylglutaryl-CoA reductase. (From Chen et al. (2020), with permission from the Oxford University Press)

liver inflammation, steatosis, and lipid metabolic disorders can occur in grass carp, if *miR-122* is overexpressed (Lu et al. 2020).

Recently, Chen et al. (2020) explored the mechanism by which miRNAs mediate HF-induced changes of cholesterol metabolism in omnivorous yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*). HFD induces hepatic cholesterol accumulation and upregulates *miR-144*. In isolated hepatocytes, *miR-144* directly targets *hydroxymethylglutaryl-CoA reductase (hmgcr)*, *cholesterol 7 α -monooxygenase A1 (cyp7a1)*, and *adenosine triphosphate binding cassette transporter A1 (abca1)*. *miR-144* inversely regulates the expression of *hmgcr*, *cyp7a1*, and *abca1* (Fig. 23.20). The action of *miR-144* can serve as a possible general mechanism for HFD-induced dysfunction in cholesterol metabolism.

23.3 High-Fat Diets

As seen above, an emerging issue in fish nutrition is high-energy (fat) diet (HFD). These diets are supposed to increase growth of fishes for a given amount of feed by maximally exploiting the protein-sparing effect of HFD and allowing as much of the dietary protein as possible to be converted into muscle protein. However, the use of increasingly HFDs is not without controversy as seen in GIFT above; and it is debatable whether the protein-sparing effect alone accounts for all of the observed WGs (Sargent et al. 2002).

Problematic in HFD diet studies is that precisely what constitutes high energy or HF remains undefined too often. Only a few data are available, and since commercial pressure to increase growth rates and reduce production times is the main rationale behind HFDs, most information comprises only studies on main species farmed in Europe: salmonids, gilthead sea bream, and European sea bass. In rainbow trout, dietary lipid at 21% increase growth better than diets with 8 and 11% lipid. In brown trout, a diet containing 29% lipid increases growth rate better than a diet with 21% lipid. In Atlantic salmon, even higher lipid levels up to 47% are successfully applied. In a study in sea bass, diets of 19 to 24% lipid, but not 30%, result in increased growth. The growth-promoting effects of dietary lipids vary with developmental stage: Increasing the dietary lipid from 12 to 20% does not alter the growth of sea bass larvae. However, in sea bass fingerlings, growth and protein sparing improve with increasing dietary lipid contents from 9 to 15% (Sargent et al. (2002) and references therein; →also Chap. 26).

High HFD levels can be detrimental and lead to reduced antioxidant capacity, induce LPO, and reduce digestion and assimilation of lipid. The consequence is decreasing growth as shown in juvenile scaleless carp (*Gymnocypris przewalskii*) (Meng et al. 2018). In rice field eel (*Monopterus albus*), Peng et al. (2019) identified another mode of action of detrimental HFD-mediated effects: disturbance in the homeostasis of intestinal microbiota which leads to dyslipidemia. One consequence is the development of diet-induced fat deposition in the liver or other tissues. The alteration of the intestinal microbiota can even be gender-specific as recently identified in zebrafish (Navarro-Barrón et al. 2019).

Confirming results are found in the intestinal microbiome of Atlantic salmon. Huyben et al. (2020) fed post-smolts diets with different lipid:protein ratios. At the genus level, *Aliivibrio*, *Streptococcus*, *Weissella*, and *Lactobacillus* are associated with low-lipid diets, while HFDs are associated with less abundant bacteria, for example, *Chromohalobacter*.

Adverse effects of HFDs are also observed in Wuchang bream. Lu et al. (2014) fed this fish diets with 5% or 15% fat. Fishes on the 15% fat diet suffer from high mortality and poor growth. Histology shows that the HF intake results in fat and glycogen accumulation and structural alterations of the hepatocytes, mitochondria, and nuclei. LPO and the ratio of oxidized to reduced glutathione indicate oxidative stress in the livers of the HFD group. Moreover, the lower leukocyte count, lysozyme and alternative complement activities, and globulin level in fishes on HFD point out suppressed immunity. Elevated plasma cortisol levels and liver *hsp70* expression (Fig. 23.21) provide evidence of increased stress on HFD. Moreover, nonspecific immune responses, such as expression of *leap2* (Fig. 23.21), are also suppressed on HFD. Collectively, the dysfunction of the mitochondria mediates oxidative stress and hepatocyte apoptosis, which in turn lead to reductions in the immune response (→Chap. 24).

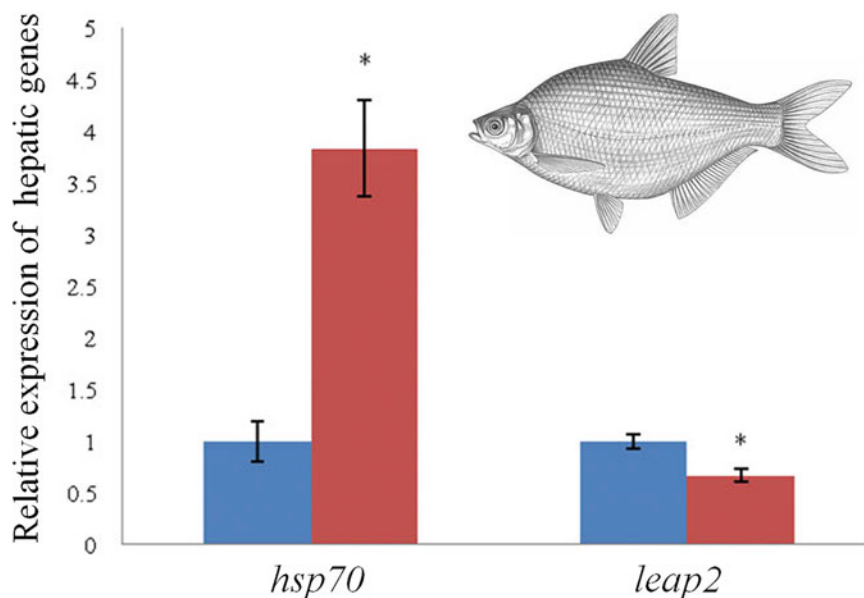


Fig. 23.21 Relative mRNA expression of the *heat shock protein 70* (*hsp70*) and *liver-expressed antimicrobial peptide 2* (*leap2*) genes in the livers of Wuchang bream fed the control (5% fat, blue columns) and high-fat (15% fat, red columns) diets (values are mean \pm SE; $n = 6$). Expression of the target genes is presented relative to the control (set to 1.0). Expression levels are normalized to β -actin. * indicates significant differences between HFD group and control ($P < 0.05$). (From Lu et al. (2014), with permission from Wiley, image credit FAO)

Subsequently, the same laboratory reported further adverse effects of HFD. Cao et al. (2019) identified endoplasmic reticulum (ER) stress⁴. Importantly, sustained ER stress aggravates fat deposition in Wuchang bream via upregulating the transcription of *srebp1*. Thus, HFD induces inflammation (upregulation of *tnf- α* and *il-6*) and depresses appetite through transcriptional upregulation of *leptin/mTOR* (Dai et al. 2018). As a cytokine, leptin has pro-inflammatory properties, and multiple inflammatory cytokines (IL-6, TNF- α) stimulate upregulation of *leptin* transcription. Leptin, in turn, plays a key role in suppressing food intake. The eventual result is oxidative stress, chronic inflammation, and apoptosis (Fig. 23.22) (Dai et al. 2019). Caspases are proteases involved in the execution of apoptosis.

⁴Endoplasmic reticulum stress: Alterations in the function of the endoplasmic reticulum (ER) can result in the accumulation of unfolded or misfolded proteins, a cellular condition referred to as ER stress. ER stress comprises the unfolded protein response (UPR), an adaptive reaction that reduces unfolded protein load to maintain cell viability and function (Hetz 2012). UPR signaling modules cross talk with pathways, which are key in the control of lipid and energy metabolism, innate immunity, and cell differentiation.

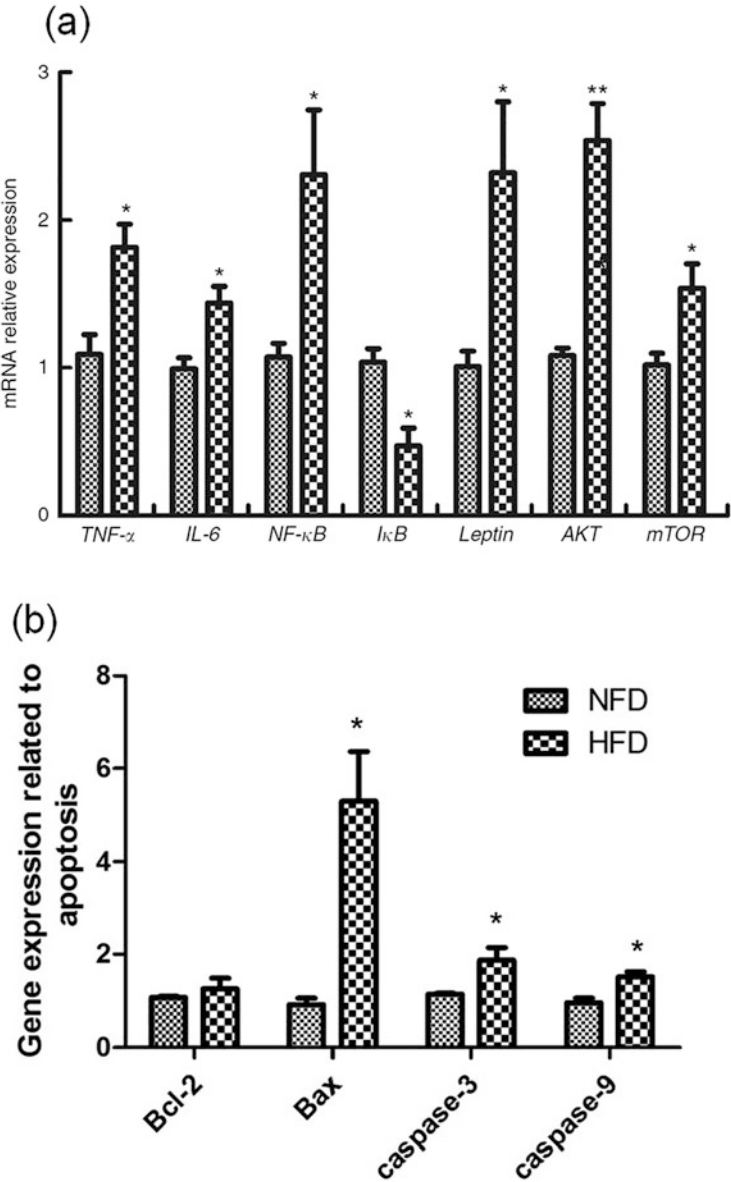


Fig. 23.22 (a) Relative gene expression of *tnf-α*, *il-6*, *nf-κb*, inhibitor of κB (*ikb*), *leptin*, mammalian target of rapamycin (*mtor*), and *protein kinase B* (*akt*) in brain of Wuchang bream fed a normal-fat diet (NFD, left columns of each pair) or high-fat diet (HFD, right columns of each pair) for 60 d. (b) Apoptosis-related indicators: Relative gene expression of *bcl-2*, *bax* (apoptotic activator), *caspase-3*, and *caspase-9* in the liver. Values are means ($n = 4$), with SE. Asterisks indicate significant differences between treatment and control groups: * $P < 0.05$, ** $P < 0.01$. (From Dai et al. (2018, 2019), with permission from the Cambridge University Press)

Reports on adverse effects of HFDs are accumulating further. Feeding HF high-carbohydrate diet (HFHCD) to carbohydrate-tolerant Wuchang bream, Prathomya et al. (2017) discovered a “congestion” of the TCA cycle, causing a decline in the numbers of amino acids entering the cycle. Excess energy causes an elevation in metabolites associated with one-carbon metabolism through high remethylation, eventually leading to oxidative stress. Later, the same laboratory reported an additional and alarming mechanism of adverse effects by HFHCD (Prisingkorn et al. 2019): Disproportionately large numbers of differentially expressed genes are associated with mitochondrial metabolism, neurodegenerative diseases (Alzheimer’s, Huntington’s, and Parkinson’s), and functional categories indicative of liver dysfunction. While the relationship between diet and neurodegenerative disorders is well established in mammals, this is the first report in fishes.

An indirect effect of HFDs has recently been identified by Ye et al. (2019). The authors studied enteroendocrine cells (EECs). These are specialized sensory cells in the intestinal epithelium that sense and transduce nutrient information.⁵ Establishing a new experimental system to investigate EEC activity in vivo, a zebrafish reporter of EEC calcium signaling was used. In fact, HFD alters morphology of EECs and converts them into a nutrient-insensitive state coupled to endoplasmic reticulum (ER) stress (→Chap. 24). Ye et al. (2019) coined the term “EEC silencing.” Gnotobiotic studies reveal that germ-free zebrafish are resistant to HFD-induced EEC silencing. HFD-altered gut microbiota composition including enrichment of *Acinetobacter* is identified sufficient to induce EEC silencing. These results establish a new mechanism by which dietary fat and gut microbiota modulate EEC nutrient sensing and signaling (Fig. 23.23) and open a door for new probiotic treatment of HFDs in aquaculture.

Using metabolomics recently, Tao et al. (2020) identified metabolic changes in GIFT tilapia with fatty liver induced by HFD. Juvenile GIFT fed an HFD accumulates more fat in the liver than do those fed an NFD. The metabolomic analyses reveals 36 differentially accumulated metabolites between the two groups, and these metabolites are involved in 25 signaling pathways. The development of a fatty liver leads to blockage of the TCA cycle, disrupts protein and carbohydrate metabolism, and eventually results in reduced growth of GIFT on HFD.

23.3.1 Mitigation

HFD-mediated oxidative stress and hepatocyte apoptosis can be attenuated by dietary supplementation of plant secondary metabolites such as berberine (Lu et al.

⁵EECs can sense nutrient stimuli derived from the diet such as glucose, amino acids, and fatty acids. Upon receptor-mediated nutrient stimulation, EECs undergo membrane depolarization that results in transient increases in intracellular calcium that in turn induces release of hormones or neurotransmitters (Goldspink et al. 2018).

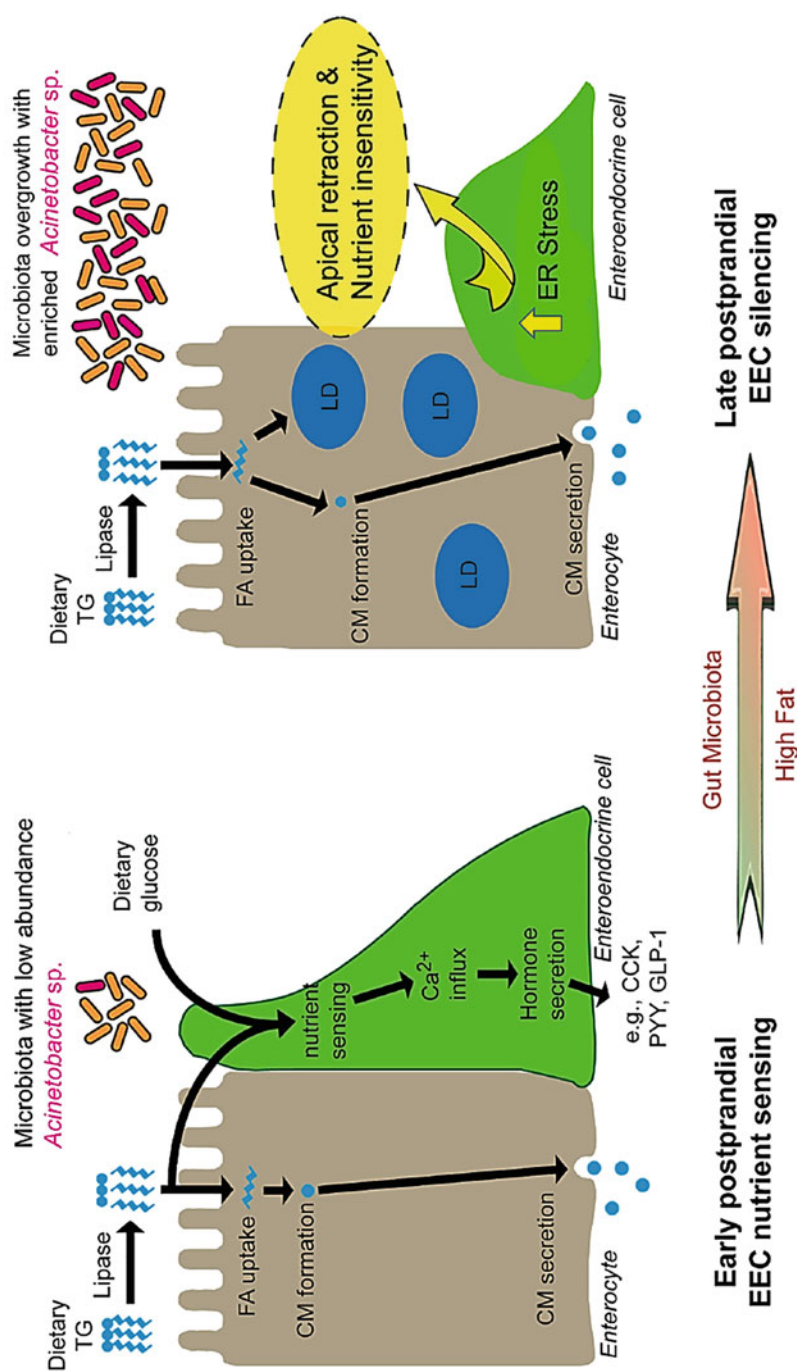


Fig. 23.23 Model for microbiota-dependent high-fat (HF) feeding-induced EEC silencing in zebrafish. At early postprandial stages after consumption of an HF meal, dietary triglyceride (TG) is hydrolyzed to monoglycerides and free fatty acids (FA) by lipases in the gut lumen. FA are taken up by enterocytes and

↓ **Fig. 23.23** (continued) re-esterified into TG, which is packaged into chylomicrons (CM) for basolateral secretion. FA and dietary glucose stimulate enteroendocrine cells (EECs), increasing $[Ca^{2+}]_i$ and inducing secretion of hormones like CCK (cholecystokinin), PYY (peptide YY is a member of the neuropeptide Y, NPY, family), and GLP-1 (glucagon-like peptide-1). During and after HF feeding, FA taken up by enterocytes are stored in cytosolic lipid droplets (LD) in addition to secreted CM. Moving into later postprandial stages, HF feeding and presence of gut microbiota lead to ER stress in EECs. HF feeding also promotes overgrowth of the gut bacterial community including enrichment of *Acinetobacter* sp. Activation of ER stress pathways by these nutritional and microbial stimuli cause EECs to retract their apical processes and reduce their nutrient sensitivity at the late postprandial stage, a process we call “EEC silencing.” (From Ye et al. (2019), credit eLife Sciences Publications)

2017); silymarin (extract from milk thistle, *Silybum marianum*), composed of isomer flavonolignans) (Xiao et al. 2017); or resveratrol (Zhang et al. 2018) (→AAN III “Plant Materials”). The latter authors provide insight into the underpinning mode of action. Resveratrol supplementation, the alleviation in Wuchang bream on HFD, takes place through an increase in *sirt1*⁶ transcription, resulting in upregulation of hepatic *atgl*, *cpt1a*, and *mtfp*⁷ transcription. This promotes liver lipolysis, β -oxidation, and lipid transport, as well as downregulation of hepatic *lpl*, *srebp-1c*, *ppary*, and *acly*⁸ transcription. This suppresses FA uptake and FA synthesis. One percent resveratrol supplementation suffices this amelioration. Supportingly, Torno et al. (2018) reported that the addition of resveratrol to very low fishmeal (FM) and FO feed of juvenile sea bream increases the hepatic long-chain ω 3 PUFA content most likely via upregulation of Δ^6 -desaturase.

Another potential attenuation of oxidative stress takes place via functional feed additives, whereby several modes of actions may apply (Hoseinifar et al. 2020). Beneficial results of specific probiotic treatments against adverse symptoms of HFD are emerging from mammalian studies since more than one decade ago (Ma et al. 2008). Corresponding studies in aquatic animals are almost completely lacking, although the antioxidative potentials of pre- and probiotics are well documented (Abdel-Tawwab et al. 2020; Hoseinifar et al. 2020). To our knowledge, this issue is empirically perfectly addressed only by Falcinelli et al. (2015, 2017, 2018), who supplemented high-lipid aquafeeds with *Lactobacillus rhamnosus*. We shall recall these studies in Chap. 25.

23.4 Concluding Remarks

Field studies, particularly of invertebrates, demonstrate a plasticity of lipid storage and metabolism that enables prevalence in competition. The plasticity, however, is no longer wanted, but still existent, if the aspect of human food production via farmed aquatic animals dominates. It can be hypothesized that an understanding of the underlying biomolecular pathways of this plasticity will have the potential of designing more sustainable aquafeeds and improving existing ones.

Excess dietary lipids reduce growth, impair immunity, and increase mortality (Fig. 23.5). One mode of action is oxidative stress; on the biomolecular level, it is beginning to be understood that epigenetics plays a central role. Evidence is

⁶Encodes sirtuin (silent mating-type information regulation 2 homolog) 1 (*S. cerevisiae*), referring to the fact that its sirtuin homolog (biological equivalent across species) in yeast (*S. cerevisiae*) is *sir2*. SIRT1 is an enzyme that deacetylates proteins that contribute to cellular regulation (reaction to stressors, longevity) (Sinclair and Guarente 2006).

⁷Encodes microsomal triglyceride transfer protein; member of a group of proteins that are able to transfer lipids between membranes.

⁸ATP citrate lyase; important step in FA biosynthesis; converts citrate to acetyl CoA and links the metabolism of carbohydrates and the production of FAs (Sun et al. 2010).

accumulating that miRNAs are major actors in this game; however, obviously as double-edged sword, they accelerate and they mitigate oxidative stress symptoms. Furthermore, there are clear indications that the genetic regulation of the lipid metabolism is species-specific and even tissue-specific (Fig. 23.15). Based on robust knowledge to come, genetics and epigenetics therefore have the potential to balance and mitigate adverse effects even of HFDs. The same holds true for functional feeds. To the best of our knowledge, supplementation of functional feeds as means against adverse effects of HFD is open to future studies; currently, only a few studies are available.

From mammalian nutrition, it is suspected that both pre- and postnatal HFDs adversely affect long-term metabolic health via epigenetic mechanisms. Importantly, the timing of HFD intake determines DNA methylation and gene expression patterns in hepatic metabolic pathways that target specific genomic contexts (Moody et al. 2019). This topic can serve as an incentive for nutritional research in farmed fishes and aquatic invertebrates. The same applies to silencing of enteroendocrine cells via HFD and gut microbiota. Questions arise: Which microbial taxa have the potential to accelerate the cellular mechanism by which the intestinal epithelium absorbs lipids from the diet? Can they be used to affect host physiology through diet? How does the microbiota change during ontogenetic development? And can these changes be used to optimize lipid utilization (Wong et al. 2015)? This topic appears to become central, because the body of evidence is growing that gut microbiota dysbiosis is one major cause for pathological changes of the central nervous system—at least in human beings (Zhang et al. 2020).

The zebrafish model offers the possibility of doing large-scale, controlled, and well-powered observational studies of microbiome composition under different dietary conditions (Stagaman et al. 2020) and to select for probiotic candidates. In addition, also the issue of prebiotics as dietary means against adverse HFD effects deserves future attention.

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Chapter 24

Lipid Homeostasis and Lipophagy—‘*The Greasy Stuff Balanced*’



Abstract With emphasis on lipid droplets and lipophagy, this chapter focuses on lipid homeostasis and its role in immune response. From mammalian studies, it is well understood that the establishment of mature adipocyte phenotypes is associated with high activity of immune genes, activated unfolded protein response, and responses to oxidative stress. Recent studies indicate that this controlling function applies also to fishes and aquatic invertebrates with lipid droplets being central. However, the understanding in invertebrates is still rather fragmentary. Promising models of the function of microbiota in lipid homeostasis as well as of lipid toxicity are emerging. Diet-induced alterations in microbiota composition influence fat absorption, providing mechanistic insight into how microbiota-diet interactions regulate host energy balance. No information is currently available on epigenetic mechanism in lipid homeostasis, but it deserves future attention.

In the context of replacing marine lipids in aquafeeds by high-carbohydrate-containing plant diets with the risk of subsequent hepatic steatosis of the farmed animals, processes of lipid homeostasis move into the study focus. In lipid homeostasis, autophagy is central. It is a catabolic process with an essential function in cellular and tissue homeostasis. It is primarily recognized for its role in the degradation of dysfunctional proteins and unwanted organelles (Panserat et al. 2019); however, in recent years, the range of autophagy substrates has been extended to glycogen (glycophagy; Kaur and Debnath (2015)) and lipids, particularly lipid droplets (LDs, also known as lipid bodies). Consequently, degradation of lipids via autophagy is termed lipophagy (Singh et al. 2009).

24.1 Lipid Homeostasis

Several examples demonstrate the significance of lipid homeostasis in fishes. Testing the hypothesis that increased lipid accumulation imposes considerable stress on adipocytes leading to adipocyte activation and production and subsequent secretion of inflammatory mediators, Todorčević et al. (2010a) pointed out the intimate

relationship between metabolic regulation and immune responses in white adipocytes of Atlantic salmon. Stress imposed on adipocytes by LD formation and expansion is prominently reflected in the endoplasmic reticulum (ER), and the activated unfolded protein response (UPR) can have an important role at visceral obesity. For the first time in fishes, Todorčević et al. (2010a) show that the establishment of mature adipocyte phenotypes is associated with high activity of immune genes, activated UPR, and responses to oxidative stress. These changes are likely to be part of the normal adipocyte development but may be accentuated when cells are overloaded with lipids. High expression of pro-inflammatory mediators implies that excessive growth of white adipose tissue can cause disturbed endocrine function with possible adverse health effects.

In the companion paper, Todorčević et al. (2010b) show that lipogenic and adipogenic processes in Atlantic salmon depend on a tight balance between factors of the redox homeostasis. These are factors that promote the generation of reactive oxygen species (ROS) and factors that inhibit it. Decreasing oxidative stress by ROS quenchers (ascorbic acid, α -tocopherol) attenuates inflammation and acts as a pro-adipogenic signal, as evidenced by increased TAG accumulation. Lipid accumulation promoted by α -tocopherol seems to cause stronger disturbance to ER homeostasis than the increased oxidative stress in antioxidant glutathione (GSH)-depleted Atlantic salmon adipocytes under high exposure to polyunsaturated fatty acids (PUFAs). On the other hand, players with regulatory functions in cell death are more sensitive to changes in the cellular redox environment. Lipid peroxidation in the GSH-depleted and PUFA-treated cells is associated with induction of genes from the apoptosis inducible factor-dependent pathway and with the occurrence of the autophagosome-like intracellular vesicles. Thus, oxidative stress-triggered adipocytes' cell death can play a role in the homeostasis of adipose tissue in animals exposed to high dietary PUFA levels (Todorčević et al. 2010b).

Moreover, fatty acids (FAs) act as signaling molecules modulating adipocyte metabolism and functions. In particular, ω 3 PUFAs can prevent TAG overload in human adipose tissue as well as adipose tissue inflammation (reviewed in Huang et al. (2016)). This effect has also been described in fishes. One of the first laboratories, Ji et al. (2011) showed that appropriate dietary amounts of ω 3 PUFA (blend of α -linolenic (ALA, 18:3 ω 3), eicosapentaenoic (EPA, 20:5 ω 3), and docosahexaenoic acids (DHA, 22:6 ω 3)) reduce hypertrophy and hyperplasia of mesenteric adipose tissue in grass carp. Liu et al. (2014) identified that the modulation of lipolysis-related genes comprises the underlying mode of action; DHA is a stronger elicitor than EPA (Fig. 24.1).

To further identify individual effects of commonly used FAs in Atlantic salmon diets, Bou et al. (2020) cultured primary adipocytes in the presence of oleic acid (OA) or DHA. DHA decreases the number and area of adipocyte lipid droplets compared to OA (Fig. 24.2a, b). The enhanced amount of lipid droplets in OA-treated adipocytes correlates well with the higher presence of the fatty acid transport protein 1 at both the protein (Fig. 24.2c, d) and transcript level (Fig. 24.2e), indicating its involvement in FA uptake and TAG synthesis (Bou et al. 2020).

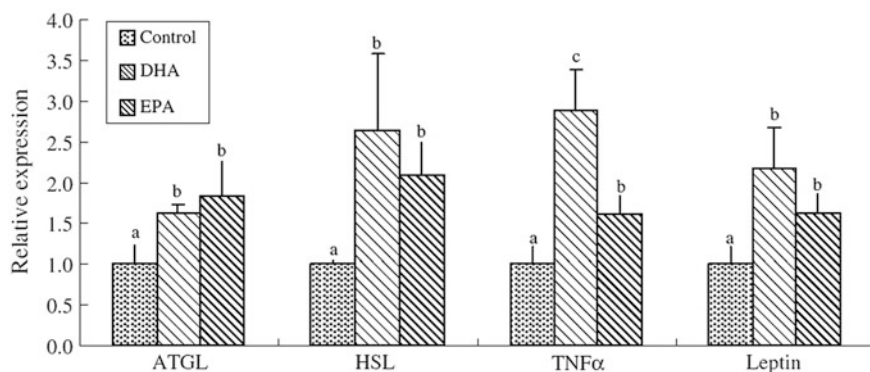


Fig. 24.1 Expression of target genes in grass carp adipocytes after incubation with DHA or EPA for 6 h. Data are presented as relative values compared to control and showed as mean \pm SD, $n = 4$. Values were analyzed by one-way ANOVA. Different letters indicate significant differences ($P < 0.05$). (From Liu et al. (2014), with permission from Springer Nature). *atgl* adipose triglyceride lipase (acts as the first step of triacylglycerol hydrolysis), *hsl* hormone-sensitive lipase (hydrolyzes diacylglycerol to monoglycerides and free fatty acids), *tnfa* tumor necrosis factor α (pro-inflammatory), *leptin* hunger hormone

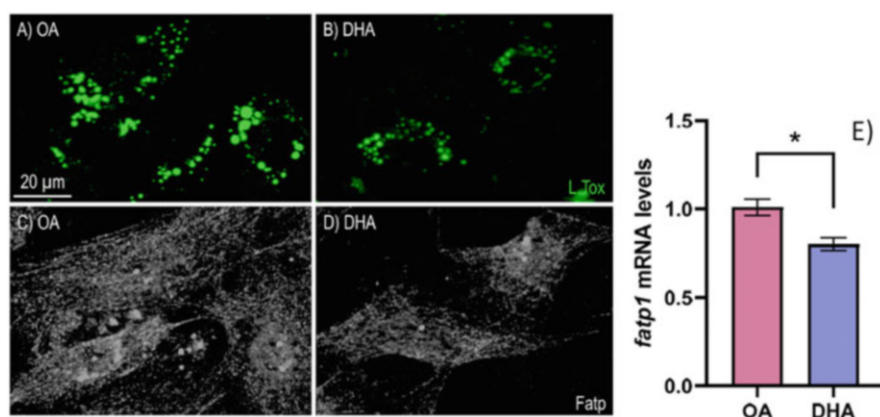


Fig. 24.2 Imaging of lipid droplets by fluorescence microscopy stained with LipidTOX (green) in mature Atlantic salmon adipocytes in vitro incubated with oleic (OA) (a) or docosahexaenoic acid (DHA) (b) for 6 days. Immunofluorescence detection of fatty acid transport protein 1 (FATP1) level in mature Atlantic salmon adipocytes in vitro incubated with oleic (OA) (c) or docosahexaenoic acid (DHA) (d) for 6 days. Thirty images were collected per treatment group (10 images per flask). One representative image for each treatment group is shown in this figure. (e) Transcript levels of *fatty acid transport protein 1* (*fatp1*) in mature adipocytes incubated for 6 days with 100 μ M OA or 100 μ M DHA. Eight samples were analyzed with real-time qPCR; data are presented as fold change \pm SEM using *ef1* as a reference gene, and the OA group was set to one ($\Delta\Delta$ method). * indicates significant differences ($P < 0.05$; Student's t-test). (From Bou et al. (2020), credit MDPI AG)

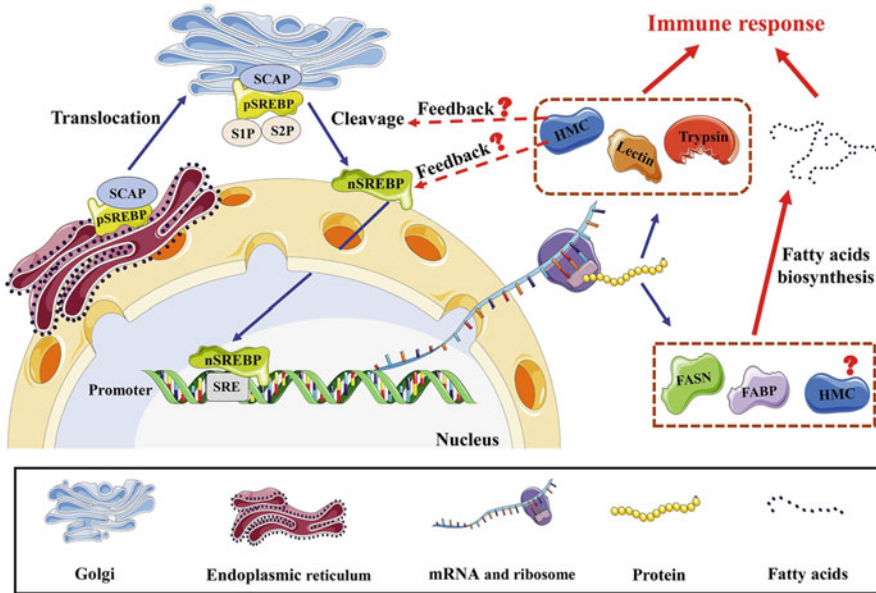


Fig. 24.3 Model of the feedback relationship between LvSREBP and LvHMC in establishing homeostasis during fatty acids metabolism in Pacific white shrimps. SREBP enters the nucleus after activation and binds to sterol response element (SRE) domains of target genes (e.g., hemocyanin (HMC), fatty acid synthase (FASN), lectin, etc.) to regulate their expression. When levels of LvHMC in the cytoplasm reach a certain threshold, it could feedback on LvSREBP to modulate its activation to establish homeostasis. LvHMC might affect LvSREBP by modulating its activation such as affecting its processing into the mature form (nSREBP) or prevent its nuclei entry. Shrimp hemocyanin (LvHMC) seems also to be directly or indirectly involved in fatty acids metabolism, as knockdown of LvHMC attenuated the expression of fatty acids metabolism-related genes and total levels of polyunsaturated fatty acids in the hepatopancreas (From Aweya et al. (2020), with permission from Elsevier) *FABP* fatty acid-binding protein

Séité et al. (2019) demonstrated that the treatment of rainbow trout hepatocytes with an autophagy inhibitor decreases the transcription of genes involved in gluconeogenesis and increases the transcription of genes involved in lipogenesis, lipid storage, and ER stress. This study highlights the strong complexity of the cross talk between ER, autophagy, and metabolism and supports the importance of considering this function in future studies on metabolic adaptation of fish to environmental stresses.

In *Litopenaeus vannamei*, sterol regulatory element binding protein (SREBP) homolog plays a dual role in FA metabolism and immune response via a feedback relationship with hemocyanin in modulating FA metabolism and homeostasis (Aweya et al. 2020). Figure 24.3 depicts a plausible working model. SREBPs integrate multiple signaling pathways to control lipogenesis as well as physiological and pathophysiological processes and pathways important also for autophagy (Shao and Espenshade Peter 2012; Shimano and Sato 2017). Wu et al. (2019) were able to

induce autophagy in *L. vannamei*. The authors challenged this shrimp with rapamycin and successfully induced autophagy in gills (→Chap. 5).

24.2 Lipophagy and Lipid Droplets

Whereas only vertebrates have specialized cells recognizable as adipocytes, lipid droplets (LD) are ubiquitous and evolutionarily conserved organelles found in almost all organisms. They are fat reservoirs with a neutral lipid core delimited by a phospholipid monolayer studded with various proteins (Thiam et al. 2013). They are involved in lipid transport, fat metabolism, lipolysis, mitochondrial FA oxidation, NF- κ B signaling, and FOXO signaling (Redhai and Boutros 2021). LDs are metabolically active, dynamic organelles with key roles in regulating storage and turnover of lipids in different cells and organisms. Proteomic studies have revealed many potential functions of cytoplasmic lipid droplets, and it is confirmed that these bona fide organelles are central not only for lipid storage and metabolism but also for development, immunity, and pathogenesis by several microbes (Hashemi and Goodman 2015). Immune response can be elicited even by pathogen mimics as demonstrated in *C. idella* kidney cells by exposing them to exogenous PUFAs (arachidonic acid (ARA), DHA) (Lei et al. 2019).

Although mobilization of LD by lipolysis has traditionally been attributed solely to the LD-associated lipases, recent studies revealed a role for autophagy in LD breakdown (Fig. 24.4). The presence of lipases in the lysosomal lumen, along with a large variety of hydrolases, such as proteases, glycases, and nucleases, is acknowledged since the early days of the discovery of this bona fide organelle. The autophagic process is coordinated by a complex network of more than 32 genes and their protein products (autophagy-related genes, *atgs*, and proteins, ATGs (Singh and Cuervo 2012)).

24.2.1 Invertebrates

First addressing the role of LDs in immune response in a (semi)-aquatic invertebrate, Barletta et al. (2016) found that LDs in *Aedes aegypti* images facilitate the response against bacteria and dengue virus. The authors identified an infection-induced accumulation of LDs in response to both bacterial and viral infections. Moreover, Jordão et al. (2016) point out that the alteration of lipid homeostasis (numbers and/or area of LDs) can be an important source of toxicity in xenobiotically exposed water fleas (*Daphnia magna*).

Most papers, however, focus on LDs as health indicator (Copeman et al. 2014) or energy storage (McLeod et al. 2004; Hirahara and Toda 2018). The following example is pointing out the involvement of lipid droplets in energy metabolism and pathogen resistance as well as in reproduction and offspring performance.

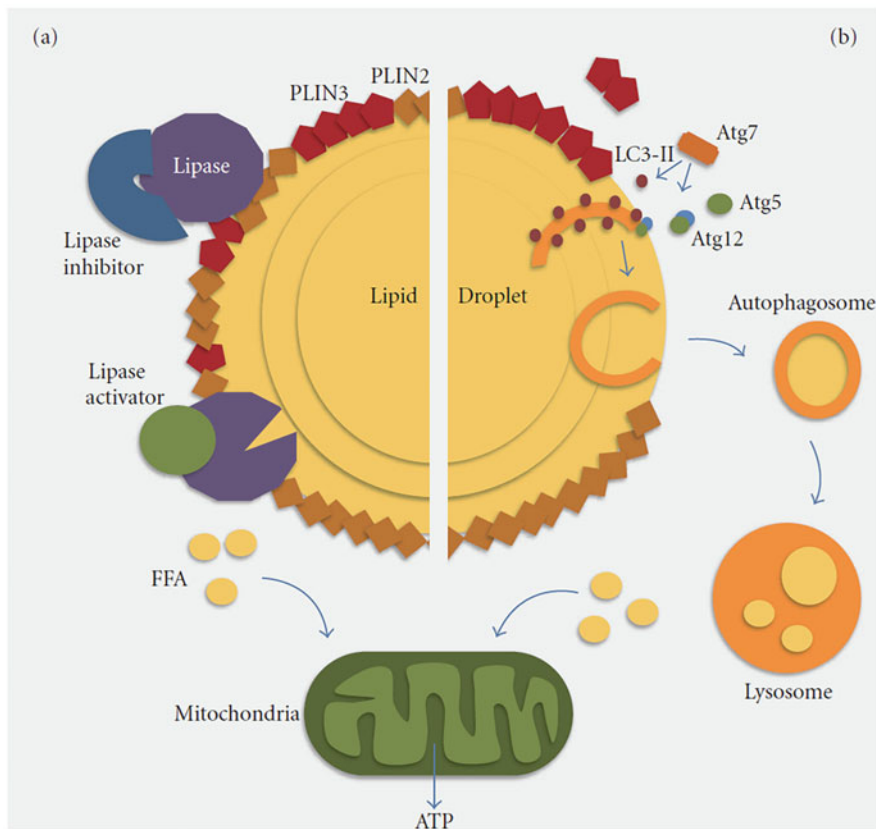


Fig. 24.4 Lipolysis of lipid droplets (LDs). **(a)** Schematic representation of the main lipid and protein components of LDs and mechanisms of lipid mobilization (lipolysis) by cytosolic lipases. **(b)** Lipolysis by lipophagy. Schematic representation of the formation of autophagic vacuoles at the surface of an LD. *PLIN* perilipin, *Atg* autophagy-related protein, *FFA* free fatty acids. (From Singh and Cuervo (2012), credit Hindawi Publishing Corporation)

The red cherry shrimp (*Neocaridina davidi*) is a popular freshwater ornamental species. Sganga and López Greco (2020) evaluated the effect of three commercial diets (A, B, and C) on offspring quality. Feed B is poor in lipids and devoid of DHA. The poor lipid content translates into reduced area of LD in the perigastric organ of Juveniles I (Fig. 24.5a, b) but not into modulated embryo survival independent of the feeding regime (Fig. 24.5c). Whether reduced LDs influence also innate immunity and resistance against pathogens has not been tested.

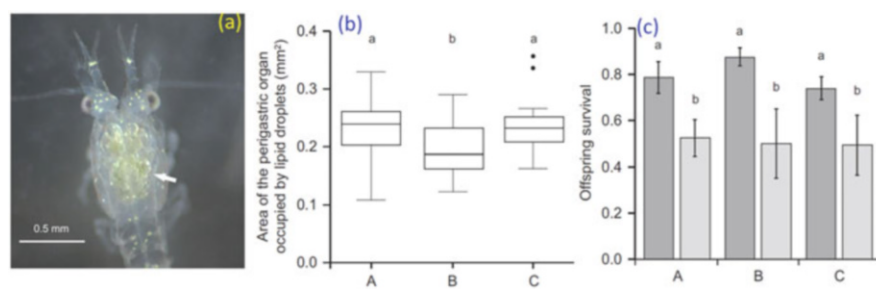


Fig. 24.5 *Neocaridina davidi*. Lipid droplets in the perigastric organ of a Juvenile I (a). The effect of three commercial diets differing in lipid and PUFA content on Juvenile I area of the perigastric organ occupied by lipid droplets (b) and offspring survival (c). (From Sganga and López Greco (2020), with permission from Wiley). Dark gray columns: daily feeding during the 32-day period; light gray columns: food deprivation for the initial 8 post-hatching days and then daily feeding until day 32

24.2.2 Fishes

In zebrafish embryos, Dutta and Sinha (2017) showed that LDs regulate embryonic ATP homeostasis to power early development. Prior to this interesting paper, Semova et al. (2012) proved that LD and the intestinal microbes are central in lipid uptake and homeostasis. The authors showed that the microbes stimulate FA uptake and LD formation in intestinal epithelium and liver. The microbes increase epithelial LD number in a diet-dependent manner. The presence of food leads to the intestinal enrichment of bacteria from the phylum *Firmicutes*. Diet-enriched *Firmicutes* and their products are sufficient to increase epithelial LD number, whereas LD size increased by other bacterial types (Fig. 24.6).

The authors propose two distinct mechanisms to explain observed diet-dependent interactions between gut microbes and host FA absorption. First, *Firmicutes* are enriched in the intestines of fed animals, where they enhance the ability of host enterocytes to absorb FAs. Second, non-*Firmicutes* bacteria that colonize the gut, irrespective of dietary status, induce increased accumulation of large LDs within host enterocytes. In fed animals, these two bacterial signals combine to stimulate FA absorption through increases in both enterocyte LD size and number (Fig. 24.6) and increased export to extraintestinal tissues. Thus, different members of the intestinal microbiota promote FA absorption via distinct mechanisms. Diet-induced alterations in microbiota composition influences fat absorption, providing mechanistic insight into how microbiota-diet interactions regulate host energy balance.

As a proof of concept, lower LD levels occur inside enterocytes and hepatocytes of Senegalese sole specimens fed diet supplemented with *Shewanella putrefaciens* compared to fishes on control diet (García de La Banda et al. 2010; Tapia-Paniagua et al. 2014). This indicates that dietary administration of this probiotic can exert beneficial effects on the intestinal bacterial community and plays a key role in the

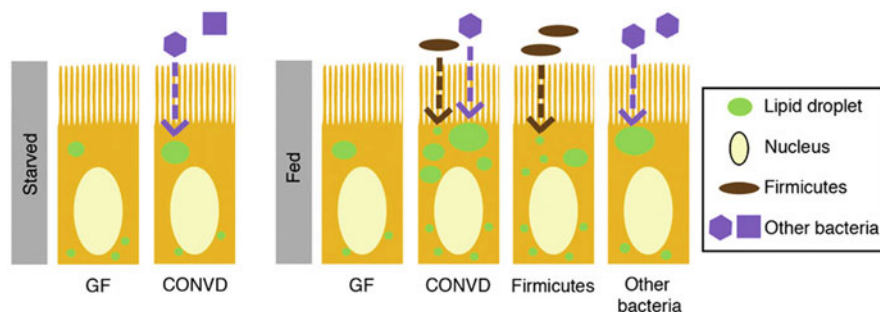


Fig. 24.6 Model for diet-dependent microbial regulation of intestinal fatty acid absorption. Microbes promote LD size in intestinal enterocytes independent of diet (dashed violet arrow). The presence of diet promotes LD number in CONVD (CONVD, conventionalized: colonized with a normal zebrafish microbiota) zebrafish and in those monoassociated with a *Firmicutes* strain (i.e., *Exiguobacterium* sp.; dashed brown arrow). Monoassociation with other bacterial strains *Chryseobacterium* sp. or *Pseudomonas* sp. promotes LD size. Although the extent to which these findings are generalizable to their respective phyla remains unclear, these data indicate two bacterial mechanisms that promote distinct LD accumulation of phenotypes: a *Firmicutes*-induced increase in LD number and a non-*Firmicutes* bacterial induction in LD size. (From Semova et al. (2012), credit Elsevier)

maintenance of lipid homeostasis, resulting in an effective tool to improve sole larviculture (Cámara-Ruiz et al. 2020).

Studies in teleosts demonstrate an adaptive strategy to maintain hepatic lipid homeostasis within certain limits. The excess of fat intake can induce abnormal lipid deposition in liver but not adipose tissue and even spare dietary proteins. In Chinese perch (*Siniperca chuatsi*), Wang et al. (2018) showed that the increase of dietary fat is propitious to reduce the consumption of protein and improve growth. It is due to the homeostasis of hepatic triglyceride (here abbreviated as TG) pool and serum glucose through promoting the FAs β -oxidation and gluconeogenesis, respectively (Fig. 24.7). In individuals fed 17% dietary fat (L17, Fig. 24.8), however, hepatic steatosis occurs—due to both the increase of lipogenesis and the absence of FA β -oxidation. Furthermore, the inhibition of gluconeogenesis can also aggravate triglyceride accumulation in the liver and induce hepatic steatosis. This study unravels the main inducement between the impaired lipid homeostasis and the aggravated hepatic lipid deposition.

24.2.3 Lipid Toxicity

In two papers, Jia et al. (2020a, b) address the often mentioned lipid toxicity by proposing two mechanistic models. Jia et al. (2020a) focused on oxidative stress as main cause of lipid toxicity combined with apoptosis and inflammation (Fig. 24.9). Feeding high-fat diet (HFD) results in severe oxidative stress, which impairs the

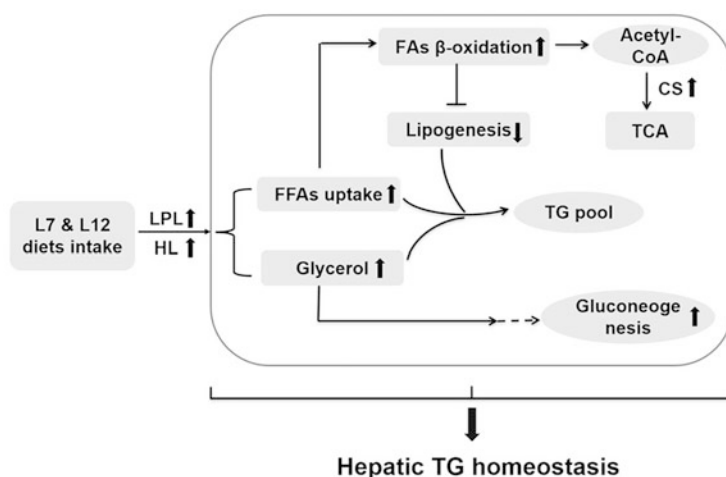


Fig. 24.7 Model of lipid metabolic strategy in Chinese perch responding to appropriate fat intake. L7 and L12 represent diets containing of 7 and 12% lipid content, which is the optimal range of lipid level for fish growth and utilization. In the liver, the elevated free FA (FFA) uptake can trigger FA β -oxidation and then inhibit the lipogenesis to maintain stabilization of TG pool. The acetyl-CoA derived from FA β -oxidation can participate in tricarboxylic acid (TCA) cycle for providing energy. The substrate glycerol derived from dietary triglyceride (TG, also TAG) hydrolysis can participate in gluconeogenesis and then prevent TG from accumulating in the liver. In this model, the homeostasis of hepatic TG pool and serum glucose takes place through the cooperation of FAs β -oxidation and gluconeogenesis. *HL* hepatic lipase, *LPL* lipoprotein lipase. (From Wang et al. (2018); credit BioMed Central)

Nrf2 pathway, weakens the antioxidant defense system, and leads to oxidative damage. In addition, apoptosis is induced by activating JNK¹ and mitochondria pathways after HFD feeding. The hepatic lipid accumulation indirectly leads to the release of pro-inflammatory factors and, thereby, worsens liver injury.

In the companion paper, Jia et al. (2020b) proposed and discussed the ER stress model. The authors investigated the hepatic steatosis. HFD induces steatosis via increased FA uptake, impaired FA β -oxidation, blocked very low-density lipoprotein (VLDL) assembly, and enhanced high-density lipoprotein particles formation (Fig. 24.10). In addition, HFD feeding induces ER stress via the inositol-requiring enzyme 1 (IRE1)² pathway and suppresses the autophagy via transcription factor EB

¹c-Jun N-terminal protein kinases are a mitogen-activated protein kinase family and responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. For instance, JNK1 is involved in apoptosis, neurodegeneration, cell differentiation and proliferation, inflammatory conditions, and cytokine production mediated by AP-1 (activation protein 1) (Oltmanns et al. 2003).

²This protein possesses intrinsic kinase activity and an endoribonuclease activity, and it is important in altering gene expression as a response to endoplasmic reticulum-based stress signals (mainly the unfolded protein response) (Tirasophon et al. 1998).

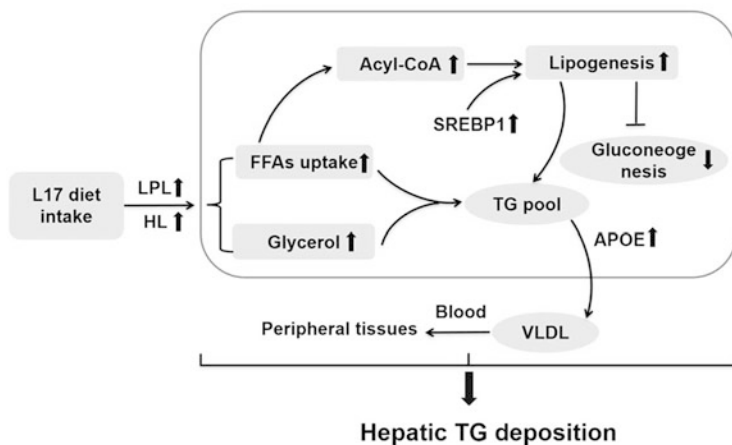


Fig. 24.8 Model of hepatic TG deposition in Chinese perch responding to high-fat intake. L17 represents diets containing of 17% lipid content, which is not beneficial for hepatic lipid utilization and metabolism. In the liver, excess dietary fat intake can accelerate the esterification of FFAs with the absence of FA β -oxidation. The extra uptake of FFAs can provide the mass acyl-CoA substrate for lipogenesis via the upregulation of *srebp1* (sterol regulatory element binding protein 1, as transcription factor, a major regulator of FA, and lipid biosynthesis). The sharply increased lipogenesis can effectively inhibit gluconeogenesis and aggravate TG deposition in liver although a part of newly synthesized TG can be delivered to peripheral tissues via very low-density lipoprotein (VLDL) in blood. For HL, LPL, see Fig. 24.7. (From Wang et al. (2018); credit BioMed Central)

(TFEB)³ and AMPK (\rightarrow Box in Chap. 5) pathways to worsen lipotoxicity in the liver (details in the caption to Fig. 24.10). It is open for studies to come whether these models are mutually exclusive or may reinforce each other.

24.3 Concluding Remarks

Available information on autophagy in fishes supports a good conservation of this function in most, if not all, species studied. However, studies on the metabolic role of this function in fish are still limited, and it remains to be determined to what extent this function, which plays a major role in mammalian hepatic metabolism, can be involved in the metabolic specificities observed in certain fish species (Panserat et al. 2019). However, neither the cross talk between different lipid degrading processes nor the cross talk between the different autophagy processes are currently well understood. The same holds true for the role of intestinal microbes in contributing to the lipid homeostasis. The few available papers point out that probiotics can significantly contribute to and modulate this process. (This issue will be revisited in Chap. 25). Moreover, also lipid toxicity deserves increased attention to unravel

³Transcription factor EB is a master gene for lysosomal biogenesis (Sardiello et al. 2009).

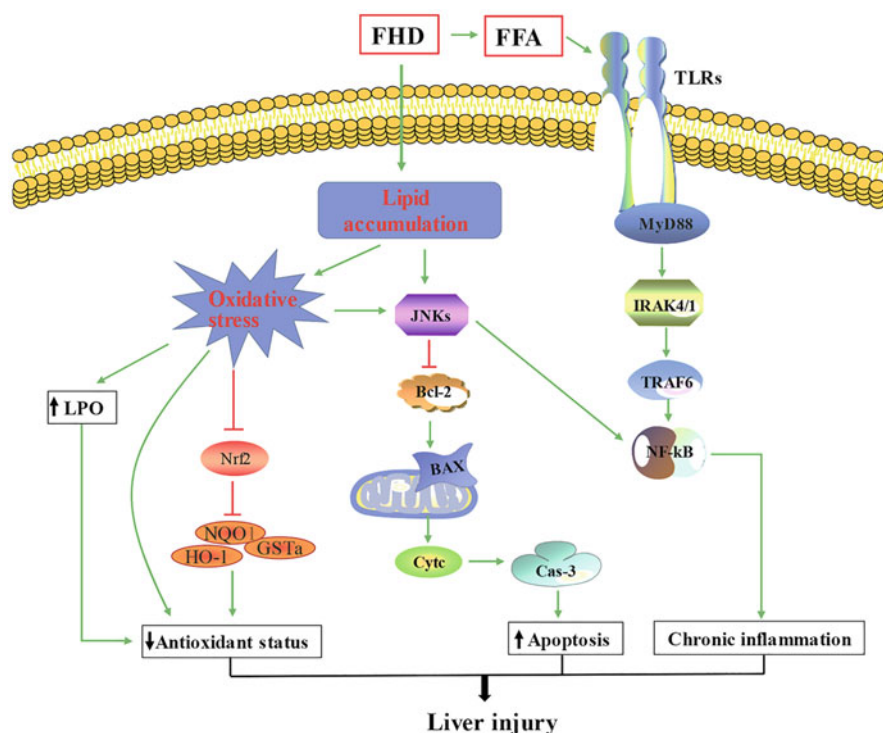


Fig. 24.9 First possible mechanisms of hepatotoxicity induced by HFD in liver of tilapia. HFD feeding increased lipid accumulation which induced oxidative stress, leading to decrease of antioxidant status and formation of lipid peroxidation (LPO). Persistent oxidative stress also impaired Nrf2 (Nuclear erythroid 2-related factor 2 is a transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation (Gold et al. 2012)) signaling pathway, blocked antioxidant protein expression, and further reduced antioxidant defenses. Oxidative stress and lipid accumulation also activated JNKs signaling pathway to initiate mitochondrial apoptotic pathway and inflammation. Moreover, in HFD-fed tilapia, the TLRs (Toll-like receptors, a class of proteins that play a key role in the innate immune system by recognizing molecules that are broadly shared by pathogens. Once pathogen microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs, which activate immune cell responses. MYD88, the myeloid differentiation factor 88, is a pivotal signaling component of the innate immune response, serving as an adaptor for the interleukin 1 receptor and the majority of TLRs (Takeda and Akira 2005). NF-κB, the nuclear transcription factor kappa-B, responds to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized low density-lipoprotein, and bacterial or viral antigens. Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development (Perkins 2007)) (TLR2 and TLR1)-MyD88-NF-κB pathway was triggered, which promoted chronic inflammation. (From Jia et al. (2020a), with permission from Elsevier)

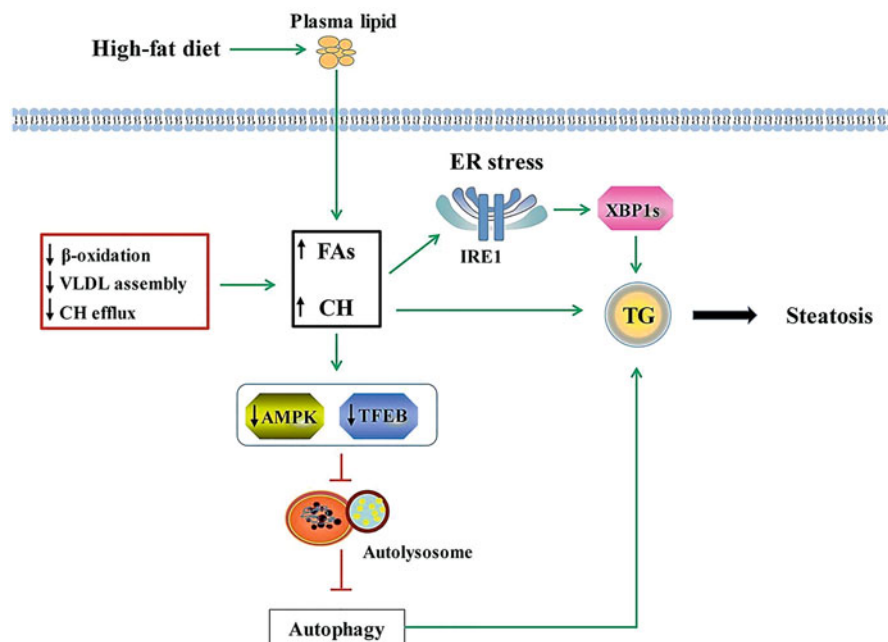


Fig. 24.10 Second possible mechanisms of lipotoxicity induced by high-fat diet (HFD) in tilapia. HFD feeding increased fatty acids (FAs) and free CH (cholesterol) via uptake from plasma. In addition, suppression of FAs β -oxidation, very low-density lipoprotein (VLDL) assembly, and CH efflux induced by HFD feeding also enhanced the hepatic FAs and CH content. Increased FAs and CH promoted TG formation which induced hepatic steatosis. Excessive free fatty acids (FFA) and free CH could cause ER stress and further accelerated steatosis by IRE1-XBP1s (Spliced X box binding protein 1 is a transcription factor that regulates the expression of genes important to the proper functioning of the immune system and in the cellular stress response. It is critical to protect cells from endoplasmic reticulum (ER) stress (Yoshida et al. 2006)) signaling pathway. In HFD-induced fatty liver, the decreased AMPK pathway inhibited formation of UKL1/Atg13 complex (A protein complex consisting of Atg1 (or Atg1 homologs, e.g., ULK1, ULK2) and Atg13 along with other proteins that regulate its function. This complex has serine/threonine protein kinase activity and is involved in autophagosome formation (Kabeya et al. 2005)), hampered autophagy proteins expression, and ultimately resulted in low autophagy. Moreover, the low autophagy was related to depressed lysosomal biogenesis mediated by TFEB pathway. Suppressed autophagy blocked decomposition of lipid droplets and worsened steatosis. (From Jia et al. (2020b), credit Frontiers Media)

underlying mechanisms. Two plausible models of the modes of action in tilapia have recently been proposed; however, they require substantiation by empirical studies in other species.

Furthermore, as part of the autophagy complex, lipid homeostasis is not only central in the catabolic process that degrades cytoplasmic constituents and organelles in the lysosome, but also it significantly contributes to immune response and stress resistance, as actual studies in fishes and particularly in crustaceans demonstrate. The specific involvement of lipid homeostasis needs to be clarified in detail.

Currently, no information is available on epigenetic mechanisms in lipid homeostasis, but they deserve future attention.

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Chapter 25

Protein Sparing by Lipids—‘*Learning from Wild Conspecifics*’



Abstract Mainly based on economical and ethical considerations, proteins are intended to be replaced by cheaper and ethically less critical macronutrients. This chapter summarizes recent papers dealing with protein and carbohydrate replacement by dietary lipids. It becomes obvious that replacement successes are evaluated one-dimensionally by production traits, such as growth performance, flesh quality, health, and pathogen resistance, and, with respect to brood stock, reproductive performance rather than ecological nutritional criteria. In particular, the capacity of aquatic animals to balance their macronutrient intake deserves attention, even under farmed conditions. Emerging studies point out the paramount importance of the axis intestinal microbiota-host welfare in lipid metabolism: Lipid diets modulate the intestinal microflora, and, vice versa, by adding appropriate probiotics, increased dietary lipid can be tolerated.

Many studies evaluate the nutritional replaceability of dietary macronutrients to identify the potential of sparing dietary protein by increasing the proportion of nonprotein energy. This dietary flexibility of farmed aquatic animals is based on adaption to fluctuations in natural systems, in which animals, due to variability in quantity and quality of food, have the ultimate goal to sustain their populations (→AAN I Chap. 4 (Steinberg 2018)). Therefore, they cannot be fastidious; instead, they have to accept suboptimal food compositions with lower shares of proteins and higher shares of lipids or, particularly, carbohydrates, if algae or macrophytes are the main food sources. However, in the medium to long term, animals have to balance their needs in macronutrients (Machovsky-Capuska et al. 2016; Machovsky-Capuska and Raubenheimer 2020). In other words, since almost all animals in the wild have to face periods of suboptimal food quality, protein sparing by lipids or carbohydrates should be a common, at least temporary, strategy in aquatic animals, if they have the eventual chance to balance their nutrient demands. Therefore, they should have developed appropriate biomolecular regulatory pathways to identify and cope with these suboptimal situations. This argument applies also to farmed species, which are able to grow and reproduce under the conditions aquaculturists are offering them. This means that the ability of protein sparing in farmed species should be the rule and not the exception to the rule—provided the animals reach a final

balanced long-term feed. It will be interesting to figure out whether this hypothesis can be verified in farmed species, if the experimental designs sufficiently address this issue. Or vice versa, it is very unlikely that farmed species really originate from stable, non-fluctuating “land of milk and honey”-like ecosystems, where they have enough valuable proteins to feed on. And if in experimental trials protein-sparing strategies cannot be identified, a critical evaluation of the applied methods should be carried out.

25.1 Replaceability of Proteins

The replacement of proteins in wild populations takes place by no means arbitrarily and without limits. Rather, in nature, many herbivores and omnivores adjust their food selection behavior to regulate the intake of multiple nutrients. Carnivores, however, are generally assumed to optimize the rate of prey capture rather than select prey according to nutrient composition. In an intriguing study, however, Mayntz et al. (2005) showed experimentally that (invertebrate) predators can forage selectively for protein and lipids to redress specific nutritional imbalances (→Chap. 2). And the chase for proteins can turn out very dramatic: Under protein-deprived conditions, omnivorous insects, such as German cockroach (*Blattella germanica*) (Lihoreau et al. 2010), or even herbivorous insects, such as desert locust (*Schistocerca gregaria*) (Bazazi et al. 2011) or flightless Mormon cricket (*Anabrus simplex*) (Simpson et al. 2006), decide to be cannibals and form marching bands seeking for protein with each insect chasing the moving meal in front and escaping the cannibals behind (Simpson and Raubenheimer 2011).

The flexibility of protein and energy requirements in farmed aquatic species recalls these natural fluctuating food conditions (Table 25.1), but the requirement of “nutrient-specific foraging” à la Mayntz et al. (2005) is mostly ignored in these studies. It is not disclosed if or how sustainable this strategy may be. In the long term, imbalanced macronutrient diets result in one or more impaired life history traits, such as reproduction, health and immune response, or longevity.

In Pacific white shrimp, Kureshy and Davis (2002) and Venero et al. (2008) demonstrated that as long as feed allowance is adjusted for dietary energy and protein density, the nutrient requirements can be met by different diets with variable levels of protein and energy. This fact applies also to fishes, such as gilthead sea bream (Lupatsch et al. 1998, 2001), Atlantic salmon (Hillestad and Johnsen 1994; Einen and Roem 1997; Karalazos et al. 2011), or red drum (McGoogan and Gatlin 1998). These species tolerate a variety of dietary energy levels, as long as energy is not limited—warm regards from their wild conspecific populations in fluctuating environments!

Really? Usually, the performance is evaluated only based on a few life history traits, mainly survival and growth performance. This means that the Darwinian fitness is only incompletely assessed. Reproductive success and maintenance of populations in the ecosystem do not count—only protein production counts.

Table 25.1 Replacement of proteins by dietary lipids with respect to effects on growth and survival in selected aquatic animals

<i>Invertebrate, common name, apparent trophic classification</i>	<i>Weight/develop. stage</i>	<i>Lipid, %</i>	<i>Protein, %</i>	<i>Growth (and surrogate proxies)</i>	<i>Apparent protein sparing</i>	<i>Survival</i>	<i>References</i>
<i>Astacus astacus</i> , noble crayfish, c	Juvenile	5.5–16	22, 40	WG↓ @ L > 10 + P22→31	✓	Optim @ P40	Ackefors et al. (1992)
<i>Cherax quadricarinatus</i> , Australian redclaw crayfish, c, (h)	0.15	4, 8, 12	26, 31, 36	Max. WG @ P31, L 8	None	↔	Cortés-Jacinto et al. (2005)
<i>C. tenuimanus</i> , hairy marron, c, (h)	1.3	0–3.5	0, 25	SGR↔	✓	↑ @ FO + sun-flower oil	Fotedar (2004)
<i>Eriocheir sinensis</i> , Chinese mitten-hand crab, c, (h)	Juvenile	2, 7, 12	30, 35, 40	WG↑ @P↑ + L↔ WG↓ @ L12 + P40	✓ only @ low P	↔	Cui et al. (2017)
<i>Fenneropenaeus merguensis</i> , banana prawn, p, d–c	~0.30	2–17	17–51	Optim WG @ L3–5 + P 34–42	✓ @ P51→34	↔	Sedgwick (1979)
<i>Jasus edwardsii</i> , southern rock lobster, c	~3.6	5, 9	24–50	Optim WG @ L5–9, P29–31	None	↔	Ward et al. (2003)
<i>Litopenaeus vannamei</i> , Pacific white shrimp, p, d–c	1.0	4–11	30, 40	WG↔	None	↔	Venero et al. (2008)
	0.09	5, 7.5, 10	30, 34, 38, 42	Optim WG @ L7.5 + P 34	None	↔	Hu et al. (2008)
	~11.0	2, 4, 8, 16	20, 30, 40, 50	SDA↑ @ P↑ + L↔	None	n.d.	Ponce-Palafox et al. (2017)
<i>Marsupenaeus japonicus</i> , kuruma prawn, p, d–c	0.4	2.5, 6, 8, 10, 12 ^a	21, 31, 41, 50, 61	Max. WG @ P42 + L8 + C11	✓	↔	Koshio et al. (1993)
<i>Macrobrachium rosenbergii</i> , giant river prawn, p, c	0.1–1.6	15, 20, 25, 35	9–17	Optim SDA @ P15–25	✓	n.d.	Clifford III and Brick (1978)
<i>Procambarus acanthophorus</i> , burrower crayfish, c, (h)	~0.014	6, 8, 10, 12	20, 25, 30, 35, 40	Max growth @ P25 + P12	None	↔	Cervantes-Santiago et al. (2010)
	~2.5	4, 7, 10	24, 27, 30		n.d.	↔	Xu et al. (2013)

(continued)

Table 25.1 (continued)

<i>P. clarkii</i> , red swamp crayfish, c, (h)						WG↑ @ L 4, 7+ P24, 27 WG↓ @ L 10 + P30			
<i>P. llamasi</i> , endemic Mexican crayfish, c, (h)	0.9–1.3	6, 12	10, 20, 30			P20 + L12 for growth P30 + L12 for maturation	✓	Best survival @ L12	Carmona-Osalde et al. (2005)
<i>Fish species, common name, apparent trophic classification</i>	<i>Weight/ develop. stage</i>	<i>Lipid, %</i>	<i>Protein, %</i>			<i>Growth</i>	<i>Apparent protein sparing</i>	<i>Survival</i>	<i>References</i>
<i>Acipenser baerii</i> , Siberian sturgeon, b–c	~30	18	38P + 25C			WG↑	✓	↔	Babaei et al. (2017)
<i>A. persicus</i> , Persian sturgeon, b–c	~10	8–26	40–50			SGR ↔	✓	n.d.	Sayed Hassani et al. (2011)
<i>Argyrosomus japonicus</i> , dusky kob, mulloway, Japanese meagre, c	~115	6, 12, 18	42, 46			Optim SGR↑ @ L18 + P 46	None	↔	Woolley et al. (2010)
	~79	3–33	25–55			Optim WG @ P ~ 48 + L ~ 11			Pirozzi et al. (2010)
	~200								Lemos et al. (2014)
<i>Betta splendens</i> , Siamese fighting fish, c	1.0	6–15	28–49			Optim WG @ P28 + L6	None	n.d.	Xu et al. (2015)
<i>Brachymystax lenok</i> , Manchurian trout, c	~12	8, 16	40, 45, 50, 55			Optim WG @ P48 + L16	✓	↓ @P40 + L8	
<i>Centropomus striatus</i> , black sea bass, c	~75	10, 15	44, 54			Optim WG @ L15 + P 44	✓	↔	Alam et al. (2009a)
<i>Channa argus</i> , northern snakehead, c	~16	9–15	45–51			WG↑ @L < 12 + P45, 48 WG↓ @L↑+P51	✓ None	↔	Sagada et al. (2017)
<i>C. striatus</i> , striped snakehead, c	3.3	6.5, 9, 11.5	35, 40, 45			WG↑ @ L6.5 + P45 WG↔ @ L11.5 + P45	✓ None	↑ @6.5 L ↓ @L↑+P↑	Aliyu-Paiko et al. (2010)
	31	7.5–23.1	43.4–55.6			Optim WG @ P52 + L < 19	None	n.d.	Hua et al. (2019)

<i>Clarias gariepinus</i> , north African catfish, o–c	10	6, 9, 12	24, 30, 35	WG↑ @L↑ or @P↑ Max 12 L + 30P or 9 L + 35 P	✓	↔	Ahmad (2008)
	Fry	12, 18	30, 35	Max WG @L12 + P35 Max FCR, PER @ L18 + P 30	✓	↑@L18 + P30	Murthy and Naik (1999)
	40	4–29	20–40	Max WG @L11–20 + P↑	(✓)	n.d.	Machiels and Henken (1985)
<i>Colossoma macropomum</i> , Tambaquí, cachama, p–o	1.1	5–20	30, 40	Protein deposition↑ with L↑	(✓)	n.d.	van der Meer et al. (1997)
	~2.5	0, 4, 10	25, 38	WG↑ @L4 + P↔	None	↔	Gao et al. (2011)
	~200	0, 2, 4, 6, 8	26	Optim WG @ L4	None	n.d.	Yuan et al. (2016)
<i>Culter alburnus</i> , topmouth culter, o	~6.5	5, 8, 11	35, 40, 45	Optim WG @ L8 + P40–45	(✓)	↔	Zhang et al. (2016)
<i>Cynoglossus semilaevis</i> , tongue sole, c	~44	12, 16	45, 50, 55	TGC↑ @L12 + P55 TGC↓ @L16	None	n.d.	Liu et al. (2013b)
<i>Cyprinus carpio</i> , common carp, o–b	~400	~8.5	28–33	WG↔, SGR↔	✓	↔	Ljubojević et al. (2015)
	~2.2	0, 3, 6, 9	24	Max WG @ L6	✓	↔	Manjappa et al. (2002)
	~121	9–18	44–59	Max WG @ L15.4	✓ Optim @L15.4	n.d.	Shimeno et al. (1995)
	Fingerling	>15	30–50	Optim WG @ L > 15 + P30–50	✓	n.d.	Steffens (1996)
	1.3	7, 14	20, 30, 40, 50	WG ↑ @ P↑ Optim. WG @ P40 + L14	✓	↔	Aminikhoei et al. (2015)

(continued)

Table 25.1 (continued)

<i>Dentex dentex</i> , common dentex, c	10, ~92	10.6–19.7	50–57	WG↑ @ L19.7 + P57	✓	n.d.	Skalli et al. (2004)
	~8	9–17	46–55	WG↔	✓@ L17	n.d.	Company et al. (1999)
	11			WG↔	↔	n.d.	
<i>Dicentrarchus labrax</i> , European sea bass, c	1.5	12–18	40–60	Optim WG @ P38–42	None	n.d.	Sá et al. (2006)
<i>Diplodus sargus</i> , white sea bream, o	~8	6–12	44–52	Max WG @ L6 + P52	✓	↔	Wang et al. (2017b)
<i>Epinephelus akaara</i> , red grouper, c	40, 80	11–61	27–75	Optim WG @ P60, L25, C15	✓	n.d.	Lie et al. (1988)
<i>Gadus morhua</i> , Atlantic cod, c	233	12, 16	48, 58	Max WG @ L12–16 + P48	✓	n.d.	Morais et al. (2001)
	<300	11, 16, 20, 23, 28	49, 54, 58, 63	Optim WG ≤ P49 FER↑@ L ≤ 20	✓@ L ≤ 20	n.d.	Grisdale-Helland et al. (2008)
	37–43	11 or 16 vs. 55 or 42		WG↔	None	n.d.	Pérez-Casanova et al. (2010)
	250	16, 31	54, 65	WG↔	✓@ P54 + L31	n.d.	Hatlen et al. (2007)
<i>Gibelion (Catla) catla</i> , Indian major carp, catla, o	Juveniles	12, 18	25, 30, 35, 40	Optim WG @ L12 + P35	(✓)	n.d.	Murthy and Naik (2000)
<i>Hephaestus fuliginosus</i> , sooty grunter, o	4.0	0, 3, 6, 9, 12	39	Optim WG @ L6	✓	↔	Song et al. (2010)
<i>Hemibarbus longirostris</i> , long-nosed barbel, o	2.3	8, 18	22, 32, 42, 52	WG↑, FER↑ @ P↑ + L↔ Max PR, PRC @ L17 + P32	✓	↔	Kim et al. (2009b)

<i>H. wyckioideus</i> , Asian red-tailed catfish, c	2.0	6, 9, 12	39, 44	WG↑ @ L↑; WG↔@P↑ Max WG @ L12 + P39	✓	↔	Hung et al. (2017)
<i>Heterotis niloticus</i> , African bonytongue, p	2.3	6, 13	28, 32, 36	Max WG @ L6 + P36	✓ @ P28	↔	Monentcham et al. (2010)
<i>Labeo rohita</i> , rohu carp, h	4.3	5, 10, 15	25, 35, 40, 45	Max WG @ L5–10 + P 45 and @L15 + P 40	✓ @ P35–40	n.d.	Satpathy et al. (2003)
	5.6	6, 7, 8	26, 28, 30	WG↑ @ L↑ + P↓ Max WG @ L7 + P26	None	n.d.	Kumar et al. (2018)
<i>Lateolabrax japonicus</i> , Japanese sea bass, c	6.3	8, 12, 16	36, 41, 46	Max. SGR @ P46 + L12 or 16	✓	↔	Ai et al. (2004)
<i>Lates calcarifer</i> , Asian sea bass, barramundi, c	1.3	5–15	38–53	Optim WG @ P43, L10, C20	None	↔ except L5	Catacutan and Coloso (1995)
<i>Leuciscus aspius</i> ♀ × <i>Rutilus frisii</i> ♂, Aspikutum, o	31	10, 15	30, 35, 40	Max WG @ L15 + P35	✓	n.d.	Haghighparast et al. (2016)
<i>Lutjanus argentimaculatus</i> , mangrove red snapper, c	21–25	6, 12	35, 42.5, 50	FCR↔, max WG @ P45–50	None	↔	Catacutan et al. (2001)
<i>Maccullochella peelii peeltii</i> , Murray cod, z–c	15	10, 17, 24	40, 50	SGR (↑) with L↑	(✓)	n.d.	De Silva et al. (2002)
<i>Megalobrama amblycephala</i> , Wuchang (blunt snout) bream, h	1.8	4, 7, 10	27, 31, 35	WG (↑) @ P↑ Optimum WG @ L7	✓ @ L4, L7	(↓)@L↑ ↔@P↑	Li et al. (2010, 2012)
<i>Melanogrammus aeglefinus</i> , haddock, c	13.5 g	11, 16	45, 50, 55	Max WG @ L11 + P55 + C16	✓ only @ P45	↔	Tibbetts et al. (2005)
	Juvenile	11 or 16 vs. 55 or 42		WG↔	None	n.d.	Pérez-Casanova et al. (2010)
<i>Micropterus salmoides</i> , largemouth black bass, c	8.7	8, 12	42, 45, 48, 51	WG↑ with P↑	None	↔	Huang et al. (2017)
	0.4	6, 12	30, 40, 50	WG↑ with P↑	None	↔	Yan et al. (2017)

(continued)

Table 25.1 (continued)

<i>Misgurnus anguillicaudatus</i> , pond loach, c	~10	6, 10, 14	34, 39, 44	Optim WG @ L10–14 + P44	✓	↔	Yuan et al. (2010)
<i>Myxocyprinus asiaticus</i> , Chinese sucker, o	12.0	5, 9, 13	40, 47, 54	WG↑, SGR↑	(✓?)	↔	Wang et al. (2018c)
<i>Nibea albiflora</i> , yellow drum, c	2.4–2.6	8, 13, 18	32, 42, 52	WG↑ @ L↑ + P↑	✓	↔	Clarke et al. (1982)
<i>Oncorhynchus kisutch</i> , coho salmon, c	94–124	16, 23, 30	37, 44	Max WG @ L23–30 + P44	✓	n.d.	Chan et al. (2002)
<i>O. mykiss</i> , rainbow trout, c	250–550	12, 24	30, 43, 52	WG↔ @ L↑	✓	n.d.	Beamish and Medland (1986)
	<4.5, >25	5, 10, 15, 20	35, 40, 45, 50	WG↑ @ L↑ <4.5 g: max @P40 + L20–30 >25 g: max @P35 + L20–23	✓	n.d.	Hecht and McEwan (1992)
	181	11–25	43–48	Optim WG @ L < 26 + P ≤ 44	✓	↔	Yigit et al. (2002)
<i>Oreochromis mossambicus</i> × <i>O. niloticus</i> , red tilapia, o–h	1.0–1.4	6, 12, 18, 24	15, 20, 30	Optim WG @ L18	✓	↔	De Silva et al. (1991)
	~2.5	0, 4, 10	25, 38	WG↔, SGR↔, FCR↔, PER↔	?	↔	Gao et al. (2011)
<i>O. niloticus</i> , Nile tilapia, o–h	55–135	2–18	5–41	SDA↑ @ P↑ + L↔	✓	n.d.	Ross et al. (1992)
<i>Pagrus pagrus</i> , red porgy, c	2.8	10, 15, 20	40–65 45, 50	WG↑ @ P ≥ 50 WG↑ @ L10–15 WG↓ @ L20	None	n.d.	Schuchardt et al. (2008)
<i>Pangasius hypophthalmus</i> , Asian catfish, o	3.5–3.8	6, 12	25, 30, 35, 40	Max. SGR @ L12 + P40	✓	n.d.	Phumee et al. (2009)
	4.7 g	5, 9		WG↑ @ P↑ or @ L↑		↑ @ P↑	Liu et al. (2011)

<i>Paralichthys lethostigma</i> , southern flounder, b–c	~1.1	10, 15	34, 38, 42, 46		Optim WG @ L10 + P50	✓ max @ P45 + L8.6	↔	Alam et al. (2009b)
<i>P. olivaceus</i> , olive flounder, bass-tard halibut, b–c	~3.1 g	10, 17	46, 51, 56		Optim WG @ L10 + P46	None	↔	Lee et al. (2000)
	114 g	10, 17	46, 51, 56		Max WG @ L17 + P56	(✓) n.s.	↔	Kim et al. (2009a)
<i>Pelteobagrus ussuriensis</i> , Ussuri catfish, c	3.4	5, 10, 15	35, 40, 45, 50		SGR ↓ @ L 5–15	None	↔	Wang et al. (2013b)
<i>Perca fluviatilis</i> , Eurasian perch, p–b, c	37	12–22	43–49		Optim WG @ P47 + L16	✓	↔	Mathis et al. (2003)
<i>Piaractus mesopotamicus</i> , pacu, o	12	4, 8	22, 25		FCR ↑ @ L ↑ + P25	✓	n.d.	Abimorad and Carneiro (2007)
<i>Platichthys stellatus</i> , starry flounder, c	31	8, 11, 14	40, 45, 50		Optim WG @ P45 + L14 or P50 + L11	✓	↔	Wang et al. (2017a)
<i>Pleuronectes americanus</i> , winter flounder, c	0.8	10, 15, 20	40, 45, 50		Max WG @ P50 + L10	None	n.d.	Hebb et al. (2003)
<i>Pomadourys commersonii</i> , small spotted grunter, c	4.2–7.5	6, 8, 12	35, 45, 55		Optim SGR @ L12 + P45	None	n.d.	Hecht et al. (2003)
<i>Protonibea diacanthus</i> , blackspotted croaker, c	12	7, 11, 15	42, 47, 52		Max. WG @ L15 + P52 Optim WG @ L11 + P47	✓	↔	Li et al. (2017)
<i>Pseudoplatystoma fasciatum</i> × <i>Leiarius marmoratus</i> , hybrid Surubim, c	8.9	5–19 (EE)	35, 39, 42, 45		Optim WG @ L10 + P42	✓	↔	Campeche et al. (2018)
<i>Pterophyllum scalare</i> , angelfish, p–c	~1.6	6, 8, 10	30, 35, 40			None	n.d.	Mohanta et al. (2012)

(continued)

Table 25.1 (continued)

						WG↑ @ P↔ + L↑ Optim WG @ L 6 + P30			
<i>Rhamdia quelen</i> , black catfish, c–o	~0.5	8, 14	30, 34, 38, 43			Optim WG @ P37 + L8–14	✓	n.d.	Salhi et al. (2004)
	~0.5	10, 13, 18	37, 42, 47, 52, 57			Optim WG @ L13 + P42	(✓)	↔	Ebrahimi et al. (2013)
	7	5.5–7.0	8–11			WG↔	n.s.	n.d.	Azevedo et al. (2002)
	100	21, 26, 30, 32	35, 37, 39, 42			Max. WG @ P37–39	✓ @ P35	n.d.	Hillestad and Johnsen (1994)
	105	26, 27	47, 50			WG ↑ @ P↑	None	n.d.	Weihe et al. (2018)
<i>Rutilus frisii</i> , Kutum, p–c	319	31, 32	43, 44						
	978	32, 36	35, 43						
	~1000	26, 31, 35, 39	38, 43, 48, 52			Optim WG @ P48 + L31	✓	↔	Einen and Roem (1997)
	~2500					Optim WG @ P43 + L35			
	~2000	35–39	29, 33, 35			Successful fish oil replacement by rape-seed oil	✓	n.d.	Karalazos et al. (2011)
<i>S. trutta fario</i> , brown trout, c	~460	9, 18	36, 39, 42, 45, 48			Max WG @ P45 + L9	✓ @ high L	↔	Wang et al. (2018a)
<i>Sander lucioperca</i> , pike perch, c	1.4	7.2–22	57–66			Max. WG @ P57 + L22	None	↔	Schulz et al. (2006)
	1.35–1.40	9, 13, 17	47, 54			Max SGR @ P47 + L17	✓	n.d.	Schulz et al. (2008)
	51	10, 16, 22	34, 43, 50			WG↑ @ P ≥ 43	None	n.d.	Nyina-Wamwiza et al. (2005)
<i>Sciaenops ocellatus</i> , red drum, c	24–28	10	35, 40, 45				None	n.d.	

					Optim WG @ P40 + L10				Serrano et al. (1992)
<i>Scophthalmus maximus</i> , turbot, c	10.5–12.1	3, 6, 9	35, 50		Optim WG @ P35	✓		n.d.	Adron et al. (1976)
	2.4–3.1	5–51	14.5–16		Optim WG @ P40 + L6	✓		n.d.	Bromley (1980)
	~10	10, 15, 20	38–70		Max SGR @ P70 + L10	✓ @ low P		n.d.	Caceres-Martinez et al. (1984)
	47	7, 17	55, 60, 65		Optim WG @ P55 + L17	✓		↔	Cho et al. (2005)
	~54	10, 13, 16, 19	50		Optim WG @ L13 WG↓ @ L > 13	None		n.d.	Sevgili et al. (2014)
<i>Sebastes schlegelii</i> , Korean rockfish, c	4.5–209	12, 16	45, 50, 55		WG↑ @ L↑ (except large individuals)	✓ in smaller individuals		↔	Liu et al. (2014)
	21.9	7, 14	37, 42, 47		Optim WG @ P42 + L14	✓		↔	Lee et al. (2002)
	7.6	9, 17	20, 30, 40		Optim WG @ P > 40, L↑	✓ @ P20–40		↔	Kim et al. (2012)
<i>S. meridionalis</i> , Chinese largemouth catfish, c	9.1	7, 10, 13	37, 40, 43, 46		Optim WG @ L43 + P7	✓		↔	Liu et al. (2013a)
	9.2				Optim WG @ L43 + P10	✓		↔	
<i>Siniperca scherzeri</i> , golden mandarin fish, c	8.3	7, 14	35, 45, 55		WG↑ @ P↑ + L↔	None		↔	Sankian et al. (2017)
<i>Solea senegalensis</i> , Senegalese sole, b	~17	4, 17	48, 54		WG↔	None		n.d.	Borges et al. (2013)
<i>Sparus aurata</i> , gilthead sea bream, c (h)	17.3	9–17	46–55		WG↔	↔		n.d.	Company et al. (1999)

(continued)

Table 25.1 (continued)

	5.3	9, 15	42–58	WG @ P† + L†	✓	n.d.	Vergara et al. (1996)
	42.5	15, 21	47, 51	WG↔	(✓)	n.d.	Santinha et al. (1999)
	~100			Optim WG @ P49 + L12			In Lupatsch et al. (2001)
<i>Stephanolepis cirrifer</i> , threadsail filefish, c (h)	3.2	7, 14	35, 40, 45, 50	WG† @ P† + L†	✓ @ P ≤ 45	↔	Khosravi and Lee (2017)
<i>Tachysurus fulvidraco</i> , yellow catfish, b–c	0.92	10, 19	22, 32, 42, 52	WG† @ P† + L10 WG† @ P ≤ 42 + L19	✓ @ P ≤ 42	↔	Kim and Lee (2005)
	~4.6	9, 13	48, 52	WG† @ P† + L†	✓ @ P48	↑ @ L†	Zhang et al. (2018)
<i>Thunnus orientalis</i> , Pacific bluefin tuna, c	~0.26	9–27	53–73	Optim WG @ P62 + L18	✓	↔	Biswas et al. (2009)
<i>Tinca tinca</i> , tench, d–o	~5.3	10, 20	17.5, 24, 35, 50	WG†P ≥ 35	None	n.d.	de Pedro et al. (2001)
<i>Tor khudree</i> , Deccan mahseer, (h)–c	1.1	3, 6, 9, 12	40	WG† @ L†	✓	↔	Muzaffar Bazaz and Keshavanath (1993)
<i>T. tambroides</i> , Malaysian mahseer, (h)–c	20.9	10, 15	30, 35, 40	Max WG @ P40 + L15	None	n.d.	Ng et al. (2008)
<i>Trachinotus falcatus</i> , permit, c	~27	10, 20, 30	40, 45, 50	WG† @ P40–50 + L10–20	✓	n.d.	Nguyen et al. (2018)
<i>T. ovatus</i> , golden pompano, c	4.7	6.5, 12.5	33, 37, 41, 45, 49	Optim WG @ P45–49 + L6.5	None	↔	Wang et al. (2013a)
<i>Trichopodus trichopterus</i> , blue gourami, z–c	0.62	6, 8, 10	30, 35, 40	Optim WG @ P35 + L8	None	n.d.	Mohanta et al. (2013)

<i>Umbrina cirrosa</i> , shi drum, b–c	7	10, 15, 20	47, 52	WG↑ @ P↑; WG↓ @ L > 10	None	n.d.	Kokou et al. (2018)
<i>Verasper variegatus</i> , spotted halibut, c	~93	8, 12, 16	40, 45, 50	Optim WG @ P50 + L8	None	↔	Lv et al. (2015)
<i>Xiphophorus helleri</i> , green swordtail ♀♀, o	1.2–1.3	8, 12, 16, 20	20, 30	WG↑ @ P↑ + L↑	✓ @ P20	n.d.	Ling et al. (2006)
<i>X. helleri</i> , green swordtail, o	0.50–0.54	6, 8, 12	30, 38, 45	Optim WG @ P45 + L6	None	↔	Kruger et al. (2001)

↑ support/increase, ↔ no apparent change/result independent of treatment, ↓ decrease/reduction, n.d. not determined, n.s. not significant, L lipid, P protein, C carbohydrate, FO fish oil, ADC apparent digestibility coefficient, TGC thermal-unit growth coefficient, more robust than SGR or WG, includes water temperature variation and initial body weight, EE ether extract, FCR feed conversion ratio, FER feed efficiency ratio, FI feed intake, LPO lipid peroxidation, PER protein efficiency ratio, PR protein retention, SDA specific dynamic action (metabolic cost of processing food), SGR specific growth rate, WG weight gain, b benthivorous (carnivorous), c carnivorous, d detritivorous, f frugivorous, h herbivorous, o omnivorous, p planktivorous, z zooplanktivorous, PPV protein productive value: protein gain (protein intake)⁻¹, gk glucokinase, pepck phosphoenolpyruvate, glut2 glucose transporter 2, fas fatty acid synthase, acca acetyl-coA carboxylase alpha

^aSimultaneously with dietary lipids, the second nonprotein nutrient has been reduced: Dextrin dropped from 28.5 to 2.0%

Studying Atlantic salmon, Einen and Roem (1997) pointed out changing demand during ontogenesis and showed that the optimal digestible protein to digestible energy ratios (DP/DE) decreases with increasing fish weight. DP/DE ratios around 19 g MJ^{-1} for individuals weighing 1 to 2.5 kg and $16\text{--}17 \text{ g MJ}^{-1}$ for fish weighing 2.5 to 5 kg are considered optimal (see Table 25.1).

Protein sparing by dietary lipids is more pronounced at suboptimal protein levels and elevated lipid levels (Schulz et al. 2007). Protein-sparing is demonstrated in many, but not all, fish species: Atlantic salmon, rainbow trout, common carp, hybrid striped bass, yellowtail, or red sea bream (Table 25.1). Such sparing effect is not found in blue gourami, pike perch, Eurasian perch, red porgy, shi drum, or grass carp (Table 25.1). The omnivorous tench, too, apparently lacks this capability.

Blue gourami is an omnivore but prefers aquatic weeds. Like grass carp, it appears to have a limited capacity to utilize dietary lipids, and therefore, it can obviously not spare protein for growth. This argument may serve as explanation, if not even carnivorous species appear in the list of fishes lacking protein-sparing capability by dietary lipids. Herbivores and omnivores should have a higher protein-sparing capability by carbohydrates than carnivores. However, the compilation in Chap. 17 reflects a picture that is less clear than anticipated. There are carnivores lacking protein-sparing by carbohydrates (Atlantic cod) but also carnivores with clear ability of protein sparing, such as Siberian sturgeon, yellowfin sea bream, Malabar grouper, stinging catfish, rainbow trout, barred sorubim, cobia, brook trout, turbot, yellow kingfish, Senegalese sole, gilthead sea bream, or yellow catfish to mention only a few.

25.2 Adipogenesis

As background of protein sparing by dietary lipids, different mechanisms of storage and mobilization for energy and metabolic purposes have been identified. Species differ in using adipose tissues (AT) or, less frequently, the liver as storage organs. Like higher vertebrates, various fishes use AT as main lipid storage. Moreover, white AT is recognized as an endocrine organ (Salmerón 2018). AT is implicated in energy homeostasis by secreting adipokines (cell signaling protein, such as leptin—a hormone inhibiting hunger); furthermore, it is a rich source of stem cells. Interest in adipogenesis has increased in aquaculture because of the excessive fat deposition experienced in some cultured fish species that compromises their welfare.

Zebrafish has emerged as model for studying early stages of adipocyte development in vivo. Differentiated adipocytes contain lipid droplets (→Chap. 24) and express adipocyte marker genes: *ppary*,¹ *CCAAT-enhancer-binding protein α*

¹Peroxisome proliferator-activated receptor γ ; regulates fatty acid storage and glucose metabolism (Jones et al. 2005) (→Chap. 23).

(*c/ebpa*),² *lipoprotein lipase* (*lpl*),³ *fatty acid synthase* (*fas*), *fatty acid binding protein 11* (*fabp11*), *fatty acid transporter protein1* (*fatp1*), *adiponectin*,⁴ and *leptin*. These adipocytes have also elevated glycerol 3-phosphate (G3P) dehydrogenase (GPDH) activity (Salmerón 2018).

The regulation of adipogenesis is well conserved across species, having similarities in fishes and mammals (e.g., insulin promotes and TNF α inhibits adipogenesis in both). In zebrafish, adipocytes appear anatomically several days postfertilization. They express the transcription factors required for the adipogenic process, as well as the enzymes involved in lipid metabolism. Noteworthy, marine PUFAs (eicosapentaenoic, docosahexaenoic acids) have an anti-adipogenic effect on adipocyte development in fishes (\rightarrow Chap. 27); current alternative diets, however, contain high levels of vegetable oils which stimulate adipogenesis (Salmerón 2018).

A few more details of adipogenesis, He et al. (2015) found in Nile tilapia that the increase of adipocyte numbers is the main metabolic solution to deal with high-fat intake. Excess ingested lipid is preferentially stored in AT through increased capability of fatty acid (FA) uptake and triglyceride (TG or TAG) synthesis. The increase in adipocyte numbers through adipocyte proliferation is mediated by PPAR γ (Fig. 25.1) (\rightarrow Chap. 23).

To comparatively elucidate the molecular mechanism of impaired lipid homeostasis and aggravated hepatic lipid deposition in apex predatory Chinese perch (*Siniperca chuatsi*), four isonitrogenous diets with different fat levels (2...17%: L2...L17, respectively) are fed to apparent satiation (Wang et al. 2018b). Maximal growth and food intake are observed in L12. The lipid content in liver and serum are comparable in L2, L7, and L12, while they increase in L17. Compared to L2, the lipolytic genes related to FA transport (*lpl* & *hl*) and FA β -oxidation (*cpt1*, *cs*) increase in L7 and L12. mRNA levels of the gluconeogenesis (*pc*, *pepck*, *g6pase*) also increase, whereas the transcription of lipogenic genes (*srebp1*, *acca*, *fas*) decreases. Compared to L12, L17 has elevated mRNA levels of FAs transport and lipogenesis. The lipolytic genes related to FA β -oxidation, however, are steady and the mRNA levels of gluconeogenesis are downregulated (Fig. 25.2).

Within certain limits, the increase of dietary fat in L7 and L12 is propitious to reduce the consumption of protein and improve growth in Chinese perch. This is due to the homeostasis of hepatic TG pool and serum glucose through promoting the FA β -oxidation and gluconeogenesis, respectively. Both the increase of lipogenesis and the absence of FA β -oxidation in L17 can trigger the esterification of FAs. The inhibition of gluconeogenesis can also aggravate triglyceride accumulation in the liver and induce hepatic steatosis (\rightarrow Chap. 24). Previous reports confirmed that

²CCAAT-enhancer-binding protein belongs to a family of transcription factors. The proteins are involved in different cellular responses, such as in the control of cellular proliferation, growth, and differentiation, in metabolism, and in immunity (Ramji and Foka 2002).

³Hydrolyzes triglycerides in lipoproteins into two free fatty acids and one monoacylglycerol.

⁴A protein hormone and adipokine, which is involved in regulating glucose levels as well as fatty acid breakdown (Diez and Iglesias 2003).

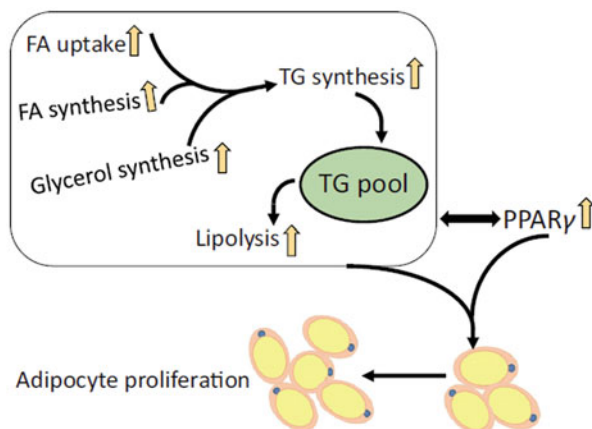


Fig. 25.1 Model of metabolic adaptation to high-fat diet (HFD) in the adipose tissue of Nile tilapia. In the adipocytes of Nile tilapia fed with HFD, the uptake of diet-sourced free fatty acids (FFA) and de novo synthesis of FA and glycerol are upregulated and contribute to increasing triglyceride (TG) synthesis. The excess cellular TG is hydrolyzed by accelerated lipolysis, and the released FFA, together with the diet-sourced circulating FFA, upregulate PPAR γ to trigger the differentiation and proliferation of visceral adipocyte. (From He et al. (2015), credit Wiley)

excess fat intake induces TG deposition and causes hepatic impairment in farmed fishes, such as haddock (Nanton et al. 2001), Atlantic salmon (Torstensen et al. 2001), hybrid striped bass (Gaylord and Gatlin 2000), or Wuchang bream (Lu et al. 2013) (potential toxicity mechanisms → Chap. 24).

Some species seem to be highly tolerant to HFDs, in which the *igf1* expression increases concomitantly to the increase of dietary lipids as shown in triploid rainbow trout (Meng et al. 2019): Increasing dietary lipid levels from 6% to 29% induces elevated growth and increased hepatic mRNA expression of *ghr*, *igf1*, and *igf2*. Although *gh* gene expression or plasma GH levels were not measured, it is conceivable that GH stimulated the hepatic expression of these transcripts (Canosa and Bertucci (2020), reference added).

A few fish species prefer using the liver as lipid storage instead of adipose tissues, for instance, tiger puffer (*Takifugu rubripes*). In juveniles, Xu et al. (2019) found that moderately high level of dietary lipid inhibits the hepatic protein secretion function—a kind of protein-sparing mechanism. Serum biochemical parameters confirm the existence of hepatic stress caused by HFD. Differentially expressed genes primarily enriched in GO terms comprise endopeptidase activity, secretion, and organic acid transport, and in KEGG pathways, they include protein processing in endoplasmic reticulum and DNA replication. The HFD tends to inhibit protein synthesis and secretion in the liver. Furthermore, HFD stimulates the transcription of genes related to liver protection and repair.

However, lipid storage is not necessarily connected with protein sparing. In carnivorous striped snakehead (*Channa striata*), Hua et al. (2019) found that increasing dietary lipid results only in significant increase of body lipid content.

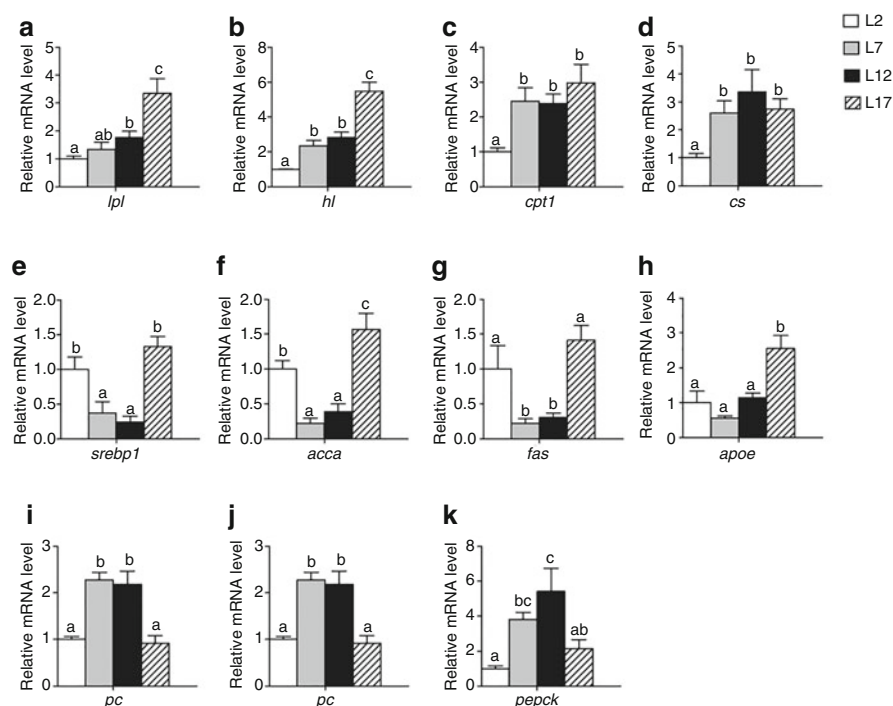


Fig. 25.2 The mRNA expression of the genes in hepatic triglyceride metabolism of Chinese perch. (a–b) *hl* hepatic lipase and *lpl* lipoprotein lipase relate to the triglyceride hydrolysis and hepatic fatty acid transport; (c) *cpt1* carnitine palmitoyltransferase 1 to FA β -oxidation; (d) *cs* citrate synthase to the conversion from acetyl-CoA and oxaloacetate into citrate; (e) *srebp1* sterol regulatory element binding protein 1 to lipogenesis in the liver; (f–g) *acca* acetyl-CoA carboxylase alpha and *fas* fatty acid synthetase to FAs biosynthesis; (h) *apoe* apolipoprotein E to triglyceride transport; (i–k) *pc* pyruvate carboxylase, *pepck* phosphoenolpyruvate carboxykinase and *g6pase* glucose-6-phosphatase to gluconeogenesis de novo. Values are means \pm SEM ($n = 6$). Different letters indicate statistical difference at $P < 0.05$ (one factor ANOVA, Duncan's post hoc test). (From Wang et al. (2018b), credit BioMed Central). L2–L17 correspond to the dietary lipid contents of 2, 7, 12, and 17%, respectively

Dietary lipid is directed toward lipid deposition rather than toward protein sparing. A lack of protein sparing is also observed in hybrid snakehead (*Channa maculata*♀ \times *C. argus*♂) but not in juvenile northern snakehead (*C. argus*) when the dietary lipid content increases from 9% to 12% (Hua et al. (2019) with references therein). However, before it may be concluded that these differences are species-specific, experimental approaches must be unified.

In contrast to previously reported investigations in pike perch (Table 25.1), Schulz et al. (2008) showed for the first time a clear protein-sparing effect in this carnivorous species by dietary lipids from 90 to 170 g kg⁻¹. In addition, Mathis et al. (2003) reported for Eurasian perch a significant protein-sparing effect up to dietary lipids of 163 g kg⁻¹ comparable with the highest dietary lipid level chosen in the pike perch study. However, the ability to spare dietary protein by lipids in other

percids like common dentex and sea bream, European sea bass, or Murray cod (Table 25.1) continues to be low. The varying growth reactions in the mentioned studies can be influenced by different feed qualities and different developmental stages tested: High dietary lipid levels better benefit fast-growing juveniles with high metabolic rates than slow growing older ones.

Also, carnivorous Atlantic cod appears to have a low-lipid-mediated protein-sparing capability. Lie et al. (1988) obtained values of 60% dietary lipid retention, in contrast to only 26% of protein retention. Obviously, cod uses protein preferentially as energy sources and is less able to utilize fat for this purpose. In contrast, Morais et al. (2001) reported protein sparing of dietary lipids in cods. The highest growth rate and feed conversion efficiency is found in individuals fed a diet with high levels of both protein (P58%) and lipid (L16%); the worst results occur with low-protein and low-lipid diet (P48%, L12%).

In hybrid African catfish (*Clarias macrocephalus* × *C. gariepinus*), Jantrarat et al. (1998) studied the interaction of lipids, proteins, and carbohydrates and found that dietary protein and energy demands are affected by the carbohydrate:lipid ratio. Fishes utilize dietary energy with a dextrin to lard ratio of 3.8:1.0 more efficiently than with a ratio of 11.3:1.0 and hence spare dietary protein.

At this stage of consideration, we have to recall that replacement successes and protein sparing are evaluated one-dimensionally by production criteria, such as growth performance, flesh quality, health, and pathogen resistance rather than nutritional ecological criteria. In particular, the capacities and the actual need of aquatic animals to balance their macronutrient intake deserve attention. This nutritional physiological strategy is never considered in the studies, and its neglected existence may be one major source or reason for lacking protein-sparing effects.

25.2.1 Microbiome Feedback

The microbiome is central in lipid metabolism (Semova et al. (2012), →Chap. 24), but the issue of how modifications of the probiotic community might affect host’s lipid metabolism is poorly understood. This applies particularly to HFDs fed to speed up growth of farmed animals (→Chap. 23).

Using a multidisciplinary approach, Falcinelli et al. (2015, 2017) addressed this knowledge gap by feeding two probiotics to zebrafishes and coupled high-throughput sequencing to biochemical, molecular, and morphological analysis in order to evaluate intestinal changes. The authors detected a novel gene network involved in lipid metabolism. Dietary *Lactobacillus rhamnosus* is able to modulate the gut microbiome of zebrafish larvae, elevating the abundance of *Firmicutes* and reducing the abundance of *Actinobacteria* (Falcinelli et al. 2015). The changed gut microbiome modulates host lipid processing by inducing transcriptional downregulation of genes involved in cholesterol and triglycerides metabolism (*fit2*, *agpat4*, *dgat2*, *mgll*, *hnf4a*, *scap*, and *cck*; Fig. 25.3) concomitantly decreasing total body cholesterol and triglyceride content and increasing FA levels. *L. rhamnosus* treatment also increases *microvilli* and enterocyte lengths and

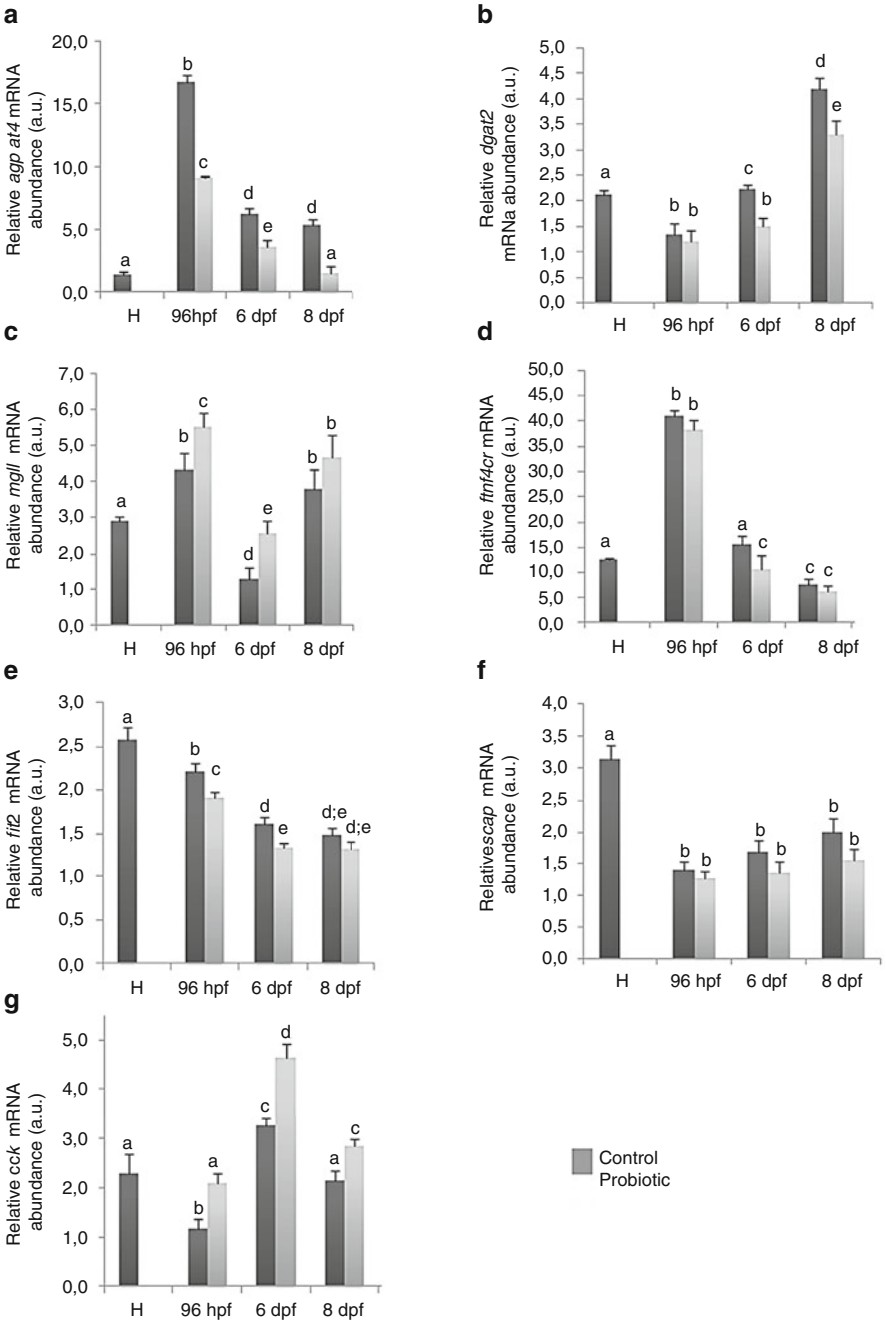


Fig. 25.3 *Lactobacillus rhamnosus* treatment modulates expression of genes involved in lipid metabolism in zebrafish larvae. Relative *agpat* (a), *dgat2* (b), *mgl1* (c), *hnf4a* (d), *fit2* (e), *scap* (f), and *cck* (g) gene expression normalized against β -act and *arp*, in pools of 15 zebrafish larvae from control and probiotic groups collected at hatching (H), 96 hpf, 6 dpf, and 8 dpf. Assays were performed in triplicate. Values with different superscript letters are significantly different

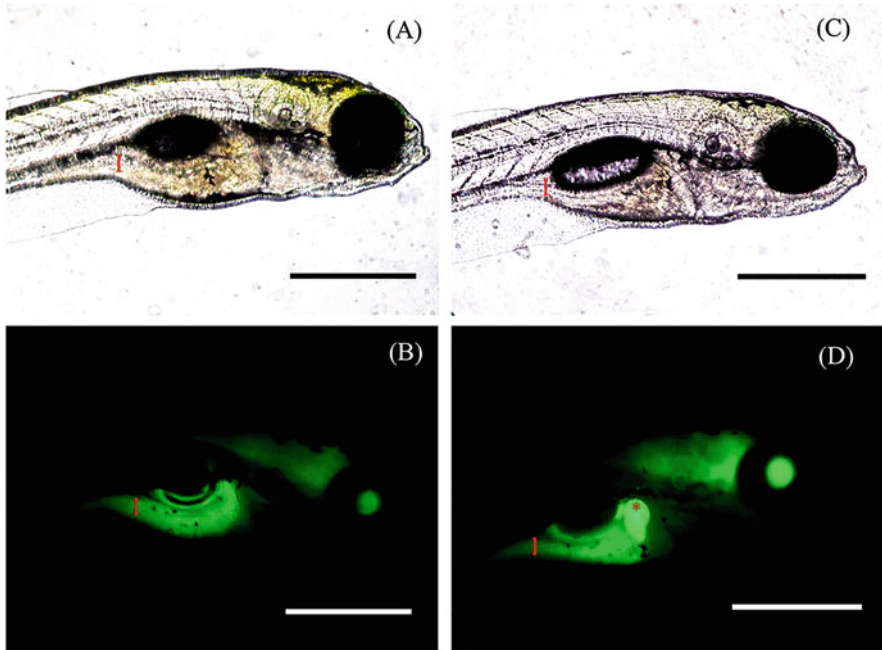


Fig. 25.4 Fluorescent (BODIPY 505/515) staining evidenced an accumulation of nonpolar fatty acids in probiotic-treated gallbladder. Representative fluorescent images of live 6 dpf zebrafish soaked in BODIPY 505/515 for 1 h. Images revealed green fluorescence in the intestine in both control (**a–b**) and probiotic-treated groups (**c–d**). Probiotic-treated larvae exhibited higher fluorescence in the gallbladder (**d**). Red asterisk: gallbladder; red arrow: intestine. Scale bar: 500 μ m. (From Falcinelli et al. (2015), credit Springer Nature)

decreases lipid droplet size in the intestinal epithelium. Eventually, these changes result in elevated growth of zebrafish larvae.

Zebrafish larvae use TAG as major source of lipids, and in order to absorb TAG in the intestine, processing by digestive enzymes and bile is required. Bile and enzymes pass from the gallbladder and arrive in the intestinal lumen where TAG are emulsified and form micelles, and finally, free FAs and monoacylglycerols are taken up by the intestine. Falcinelli et al. (2015) used fluorescent staining as a noninvasive in vivo method in order to visualize the dynamics of nonpolar FAs in living tissues by confocal microscopy. Imaging reveals similar green fluorescence intensity in the intestine of both control (Fig. 25.4a–b) and probiotic-treated zebrafish larvae at 6 dpf

Fig. 25.3 (continued) ($P < 0.05$). (From Falcinelli et al. (2015), credit Springer Nature). *fit2* → protein involved in fat storage; *agpat4* → involved in biosynthesis of TAG; *dgat2* → protein catalyzing a reaction which produces TAG from DAG and fatty acyl-CoA; *mgll* → serine hydrolase involved in the breakdown of TAG to free fatty acids and monoacylglycerols; *hnf4a* → transcription factor implicated in the metabolism of cholesterol, FAs, and AAs; *scap* → SREBF chaperone essential in maintaining and controlling de novo synthesis of cholesterol; *cck* → cholecystokinin, induces the contraction of the gallbladder to release bile into the intestine and solubilize lipids

(Fig. 25.4c–d). However, probiotic-treated larvae exhibit higher fluorescence in the gallbladder than the control, highlighting an accumulation of nonpolar FAs. This nonpolar lipid can be absorbed by the intestine, successively taken up by the liver, and secreted into the gallbladder.

Later, Falcinelli et al. (2017) explored whether the dietary lipid content influences the gut microbiome in adult zebrafish and, vice versa, whether dietary probiotic amendment has the potential to alleviate adverse effects of HFDs. Therefore, diets containing three different lipid levels (high, HFD; medium, MFD; and low, LFD) were administered with or without the probiotic *L. rhamnosus* (P). In fact, the dietary lipid content shifts the gut microbiome structure. The addition of *L. rhamnosus* induces transcriptional reduction of orexigenic genes, upregulation of anorexigenic genes (Fig. 25.5), and transcriptional decrease of genes involved in cholesterol and TAG metabolism (Fig. 25.5), resulting in lower body contents of cholesterol and TAGs. Probiotic feeding also decreases anorexigenic *nesfatin-1* peptide in HFD-P and attenuates weight gain in HFD-P- and MFD-P-fed zebrafish but not in the LFD-P group. Intestinal ultrastructure is not affected by dietary fat level or probiotic inclusion. In sum, this paper underpins the role of dietary fat in altering the gut microbiota community and highlights the potential of probiotics to attenuate HFD-related metabolic disorders.

Without probiotic supplementation, energy-dense feeds and overnutrition, however, bear the risk of altering lipid metabolism and gut microbiota and, thereby, causing systemic inflammation. The consumption of the HFD results in microbial dysbiosis in zebrafish, characterized by an increase in the relative abundance of the phylum *Bacteroidetes* (Arias-Jayo et al. 2018). Moreover, an emerging intestinal inflammation via NF- κ B activation is confirmed by the overexpression of several genes related to signaling receptors, antimicrobial metabolism, and the inflammatory cascade (Fig. 25.6). Moreover, the intestinal barrier is damaged, with an increase of goblet cell mucin production. Collectively, the consumption of a diet enriched with 10% fat changes the intestinal microbial community composition resulting in low-grade inflammation.

25.3 Concluding Considerations

This chapter has disclosed that replacement successes of proteins are evaluated almost exclusively one-dimensionally by production criteria, such as growth performance, flesh quality, pathogen resistance, and, with respect to brood stock, reproductive performance rather than by nutritional ecological criteria. In particular, the capacities of aquatic animals to balance their macronutrient intake deserve attention. This means that for reasons of tractability, studies often concentrate on a small subset of such factors, and it is important to use models that take into account several attributes both of the environment and of the animal. The geometrical models derived from nutritional ecology (\rightarrow Chap. 2) offer tools for doing this. As Raubenheimer et al. (2012) nicely summarized, in this approach, the animal has

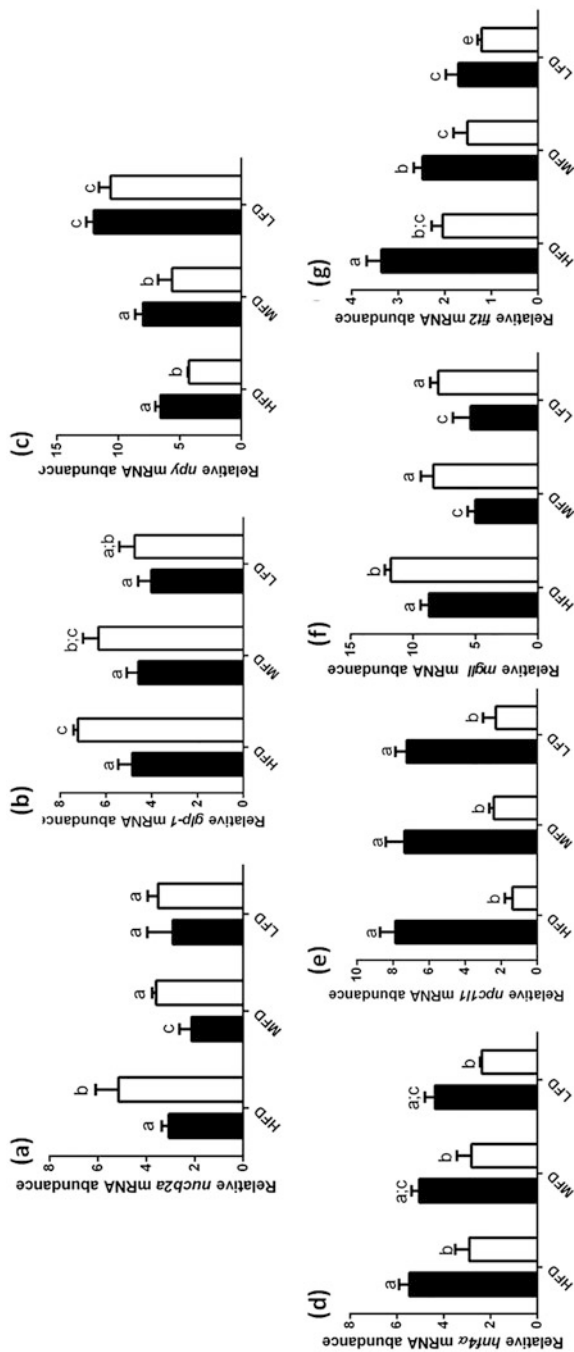


Fig. 25.5 *Lactobacillus rhamnosus* modulates the expression of genes involved in the regulation of appetite (a–c) and lipid metabolism (d–g). Relative anorexigenic *nucb2a* (a), anorexigenic *gcpa* (b), and orexigenic *npv* (c) and relative *hnf4a* (d), *npc11* (e), *mgll* (f), and *fit2* (g) gene expression normalized against β -act and *mp1p* in fishes fed a HFD-C, MFD-C, and LFD-C and fishes fed a HFD-P, MFD-P, and LFD-P. Assays were performed in triplicate. Different superscripts indicate significant differences ($P < 0.05$). (From Falcinelli et al. (2017), credit Springer Nature). HFD high-fat diet, MFD medium-fat diet, LFD low-fat diet, Black control, white probiotic supplementation

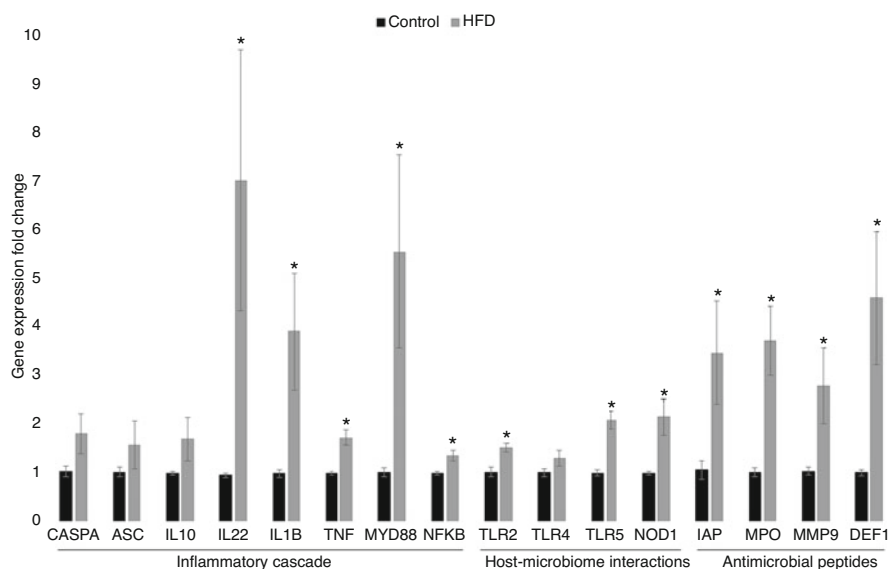


Fig. 25.6 Gene expression fold change analysis after 25 days of diet exposure (30 dpf) in control and HFD zebrafish larvae ($n = 240$) (highlighted with different gray gradients). Error bars indicate SEM. Differences were considered statistically significant at $P < 0.05$ (*). (From Arias-Jayo et al. (2018), with permission from Springer Nature)

defined optimal nutrient needs and is assumed to assess current nutritional state in relation to those needs. The behavior and physiology of the animal are directed toward reducing differences between current nutritional state and optimal nutritional state. Effective fish culture is dependent on the nutritional elements that fishes need, the behavioral mechanisms that they use to acquire these, and the physiological systems that they use to process and assimilate the food once eaten. Therefore, the aims of nutritional ecology (to understand and predict the nutritional interactions of animals with their environment) and aquaculture (to manage these interactions for optimal outcomes) have much in common. Identifying the combination of nutrients that promotes growth and at the same time reduces mortality, excreted pollutants, and the cost of production is most relevant to fish culture. The relationship between diet, longevity, and lifetime reproduction is relevant when fish are reared as brood stock.

The use of nutritional ecology as a tool for identifying optimal dietary composition is illustrated by a study conducted on the European whitefish (*Coregonus lavaretus*; Ruohonen et al. (2007)). The authors show how a combination of mixture design theory and state-space models of nutrition (the Geometric Framework, GF) can be used to derive a five-step protocol for multi-criterion diet optimization. Step 1 involves selecting the focal nutritional axes for modelling; step 2 uses mixture theory to choose an optimal selection of experimental diets to test in experiments; step 3 entails using GF to plot and interpret intake and growth arrays; step 4 involves

plotting response variables onto intake arrays, and step 5 uses multi-criterion optimization to combine and weigh several relevant response variables.

This ecology-based approach of designing optimal aquafeed for selected farmed animals is waiting to be applied.

Moreover, time has come that future studies should aim at deciphering precise profiles, underpinning regulatory mechanisms, and networks that drive effects of dietary lipids rather than extend the existing catalog of adverse HFD-mediated phenomena. This chapter has provided several excellent role models, which can serve as orientation for future studies. Nevertheless, some aspects remain uncovered. In the last decade, several studies have shown that probiotics beneficially modulate and interact with gut microbiota in mammals, which, in turn, modify host nutrient metabolism and energy balance. However, dietary probiotic approaches in aquatic animals are inexplicably sparse.

Based on the “superorganism concept” of Wilson and Sober (1989), Limborg et al. (2018) proposed a comprehensive forward-looking approach to solve scientific and practical problems emerging from traditional fragmented approaches of the different disciplines. The authors advocate that the adoption of holo-omic approaches would overcome the limited functional insights of current analytical strategies by simultaneously considering the holobiont at multiple molecular levels. This involves deciphering interactions between not only the host genome but also its epigenome and transcriptome, as well as its microbial metagenome and metatranscriptome. Studies would ideally also incorporate analyses of the associated proteomes and metabolomes, and metaproteomes and metametabolomes, to recover the functional pathways controlling the host phenotype.

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Chapter 26

Fatty Acids—‘Fueling Versus Steering’



Abstract After a brief description of basic features of fatty acids (FAs), this chapter summarizes recent findings of FAs in terms of sensing, uptake, and transport. Major transporter families are cluster of differentiation 36 (CD36), fatty acid transport proteins (FATPs), fatty-acid-binding proteins, ATP-binding cassettes, and intracellular lipid-binding proteins (iLBPs). Besides transport, these proteins are crucial in immunity, pathogen defense, and trafficking further essential biological cell functions. Furthermore, the fine-tuning of FA transport is briefly outlined. Recent studies signify short-chain FAs and lipoic acid as immune and broad health modulators. Based on metabolomics, evidence is accumulating that several saturated fatty acids (SFAs) are deficient during pathogen challenges; therefore, the defense response of the host can be boosted by exogenous supplementation of these SFAs. The role of the intestinal microbiota is beginning to be well understood, particularly with respect to short-chain FAs; the first information of controlling function via epigenetics is available.

Fatty acids (FAs) are carboxylic acids that are major components of lipids. Over 70 different FAs are known in nature (Brody 1999). The properties of the FAs are most important to the properties of the conjugated fat. FAs are usually not found in organisms in their standalone form but exist instead as three main classes of esters: triglycerides, phospholipids, and cholesteryl esters (Brody 1999). FAs are named based on:

1. The number of carbon atoms: Short-chain FAs (SCFAs) are 2–6 C atoms long; medium-chain FAs (MCFAs) comprise chain lengths of 7–12 C. Long-chain FAs (LCFAs) have a chain length of 13–21 C, of which C_{16} and C_{18} FAs are the most abundant ones in aquatic animals. FAs longer than C_{21} ($C > 21$) are called very-long-chain FAs (VLCFAs) and less abundant than LCFAs. VLCFAs with $C \geq 26$ are often subclassified as ultra-long-chain FAs (ULCFAs) (Brody 1999).
2. The number (if any) of the unsaturations and their positions: For example, 18:3 ω 3 (also written as 18:3n – 3 or 18:3 ω – 3) denotes an unsaturated FA (USFA) containing 18 carbons and three double bonds with the first one at the number 3 carbon atom from the methyl end (ω) of the molecule (Fig. 26.1):

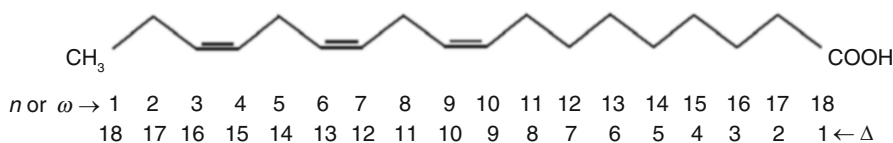


Fig. 26.1 Fatty acid nomenclature: polyunsaturated fatty acid α -linolenic acid or *all cis* $\Delta 9,12,15$ -octadecatrienoic acid (18:3n – 3; 18:3 ω 3) (From Castro et al. (2016), with permission from Elsevier). The carbon position at which the incipient double bond locates is counted from methyl (ω) and front (Δ) ends of the fatty acyl chain

FAs are generally unbranched and can be classified as:

- Saturated FAs (SFAs): Every carbon atom has two hydrogens attached to it except for the terminal carbon with three hydrogens. All carbon atoms are connected by single bonds.
- Unsaturated FAs (UFAs): Some of the carbons are attached by at least one double bond. Monounsaturated FAs contain one carbon-to-carbon double bond, and polyunsaturated FAs (PUFAs) contain two or more carbon-to-carbon double bonds. These FAs may be liquid at room temperature because of their double bonds.

The position of the double bond is important for its biological activity. Often, highly unsaturated FAs (HUFAs) are differentiated from PUFAs; they possess 20 or more C atoms and three or more double bonds (Lands 2017). We combine both classes as PUFAs.

Figure 26.2 presents an overview of common and most abundant FAs and the elongation and desaturation pathways of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and ultra-long-chain fatty acids (ULCFAs). Essential FAs are highlighted in this figure.

UFAs have lower melting points than SFAs. For instance, the melting point of stearic acid (C₁₈ saturated) is 69.6 °C; in contrast, the melting points of corresponding C₁₈ UFAs oleic (one double bond), linoleic (two double bonds), and α -linolenic acid (three double bonds) are 13, –5, and –16 °C, respectively (Ouellette and Rawn 2018). Stearic acid is a straight chain; oleic acid and α -linolenic acid are kinked wherever a carbon-to-carbon double bond appears (Ouellette and Rawn 2018). To maintain membrane fluidity, poikilothermic animals make use of these features through “homeoviscous adaptation” and change the FA composition of membranes in favor of PUFAs at decreasing temperatures (De Carvalho and Caramujo 2018). Homeoviscous adaptation is the homeostatic process that regulates the viscosity of membrane lipids by specific membrane phospholipids and LC-PUFAs to counteract the tendency of cell membranes to become rigid at low temperatures or high salt contents (Sinensky 1974; Farkas and Nevenzel 1981; Ernst et al. 2016).

For the elongation and desaturation of both α -linolenic (ALA, an ω 3 PUFA) and linoleic acids (LIN, an ω 6 PUFA) to produce eicosapentaenoic (EPA) and

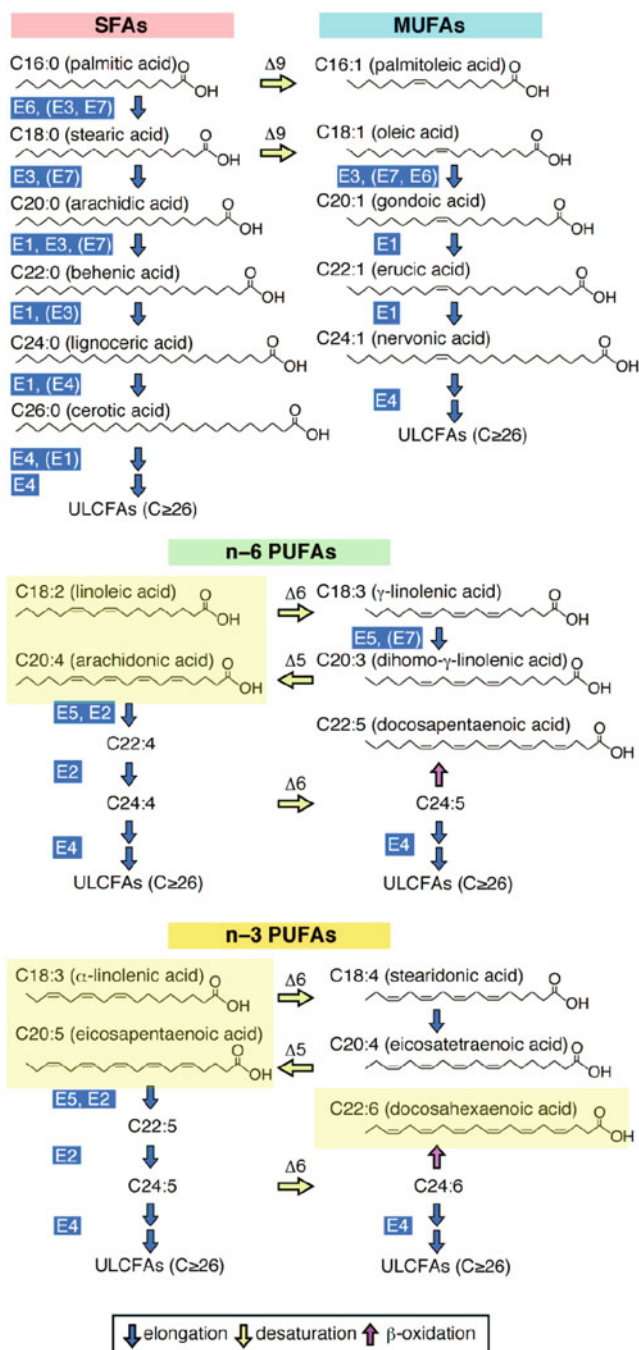


Fig. 26.2 Fatty acid elongation pathways of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and ultra-long-chain fatty acids (ULCFAs) are illustrated. Elovl isozyms (E1–E7) responsible for each elongation step are indicated.

arachidonic (ARA) acids (Fig. 26.2), the same set of enzymes (Δ^6 and Δ^5 desaturases) is required. Therefore, a competition emerges between the two FA families for these enzymes (\rightarrow Chap. 28). Moreover, during the conversion of EPA to docosahexaenoic acid (DHA), also Δ^6 desaturase is needed resulting in additional competition for this enzyme. Consequently, both the absolute concentrations and the dietary proportions between $\omega 3$ and $\omega 6$ PUFA are important. If one FA is greatly in excess of the other, suppression of the desaturation process of the less abundant FA will take place (Ahlgren et al. (2009) and references therein).

Due to the double bonds, there are two types of FAs: (1) *cis* and (2) *trans*. Because the *trans* configuration does not produce a bend at the site of the double bond, *trans* FAs are more solid at room temperature, like SFAs (Solomon et al. 2008).

A major role of FAs in all organisms is to generate metabolic energy in the form of ATP via mitochondrial β -oxidation. This energy is used as major source for growth from egg to adult fish or adult invertebrate and also for reproduction (Brody 1999; Sargent et al. 2002).

Saturated FAs, MUFAs, and PUFAs have nutritional values; however, the apparent digestibility of the different types differs (Hua and Bureau 2009): A meta-analysis of data from 16 studies in salmonids shows that variations in apparent digestibility of dietary lipid can be primarily explained by the proportion of SFA in total FAs. Therefore, SFAs can be incorporated into diets at levels only below $\sim 23\%$ of total FAs without adversely affecting lipid digestibility. Furthermore, a multiple regression analysis of data from these 16 studies examined the relationship between digestible lipid content of the diet and inclusion levels of SFAs, MUFAs, PUFAs, and water temperature (Hua and Bureau 2009). This analysis yields the following model:

$$\begin{aligned} \text{Digestible lipid content (\% of diet)} = & 0.45 \text{ SFA} - 0.08 \text{ SFA}^2 + 0.86 \text{ MUFA} \\ & + 0.94 \text{ PUFA} + 0.03 \text{ SFA} \times \text{MUFA} \\ & + 0.04 \text{ SFA} \times \text{PUFA} + 0.03 \text{ temperature} \\ & \times \text{SFA} \quad (P < 0.0001, R^2 = 0.98). \end{aligned}$$

The model shows that there is an apparent threshold of SFA concentration on lipid digestibility, above which reduction of lipid digestibility is observed with an increase in dietary SFA concentration. The model also indicates that MUFA, PUFA, and water temperature positively affect the digestibility of SFAs. A comparison of the predictions obtained by this model to experimental observations from

Fig. 26.2 (continued) Parentheses denote Elovls that exhibit weak activity toward the indicated substrates. (From Sassa and Kihara (2014), credit The Korean Society of Applied Pharmacology). Δ^5 , Δ^6 , and Δ^9 are Δ^9 desaturases. Δ^n indicates the position (n) of the double bond from the carboxyl end of the FA chain. Essential FAs of many marine species are highlighted

independent groups of data indicates that it can be a useful tool in practical feed formulation (Hua and Bureau 2009).

26.1 Fatty Acid Sensing in Fishes

26.1.1 Sensing

Several hormones regulating energy homeostasis (→ “Dietary Restriction, Starvation, Compensatory Growth” in AAN I (Steinberg 2018)) are involved in the modulation of FA sensing systems in fishes as excellently reviewed by Conde-Sieira and Soengas (2017). Anorexigenic/anabolic hormones activate nutrient sensing systems, whereas orexigenic/catabolic hormones inhibit them. Recent evidence provides information of the presence and functioning of putative nutrient sensing systems either in peripheral or central areas mainly in rainbow trout, as summarized in Fig. 26.3. The known mechanisms are comparable to those of mammals in several aspects, but clear differences arise in others, such as the capacity of detecting changes in circulating levels of MCFAs or PUFAs. These differences between fishes and mammals might relate to at least three different reasons. A first reason might relate to the large importance of amino acids (AAs) for metabolic purposes in fishes. A second reason may be due to the high variety of dietary fish habits resulting in large differences in gastrointestinal morphology and function (→ “Diets and Digestive Tracts” in AAN I (Steinberg 2018)). A third reason may rely on the existence of multiple gene variants in neuropeptides, hormones, and metabolic effectors resulting from the additional gene duplication of actinopterygians (Conde-Sieira and Soengas 2017).

Sensing and adjusting to fluctuations in environmental nutrient levels is a requisite for life (Grainger et al. 2020). Different organisms are able to detect extracellular and intracellular lipid levels. Evidence obtained in rainbow trout supports the presence of FA sensing mechanisms that seem to be present in hypothalamus, liver, and Brockmann bodies. Bony fishes have a conventional exocrine pancreas, but the endocrine tissue is often gathered in separate structures called Brockmann bodies (Youson and Al-Mahrouki 1999).

FA sensing capacity relates to food intake regulation and metabolism in fishes (Fig. 26.5). Besides responding to LCFAs, these systems also respond to MCFAs or PUFAs. However, the capacity of PUFAs to activate FA sensing systems appears to be specific of certain PUFAs, since eicosapentaenoic acid (EPA) does not induce any significant change in rainbow FA sensing systems (Conde-Sieira et al. 2015). The hypothalamus acts as signaling integrator in a way that detection of increased levels of nutrients results in food intake inhibition through changes in the expression of anorexigenic and orexigenic neuropeptides (Conde-Sieira and Soengas 2017).

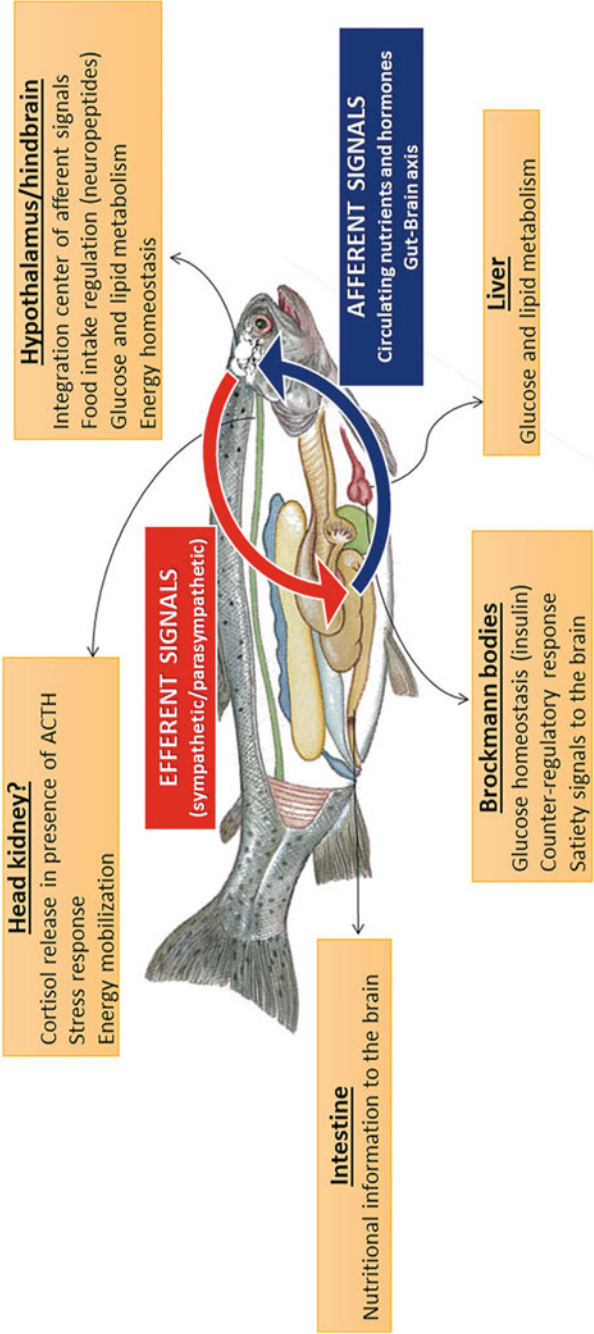


Fig. 26.3 Schematic drawing summarizing functions of nutrient sensing systems in central and peripheral tissues of fishes (From Conde-Sieira and Soengas (2017), credit Frontiers Media)

26.2 Fatty Acid Transport

The solubility of LCFAs in aqueous solutions is in the range of 1–10 nM (Vorum et al. 1992). Because of this lipophilicity, it was initially thought that they traverse the lipid bilayer by diffusion without involvement of protein mediators. However, many organs and cell types display a rapid, saturable, substrate-specific, and hormonally regulated LCFA uptake, and intracellular mechanisms are indicative of protein-(transporter)-mediated processes.

26.2.1 Fatty Acid (Uptake) Transporters

Fatty acid transport proteins (FATPs, SLC27A1–6) form a family of six related proteins with representatives in all vertebrate and invertebrate species (Gimeno 2007). FA transporters are not limited to facilitate the traversing of membranes; however, they are also responsible for cytoplasmic transport.

Focusing on zebrafish, Salmerón (2018) mentions that FAs cross the plasma membrane using FA transporters, such as cluster of differentiation 36 (CD36), FATP, and plasma membrane fatty-acid-binding protein (FABPm). Moreover, cytoplasmic FABP (FABPc) carries FAs from the membrane to acyl-CoA synthase to be esterified as acyl-CoA. Then, this acyl-CoA is carried by an acyl-CoA-binding protein (acyl-CoA-BP) to the endoplasmic reticulum to be esterified with glycerol 3-phosphate (G3P) to form triacylglycerol (TAG, also abbreviated as TG, triglyceride).

Fatty acid transport proteins and receptors CD36 have been identified, for instance, in Akoya pearl oyster (*Pinctada fucata martensii*) (Lei et al. 2017) and Japanese carpet shell (*Ruditapes philippinarum*) (Yang et al. 2019), as well as finfishes, such as *Ctenopharyngodon idella* (Lei et al. 2019), *Cyprinus carpio*, *Danio rerio* (Fink et al. 2015), *Larimichthys crocea* (Yan et al. 2015), *Lateolabrax japonicus* (Xu et al. 2017), *Megalobrama amblycephala* (Jiang et al. 2018), *Misgurnus anguillicaudatus* (Zhang et al. 2017b), *Oncorhynchus mykiss* (Cleveland and Weber 2016), *Oreochromis niloticus* (Xie et al. 2017), *Pampus argenteus* (Liao et al. 2017), *Salmo salar* (Torstensen et al. 2009), *Scophthalmus maximus* (Li et al. 2018), or *Solea senegalensis* (Velasco et al. 2017).

Members of the third transporter family, FABPs, have been characterized, inter alia, in the oysters *Crassostrea brasiliana* (Zacchi et al. 2018) and *C. gigas* (Medeiros et al. 2008); the crustaceans *Calanus finmarchicus* (Tarrant et al. 2008), *Cherax quadricarinatus* (Li et al. 2012), *Pacifastacus leniusculus* (Söderhäll et al. 2006), *Macrobrachium nipponense* (Ding et al. 2014), *Fenneropenaeus chinensis* (Ren et al. 2009), *Eriocheir sinensis* (Gong et al. 2010), and *Portunus trituberculatus* (Tang et al. 2018); and the finfishes *D. rerio* (Her et al. 2004), *Dichotomylctere nigroviridis* (Thirumaran and Wright 2014), *Fundulus heteroclitus* (Roling et al. 2006), *O. mykiss* (Venold et al. 2012), *S. salar* (Venold et al. 2013),

Schizothorax prenanti (Zhang et al. 2017a), *Trachinotus ovatus* (Lei et al. 2020), or the ancient, air-breathing spotted gar (*Lepisosteus oculatus*) (Venkatachalam et al. 2018). Interestingly, FABPs participate even in antibacterial responses as identified in *E. sinensis* (Cheng et al. 2013), orange-spotted grouper (*Epinephelus coioides*) (Luo et al. 2014b), and goldfish (Chen et al. 2018).

26.2.2 ATP-Binding Cassettes

In addition to FA (uptake) transporters, peroxisomal ABC (ATP-binding cassette) transporters mediate the transport of VLCFAs and long-chain fatty acid (LCFA)-CoA into peroxisomes and trafficking of further essential cell biological functions (Theodoulou et al. 2006).

Sturm et al. (2009) identified members of the subfamily ABCD in *Daphnia pulex*, which participate in VLCFA transport, gene transcription, and protein translation. Importantly, the simultaneous posttranscriptional silencing of three ABCD transporters disrupts offspring production in *Daphnia*, pointing out developmental roles of peroxisomal ABC transporters (Sturm et al. 2009). In a follow-up study, Valenzuela-Munoz et al. (2015) found this ABC subfamily overexpressed in several larval stages of the salmon louse *Caligus rogercresseyi*. Congruent with this observation, this transporter subfamily is expressed during all developmental stages in the intertidal copepod *Tigriopus japonicus* (Jeong et al. 2014). The same laboratory identified ABCD transporters in *Brachionus koreanus* (Jeong et al. 2017b) and the copepod *Paracyclopsina nana* (Jeong et al. 2017a) (→AAN III Chapters on Fish Oil Replacement).

ABCD transporters play a central role in FA transport also in chordates. Dean and Annilo (2005) reported four ABCD genes in zebrafish and three in sea squirts (*Ciona intestinalis*, a non-vertebrate chordate). Later, Liu et al. (2013) characterized ABCD proteins in channel catfish (*Ictalurus punctatus*) and Ren et al. (2015) in sea lamprey (*Petromyzon marinus*) and Arctic lamprey (*Lethenteron camtschaticum*). In addition, Jeong et al. (2015) listed ABCD proteins in two medakas (*Oryzias latipes*, *O. melastigma*), *Oreochromis niloticus*, and spotted green puffer (*D. nigroviridis*) and Liu et al. (2016) in common carp (*Cyprinus carpio*).

26.2.3 Intracellular Transporters

Intracellular lipid-binding proteins (iLBPs) overlap in parts with FATPs. They, too, play a role in transport and cellular uptake of FAs and gene expression regulation. de Toledo-Silva et al. (2017) characterized the *ilbp* gene family in Pacific oyster (*Crassostrea gigas*) with 26 transcripts. The different gene transcription profiles and reported docking systems indicate that the *ilbps* are a non-generalist ligand-binding protein family with specific functions. Oyster gills are directly in contact

with the external environment, and it is known that bivalves can uptake lipids directly from water (de Toledo-Silva et al. 2017).

Venkatachalam et al. (2017) characterized the *ilbp* multigene family in salmonids (*S. salar*, *O. mykiss*), *D. rerio*, *O. latipes*, *Gasterosteus aculeatus*, and *D. nigroviridis*. iLBPs transport essential signaling molecules to nuclear receptors that modulate the transcription of other genes involved in growth, development, differentiation, and reproduction. By duplication of *ilbp* genes, the repertoire and complexity of the signaling, genetic, and metabolic networks is enhanced combined with increased flexibility to respond to stimuli. This confirms findings by Zhang et al. (2012) that, due to the stressful environment of the Pacific oyster, several families of defense genes are multiplied.

26.2.4 Fine-Tuning of Fatty Acid Transport

In order to assure cellular FAs demand and avoid lipotoxicity (→Chap. 24), FA uptake into cells and tissues must be fine-tuned by transporter proteins. To identify mechanisms, Wang et al. (2019) investigated how zebrafish coordinates the expression of FA transporters, such as CD36, FABPpm, and FATPs, in response to changes of dietary lipid level and FA patterns. Therefore, five diets were formulated with fish oil (FO, rich in ω 3 PUFA), palmitic acid (PA, 16:0), olive oil (OO; rich in oleic acid, 18:1 ω 9), sunflower oil (SO; rich in LIN, 18:2 ω 6), or perilla oil (PO; rich in ALA, 18:3 ω 3) as the sole lipid source.

Figure 26.4 shows that FA transporters are coordinating the mobilization of fuel substrates in response to diet composition. The hepatic *fatp2* mRNA is higher on PA than on FO diet, while the intestine *fatp2* transcription increases on OO diet. These data point out that 16:0-enriched PA diet promotes hepatic FA uptake, while 18:1 ω 9-enriched OO diet stimulates intestinal FA transport. An increased hepatic and intestine *fatp4* transcription on PA diet, compared with FO diet, indicates a preferential uptake of this C₁₆ FA in these organs/tissues. The function of FATP6 remains obscure and needs additional studies (Wang et al. 2019). This appears to be the only available study on this issue so that different aquatic animals may have different fine-tuning patterns of FA transport based on gene activation.

26.3 Regulation of Feed Intake

In fishes on lipid-enriched diets or with enhanced lipid storage, usually food intakes decrease (Conde-Sieira and Soengas 2017; Johansen et al. 2002, 2003). And vice versa, reducing levels of circulating FA through pharmacological treatment, food intake will increase as demonstrated in rainbow trout (Librán-Pérez et al. 2014).

Pathways of lipid metabolism relate to the action of proteins involved in cell signaling like mechanistic target of rapamycin (mTOR) and protein kinase B (Akt),

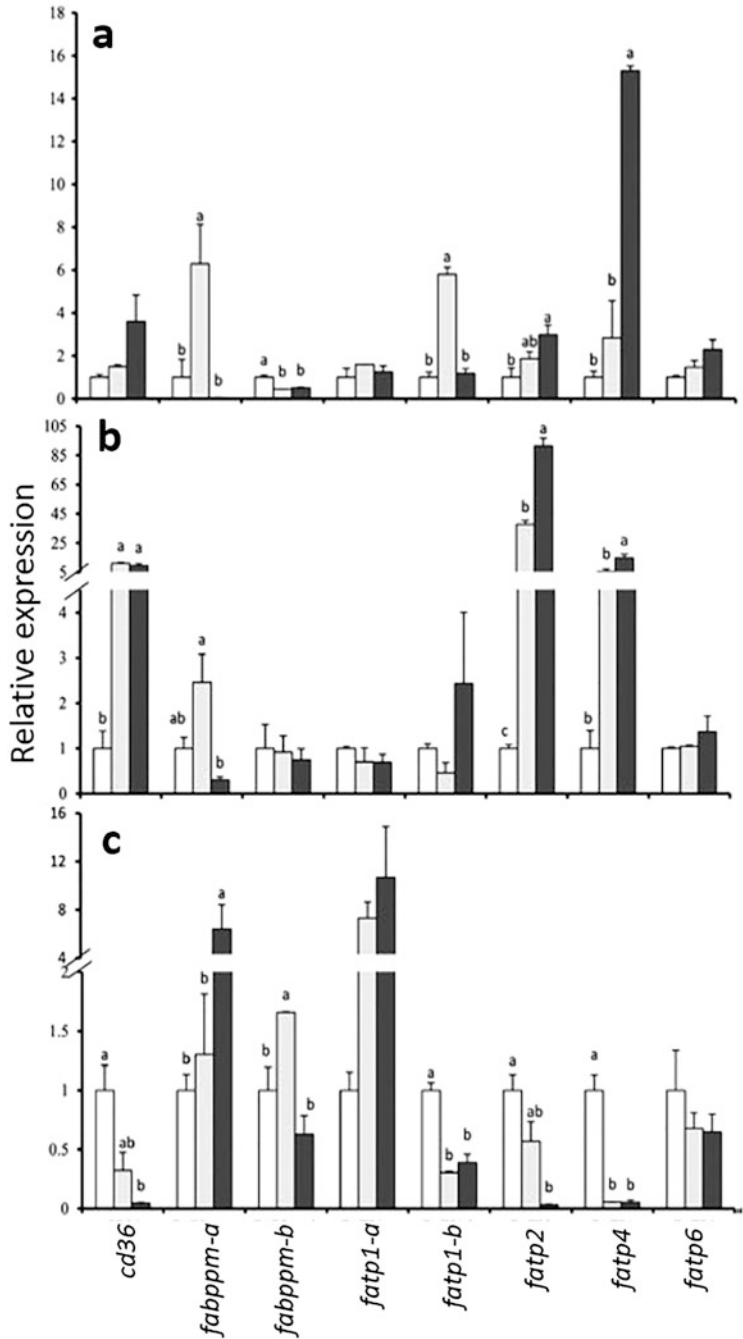


Fig. 26.4 Effects of dietary vegetable oils with different fatty acid profiles on the expression of fatty acid transporters in the liver (a), muscle (b), and intestine (c) of zebrafish. Values are means \pm SEM ($n = 3$). Statistical significance was evaluated by one-way ANOVA, followed by Duncan’s multiple range test. Labeled means without a common letter differ, $P < 0.05$. (From Wang et al.

energy sensing like 5-AMP-activated protein kinase (AMPK) (→Box “*Yin and Yang of Energy Regulation*,” Chap. 5), and transcription factors like forkhead box01 (FoxO1) and cAMP response-element binding protein (CREB) (Velasco et al. 2018). Treatment of rainbow trout liver with oleate or octanoate results in the modulation of phosphorylation status of proteins involved in cell signaling (increased Akt and mTOR) and energy sensing (decreased AMPK) (Velasco et al. 2018). These changes occur simultaneously with increased transcription of *fas*, *sreb1c*, and *ppara* as well as decreased mitochondrial activity.

Changes of the appetite are modulated by neuropeptides (→“Dietary Restriction, Starvation, Compensatory Growth” in AAN I (Steinberg 2018)). Feeding fishes with lipid-enriched diets results in increased *pomc*, *agrp*, and *cart* transcription shown in rainbow trout (Figueiredo-Silva et al. 2012; Librán-Pérez et al. 2015) and Atlantic salmon (Hevrøy et al. 2012). In several other studies, however, high-lipid diets do not change the gene transcription: neither *npy* in rainbow trout and orange-spotted grouper (Figueiredo-Silva et al. 2012; Tang et al. 2013) nor *agrp* in Atlantic salmon (Hevrøy et al. 2012). This indicates that only one or a few neuropeptides facilitate the regulations as sketched in Fig. 26.5.

26.4 Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are produced from monosaccharides, disaccharides, and polysaccharides that are subject to digestion by endogenous enzymes. In addition, they are produced by microbes from those plant polysaccharides that are not digested by host enzymes, e.g., cellulose (Stevens and Hume 1998). Clements et al. (1994) identified SCFAs in the gut of three marine herbivores and Kihara and Sakata (2002) in common carps. The authors considered SCFAs mainly as source of energy and lipid synthesis for the hosts. In fact, SCFAs contribute considerably to the host energy requirements (Mountfort et al. 2002). However, this is only part of the controlling function of SCFAs.

Moreover, several prebiotics, such as arabinoxylan, soybean oligosaccharides, isomalto-oligosaccharides, raffinose, gentiobiose, lactosucrose, arabinoxylan-oligosaccharides, oligofructose, xylose, fructose, and resistant starch, have been shown to enhance SCFA levels via gut microbes in, among others, common carp (Kihara and Sakata 2002), Nile tilapia, European seabass (Leenhouwers et al. 2008), Siberian sturgeon (Geraylou et al. 2013), African catfish (Romano et al. 2018), rainbow trout (Hoseinifar et al. 2017a), and grass carp (Li et al. 2019).



Fig. 26.4 (continued) (2019), with permission from Wiley). Dietary groups (columns) from left to right in each gene group: *FO* fish oil; *PA* palmitic acid; *OO* olive oil; *SO* sunflower oil; *PO* perilla oil; *cd36* scavenger receptor; *fabppm-a*, *fabppm-b* plasma membrane fatty acid-binding proteins a, b, *fatp1*...6 fatty acid transport protein family

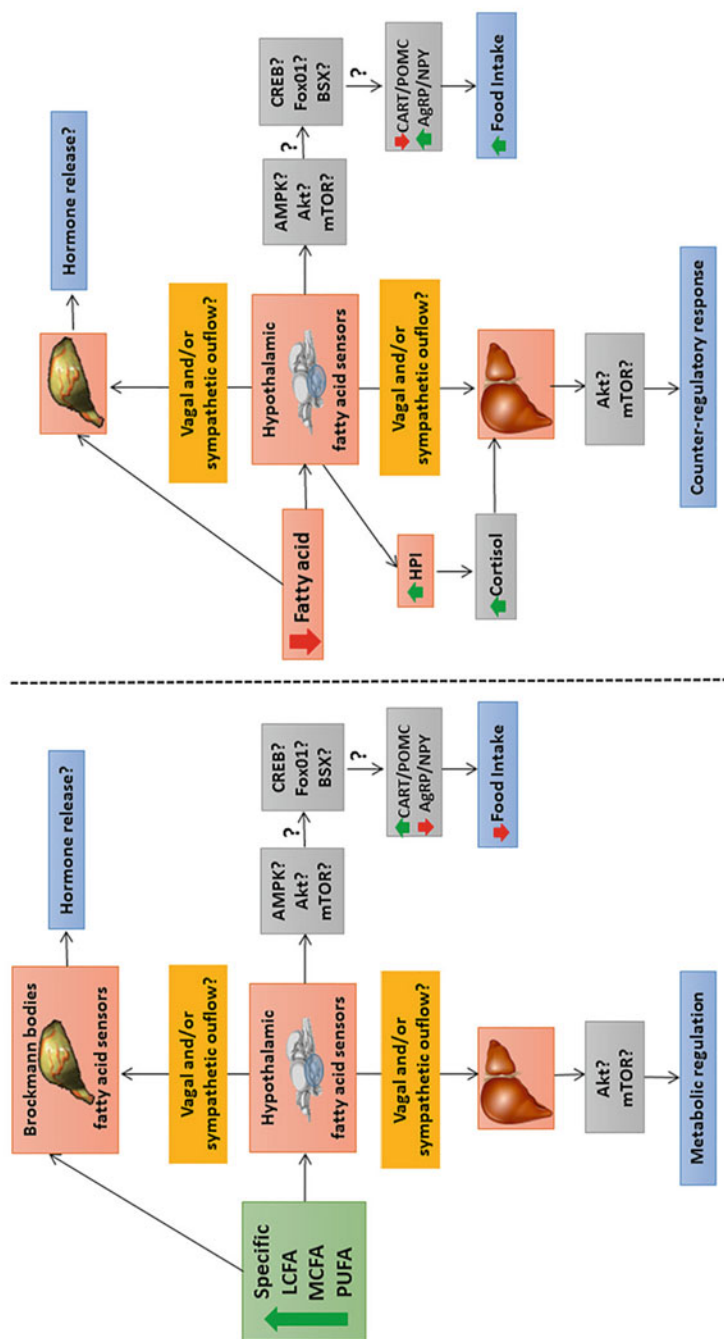


Fig. 26.5 Hypothetical model of integrative responses to an increase (left panel) or decrease (right panel) in levels of specific fatty acids of fatty acid sensing systems in different fish tissues. ↑ increase; ↓ decrease; ? unknown; *AgRP* agouti-related peptide (appetite-stimulating neuropeptide); *Akt* protein kinase B; *AMPK* AMP-activated protein kinase; *BSX* hypothalamic homeobox transcription factor; *CART* cocaine- and amphetamine-related transcript (involved in appetite regulation); *CREB* cAMP response-element binding protein; *FoxO1* forkhead box protein O1; *HPI* hypothalamus-pituitary-interrrenal axis; *LCFA* long-chain fatty acid; *MCFA* medium-chain fatty acid; *mTOR* target of rapamycin; *NPY* neuropeptide Y (produces one of strongest orexigenic signals); *POMC* pro-opiomelanocortin (POMC neuron stimulation results in satiety; Varela and Horvath 2012); *PUFA* polyunsaturated fatty acid (From Conde-Sieira and Soengas (2017), credit Frontiers Media)

Sugar alcohols, such as mannitol, are not efficiently digested by vertebrates; however, mannitol can serve as substrate for fermentation to SCFAs. It is the primary photosynthate of Phaeophyta (brown algae) and, as such, an important dietary constituent for many marine herbivores. White et al. (2010) tested eight New Zealand herbivores and found that mannitol is utilized as an indirect nutrient via fermentation by hindgut microorganisms. This fact is nutritionally particularly important for adult silver drummer (*Kyphosus sydneyanus*) and butterfish (*Odax pullus*).

As new function in addition to energy provision, the production of SCFAs improves host's immunity (Fig. 26.6) by several modes of action. SCFAs increase the solubility of minerals, potentially making them more easily absorbed (Merrifield and Rodiles 2015). Production of SCFAs by intestinal microbes reduces the intestinal pH and makes the environment less favorable to some potential pathogens, since it can lead to internal acidification of microbial cells. The subsequent export of excess protons requires consumption of cellular ATP and may result in depletion of cellular energy and decreased virulence (Ricke 2003). Moreover, SCFA and their salts can serve as antibacterial feed supplements: formic, acetic, propionic, butyric, valeric, or lactic acids are well known to possess antibacterial activity (Bergeim 1940; Defoirdt et al. 2006).

Further mechanisms of improved host's immunity are upregulation of beneficial immune components and, simultaneously, reduction of immune parameters that are detrimental to the host, such as liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Hoseinifar et al. 2017c). Furthermore, SCFAs support gut health by decreasing epithelial permeability and modulating cytokines in the intestine (Macia et al. 2012). SCFAs also alter the intestinal microbial community, which can produce variations in epithelial morphology and serum immunoglobulin concentration (McCracken and Lorenz 2001; Guarner and Malagelada 2003). All these immune responses work synergistically against pathogens. In invertebrates, the biomolecular pathways are less well documented than in finfishes, but they may be similar (Fig. 26.6).

In educational depth, Tian et al. (2017) show that sodium butyrate (SB or NaB) supplementation improves not only growth performance, intestinal growth, and the function of digestive enzymes in young grass carps but also increases beneficial bacteria, such as *Lactobacillus*, and butyrate concentrations, decreases baneful bacteria, such as *Aeromonas* and *Escherichia coli*, and reduces acetate and propionate concentrations. Additionally, the authors show that (Fig. 26.7):

1. SB supplementation increases enteritis resistance up to the dietary optimum ranging from ~160 to 340 mg kg⁻¹ diet.
2. SB supplementation enhances intestinal immune function:
 - (i) Increase of lysozyme and acid phosphatase activities, C3, C4, and IgM contents, and upregulation of antimicrobial peptides and proteins (*β-defensin-1*, *hepcidin*, *leap-2b*, and *mucin2*)
 - (ii) Downregulation of pro-inflammatory cytokines (*tnf-α*, *ifn-γ2*, *il-1β*, *il-6*, *il-8*, *il-15*, *il-17d*, *il-12 s*) and upregulation of anti-inflammatory cytokines (*il-10*, *il-11*, *tgf-βs*, *il-4/13 s*) in the three intestinal segments.

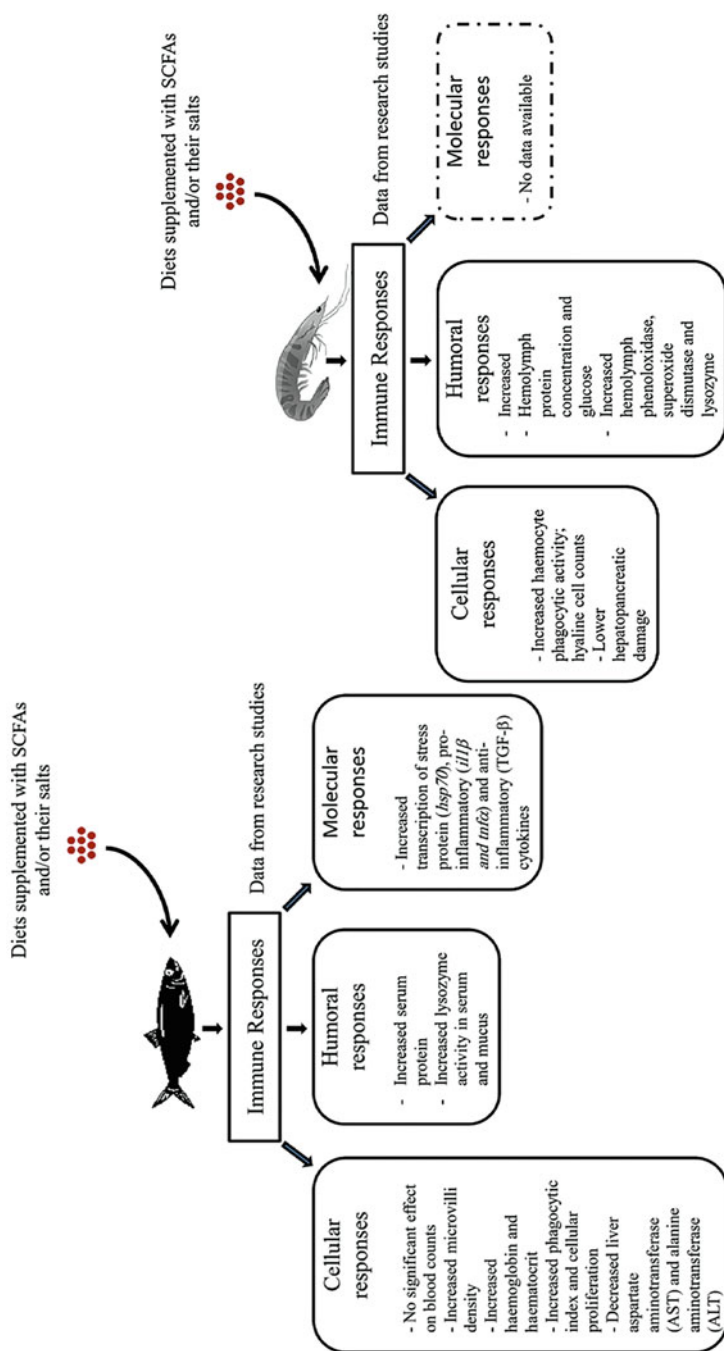


Fig. 26.6 Modulation of the immune response in fishes and shrimps following dietary supplementation with various SCFAs and their salts (From Hoseinifar et al. (2017c), with permission from Wiley)

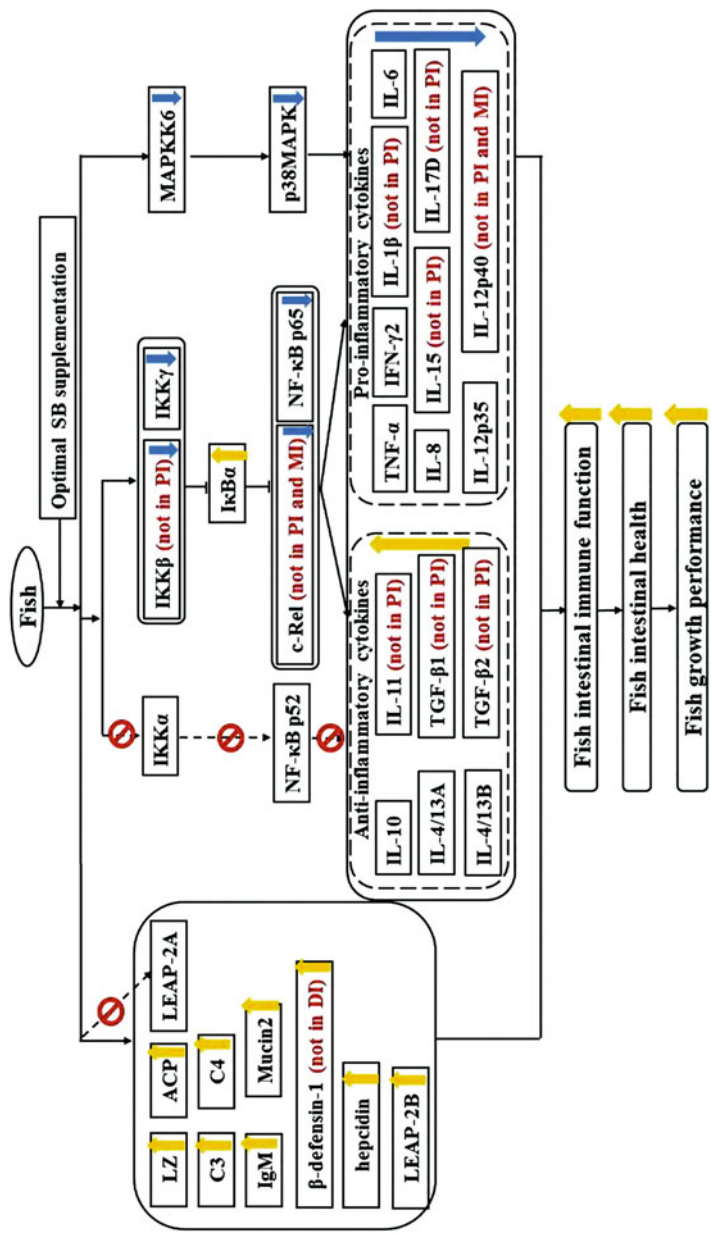


Fig. 26.7 Effect of sodium butyrate (SB) on immune function and its potential signaling pathways in the intestine of young grass carps. —●— not through; —■— inhibit; —▶— might through. (From Tian et al. (2017), with permission from Elsevier). *PI* proximal intestine, *MI* middle intestine, *DI* distal intestine

In a companion paper, Wu et al. (2018) report that optimal SB supplementation improves the intestinal physical barrier function of young grass carp due to:

1. Inhibition of intestinal cell apoptosis, thereby maintaining the intestine cell structure integrity: downregulation of *cysteine-aspartic protease-2 (caspase-2)*, *caspase-3*, *caspase-7*, *caspase-8*, *caspase-9*, *fatty acid synthetase ligand (fasl)*, *apoptotic protease activating factor-1 (apaf-1)*, *B cell lymphoma 2-associated X protein (bax)*, and *c-Jun N-terminal protein kinase (jnk)* and upregulation of *B-cell lymphoma protein-2 (bcl-2)*, *inhibitor of apoptosis proteins (iap)*, and *myeloid cell leukemia-1 (mcl-1)*.
2. Increasing antioxidant capacity referring to increased CuZnSOD and other antioxidant enzyme activities which are related to their gene expression under the regulation of the Keap1b/Nrf2 signaling pathway, thus enhancing the ability of scavenging reactive oxygen species (ROS), decreasing lipid and protein peroxidation, and alleviating further oxidative damage.
3. Improving the integrity of intercellular structures by upregulation of *zonula occludens-1 (zo-1)*, *zo-2*, *occludin*, *claudin-b*, *claudin-c*, *claudin-f*, *claudin-3c*, *claudin-7a*, *claudin-7b*, and *claudin-11* and downregulation of *claudin-12*, *claudin-15a*, and *claudin-15b*, which are related to decreasing the molecule *myosin light chain kinase (mlck)* expression.

Similarly, Zhou et al. (2019a) identified in juvenile golden pompano (*Trachinotus ovatus*) that dietary SB remarkably upregulates the expression of *creb* and *cdx2*. These transcription factors are crucial for the butyrate-induced transcription of the intestinal epithelial apical di-/tripeptide transporter, pointing out the involvement of SB in protein synthesis. Overall, SB is a beneficial dietary supplement for improving growth performance and intestinal proliferation. With 2 g kg⁻¹ dietary SB, the optimal level for golden pompanos is roughly six times above that of young grass carps.

Butyrate is a histone deacetylase inhibitor that plays a predominant role in the epigenetic regulation of gene expression and cell function. To evaluate this effect, Terova et al. (2016) fed a plant-based diet to European seabass. The trial reveals no significant differences in WG and SGR of individuals, which received 0.2% SB supplementation. Dietary SB increases the acetylation state of histone H4 but not of histone H3 at Lys9. Dietary SB causes significant changes in vivo in the expression of genes related to epigenetic regulatory mechanisms such as *hdac11*, *ehmt2*, and *dicer1* (Fig. 26.8). The expression of four (*il1β*, *il8*, *irf1*, and *tnfa*) out of seven target genes related to mucosal protection and inflammatory response is significantly different between liver and intestine, but only for the *il10* gene differences are observed in the expression due to the SB treatment.

More effects of SB and other SCFA in selected farmed species are listed in Table 26.1. The applied approaches lack a common protocol and, therefore, translate into incomparability of the studies.

One often supplemented SCFA derivative is the bacterial storage polymer polyhydroxy butyric acid (PHB, Fig. 26.10) (Reddy et al. 2003). Studying sea cucumbers, Sawabe et al. (2016) identified a link between microbial PHB producers and

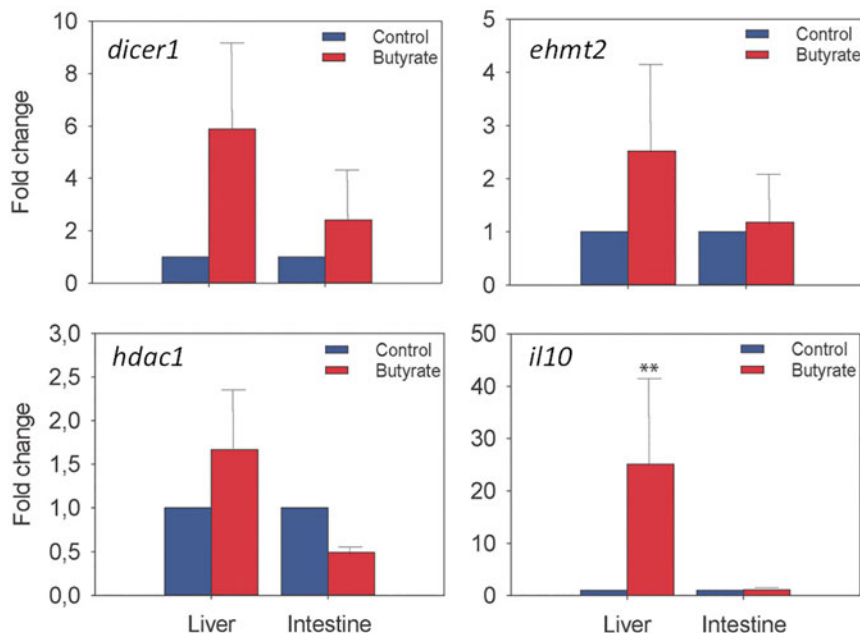


Fig. 26.8 Effects of dietary butyrate on gene expression in two tissues of the European seabass: liver and intestine, as determined by qRT-PCR analysis. Only those genes that showed statistical differences for the interaction between tissue and treatment (*dicer1*, *ehmt2*, and *hdac11*) or differences in expression solely due to the treatment (*il10*) are depicted. Fish were fed for 8 weeks two different diets, a control diet, similar to feed commercially available for growing European seabass, and the experimental diet, which was the control diet supplemented with 2 g/kg (0.2%) of Na butyrate. The means of six animals in each group are shown (From Terova et al. (2016), credit Public Library of Science)

potential growth promotion in this marine invertebrate. Selected effects of dietary PHB effects are gathered in Table 26.1.

Even antidote activity is attributed to NaB: it improves intestinal integrity and immunity in juvenile Chinese mitten crab fed glycinin (\rightarrow AAN III Fishmeal Replacement: Terrestrial Plants). Han et al. (2020) fed crabs diets containing glycinin without NaB (B0 in Fig. 26.9), which then suffer from oxidative stress (reduced glutathione but increased lipid peroxidation) in the intestine. Moreover, dietary glycinin decreases lysozyme and phenoloxidase activities and increases the level of histamine, while NaB supplementation counteracts all these adverse effects. The NaB addition also restores the impaired immunity and morphological structure of the intestine. Dietary NaB decreases the content of pro-inflammatory TNF- α and increases the transcription of antimicrobial peptides *anti-lipopolysaccharide factor 1* and 2 (*alf1*, *alf2*) (Fig. 26.9). Furthermore, NaB restores the intestinal microbial community disorganized by glycinin: the abundance of pathogenic bacteria (*Aeromonas*, *Vibrio*, and *Pseudomonas*) decreases and of potential probiotic bacteria (*Bacillus*, *Lactobacillus*, *Chitinibacter*, and *Dysgonomonas*) increases on dietary 10 g kg⁻¹ NaB.

Table 26.1 Effect of dietary short-chain fatty acid, including formiate and PHB, in selected farmed species

Species	Short-chain fatty acid	Level, g kg ⁻¹	Affected trait	References
<i>Invertebrates</i>				
<i>Litopenaeus vannamei</i>	NaB	0, 20	WG↔, immunity↔, pathogen resistance↑	Ramírez et al. (2017)
	NaB, NaP	0→20	WG↑, microbial diversity↑, nitrogen retention↑, PER↑	da Silva et al. (2016c)
	NaB or PHB	0, 20	Gut morphology↑, digestive capacity↑, NaB > PHB	da Silva et al. (2016b)
	Apple cider vinegar Propionic acid	0→4 0.5	Albumin↑, hematocytes↑ @ 2; 4 Albumin↑, hematocytes↑	Pourmozaffar et al. (2019)
	PHB	0→0.5	<i>Vibrio harveyi</i> resistance↑	Situmorang et al. (2020)
<i>Penaeus monodon</i>	Butyrate	1	Survival↑, feed intake↑, production↑	Rombenso et al. (2020)
<i>Fishes</i>				
<i>Acipenser baerii</i>	PHB	0, 20, 50	@20: WG↑, SGR↑, survival↑, intestinal microbiota↑	Najdegerami et al. (2012)
		PHB- <i>Artemia</i>	WG↔, environmental stress resistance↓	Najdegerami et al. (2015)
	Acetic acid	20	WG↑, innate immunity↑, LC-PUFA↑	Zare et al. (2021)
<i>Andinoacara rivulatus</i>	Apple cider vinegar	0→40	WG↑, serum immunity↑, skin mucus immunity↑, pathogen resistance↑	Ahmadniaye Motlagh et al. (2019)
<i>Arapaima gigas</i>	NaB	1.2	WG↑, glycogen↑, environmental stress resistance↑	Luz et al. (2019)
<i>Carassius auratus</i>	NaP	0→5	WG↑, immunity↑, <i>Ichthyophthirius</i> resistance↑	Sheikhzadeh et al. (2021)
<i>Centropomus parallelus</i>	NaA NaC	30	Survival↔, WG↑ Survival↔, WG↔	da Silva et al. (2016a)

(continued)

Table 26.1 (continued)

Species	Short-chain fatty acid	Level, g kg ⁻¹	Affected trait	References
<i>Colossoma macropomum</i>	Citric acid	0→30	Survival↔, WG↔; mineral availability↑	Nascimento et al. (2021)
<i>Ctenopharyngodon idella</i>	NaB	0→2.0	Enteritis resistance↑, intestinal immune function↑ (Fig. 26.7)	Tian et al. (2017)
	NaB, microencapsulated	0→0.2	Protein carbonyl content↓, intestinal physical barrier function↑	Wu et al. (2018)
	NaB→lipid-coated-NaB	0.5	<i>myd88</i> ↑, <i>tlr22</i> ↑, healthy bacteria↑	Zhou et al. (2019b)
<i>Cyprinus carpio</i>	NaB, microencapsulated	0, 15, 30	<i>hsp70</i> ↑, <i>il-1β</i> ↑, <i>tnfa</i> ↑, <i>tgfb</i> ↑	Liu et al. (2014)
	NaP	0, 5, 10, 20	Serum immunity↑, skin mucus immunity↑	Safari et al. (2017)
<i>Danio rerio</i>	NaP	0, 5, 10, 20	<i>Gh</i> ↑, <i>igf1</i> ↑, <i>mystn</i> ↑, <i>ghrl</i> ↑, <i>ig</i> ↑, protease↑	Hoseinifar et al. (2017b)
	Apple cider vinegar	0→45	@45: <i>Lyz</i> ↔, <i>tnfa</i> ↔, <i>il-1β</i> ↑, <i>il-8</i> ↑, <i>igf1</i> ↔, <i>ghrl</i> ↑, <i>gh</i> ↔	Ahmadifar et al. (2019)
	NaA	1.5	FI↑, WG↑, EM↑, GM↑, <i>ghrl</i> ↑, <i>npv</i> ↑, <i>npv7R</i> ↑	Zhang et al. (2020a)
<i>Dicentrarchus labrax</i>	NaB	2	<i>tnfa</i> ↑, <i>il-1β</i> ↔, <i>il-6</i> ↔, <i>il-8</i> ↔, <i>il-10</i> ↔	Rimoldi et al. (2016)
	NadF	0→5	@3→4: Max WG, protein utilization, survival	Wassef et al. (2017)
	NaB, microencapsulated	0→3	Immunity↑, intestinal cell-proliferation↑	Abdel-Mohsen et al. (2018)
<i>Epinephelus lanceolatus</i>	Butyric acid @ 50% FM replacement by soybean meal	0, 1.0	Tendency of inflammation mitigation	Yong et al. (2020)
<i>E. fusoguttatus</i> ♀ × <i>E. lanceolatus</i> ♂	NaB	0, 13		Yin et al. (2021)

(continued)

Table 26.1 (continued)

Species	Short-chain fatty acid	Level, g kg ⁻¹	Affected trait	References
			Serum immunity↑, inflammation by soy glycinin↓	
<i>Lates calcarifer</i>	Butyric acid	0→10	≥5: WG↑, IG↑, hematol. parameters↑, humoral immunity↑	Aalamifar et al. (2020)
	NadF	0→20	@5: WG↑, LYZ↑, FI↑	Reyshari et al. (2019)
	NaF and NaB	10	WG↔	Gao et al. (2011)
	KdF	12	WG↑	Naderi Farsani et al. (2019)
<i>Monopterus albus</i>	NaB	0→1.0	WG↑, LPO↓	Zhang et al. (2020b)
<i>Oncorhynchus mykiss</i>	NaB (Butirex [®] C4)	0→5	Disease resistance↑	Mirghaied et al. (2019)
<i>Oreochromis</i> sp.	NaC	0→4	WG↓, health↓, SCFAs↓, liver damage↑	Romano et al. (2016)
<i>O. mossambicus</i> × <i>O. niloticus</i>	NaB, NaP, NaA, NaF	20	WG↔, intestin. SCFA↓, LPO↓	Ebrahimi et al. (2017)
<i>O. niloticus</i>	NaB	0→30	Liver steatosis↑ @ 20 and 30	El-Sayed et al. (2018)
	KdF	≤3	WG↑, pathogen resistance↑	Abu Elala and Ragaa (2015)
	NaB	0→10	WG↑, SGR↑, pathogen resistance↑ @ 7–10	Abd El-Naby et al. (2019)
	NaA	0.1; 0.2; 0.4	Intestinal inflammation↓ @ high-carbohydrate diet (→Chap. 17)	Li et al. (2020)
<i>O. niloticus</i> ♀ × <i>O. aureus</i> ♂	KdF	≤12	WG↑	Zhou et al. (2009)
<i>Paralichthys olivaceus</i>	NaB	4	WG↔, survival↔, MPO↑, LYZ↔; villi length↑	Bae et al. (2020)
<i>Salmo (trutta) caspius</i>	NadF	0, 0.5, 1.0, 1.5	WG↑, immunity↑	Mohammadian et al. (2020)
<i>S. salar</i>	NaB + NaP + NaA	≤2.0	WG↔	Bjerkeng et al. (1999)

(continued)

Table 26.1 (continued)

Species	Short-chain fatty acid	Level, g kg ⁻¹	Affected trait	References
<i>Salvelinus alpinus</i>	NaL NaP	10	WG↑, diarrhea↓ WG↓	Ringø (1991)
<i>Scophthalmus maximus</i>	Citric acid	15, 30	Phosphorus digestibility↑ @ 30	Dai et al. (2018)
	NaB	2	Intestinal health↑	Liu et al. (2019)
<i>Sparus aurata</i>	NaB	4	Anti-inflammation↑: TNFα↓	Benedito-Palos et al. (2016); Estensoro et al. (2016)
<i>Trachinotus ovatus</i>	NaB	0.25→4	WG↑, glucose↑	Zhou et al. (2019a)

↑ supporting; ↔ indifferent; ↓ adverse/reducing effect
KdF K “di”formiate (NadF and KdF contain free formic acid), *NaA* Na acetate, *NaB* Na butyrate (=SB), *NaC* Na citrate, *NadF* Na “di”formiate, *NaF* Na formiate, *NaL* Na lactate, *NaP* Na propionate
AMPs antimicrobial peptides, *IG* immunoglobulin, *LYZ* lysozyme, *MPO* myeloperoxidase, a peroxidase with antimicrobial activity, *PER* protein efficiency ratio, *TNFα* tumor necrosis factor alpha, *WG* weight gain, *WSSV* white spot syndrome virus, *EM* energy metabolism, *FI* food intake, *GM* gut microbiota, *LPO* lipid peroxidation, *SGR* specific growth rate
gh growth hormone, *ghrl* ghrelin, *igf1* insulin-like growth factor 1, *lyz* lysozyme, *myd88* myeloid differentiation factor 88, *mystn* myostatin, *npv* neuropeptide Y, *npv7R* neuropeptide Y receptor, *tlr22* toll-like receptor 22

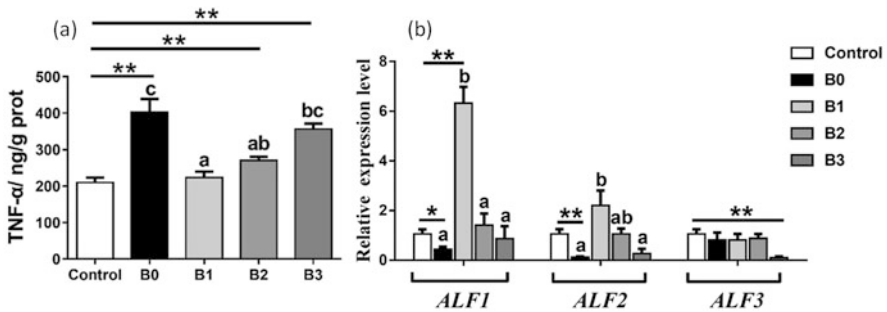


Fig. 26.9 The content of the pro-inflammatory tumor necrosis factor-α (a) and relative mRNA expression levels of three anti-lipopolysaccharide factors (ALF) (b) in the intestinal tract of Chinese mitten crabs exposed to different treatment diets. * indicates significant difference of $P < 0.05$ and ** of $P < 0.01$ between treatment and control. Different letters show significant difference ($P < 0.05$) among the B0 (=0), B1 (=10), B2 (=20), and B3 (=40 g kg⁻¹ NaB) groups (From Han et al. (2020), with permission from Elsevier)

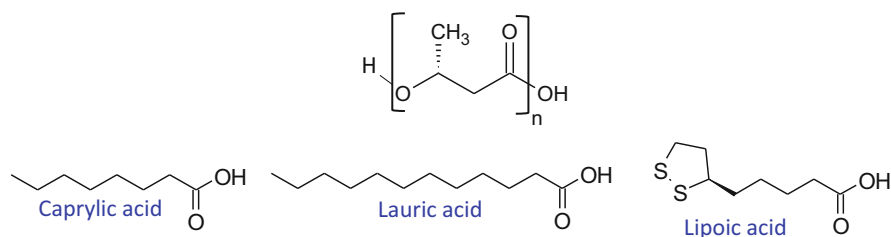


Fig. 26.10 Structures of poly-(R)-3-hydroxybutyrate (P3HB), caprylic acid (CaA), lauric acid (LauA), and (R)-lipoic acid, a caprylic acid derivative

26.5 Medium-Chain Fatty Acids

Medium-chain fatty acids (MCFAs) are SFAs or USFAs. Lauric acid (LauA) (Fig. 26.10) is one of the nutritionally prominent MCFAs, found in high concentrations (~50%) in coconut oil (Brody 1999). Its name is derived from laurel (*Laurus nobilis*). Besides coconut oil and LauA, caprylic acid (CaA) (Fig. 26.10) has often been studied, mainly in terms of antiparasitic actions. CaA is the common name for the eight-carbon SFA known by the systematic name octanoic acid, whereas LauA is the common name for dodecanoic acid. For membrane passage, MCFAs do not require carrier molecules (Denstadli et al. 2011).

In tiger puffer (*Takifugu rubripes*), dietary CaA has an anthelmintic efficacy against monogenean *Heterobothrium okamotoi* infections (Hirazawa et al. 2000). Furthermore, CaA has a parasitocidal activity against ciliate *Cryptocaryon irritans* theronts, monogenean *Benedenia seriola* oncomiracidia, myxosporean *Kudoa shiomitsui* spores, and a contractile effect against *Benedenia seriola* adults but shows no clear result against the copepod *Pseudocaligus fugu* (Hirazawa et al. 2001b). Rigos et al. (2013) enlarged this catalog by showing that dietary CaA alone and in combination with organic iron and mannan oligosaccharide can reduce the infection with *Sparicotyle chrysophrii* in gilthead seabream.

In addition to antiparasitic potentials, Rimoldi et al. (2018) communicated that MCFAs act as prebiotics. They beneficially modulate fish intestinal microbiota by promoting lactic acid bacteria, namely, *Lactobacillus*, and reducing *Gammaproteobacteria*, which include several potential pathogens.

Further effect studies of dietary MCFAs are listed below (Table 26.2) indicating that MCFAs are not an explicit energy source for larvae and juveniles of many fishes—they are not preferentially directed toward β -oxidation with the obvious exception of Atlantic salmon leading to a decreased liver lipid storage (Belghit et al. 2019). Denstadli et al. (2011) showed that this fish uses dietary decanoic acid in heart and skeletal muscles as a source for rapid energy production. However, the studies in Atlantic salmon are not yet consistent, since Røsjø et al. (2000) found hepatic accumulation of 18:1 and reduced β -oxidation of dodecanoic acid (LauA) in hepatic mitochondria.

Prawns do not obviously accumulate MCFAs from triacylglycerols in their lipids (Table 26.2), as far as these two studies allow this conclusion; the reason, however, remains undiscovered.

Table 26.2 Effect of dietary medium-chain fatty acid, including medium-chain triacylglycerols, in selected aquatic species

Species	MCFA source	Level, g kg ⁻¹	Affected trait	References
<i>Invertebrates</i>				
<i>Penaeus monodon</i>	MTCs	40	MCFAs not accumulated	Deering et al. (1997)
<i>Marsupenaeus japonicus</i>		50		Kayama et al. (1980)
<i>Fishes</i>				
<i>Cyprinus carpio</i>	Coconut oil (C ₁₂) Triolein (C ₁₈) Tricaprylin (C ₈)	50, 100	Survival↑, growth↑ Survival↑, growth↑ Survival↓, growth↓	Fontagné et al. (1999)
	MTCs: Tricaproin, tricaprylin, tricaprin, or trilaurin	5	Growth: Tricaproin, tricaprin, trilaurin>tricaprylin	Fontagné et al. (2000b)
	LaAs	1→10	Growth↓ @ < 2	Fontagné et al. (2001)
	MTCs: Tricaprylin Tricaproin	30	Survival↑ @ both sources No MCFA in body MCFA in body	Fontagné et al. (2000a)
<i>C. carpio</i> var. Jian	LaA in black soldier fly larvae oil	0→100% soybean oil substitution	Growth↔, hepatic health↔, serum biochemistry↔	Li et al. (2016)
<i>Lateolabrax japonicus</i>	C ₈ , C ₁₀	100	Feed intake↓	Xu et al. (2016)
<i>Morone chrysops</i> ♀ × <i>M. saxatilis</i> ♂	Maize oil	8.5	WG↔	Trushenski et al. (2011)
<i>Oncorhynchus mykiss</i>	Coconut oil	50, 150	Serum biochemistry↔	Luo et al. (2014a)
<i>Oreochromis niloticus</i>	Virgin coconut oil	0→30	WG↑, resistance against <i>Streptococcus iniae</i> ↑	Apraku et al. (2017)
<i>Pagrus major</i>	Various MCFAs	0, 1.25, 2.5	C8 antiparasitic effect against <i>Cryptocaryon irritans</i>	Hirazawa et al. (2001a)
<i>Rhamdia quelen</i>	Coconut oil	80	<i>Aeromonas hydrophila</i> resistance↑	Vargas et al. (2013)
<i>Salmo salar</i>	LaA in black soldier fly larvae oil	60 (insect meal)	Hepatic lipids↓, no health effects	Belghit et al. (2019)
	MTCs	0, 100	Hepatic 18:1↑, mitochondrial β-oxidation of 16:0↓	Røsjø et al. (2000)
		100		Nordrum et al. (2000)

(continued)

Table 26.2 (continued)

Species	MCFA source	Level, g kg ⁻¹	Affected trait	References
			Protein and starch digestibilities↑, nitrogen retention↑	
	Decanoic acid	¹⁴ C-labeled	Source for rapid energy production in heart and skeletal muscle	Denstadli et al. (2011)
<i>Sciaenops ocellatus</i>	Coconut oil	30→80% of dietary lipids	WG↑, hepatic lipids↑	Craig and Gatlin (1995)
<i>Sparus aurata</i>	Coconut oil	3	FI↑, SGR↑, intestinal surface↑, <i>alpi</i> ↑	Simó-Mirabet et al. (2017)
	CaA	200 mg kg ⁻¹ for 60 d	<i>Sparicotyle chrysophrii</i> infection↓	Rigos et al. (2013)
<i>Takifugu rubripes</i>	CaA	2.5	Antiparasitic effect against <i>Heterobothrium okamotoi</i>	Hirazawa et al. (2000)

MCT medium-chain triacylglycerol, ↑ supporting, ↔ indifferent, ↓ adverse/reducing effect, FI feed intake, SGR specific growth rate, WG weight gain, *alpi* intestinal-type alkaline phosphatase, *LauA* lauric acid, CaA caprylic acid

26.6 Lipoic Acid

Lipoic acid (LiA, Fig. 26.10) is a natural ubiquitous sulfur-containing FA derived from CaA and distributed in every cell of all organisms (Brody 1999). A coenzyme, it is essential for aerobic metabolism (Packer et al. 2001). With metal-chelating and antiglycation capabilities, dietary LiA is often used as powerful antioxidant to mitigate any kind of oxidative stress (Lobato et al. 2018). LiA is present in almost all foods, slightly elevated in spinach, broccoli, yeast, and wheat flour (Sontag and Schwartz 2012).

Feeding trials in farmed invertebrates and fishes generally improve growth performance and, as expected, the antioxidant capacity. This is shown in invertebrates, such as *Haliotis discus hannai* (Huang et al. 2019), *Eriocheir sinensis* (Xu et al. 2018), *Litopenaeus vannamei* (Lobato et al. 2018), and fishes, such as *Corydoras paleatus* (Monserrat et al. 2008), *Cyprinus carpio* (Amado et al. 2011), *Ctenopharyngodon idella* (Shi et al. 2018), *Jenynsia multidentata* (Montserrat et al. 2014), *Oreochromis niloticus* (Lu et al. 2019), *Piaractus mesopotamicus* (Park et al. 2006), *Trachinotus marginatus* (Kütter et al. 2012), and *Osphronemus goramy* (Samuki et al. 2020). Also oxidative stress by consumption of excess lipids can be ameliorated by LiA as shown in mitten crab (Xu et al. 2018) or GIFT tilapia (Xu et al. 2019).

As a specific antioxidant effect of LiA, Park et al. (2006) reported that its enrichment partially reverses tissue vitamin C depletion in pacu (*Piaractus mesopotamicus*) fed vitamin C-devoid diets. In the same species, dietary LiA

reduces plasma free essential AAs but elevates cystine content (Terjesen et al. 2004). Similarly, also FA profiles are changed by dietary LiA supplementation: muscle and brain EPA contents increase (Trattner et al. 2007). The picture becomes even more complex, since Bou et al. (2017) discovered in Atlantic salmon hepatocytes that LiA affects the ω 3 PUFA metabolism by enhancing the production of DHA but not of EPA. The molecular mechanisms for these findings remain to be studied in detail.

Noteworthy, Shi et al. (2018) identified a protein-sparing effect of LiA in juvenile grass carps. LiA promotes lipolysis without loss of body weight, promotes FA β -oxidation to increase energy supply from lipid catabolism, and spares the protein from energy production to increase protein deposition.

Liu et al. (2018) reported that optimal LiA supplementation (203 mg kg⁻¹) promotes growth and strengthens immunity by enhancing the immune function of head kidney and spleen:

1. Optimal LiA improves the innate and adaptive immune responses related to increase the immune components including lysozyme, acid phosphatase, C3, C4, antimicrobial peptides, and IgM.
2. Optimal LiA supplementation alleviates inflammatory responses via upregulating anti-inflammatory cytokines and downregulating pro-inflammatory cytokines.
3. Most interestingly, Liu et al. (2018) identified that excessive LiA inhibits growth and impairs immune function of head kidney, spleen, and skin.

26.7 Saturated Fatty Acids

By applying functional metabolomics, several metabolites are identified that promote fish survival after bacterial challenges. Interestingly, several SFAs are among these metabolites. Stearate and palmitate exert a protective activity against bacterial challenge in zebrafish (Zhao et al. 2014). Later, the same laboratory applied reprogramming of the metabolome to understand the mechanisms by which exogenous glucose elevates Nile tilapia anti-infectious ability to cope with invaded *Edwardsiella tarda* (Zeng et al. 2017). Exogenous glucose does not flux to the TCA cycle. Instead, it promotes stearic acid and palmitic acid biosynthesis to potentiate the host against bacterial infection. This hypothesis is validated by the finding that exogenous stearic acid increases immune protection in Nile tilapia against *Edw. tarda* infection (Fig. 26.11) via the Mx protein. The Mx protein belongs to the small GTPase family, which are implicated in antiviral activity (Haller et al. 2007). Mx proteins are antiviral “gatekeepers that restrain the uninvited” (Verhelst et al. 2013). Exogenous glucose reprograms tilapia’s anti-infective metabolome characterized by elevation of stearic acid and palmitic acid and attenuation of the TCA cycle. This is obviously the first report that exogenous glucose takes anti-infective action through promotion of SFA biosynthesis (→Chap. 13).

In crucian carp, Jiang et al. (2019) identified important metabolites that promote the survival upon bacterial infection at 30 °C, a water temperature above the thermal

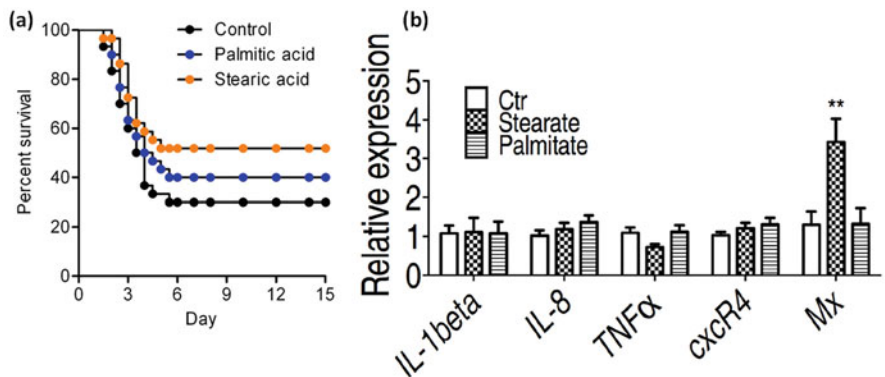


Fig. 26.11 Exogenous fatty acids enhance *Oreochromis niloticus* immune response and protect from infections by *Edwardsiella tarda*. *O. niloticus* in tested groups were intraperitoneally injected with 30 mg palmitic acid or stearic acid for 3 days and in control group were used with saline. (a) Fishes were intraperitoneally challenged by 2.0×10^5 CFU fish⁻¹. Significance was obtained by chi-square test ($n = 60$, $df = 1$): P -value (palmitic acid) = 0.15, P -value (stearic acid) = 0.04. (b) mRNA levels of *il-1 β* , *il-8*, *tnf- α* , *cxcR4*, and *mx* were quantified with qPCR. Results were represented by three independent replicates (From Zeng et al. (2017), with permission from Elsevier)

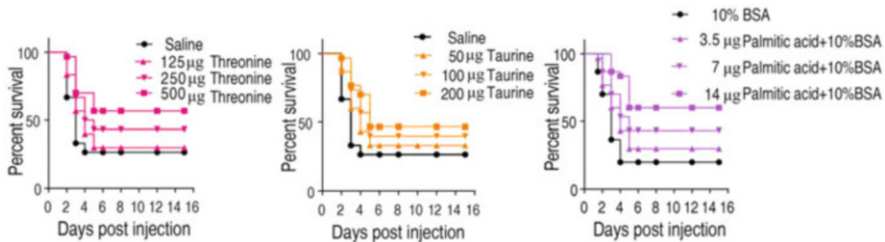


Fig. 26.12 Crucial biomarkers modulate the metabolism of crucian carps and promote their survival against bacterial infections: The survival of crucian carps in the presence of crucial biomarkers upon *Edwardsiella tarda* infection. *Carassius carassius* was treated with saline control or different dose crucial biomarkers at 30 °C for 3 days, followed by bacterial challenge through intraperitoneal injection (1×10^5 CFU). The accumulative mortality was monitored for 15 days post-infection ($n = 30$ per group) (From Jiang et al. (2019), credit Frontiers Media)

optimum of this species. Individuals grown at 30 °C demonstrate a metabolome that is characterized by enhanced TCA cycle but reduced abundance of palmitate, threonine, and taurine. Importantly, exogenous palmitate, threonine, and taurine slightly inhibit the TCA cycle and restore the altered innate immune responses repressed at the elevated temperature. Consequently, challenged fishes supplied with these three acids survive better (Fig. 26.12) than those without supplementation.

26.8 Concluding Remarks

Innovative “omics” and bioinformatic techniques enable the identification of FAs not only as substrate of energy and lipid metabolism but also as key molecules of central pathways of metabolism and host’s immunity—including first information about the involvement of epigenetics. Based on functional metabolomics, even several SFAs, considered as pure energy substrates since long, turn out to be direct and indirect key metabolites in pathogen defense. It can be expected that the number of such key metabolites will increase by corresponding studies to come.

Although already rather complex, so far, only individual pathways have been identified, the mutual interrelationships between these pathways and traits have not been considered—simply because of the sake of feasibility. Therefore, questions remain to be answered: Can boosted immunity be translated to improved growth performance or increased reproductivity? Can strengthened immunity be transmitted to succeeding generations? Which role does epigenetics play in the action of FAs, other than SCFA, and can it be modulated?

Due to the fast technological progress in molecular biology, biochemistry, and bioinformatics, the combined elucidation of different pathways and traits becomes feasible. Even the ultimate goal of considering microbiota and host as a superorganism in the sense of Wilson and Sober (1989) and Limborg et al. (2018) is moving closer to its solution (→Chap. 25).

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Chapter 27

Essential Fatty Acids—‘Fueling Versus Controlling’



Abstract This chapter focuses on mono- and polyunsaturated fatty acids as essential nutrients with emphasis on linoleic (LIN), α -linolenic (ALA), arachidonic (ARA), eicosapentaenoic (EPA), and docosahexaenoic acids (DHA). The common classification of LC-PUFA requirements of fishes, based on dichotomies of warmwater vs. coldwater and freshwater vs. marine, is challenged and reconfirmed—at least in parts. The Janus-faced character of ARA is revisited, and the essentiality of this PUFA becomes increasingly obvious, almost comparable to EPA or DHA. Studies are beginning to show that epigenetics is essential in controlling action of EFAs and in inheriting of metabolic features to offspring. Less well studied is the role of intestinal microorganisms in host’s growth, health, immunity, and further traits.

Fatty acids (FAs) that are required, but cannot be synthesized in sufficient quantity from other substrates, and therefore must be obtained from food, are called essential fatty acids¹ (EFAs). There are two series of EFAs: one has a double bond three carbon atoms away from the methyl end ($\omega 3$); the other has a double bond six carbon atoms away from the methyl end ($\omega 6$). The requirement of these two EFA types and the effects of the corresponding metabolites differ considerably between species and EFAs and deserve detailed consideration.

Docosahexaenoic acid (DHA, 22:6 $\omega 3$) provides the greatest EFA value to most species. However, the nutritional value of eicosapentaenoic (EPA, 20:5 $\omega 3$) and arachidonic (ARA, 20:4 $\omega 6$) acids is also significantly greater than that exhibited by α -linolenic (ALA, 18:3 $\omega 3$) and linoleic (LIN, 18:2 $\omega 6$) acids (Glencross 2009) (\rightarrow Chap. 26). All five FAs have been shown to provide EFA value to most aquaculture species, although the optimal dietary inclusion levels and balance among the FA classes ($\omega 3$ and $\omega 6$) and FA chain lengths (C_{18} , C_{20} , or C_{22}) vary among species. Environmental origin (freshwater, estuarine (brackish), or marine) has been considered the primary factor influencing the difference in EFA requirements (however, refer to the concern by Trushenski and Rombenso (2020) below).

¹Erucic acid, a monounsaturated $\omega 9$ C_{22} FA, is considered an anti-nutritional factor in seeds of *Brassica* sp., and corresponding papers will be reviewed in AAN III “Fish Oil Replacement.”

The influence of EFAs on growth appears to be greatest in larval fishes and crustaceans, possibly because of their reduced ability to digest and absorb lipids but also because of a proportionally higher demand for EFA in the development of, in particular, neural tissues (Glencross 2009).

The paramount importance of EFAs stems not only from their effect on growth but also on other traits, such as reproduction, immunity, and health. Some examples are as follows: Zeng et al. (2016a, b) documented that an optimal dietary ALA/LIN ratio regulates mRNA levels of tight junction proteins, improves mucosal immunity, ameliorates intestinal antioxidant capacities, and improves digestive and absorptive capacities and target of rapamycin gene (*tor*) expression as shown in juvenile grass carps. Consistently, juvenile Wuchang breams (*Megalobrama amblycephala*) fed an ALA-deficient diet show poor growth and feed utilization as well as impaired immunity (Zhang et al. 2017). Furthermore, the expression of genes regulating homocysteine synthesis from methionine is sensitive to either high dietary $\omega 3$ or $\omega 6$ polyunsaturated fatty acids (PUFAs) indicating that dietary PUFAs affect the one-carbon metabolism with epigenetic inheritance to succeeding generations (Adam et al. 2019) (Table 27.3 and Fig. 27.14). Further evidence indicates also a role of PUFAs in nutritional programming of offspring (Perera et al. 2020) (→ Sect. 27.1).

The high requirements of $\omega 3$ FAs in fishes are necessary for the functioning of membranes; they must maintain proper fluidity, particularly at changed salinity and lower environmental temperatures than many terrestrial species experience (Farkas et al. 1984) (homeoviscous adaptation). For instance, zooplankton exposed to cold stress increases the accumulation of $\omega 3$ PUFAs (Brett et al. 2009). Vice versa, two PUFAs, LIN and ALA, decrease in the small-bodied cladoceran, *Moina macrocopa*, with increasing temperature from 20 °C to 30 °C (Gama-Flores et al. 2015). In addition to maintaining membrane fluidity, ALA affects life history traits. In the same cladoceran species, dietary ALA triggers longevity (Bouchnak and Steinberg 2014). This effect of ALA does not occur in *Daphnia magna* (Bouchnak and Steinberg 2013). Thus, ALA-mediated longevity appears to be species-specific, so that the functioning of dietary ALA deserves detailed studies, not only in cladocerans.

27.1 Molecular Interplay

In their pioneering goldfish in vitro hepatopancreas study, Bertucci et al. (2017a, b) demonstrated PUFA-mediated dynamic modulations of genes. This modulation comprises the transcription of *ghr-I*, *ghr-II*, *igf-I*, *igf-II*, and the appetite-regulating peptide hormones *preproghrelin* (precursor of *ghrelin*) and *nucb2/nesfatin-1*.² Hepatopancreas sections are incubated with different doses of oleic acid (OA, 18:1 ω 9),

²*ghr-I*, *ghr-II* growth hormone receptors I and II; *igf-I*, *igf-II* insulin-like growth factors I and II. Nesfatin-1-like peptide is a novel anorexigen modulated by sex steroids, macronutrients, and daily rhythm. Increased nesfatin-1 in the hypothalamus contributes to diminished hunger and provides a “sense of fullness.” Furthermore, nesfatin-1 suppresses fish reproductive axis and gonadal steroidogenesis (Rajeswari and Unniappan 2020).

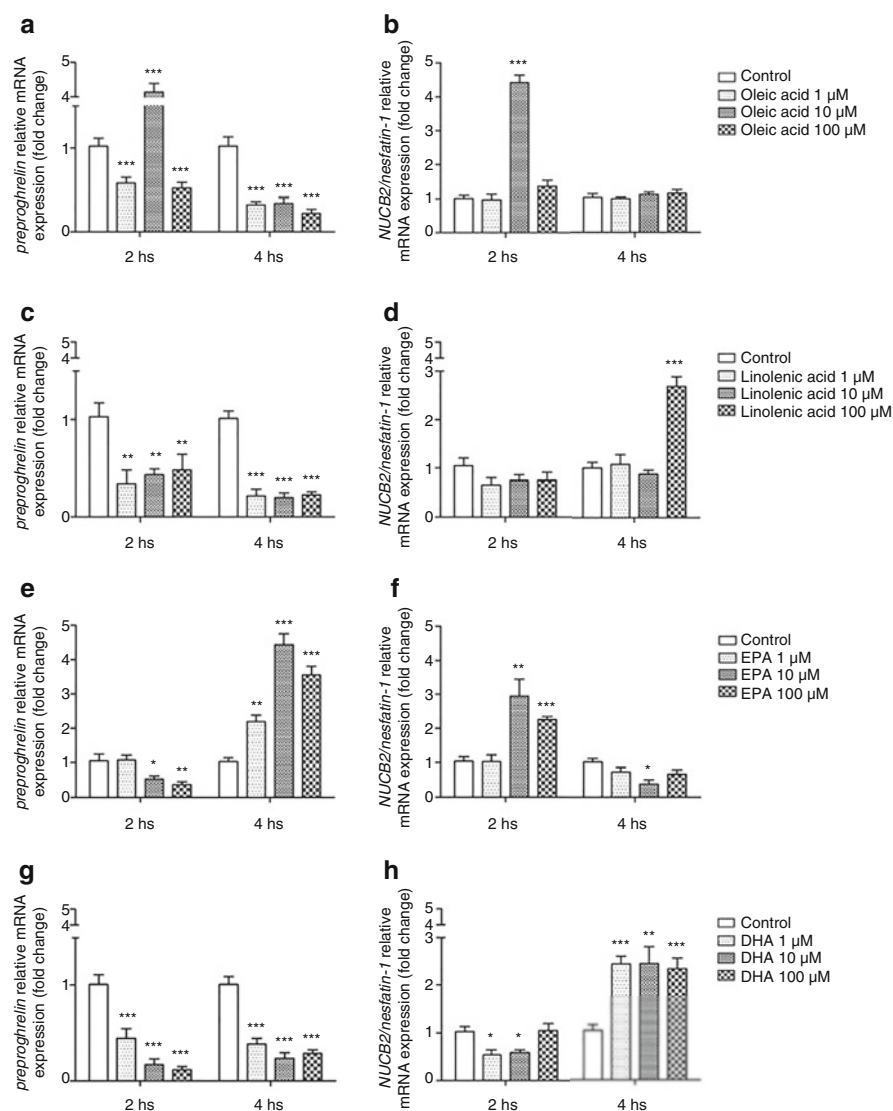


Fig. 27.1 *Preproghrelin* and *nucb2/nesfatin-1* transcription in hepatopancreas of goldfish after 2 h and 4 h of treatment with 1, 10, and 100 mM oleic acid (a, b), linolenic acid (ALA; c, d), eicosapentaenoic acid (EPA; e, f), and docosahexaenoic acid (DHA; g, h). Data are mean + SEM. Asterisks denote statistical differences between control and treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (From Bertucci et al. (2017b), with permission from Elsevier)

ALA, EPA, and DHA. The FAs modulate *preproghrelin* and *nucb2/nesfatin-1* transcription differently in a time- and concentration-dependent manner (Fig. 27.1). This indicates that both appetite-regulating peptides act together in controlling metabolic processes. Moreover, the transcription assay indicates a global

decrease in the expression of *preproghrelin* and an increase in the expression of *nucb2/nesfatin-1* in response to the FAs, indicating reduced appetite.

An intriguing transgenerational effect of dietary FAs has been discovered by Perera et al. (2020). The authors show that *stearoyl-CoA desaturase 1a* (*scd1a*) can be programmed through broodstock nutrition by increasing the contents of OA, LIN, and ALA. Stearoyl-CoA desaturases (Δ^9 desaturases, *scd*) are endoplasmic reticulum enzymes that catalyze the rate-limiting step in the formation of monounsaturated fatty acids (MUFAs) (\rightarrow Chap. 28).

The results of DNA methylation and gene expression profiles of *scd1a* (Fig. 27.2) support, for the first time in fishes, a reliable epigenetic mechanism by which parental nutrition shapes the *scd1a* gene expression in offspring. ALA enrichment in parental diet leads to offspring with increased methylation at a regulatory region in

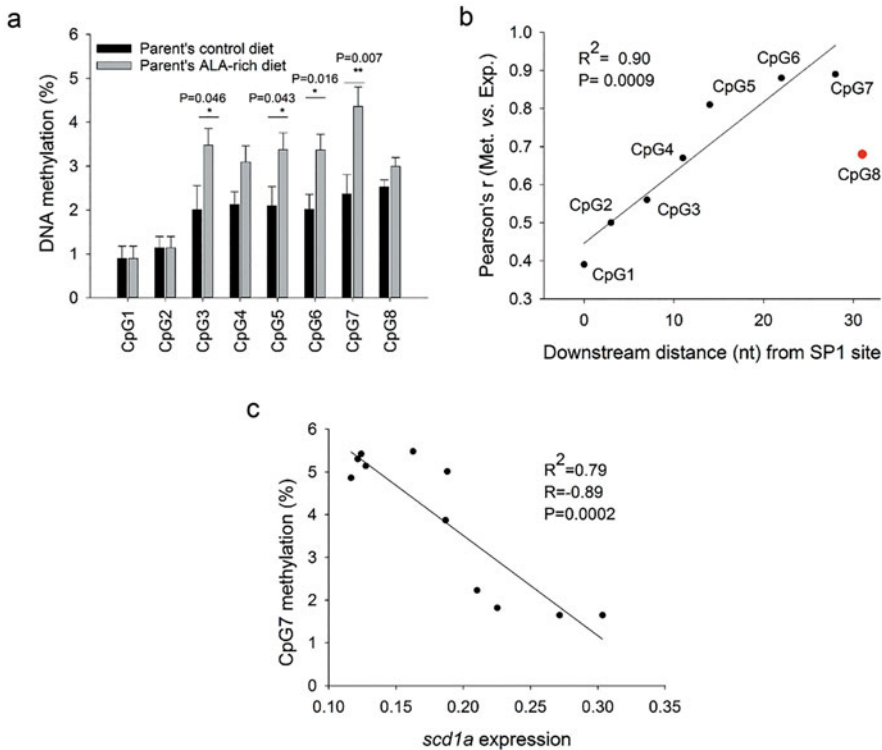


Fig. 27.2 Cytosine methylation in examined promoter regions of *scd1a* in juvenile gilthead seabreams fed a low FM/FO diet, whose parents were fed with a control or an ALA-rich diet. Data represent the mean \pm SEM of nine individuals per condition. Differences found between groups by Student t-test ($P < 0.05$) are indicated (a). Correlation between gene expression and the methylation level of CpGs at different positions relative to an SP1 site within the *scd1a* promoter; correlation is maximal for CpG6 and 7 (b). Correlation between methylation at CpG7 in the *scd1a* gene promoter and hepatic *scd1a* gene expression. Significance for correlation is shown (c) (From Perera et al. (2020), with permission from Taylor & Francis)

the *scd1a* promoter, leading to reduced gene expression and likely to reduced fat accumulation under highly dietary lipogenic conditions, often associated with reduced supply of $\omega 3$ LC-PUFA (Perera et al. 2020). It remains to be explored how the response of offspring is further modulated by a combined effect of changes in dietary ALA and EPA/DHA levels, targeting liver and other tissues. From mammals, several interesting hints are available: In rats, maternal high-fat feeding during suckling alters the DNA methylation of *scd1* promoter in adipose tissue and programs visceral adiposity. Additionally, in pigs, *scd1* expression is regulated by miRNAs, and this opens new alternative epigenetic regulatory mechanisms of lipid metabolism and offspring management approaches in fishes (Perera et al. (2020) and reference therein).

Box: Eicosanoids

Eicosanoids (*eicosi* = twenty) are the collective term for derivatives of C_{20} precursor FAs and include prostaglandins and others. They are local hormones, because they are short-lived and cannot be transported over long distances. Prostaglandins regulate blood flow to particular organs, control ion transport across membranes, modulate synaptic transmission, and induce sleep (Berg et al. 2015).

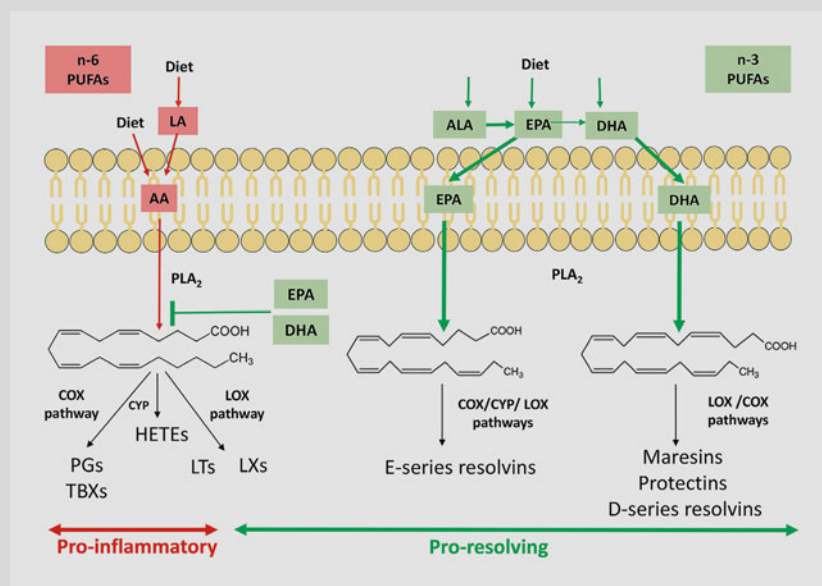
There is ample evidence for an anti-inflammatory and pro-resolution effect of long-chain $\omega 3$ PUFAs (LC-PUFAs) after they incorporate into cell membrane phospholipids. They disrupt membrane rafts and, when released from the membrane, suppress inflammatory signaling by activating PPAR- γ and free fatty acid receptor 4; furthermore, they shift the lipid mediator profile from pro-inflammatory eicosanoids to specialized pro-resolving mediators. The allocation of LC-PUFAs also leads to a higher microbiome diversity in the gut, increases short-chain fatty acid (SCFA)-producing bacteria, and improves intestinal barrier function by sealing epithelial tight junctions (Wawrzyniak et al. 2020).

LC-PUFAs form a pool of precursors that are metabolized to distinct bioactive lipid mediators by three major pathways: the cyclooxygenase (COX-1, a constitutive enzyme, or COX-2, an inducible enzyme) pathway, the lipoxygenase pathways (ALOX-5, ALOX-12, or ALOX-15), and the cytochrome P-450 monooxygenase pathway (Box Fig. 1). The exact profile and balance of bioactive products derived from the $\omega 3$ and $\omega 6$ LC-PUFAs pool depends on the cell and tissue type and is determined by environmental and physiological contexts (Wawrzyniak et al. 2020).

Many eicosanoids are ARA-derived and produced by the COX and ALOX pathways from membrane-incorporated phospholipids. Dietary EPA and DHA lead to their incorporation into phospholipids of the cellular plasma membrane. This takes place largely at the expense of the $\omega 6$ ARA. EPA, and to a lesser extent DHA, can be metabolized by the same COX and ALOX enzymes

(continued)

as ARA to give rise to a different series of lipid mediators (Box Fig. 1). The EPA-derived eicosanoids are less potent pro-inflammatory than those from ARA. Besides these general anti-inflammatory and pro-resolving effects, the allocation of $\omega 3$ LC-PUFAs also leads to a higher microbiome diversity in the gut, increases SCFA-producing bacteria, and improves intestinal barrier function by sealing epithelial tight junctions (Wawrzyniak et al. 2020).



Box Fig. 1 The metabolism of $\omega 6$ and $\omega 3$ PUFAs into either pro-inflammatory or pro-resolving lipid mediators. Long-chain PUFAs from diet become incorporated in the plasma membrane and are liberated by phospholipase A2 (PLA2). Free $\omega 3$ PUFAs such as EPA and DHA compete with $\omega 6$ ARA (here AA) for metabolism by the same classes of enzymes (COX, LOX, CYP) to produce lipid mediators with pro-resolving powers like the E-series resolvins, maresins, protectins, and the D-series resolvins, rather than the pro-inflammatory lipid mediators derived from ARA like the prostaglandins (PGs), leukotrienes (LTs), and thromboxanes (TBXs). Supplementation with $\omega 3$ PUFAs lowers the ratio of $\omega 6/\omega 3$ PUFAs in the membrane and thus produces more of the lipid mediators that favor resolution of inflammation. **LXs** lipoxins, **AA** arachidonic acid (used abbreviation in this book is ARA); **ALA** α -linolenic acid; **COX** cyclooxygenase; **DHA** docosahexaenoic acid; **EPA** eicosapentaenoic acid; **HETEs** hydroxyeicosatetraenoic acids; **LA** linoleic acid (=LIN); **LOX** lipoxygenase; **CYP** cytochrome P450 (From Wawrzyniak et al. (2020), with permission from Wiley)

27.2 ω 3 or ω 6 PUFAs

27.2.1 *Invertebrates*

Information on FA nutrition of invertebrates is available particularly for crustaceans (Table 27.1). Much of the pioneering work focused on requirements and metabolism of EFAs in marine, cultivable shrimps, most notably the kuruma shrimp (*Marsupenaeus japonicus*). It is noteworthy that individual life history traits may be differently affected by dietary PUFAs, even at optimal PUFA compositions. For instance, in red king crab (*Paralithodes camtschaticus*), Beder et al. (2018) found that elevated dietary EFAs (ARA, EPA, DHA) result in increased growth, energy storage, and stress resistance but not improved survival during early ontogenesis.

An interesting paper reported that dietary ARA promotes growth, improves immunity, and regulates the expression of immune-related signal molecules in freshwater *Macrobrachium nipponense* (Ding et al. 2018). Different results are obtained in the marine *Litopenaeus vannamei*. ARA inclusion in fish oil (FO)-free diets adversely affects growth of juveniles and influences transcription of lipid-relevant genes, mainly those related to eicosanoid synthesis (\rightarrow Box Eicosanoids) (Araújo et al. 2020). Additionally, a reduced hepatopancreas lipid deposition occurs in ARA-fed animals.

27.2.1.1 Mollusks

Parental size and nutritional state are interrelated parameters determining reproductive investment in marine gastropods. In the sacoglossan sea slug *Elysia viridis*, Cartaxana et al. (2019) investigated the availability of the macroalga *Codium tomentosum* and its FAs on the reproductive investment of the slug. Starved sea slugs produce eggs enriched in saturated FAs, namely, stearic acid (18:0), whereas sea slugs with unlimited access to food spawn produce eggs rich in PUFAs, such as LIN, eicosatrienoic acid (20:3 ω 3), and EPA. This indicates that the nutritional state affects resource allocation to reproductive traits in *E. viridis* and its offspring: Food limitation reduces fecundity and affects FA composition of individual eggs with clear reduction of EPA, central in embryonic development and early larval fitness. How this PUFA deficit translates into offspring life history traits deserves future attention.

27.2.1.2 *Daphnia* and Farmed Shrimps

Undeniably, the strongest underlying motivation of the function of PUFAs discussed above is protein production and its optimization in aquaculture industries. However, the question of how different PUFA compositions translate into ecological terms,

Table 27.1 Quantitative essential fatty acid (EFA) requirements of selected crustacean species

Species	Essential fatty acid	Optimum g kg ⁻¹ diet	References
ω3/ω6 PUFA requirement			
<i>Astacus leptodactylus</i>	18:1ω9	24.73	Harlioğlu et al. (2012)
	18:2ω6 (LIN)	45.39	
	20:4ω3 (ETA) (Eicosatetraenoic acid)	1.44	
	20:5ω3 (EPA)	8.07	
	22:5ω3 (DPA) (Docosapentaenoic acid)	2.03	
	22:6ω3 (DHA)	5.21	
<i>Penaeus monodon</i>	LIN	11–16	In Glencross (2009)
	ALA	16–28	
	EPA	2–9	
	DHA	2–15	
<i>Macrobrachium rosenbergii</i>	ARA	10	In Glencross (2009)
	DHA	10	
<i>Scylla</i> sp.	ω6/ω3	3:1	
<i>Scylla paramamosain</i>	PUFA + EPA:DHA	30% + 0.25	Nghia et al. (2007) Zhao et al. (2016)
	ω6/ω3	<1.0	
<i>S. serrata</i>	ω6/ω3	4.93–5.35	Unnikrishnan et al. (2010)
ω3 PUFA preference			
<i>Fenneropenaeus chinensis</i>	ALA	10	In Glencross (2009)
	DHA	10	
<i>Litopenaeus vannamei</i>	EPA	2	In Glencross (2009)
	DHA	1	
	ALA	>1.0 after microsporidian infection	
<i>Marsupenaeus japonicus</i>	ALA	10	In Glencross (2009)
	EPA	10	
	DHA	20	
ω3 PUFA preference	Composition of storage lipids (major FAs, % of total FAs) (× indicates significance, ×× high significance)		
<i>Calanus finmarchicus</i>	EPA	××	In Jónasdóttir et al. (2005)
<i>C. glacialis</i>	EPA	6.5–17.5	Søreide et al. (2010)
	DHA	2.2–19.7	
<i>C. helgolandicus</i>	EPA	×	In Jónasdóttir et al. (2005)
	DHA	×	

(continued)

Table 27.1 (continued)

Species	Essential fatty acid	Optimum g kg ^{−1} diet	References
<i>Daphnia magna</i>	ALA	× ×	Sperfeld and Wacker (2015)
	EPA	×	
Preference of rare PUFAs	Composition of storage lipids (major FAs, % of total FAs)		
<i>Temora longicornis</i> (copepod)	16:1ω7	10.1–25.0	Peters et al. (2007)
	EPA	16.4–31.3	
	DHA	3.4–8.7	

such as Darwinian fitness, population dynamics, population maintenance, or fuel for higher trophic levels, often remains unanswered.

This question is going to be partly answered by zooplankton studies. Pioneering papers by Lovern (1935, 1936) demonstrated a biochemical bottom-up effect from phytoplankton to planktonic crustaceans. In their review, Brett et al. (2009) confirmed that studies indicate that *Daphnia* spp. have quite plastic FA profiles strongly influenced by their diets. This plasticity is based on plastic transcriptional responses to different food types (Windisch and Fink 2019). Other zooplankters show less plasticity in response to FA compositions of their diets.

Lovern’s finding has been re-“invented” several times. D’Abramo (1979) proposed that the FA content of phytoplankton determines its quality as food for *M. macrocopa*. Later, Goulden and Place (1990) confirmed that at least 98% of the accumulated FAs in *D. magna* and *D. pulex* are dietary in origin. This dependence on dietary lipids would have little ecological significance, if dependable sources of FAs were available. However, this is seldom the case in nature; rather, during summer periods, the growth of *D. galeata* is limited by dietary EPA (Müller-Navarra 1995). Furthermore, the dietary EPA content controls not only growth but also the fertility of *D. magna* (Fig. 27.3) (Müller-Navarra et al. 2000).

Comparing the FA composition of sexual and asexual eggs of *D. magna* as a function of food quality, Putman et al. (2014) found that food quality and distinct allocation strategies influence the FA composition of asexual and sexual eggs, with asexual eggs having higher concentrations of FAs than sexual eggs with an enrichment of ARA and EPA. This enrichment takes place, even when the mothers are fed a diet lacking these LC-PUFAs. The authors assume that these PUFAs, presumably together with heat-shock proteins and glycerol (Pauwels et al. 2007), are involved in mediating the striking resistance of *Daphnia* dormant eggs to harsh environmental conditions.

Free LC-PUFAs are hardly available in natural diets. PUFAs are bound to phospholipids and triglycerides. Therefore, Denoux et al. (2017) evaluated the potential of free and phospholipid-bound dietary EPA to support somatic growth and fecundity of *D. magna*. Supplementing C₂₀ PUFA-deficient diet with free or phospholipid-bound EPA improves somatic growth of *D. magna* equally. However, the increase in fecundity is more pronounced when phospholipid-bound EPA is

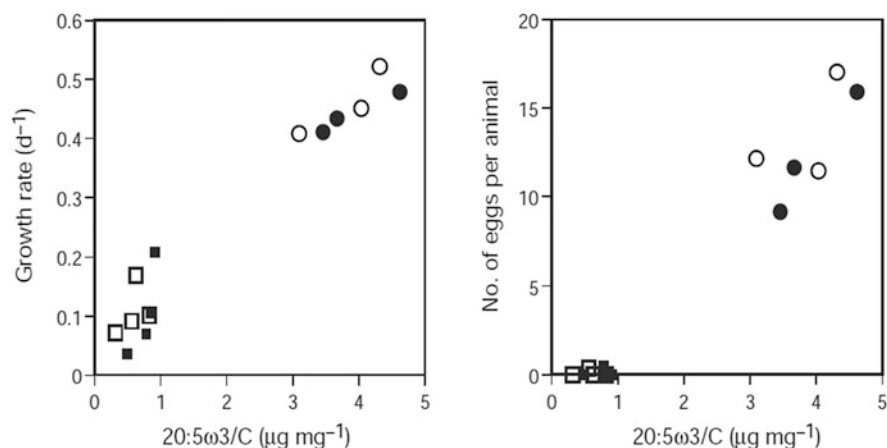


Fig. 27.3 The relationship between the seston EPA (20:5ω3)/carbon ratio, growth rate, and mean number of eggs produced by *Daphnia magna* (mean clutch size). Squares denote the cyanobacteria-dominated summer assemblages, and circles denote the diatom- and cryptophyte-dominated winter/spring assemblages. Open symbols represent the seston fraction less than 30 μm, and closed symbols the total seston (From Müller-Navarra et al. (2000), with permission from Springer Nature)

provided. This finding stresses the need to consider the distribution of dietary PUFAs in different lipid classes to gain a better understanding of how PUFAs influence life history traits (Denoux et al. 2017).

To elucidate the genetic network behind the positive relationship between phytoplankton EPA content and zooplankton life history traits, Windisch and Fink (2018) analyzed gene expression changes of *D. magna* under various dietary EPA regimes. The authors identified specific candidate genes of yet unknown function involved in EPA metabolism and potential immune functions (Windisch and Fink 2019). In a companion paper, Ilić et al. (2019) found that even ARA limits the fitness of *Daphnia* species to an equal extent as EPA does.

The ARA-derived eicosanoid issue³ is further emphasized by a *Daphnia magna* study. ARA appears to be less important for *Daphnia* nutrition than EPA or DHA, since it does not improve somatic growth (von Elert 2002); however, it affects reproductive traits (Martin-Creuzburg et al. 2010) and host–pathogen interactions (Schlotz et al. 2013) and interacts within the EPA metabolism. To check the biomolecular relevance of EPA in the eicosanoid pathway, Fink and Windisch (2019) conducted feeding experiments with *D. magna* using diets differing only in their EPA contents. Interestingly, some genes related to the ARA-eicosanoid metabolism of *D. magna* are significantly upregulated when EPA is available. This study demonstrates that dietary ω3 PUFAs affects the expression of genes typically considered part of the ω6 PUFA-dependent eicosanoid metabolism. This finding in

³Eicosanoids in reproduction and behavior →Chap. 29.

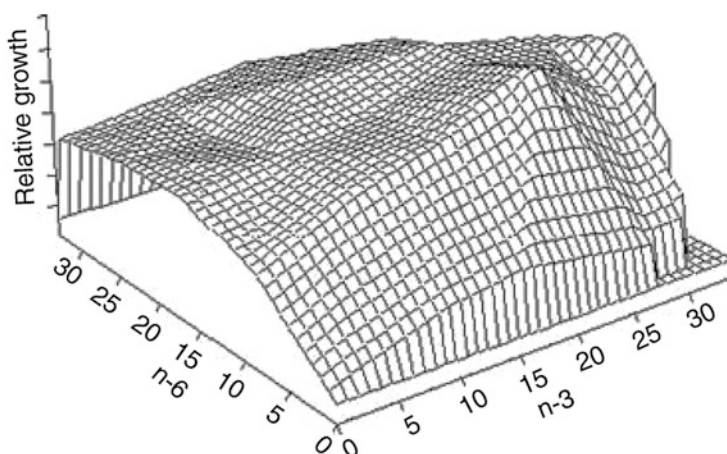


Fig. 27.4 Graphical representation of the interactive effects between dietary ω 3 and ω 6 fatty acids on growth of *Penaeus monodon* (From Glencross (2009), with permission from Wiley). Notably, the multiple peaks possibly are indicative of further interactive effects, such as ARA:EPA and EPA:DHA ratios

Daphnia contrasts that in humans (Obata et al. 1999) and points out the urgent need for detailed and comprehensive studies in other aquatic animals.

Glencross and Smith (2001) examined the relationship between growth and ω 6/ ω 3 ratio in *Penaeus monodon*, where several FAs have been modified to change the FA ratio. By modeling, Glencross (2009) identified several interactions (Fig. 27.4).

It is hypothesized that one mechanism for the interactions is due to the Δ^6 enzyme system, which has an affinity hierarchy to the FAs. The proposed affinity hierarchy is $18:1\omega 9 < 18:2\omega 6 < 18:3\omega 3 < 20:4\omega 6 < 20:5\omega 3 < 22:6\omega 3$. Such a hierarchy provides a possible mechanism for the observed interactive effects between the EFAs. Studies in mammals show that interactions of ω 3, ω 6, and saturated FAs can influence the ARA metabolism (Glencross (2009) and references therein); most likely this interaction applies also to aquatic animals.

As shown already several times for various organic nutrients, also dietary ARA follows an optimum curve as documented in *M. nipponense*. This applies to gene transcription (Fig. 27.5a–f) as well as survival after *Aer. hydrophila* infection (Fig. 27.5g). *tlr* and *myd88* play important roles in disease recognition and defense. The Toll-like receptor (TLR) family of pattern recognition receptors is central in the innate immune system and the myeloid differentiation factor 88 a pivotal signaling component of the innate immune response, serving as an adaptor for the interleukin 1 receptor and the majority of TLRs.

The nonspecific immunity of this shrimp improves on a proper (~1%) dietary ARA content (Fig. 27.5g). A high level of ARA gives rise to an immune inhibitory effect (Ding et al. 2018). This phenomenon is also reported in the Mexican oyster (*Crassostrea corteziensis*): feeding moderate levels of ARA boosts the immune



Fig. 27.5 Expression profiles of *tlr3* Toll-like receptor 3 (a), *myd88* myeloid differentiation primary response protein D88 (b), *irak4* interleukin 1 receptor-associated kinase 4 (c), *traf6* *tnf* receptor-associated factor 6 (d), *map3k7* mitogen-activated protein kinase kinase 7 (e), and *mapk14* mitogen-activated

↓ **Fig. 27.5** (continued) protein kinase 14 (f) in the hepatopancreas of juvenile *Macrobrachium nipponense* with graded ARA levels measured by real-time quantitative PCR. (g) Effects of graded dietary ARA levels on post-challenge mortality of juvenile shrimps after infection with *Aer. hydrophila* for 48 h. Values are mean \pm SD. Different letters indicate significant differences ($P < 0.05$) (From Ding et al. (2018), with permission from Elsevier; image credit Regional Euro-Asian Biological Invasions Centre)

response as well as the oocyte production. High levels of ARA favor final maturation and advanced stages of vitellogenesis but at the expense of immunity (Hurtado et al. 2009).

With a series of highly bioactive eicosanoid metabolites, ARA regulates the expression of signaling molecules in the Toll pathway, thus modulating the immune function of *M. nipponense* by upregulating *irak4*, *traf6*, *map 3k7*, and *mapk14*⁴ (Fig. 27.5c–f) (Ding et al. 2018). The same laboratory replaced dietary DHA with ALA and found that this is unlikely to be an effective strategy, because increasing dietary ALA levels alters the transcription of lipid metabolism-related genes involved in β -oxidation and FA synthesis, resulting in decreased liver lipid content (Luo et al. 2018).

27.2.2 Fishes

A pioneer in lipid and FA nutrition of fishes, Takeuchi (1996) summarized the FA demand and established three groups of FA requirement:

- Species with a high demand for ω 6 PUFAs (e.g., *Coptodon (Tilapia) zillii* (Kanazawa et al. 1980))
- Species with equal demand for both ω 3 and ω 6 PUFAs (e.g., *Anguilla japonica*)
- Species with a high demand for ω 3 PUFAs (e.g., *Oncorhynchus mykiss* (Watanabe 1982))

A more recent, updated survey of the PUFA requirements is presented in Table 27.2. Most fish species depend on dietary ω 3 LC-PUFAs. Noteworthy, freshwater largemouth bass (*Micropterus salmoides*) is able to synthesize EPA and DHA from C₁₈ precursors to meet physiological needs for adequate growth and health (Yadav et al. 2020). Nevertheless, the positive responses of this fish to supplemental EPA + DHA with increased weight gain (WG) and feed efficiency support the applicability of ω 3 PUFA supplementation to high-performing diets for rapidly growing juvenile fishes.

In their recent synthesis, Trushenski and Rombenso (2020) point out that defining nutritional FA essentiality in fish nutrition is complex given the wide range of taxonomic (e.g., divergence among species subjected to different selective pressures), biological (e.g., trophic levels and environmental tolerances), and external

⁴Interleukin 1 receptor-associated kinase 4 is involved in signaling innate immune responses from Toll-like receptors; it also supports signaling from T-cell receptors (Takaesu et al. 2001). TNF receptor-associated factors (TRAF proteins) are associated with signal transduction from members of the TNF receptor superfamily (Xie 2013). Mitogen-activated protein kinase kinase kinase 7 mediates the signal transduction induced by TGF- β and morphogenetic protein and controls a variety of cell functions including transcription regulation and apoptosis. Mitogen-activated protein kinase 14 belongs to stress-activated serine/threonine-specific kinases and is activated by various environmental stresses and pro-inflammatory cytokines (Long et al. 2013).

Table 27.2 Quantitative essential fatty acid (EFA) requirements of selected juvenile and subadult freshwater, diadromous, and marine fish species as well as larval and early juvenile(*) fish species

Species	Habitat (according to FishBase)	Fatty acid	Optimum (% dry matter diet)	References
ω6 PUFA preference				
<i>Bidyanus bidyanus</i>	Freshwater	LIN	27% of lipids	In Glencross (2009)
<i>Coptodon (tilapia) zillii</i>	Freshwater, brackish	18:2 ω 6 and 20:4 ω 6	1.0 of each	Kanazawa et al. (1980)
<i>Cyprinus carpio</i> *	Freshwater, brackish	$\Sigma\omega$ 6 PUFAs	1.0	In Tocher (2010)
<i>Danio rerio</i> *	Freshwater	ω 3/ ω 6 PUFAs	<1.0	Meinelt et al. (1999)
<i>Dicentrarchus labrax</i>	Marine, brackish, freshwater	ARA	1.2	Atalah et al. (2011)
<i>Gadus morhua</i>	Marine, brackish	ARA	0.5	In Izquierdo and Koven (2011)
<i>Oreochromis niloticus</i>	Freshwater, brackish	18:2 ω 6	0.5	Ng et al. (2001)
<i>Paralichthys dentatus</i>	Marine	ARA	0.1	In Izquierdo and Koven (2011)
<i>Poecilia reticulata</i>	Freshwater	ARA	2.5	
<i>Trachinotus ovatus</i>	Marine, brackish	$\Sigma\omega$ 3 PUFAs $\Sigma\omega$ 6 PUFAs ARA	1.0–0.22 2.02–2.88 0.53	Qi et al. (2016)
ω3/ω6 PUFA requirement				
<i>Chanos chanos</i>	Marine, freshwater, brackish	18:3 ω 3 and 18:2 ω 6	1.0 of each	In Tocher (2010)
<i>Ctenopharyngodon idella</i>	Freshwater	18:3 ω 3 18:2 ω 6	0.5 1.0	
<i>Cyprinus carpio</i>	Freshwater, brackish	18:3 ω 3 18:2 ω 6	0.5–1.0 1.0	
		18:3 ω 3:18:2 ω 6	~1:1	
<i>Oncorhynchus keta</i>	Anadromous	18:3 ω 3 and 18:2 ω 6	1.0 of each	In Tocher (2010)
<i>O. kisutch</i>		18:3 ω 3 and 18:2 ω 6	1.0 of each	
<i>Oreochromis niloticus</i> \times <i>O. aureus</i>	Freshwater, brackish	$\Sigma\omega$ 3 PUFAs $\Sigma\omega$ 6 PUFAs	0.76 1.01	Chou and Shiau (1999)
ω3 PUFA preference				
<i>Acanthochromis polyacanthus</i>	Marine	EPA DHA	1.0 0.5	In Izquierdo and Koven (2011)
<i>Centropomus parallelus</i>	Marine, freshwater, brackish	EPA DHA	1.3 >0.6	

(continued)

Table 27.2 (continued)

			Optimum (% dry matter diet)		
Species	Habitat (according to FishBase)	Fatty acid		References	
<i>Chanos chanos</i>	Marine, freshwater, brackish	18:3ω3 EPA DHA	1.0 0.5 0.5	Borlongan (1992)	
<i>Coregonus lavaretus</i>	Freshwater, brackish	18:3ω3 Σω3 PUFA	>1.0 0.5–1.0	In Tocher (2010)	
<i>C. hippurus</i> *	Marine, brackish	Σω3 PUFA	0.6–1.0		
<i>Dicentrarchus labrax</i>	Marine, freshwater, brackish	Σω3 PUFA	1.0		
<i>Epinephelus malabaricus</i>	Marine, brackish, amphidromous	Σω3 PUFA	4% of lipids	Lin and Shiau (2007)	
<i>Gadus morhua</i> *	Marine, brackish	DHA EPA	~1.0 ?	In Tocher (2010)	
<i>Hippoglossus hippoglossus</i>	Marine	DHA	2.5	In Izquierdo and Koven (2011)	
<i>Ictalurus punctatus</i>	Freshwater	18:3ω3 Σω3 PUFA	1.0–2.0 0.5–0.75	In Tocher (2010)	
<i>Lates calcarifer</i>	Marine, freshwater, brackish	Σω3 PUFA	1.0	In Turchini et al. (2009)	
<i>Limanda ferruginea</i>	Marine	ARA DHA	<1.3 ~2.5	In Izquierdo and Koven (2011)	
<i>Morone chrysops</i> × <i>M. saxatilis</i>	Freshwater	Σω3 PUFA	1.0	In Tocher (2010)	
<i>M. chrysops</i> × <i>M. saxatilis</i> *		Σω3 PUFA	<0.5		
		18:3ω3	?		
<i>Oncorhynchus masou</i>	Anadromous	18:3ω3 or Σω3 PUFA	1.0	Watanabe (1982)	
<i>O. mykiss</i>		18:3ω3 Σω3 PUFA	20% of lipids 10% of lipids		
		18:3ω3	0.7–1.0		In Tocher (2010)
		DHA	?		
<i>Pagrus major</i>	Marine	Σω3 PUFA or EPA	0.5–1.0	In Tocher (2010)	
<i>P. major</i> *		Σω3 PUFA DHA EPA	2.1 1.0–1.6 2.3		
<i>Paralichthys olivaceus</i>		Σω3 PUFA	1.4		
<i>Platichthys stellatus</i>		Marine, freshwater, brackish	Σω3 PUFA		0.9
<i>Plecoglossus altivelis</i>		18:3ω3 or EPA Σω3 PUFA	1.0 1.7	In Turchini et al. (2009)	

(continued)

Table 27.2 (continued)

Species	Habitat (according to FishBase)	Fatty acid	Optimum (% dry matter diet)	References
<i>Pleuronectes ferrugineus</i>	Marine	$\Sigma\omega$ 3 PUFA	2.5	In Tocher (2010)
<i>Pseudocaranx dentex</i>	Marine, brackish	DHA	1.7	
<i>P. dentex</i> *		DHA EPA	1.6–2.2 <3.1	
<i>Rhabdosargus sarba</i>		$\Sigma\omega$ 3 PUFA	1.3	
<i>Salmo salar</i>	Anadromous	18:3 ω 3 or $\Sigma\omega$ 3 PUFA	1.0	
<i>Salvelinus alpinus</i>		18:3 ω 3	1.0–2.0	
<i>Sciaenops ocellatus</i>	Marine, brackish	$\Sigma\omega$ 3 PUFA EPA or DHA	0.5–1.0 0.3–0.6	
<i>Scophthalmus maximus</i>	Marine	$\Sigma\omega$ 3 PUFAs ARA	0.8 ~0.3	
<i>S. maximus</i> *		DHA	Required	
<i>Sebastes schlegelii</i>	Marine	$\Sigma\omega$ 3 PUFA EPA or DHA	0.9–1.0 1.0	
<i>Seriola quinqueradiata</i>	Marine	$\Sigma\omega$ 3 PUFA DHA EPA	3.9 1.4–2.6 3.7	
<i>Silurus glanis</i>	Freshwater, brackish	18:3 ω 3	1.0	
<i>Sparus aurata</i>	Marine, brackish	$\Sigma\omega$ 3 PUFA	0.9–1.9	
<i>S. aurata</i> *		$\Sigma\omega$ 3 PUFA	1.5–5.5	
<i>Thunnus thynnus</i>	Marine	DHA	1.1	Koven et al. (2018)

Amphidromous fishes regularly migrate between freshwater and the sea (in both directions) but not for breeding; *anadromous* fishes migrate from the sea up into freshwater to spawn; examples are salmon and striped bass (Moyle and Cech 2004)

(e.g., experimental conditions and differences in feed formulation and manufacturing) factors involved, all of which can influence absolute requirements. Fishes vary in their ability to meet the physiological demand for LC-PUFAs via elongation and desaturation of C_{18} PUFA (i.e., 18:2 ω 6 and 18:3 ω 3) precursors (\rightarrow Chap. 29). Previous attempts to generalize dietary FA requirements have been focused on thermal and salinity preferences, suggesting that coldwater and/or marine fish exhibit dietary requirements for LC-PUFAs, but warmwater and/or freshwater species do not. Instead, Trushenski and Rombenso (2020) show that PUFA essentiality does not adhere to any obvious patterns related to temperature or salinity preferences (Fig. 27.6): there are warm-, cool-, and coldwater fishes that can utilize C_{18} PUFAs and those that cannot; similarly, there are freshwater, euryhaline, and saltwater fishes that can utilize C_{18} PUFAs and others that cannot. However, demand for LC-PUFAs

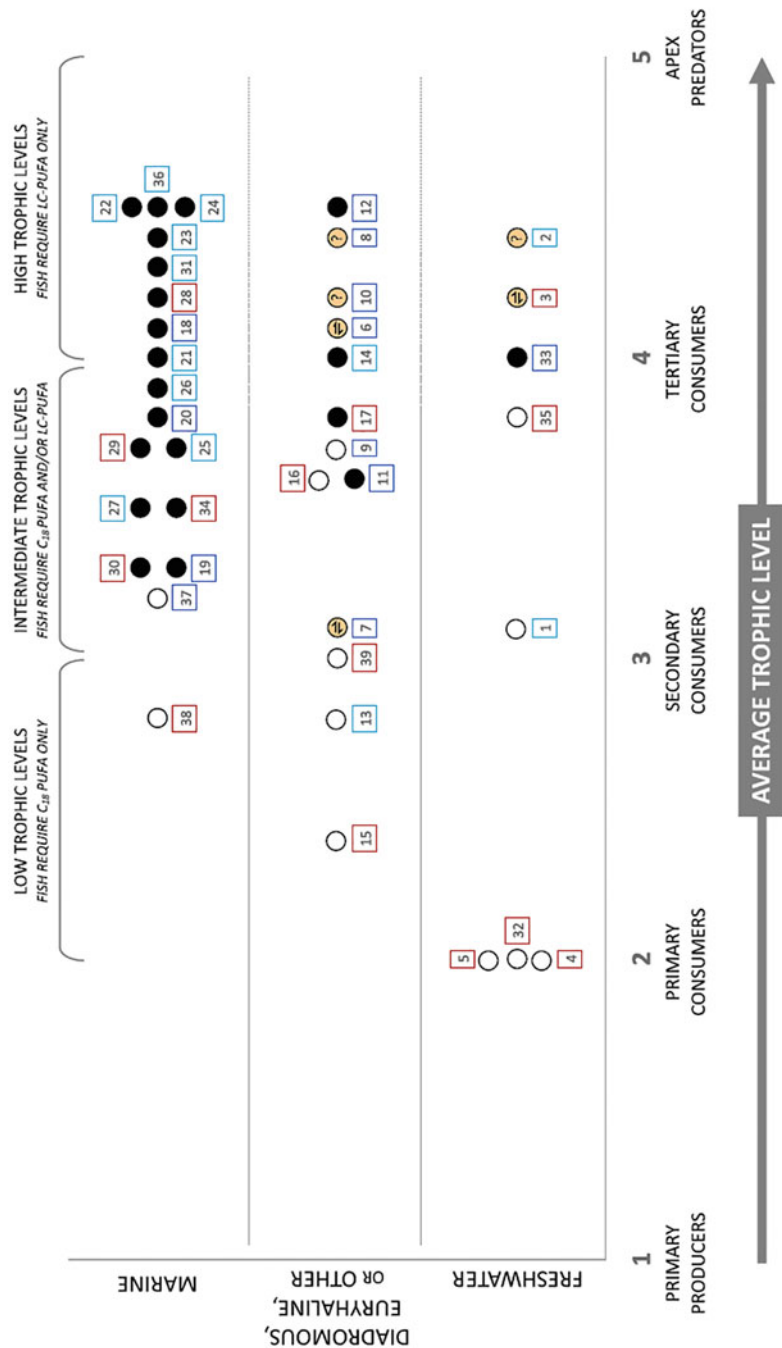


Fig. 27.6 Dietary PUFA essentiality for selected fishes according to salinity and temperature preferences and average trophic level. Each circle represents a single taxon, indicated by the numeric label: **1** common carp (*Cyprinus carpio*); **2** wels (*Silurus glanis*); **3** channel catfish (*Ictalurus punctatus*); **4** Nile tilapia (*Oreochromis niloticus*); **5** grass carp (*Ctenopharyngodon idella*); **6** rainbow trout/steelhead (*Oncorhynchus mykiss*); **7** pike (*Esox lucius*); **8** Arctic charr (*Salvelinus alpinus*); **9** chum salmon (*Oncorhynchus keta*); **10** coho salmon (*Oncorhynchus kisutch*); **11** masu salmon (*Oncorhynchus masou*); **12** Atlantic salmon (*Salmo salar*); **13** ayu (*Plecoglossus altivelis*); **14** hybrid striped bass (*Morone chrysops* × *M. saxatilis*); **15** milkfish (*Chanos chanos*); **16** Japanese eel (*Anguilla japonica*).

(*Anguilla japonica*); **17** barramundi (*Lates calcarifer*); **18** Atlantic cod (*Gadus morhua*); **19** yellowtail flounder (*Limanda ferruginea*); **20** Korean rockfish (*Sebastes schlegelii*); **21** buri (*Seriola quinqueradiata*); **22** olive flounder (*Paralichthys olivaceus*); **23** turbot (*Scophthalmus maximus*); **24** madai (*Pagrus major*); **25** gilthead seabream (*Sparus aurata*); **26** white trevally (*Pseudocaranx dentex*); **27** European sea bass (*Dicentrarchus labrax*); **28** groupers (*Epinephelus* spp.); **29** red drum (*Sciaenops ocellatus*); **30** goldlined seabream (*Rhabdosargus sarba*); **31** meagre (*Argyrosomus regius*); **32** tambaqui (*Colossoma macropomum*); **33** zander (*Sander lucioperca*); **34** sobaity seabream (*Sparidentex hasta*); **35** sharptooth catfish (*Clarias gariepinus*); **36** dentex (*Dentex dentex*); **37** Ballan wrasse (*Labrus bergylta*); **38** white-spotted spinefoot (*Siganus canaliculatus*); **39** spotted scat (*Scatophagus argus*). Average trophic level is indicated horizontally, with primarily producers on the left (trophic level = 1) and apex predators on the right (trophic level = 5). Salinity preference is indicated vertically, with marine fish at the top, freshwater fish at the bottom, and fish exhibiting other salinity preferences/tolerances (e.g., amphidromy, anadromy, catadromy, etc., in at least one life history form) in the middle. The color of the numeric label border indicates temperature preference for cold water (<18 °C; dark blue), cool water (18–25 °C; light blue), or warm water (>25 °C; red). Polyunsaturated fatty acid essentiality is expressed in terms of dietary requirements for C₁₈ PUFAs (open circles) or long-chain (LC) PUFAs (black-filled circles); yellow-filled circles indicate conflicting reports regarding C₁₈ PUFA versus LC-PUFA nutritional essentiality (yellow-filled circles with opposing arrow symbols) or studies with incomplete experimental designs (i.e., studies which examined either C₁₈ PUFAs or LC-PUFAs but not both; yellow-filled circles with question marks). Nutritional PUFA essentiality does not adhere to any obvious patterns related to temperature or salinity preferences but is strongly related to trophic level. Fish occupying low trophic levels (i.e., trophic level < 3) require C₁₈ PUFAs only, whereas those occupying high trophic levels (i.e., trophic level > 4) require LC-PUFAs. Fish occupying intermediate trophic levels (i.e., 3 < trophic level < 4) may require either class of PUFA, depending on other aspects of their ecological niche and life history (From Trushenski and Rombenso (2020) and references to original papers therein, with permission from Wiley)

does appear to be a function of trophic level (organism’s position in the food web hierarchy; scale of 1–5, where 1 = primary producers and 5 = apex predators). Regardless of thermal or salinity guild, fishes occupying low trophic levels (i.e., trophic level < 3) are capable of de novo LC-PUFA synthesis and require only C₁₈ PUFAs in their diet, whereas those occupying high trophic levels (i.e., trophic level > 4) are unable to synthesize LC-PUFAs from C₁₈ precursors and therefore require a dietary source of intact LC-PUFA (Fig. 27.6). Like the coldwater–warmwater and freshwater–saltwater dichotomies, this concept is based on the working hypothesis that de novo synthesis of LC-PUFAs is unnecessary among organisms, whose natural diet is rich in these nutrients, but the concept differs in the recognition that high-level carnivores consume an LC-PUFA-rich diet regardless of their environment.

The trophic level generalization of PUFA essentiality may be subject to “exceptions to the rule” (→AAN I “Diets and Digestive Tracts” (Steinberg 2018)); indeed, fishes occupying intermediate trophic levels (i.e., 3 < trophic level < 4) may require either C₁₈ PUFAs or LC-PUFAs depending on other aspects of their ecological niche and life history (Fig. 27.6). Nonetheless, trophic level appears to explain much of the variation among species with regard to utilization of C₁₈ PUFAs versus LC-PUFAs and may provide considerable predictive value in the context of FA essentiality. However, if this conceptual model is to be used effectively, it is necessary to redefine FA essentiality and quantitative requirements in terms of individual C₁₈ PUFAs and LC-PUFAs (i.e., 18:2 ω 6, 18:3 ω 3, 20:4 ω 6, 20:5 ω 3, and 22:6 ω 3) rather than in terms of FA groupings (e.g., total ω 6 FAs or ω 3 LC-PUFAs) as has been standard in the past (NRC 2011).

Although some valuable published research on this topic still relies on this traditional approach, Trushenski and Rombenso (2020) strongly recommend a transition toward individual C₁₈ PUFA and LC-PUFA assessment as recently done in *Larimichthys crocea* (Zuo et al. 2015), *Lates calcarifer* (Salini et al. 2016), *Salmo salar* (Emery et al. 2016; Bou et al. 2017), *Oreochromis niloticus* (Nobrega et al. 2017), *Dicentrarchus labrax* (Torrecillas et al. 2017, 2018), *Oncorhynchus mykiss* (Colombo et al. 2018), *Sander lucioperca* (Lund et al. 2019), or *Dentex dentex* (Giménez Papiol and Estévez 2019) (from Trushenski and Rombenso (2020) with permission from Wiley, references added).

As with any emerging paradigm, it has to be able to explain most (but necessarily not all) corresponding phenomena, and it is challenged with contrasting findings. On the small database of three studied fish species, Galindo et al. (2021) state that the phylogeny of the fish species, instead of trophic level, might be a more relevant factor in the LC-PUFA biosynthetic capacity. Herbivorous *Sarpa salpa* and carnivorous *Pegusa lascaris* show lipid metabolism characteristics similar to two established commercial species, namely, *Sparus aurata* and *Solea senegalensis*. The LC-PUFA biosynthetic capacity of wild *S. salpa*, *P. lascaris*, and omnivorous *Chelon labrosus* resembles that of their phylogenetically close species *S. aurata*, *S. senegalensis*, and *Liza aurata*, respectively. The desaturase activities observed in this study include Δ^5 , Δ^6 , and Δ^8 activities in herbivorous *S. salpa*, Δ^5 and Δ^8 activity in omnivorous *C. labrosus*, and Δ^6/Δ^5 residual activity and Δ^4 in carnivorous *P. lascaris*, thus confirming the ability of the three studied species to

biosynthesize 22:6 ω 3 from 18:3 ω 3. Doubtless, the solution of this contradiction comprises a process, whose successive stages are characterized by an increasingly detailed and refined understanding of the PUFA requirement and biosynthesis in aquatic animals—a process excellently outlined for physics by Kuhn (2012).

Moreover, concerns have also been raised by Parzanini et al. (2020) who used 1382 fish FA profiles and provided a first comprehensive survey of FA abundance and distribution in freshwater vs. marine fishes. This analysis detects fundamental differences between freshwater and marine fishes. The former is characterized by high contents of ω 6 PUFA, mainly LIN and ARA, indicative of freshwater algae and terrestrial dietary sources. This highlights primary differences at the base of freshwater vs. marine food webs and the differential ability of fish to synthesize certain essential FA de novo. In contrast, the FA composition of marine fish species is mostly driven by the calanoid copepod/zooplankton biomarkers 20:1 ω 9 (eicosenoic acid) and 22:1 ω 11 (docosenoic acid or erucic acid) and ω 3 PUFA (e.g., EPA and DHA).

Based on patterns of PUFAs in fishes, rather than on their nutritional essentiality or capability of de novo biosynthesis, Parzanini et al. (2020) successfully established a new FA metric to aid in the identification of marine (M) vs. freshwater (F) fishes:

$$M/F = (20 : 1\omega 9 + 22 : 1\omega 11 + \text{EPA} + \text{DHA}) / (\text{ALA} + \text{LNA} + \text{ARA}).$$

For this metric, higher values (≥ 11) represent “true” marine fish species, as these are characterized by greater proportions of 20:1 ω 9, 22:1 ω 11, EPA, and DHA overall ($\sim 30\%$). In contrast, lower values of this ratio (< 2) are indicative of “true” freshwater species as these overall contain high proportions of ALA, LNA, and ARA ($\sim 13.0\%$) in their tissues (Parzanini et al. 2020).

Furthermore, ontogenetic changes of the dietary demands have been documented in several species: larval and early juvenile carps prefer ω 6 PUFAs, whereas juvenile and subadult individuals require 18:3 ω 3 and 18:2 ω 6. Also, some classifications need empirical re-evaluation: European sea bass is listed as ω 3 PUFA type (Tocher 2010), whereas Atalah et al. (2011) mention it as ARA-dependent species. In a lipid-replacement trial, Eroldoğan et al. (2013) reported that this species seems to be able to efficiently use ω 6 PUFA as energy substrate, and this minimizes β -oxidation of ω 3 LC-PUFA. Furthermore, the classification of the milkfish (*Chanos chanos*) should be revised, since it is listed as ω 3/ ω 6 as well as ω 3 type in Table 27.2.

Overall, newly emerging paradigms and extensive discussions are beginning, and independent of the outcome, some recent studies of regulatory networks in fishes on different LC-PUFA diets are worth being discussed.

27.2.2.1 ω 3 Requirement

Jin et al. (2017) evaluated the effects of dietary DHA/EPA ratios on growth and expression of lipid metabolism-related genes of juvenile carnivorous black seabream (*Acanthopagrus schlegelii*). DHA/EPA ratios clearly affect genes encoding the

enzymes of LC-PUFA biosynthesis, with both elongase of *very-long-chain fatty acids* 5 (*elovl5*) and *fatty acid desaturase* 2 (*fads2*)⁵ showing decreasing expression with increasing dietary DHA/EPA ratio (Fig. 27.7c). Expression of *acetyl-CoA carboxylase alpha* (*accα*) and *carnitine palmitoyltransferase 1A* (*cpt1a*) are upregulated by high DHA/EPA ratios (Fig. 27.7b), whereas *sterol regulatory element-binding protein-1* (*srebp-1*) and *hormone-sensitive lipase* (*hsl*) are downregulated. *Fatty acid synthase* (*fas*), *6-phosphogluconate dehydrogenase* (*6pgd*), and *peroxisome proliferator-activated receptor α* (*ppara*) show the highest expression in fishes fed an intermediate (1.16) DHA/EPA ratio (Fig. 27.7a). This study shows that, although the dietary ratio of DHA/EPA does not modulate growth or feed utilization, it does affect tissue FA profiles and expression of lipid-related genes.

In a subsequent study, Jin et al. (2019) found that also optimal dietary ω3 LC-PUFA/ω6 C₁₈-PUFA ratios enhance juvenile black seabream growth and feed utilization with an optimum ω3 LC-PUFA/ω6 C₁₈-PUFA ratio of 0.7, indicating a slightly higher requirement of ω3 LC-PUFAs than ω6 C₁₈-PUFAs. Higher dietary ratio of ω3 LC-PUFA/ω6 C₁₈-PUFA can induce liver damage by boosting the activities of aspartate transaminase and alanine aminotransferase and increasing the cholesterol content.

The nutritional requirement for ω3 LC-PUFAs in fast-growing Atlantic salmon during grow-out in the sea has been studied by Rosenlund et al. (2016). This species has a specific requirement for EPA + DHA for optimal growth with a level of >2.7% of ΣFA. Furthermore, salmon can be a net producer of DHA dependent on dietary supply, and the dietary PUFA composition does not influence the ability of salmon to autochthonously produce DHA (Sissener et al. 2017).

A commercially important cyprinid in Southeast Asia, silver barb (*Barbonymus gonionotus*) has a reasonable deposition of the physiologically important ω3 LC-PUFAs, implying the existence of a functional LC-PUFA biosynthesis pathway. In fact, *fads2* and *elovl5* occur in this fish (Nayak et al. 2018). Furthermore, Janaranjani et al. (2018) showed the presence of all required enzymes for biosynthesis of EPA and ARA from ALA and LIN, respectively. *Fads2* and *elovl5* are highly expressed in the brain and liver. Juvenile fishes fed ALA-rich diet show higher expression of hepatic *fads2* and *elovl5* than to those fed LC-PUFA-rich diet pointing out endogenous EPA and DHA biosynthesis.

⁵ Also Delta 6 desaturase (D6D or Δ⁶ desaturase) named so because it was thought to convert only ω6 FAs but actually converts also some others. It is obligatory to build the longer chain ω3 FAs from other simpler FAs (Glaser et al. 2010). Furthermore, the conversion of hexadecanoic acid (palmitic acid, 16:0) to hexadecenoic acid (16:1ω10) was observed (Guillou et al. 2003).

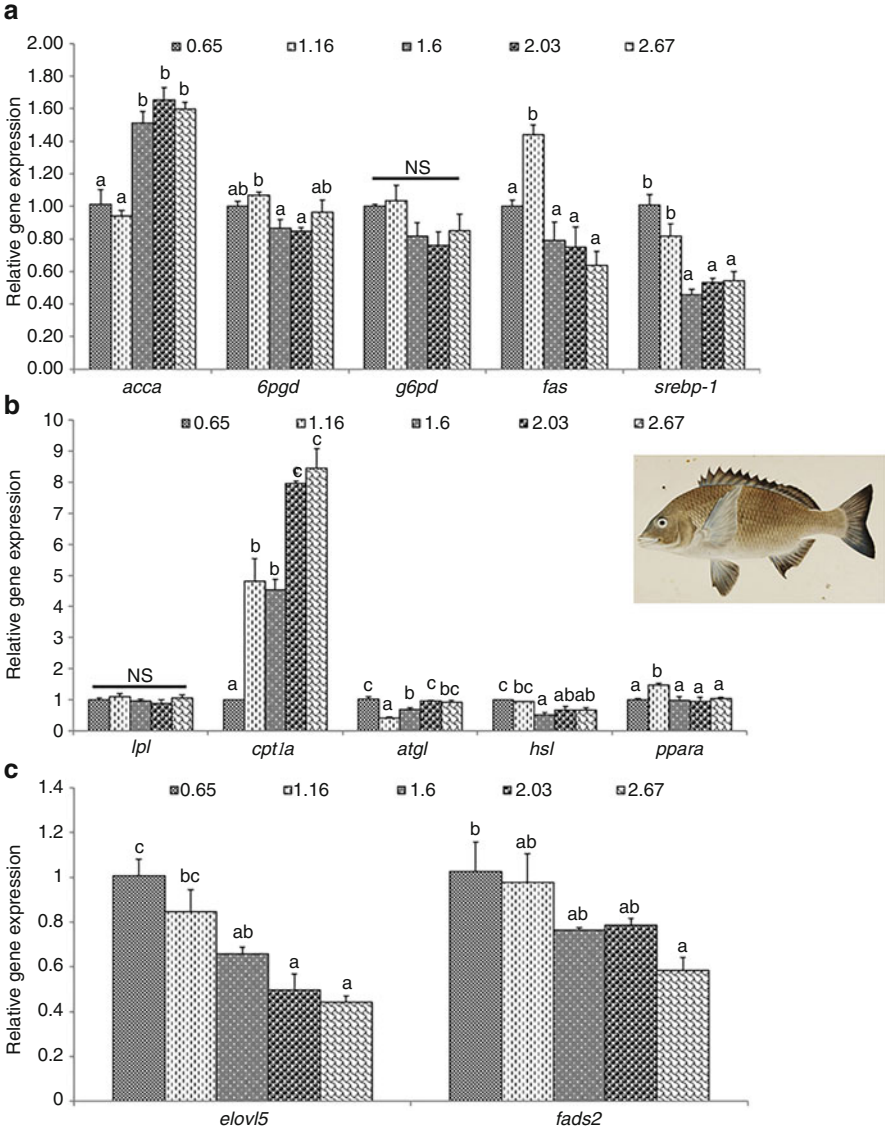


Fig. 27.7 Effects of dietary DHA/EPA ratio on relative mRNA expression of genes involved in lipid metabolism pathways including anabolism (a), catabolism (b), and LC-PUFA biosynthesis (c) in the liver of juvenile black seabream. The control group (0.65 DHA/EPA) is used as reference group, and the mRNA expression levels of target genes are normalized relative to the expression of β -actin. Values are means \pm SE. Mean values for the same gene with different letters are significantly different ($P < 0.05$) (From Jin et al. (2017), credit Public Library of Science; image credit Kawahara Collection at Naturalis Biodiversity Center). *acca* acetyl-CoA carboxylase alpha; *6pgd* 6-phosphogluconate dehydrogenase; *g6pd* glucose 6-phosphate dehydrogenase; *fas* fatty acid synthase; *srebp-1* sterol regulatory element-binding protein-1; *lpl* lipoprotein lipase; *cpt1a* carnitine palmitoyltransferase 1A; *atgl* adipose triglyceride lipase; *hsl* hormone-sensitive lipase; *ppara* peroxisome proliferator-activated receptor alpha; *fads2* fatty acyl desaturase 2; *elovl5* elongase of very-long-chain fatty acids 5. NS not significant

Broodstocks

EFA are particularly important in broodstock nutrition and in performance of offspring (Table 27.3). Their demand of broodstock during reproduction is higher than those of juvenile fishes, but excess amounts or an imbalance can be detrimental for reproduction (Izquierdo et al. 2001). To optimize the reproductive potential in white bass (*Morone chrysops*), Fuller et al. (2017) evaluated the FA composition of broodstock ova. The authors provided commercial diets with $\omega 6/\omega 3$ ratios from 0.36 to 1.12 to the broodstock and found that females have the ability to incorporate preferentially $\omega 3$ PUFAs, mainly DHA. Therefore, the authors recommend the inclusion of large quantities of $\omega 3$ PUFA in diets fed to female white bass broodstock. The same applies to Nile tilapia (Table 27.3). (More details can be found in AAN III Fish Oil Replacement.)

Most of the tested invertebrate and fish species prefer $\omega 3$ PUFAs for reproductive performance. However, the Pacific oyster appears not to benefit from dietary EPA and DHA for its reproduction. Of the fish species, only common sole and zebrafish (Fig. 27.8) improve reproductive traits if fed increasing $\omega 6$ PUFA contents in their diets. Although the tested life history traits in Nile tilapia are not identical in the two studies listed in Table 27.3, it is obvious that the Malaysian strain prefers $\omega 6$ for fry production, whereas the Brazilian one increases spawning on increasing dietary $\omega 3$ PUFA contents. Future studies have to solve this apparent contradiction.

27.2.2.2 $\omega 3 + \omega 6$ Requirement

In *Danio rerio*, Meinelt et al. (1999, 2000) showed that the dietary FA composition influences egg fertilization rate and larval growth: Elevated fertilization occurs with low $\omega 3/\omega 6$ ratio (relative high $\omega 6$ share, Fig. 27.8). Consequently, also larval growth is accelerated by elevated dietary $\omega 6$ PUFA contents. However, excess dietary ARA content can be detrimental (Fig. 27.9). Samaee et al. (2019) confirmed that FA composition contributes to protection and improvement of the reproductive capacity of zebrafish and attributed this to a certain ratio of OA (18:1 $\omega 9$) to LIN (18:2 $\omega 6$). These findings confirm the requirement of fishes on an intermediate trophic level: their demand depends not only on a fixed PUFA pattern but is also determined by aspects of their ecological niche and life history (Trushenski and Rombenso 2020).

Supporting the above results, Fowler et al. (2020) found that female zebrafishes fed a $\omega 3:\omega 6$ PUFA ratio of 1:5 produce higher proportions of viable embryos than those on a 1:1.4 ratio. Adam et al. (2017) specifically explored the impact of high dietary ARA on life history traits in zebrafish. Low dietary $\omega 3/\omega 6$ PUFA ratio is a health concern, as $\omega 6$ PUFA gives rise to ARA-derived eicosanoids, which are considered pro-inflammatory. Juvenile zebrafishes on high-ARA diets have a lower whole-body $\omega 3/\omega 6$ PUFA ratio than individuals on low-ARA diets. Metabolomics reveals altered levels of eicosanoids, PUFAs, dicarboxylic acids, and complex lipids, such as glycerophospholipids and lysophospholipids. ARA-derived hydroxylated eicosanoids, such as hydroxyeicosatetraenoic acids,

Table 27.3 Impact of PUFAs in broodstock diets on reproductive performance of selected farmed species

Species, common name	Broodstock PUFA	Effect	References
Invertebrates			
<i>Argopecten purpuratus</i> , Peruvian calico scallop	<i>Isochrysis galbana</i> , PUFA-rich (\rightarrow AAN III “Fish oil replacement—Single cell factories”)	Parental HSP70 \uparrow , offspring HSP70 \uparrow , larval survival \uparrow	Pérez et al. (2016)
<i>Astacus leptodactylus</i> , Narrow-clawed crayfish	DHA \uparrow	Quality of pleopodal eggs \uparrow and stage 1 juveniles \uparrow	Harlioğlu et al. (2012)
<i>Crassostrea gigas</i> , Pacific oyster	EPA \uparrow , DHA \uparrow	Egg number \leftrightarrow , egg size \leftrightarrow	Hendriks et al. (2003)
<i>Eriocheir sinensis</i> , Chinese mitten crab	EPA \uparrow DHA \uparrow	Fecundity \uparrow Hatchability \uparrow	Wen et al. (2002)
<i>Fenneropenaeus indicus</i> , Indian white shrimp	Dietary ω 3/ ω 6 ratio \uparrow	Egg quality \uparrow , nauplii quality \uparrow , zoeae \uparrow	Regunathan (2008)
<i>Macoma balthica</i> , Baltic clam	EPA \uparrow , DHA \uparrow	Egg number \uparrow , egg size \uparrow	Hendriks et al. (2003)
<i>Litopenaeus vannamei</i> , Pacific white shrimp	PUFA \uparrow	Fertilization \uparrow , egg production \uparrow	Wouters et al. (1999)
<i>Pecten maximus</i> , Great Atlantic scallop	ARA \uparrow DHA \uparrow	Gametogenesis \uparrow Embryogenesis \uparrow	Soudant et al. (1996a, 1996b)
	EPA \uparrow	Spermatogenesis \uparrow	Soudant et al. (1997)
<i>Penaeus monodon</i> , Tiger shrimp	ARA \uparrow , DHA \uparrow	Oogenesis \uparrow , spermatogenesis \uparrow	Paibulkichakul et al. (2008)
<i>Pinctada margaritifera</i> , Pacific pearl-oyster	DHA \uparrow	Oogenesis \uparrow , embryogenesis success \uparrow	Ehteshami et al. (2011)
Fishes			
<i>Acanthopagrus latus</i> , Yellowfin seabream	Dietary ω 3/ ω 6 ratio \rightarrow hatching success	2.0 \rightarrow 59.8 1.4 \rightarrow 43.6 1.1 \rightarrow 39.1	Zakeri et al. (2011)
<i>Acipenser baerii</i> , Siberian sturgeon	DHA \uparrow	Fecundity \uparrow , egg hatchability \uparrow , larval quality \uparrow	Luo et al. (2015)
	EPA + DHA \uparrow	♀ + ♂ reproductive performance \uparrow	Luo et al. (2017)
	DHA/EPA \uparrow	Growth performance \uparrow	Luo et al. (2019)

(continued)

Table 27.3 (continued)

Species, common name	Broodstock PUFA	Effect	References
<i>Anguilla anguilla</i> , European eel	ARA↑	Sperm velocity↓	Baeza et al. (2015)
	ω3 PUFA↑	Oocyte development↑	da Silva et al. (2016)
<i>Centropomus undecimalis</i> , common Snook	DHA↑	Fertilization↑, hatching↑, larval survival↑	Yanes-Roca et al. (2009)
<i>Channa striata</i> , striped snakehead	ω3 PUFA↑ + ω6 PUFA↑	Fecundity↑	Ghaedi et al. (2016)
<i>Cynoglossus semilaevis</i> , tongue sole	Dietary ω3/ω6 ratio between 2.8 and 5.2	Best spawning, egg, larval performance	Liang et al. (2014)
<i>Danio rerio</i> , zebrafish	TAG ω3/ω6 ratio	Ratio↓ → fertilization↑ (Fig. 27.8)	Meinelt et al. (1999)
	Dietary ω3/ω6 ratio → eggs/♀	11.2 → 3680 5.5 → 5000 4.1 → 4460	Jaya-Ram et al. (2008)
	ARA↑	F ₁ hepatic genes↑ (Fig. 27.13), DNA methylation↑	Adam et al. (2018, 2019)
<i>Dicentrarchus labrax</i> , European sea bass	Dietary ω3/ω6 ratio↑	Egg quality↑, hatching rate↑	Navas et al. (1997)
	PUFA↑	Milt production↑, fertilization success↑	Asturiano et al. (2001)
<i>Gibellion (Catla) catla</i> , Indian major carp	Dietary ω3/ω6 ratio → fertilization rate	0.02 → 49.0 0.12 → 22.7 0.7 → 73.4 4.87 → 56.8	Nandi et al. (2001)
<i>Morone chrysops</i> , White bass	EPA↑ or ALA↑	Reproductive success↑	Lewis et al. (2010, 2011)
<i>Oncorhynchus mykiss</i> , Rainbow trout	ω3 PUFAs↑	Testis quality↑, semen quality↑	Köprücü and Özcan (2019); Yonar et al. (2020)
<i>Oreochromis niloticus</i> , Nile tilapia, ♀♀	Crude palm oil → ω3 PUFAs↓	Fry production↑	Ng and Wang (2011)
	Dietary ω3/ω6 ratio↑	<i>cyp17</i> ↑, spawning performance↑	Orlando et al. (2020)
<i>Pampus argenteus</i> , Silver pomfret	ω3 PUFA↑	Ovarian development↑	Peng et al. (2015)
<i>Seriola dorsalis</i> , Yellowtail amberjack	ARA↑	Egg quality↑	Stuart et al. (2018)

(continued)

Table 27.3 (continued)

Species, common name	Broodstock PUFA	Effect	References
<i>S. dumerili</i> , Greater amberjack	Dietary ω 3/ ω 6 ratio \rightarrow fertilization; 3 dph larval survival %	2.2 \rightarrow 86.3; 38.1 2.9 \rightarrow 91.8; 38.3 3.6 \rightarrow 69.0; 11.6 5.0 \rightarrow 52.4; 16.1	Sarih et al. (2020)
<i>Solea senegalensis</i> , Senegalese sole	DHA \uparrow	Sperm quality \uparrow	Beirão et al. (2015)
<i>S. solea</i> , Common sole	Dietary ω 3/ ω 6 ratio \downarrow	Larval survival \uparrow	Parma et al. (2015)
<i>Sparus aurata</i> , Gilthead seabream	ω 3 PUFA \uparrow	Offspring nutritional programming \uparrow , immunity \uparrow	Turkmen et al. (2017, 2019)
	ALA \uparrow	Methylation of <i>scd1a</i> promoter \uparrow , fat accumulation \downarrow (Fig. 27.2)	Perera et al. (2020)
<i>Trichopodus (Trichogaster) leeri</i> , Pearl gourami	Dietary ω 3/ ω 6 ratio \rightarrow fertilization rate	15.0 \rightarrow 75 5.3 \rightarrow 86 2.0 \rightarrow 79	Ghosi Mobaraki et al. (2020)

\uparrow increase/support; \downarrow reduction/decrease; TAG triacylglycerol (triglyceride) fraction; *cyp17 17 α -hydroxylase and 17,20 lyase* (key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens)

are elevated in response to high-ARA feed. In addition, increased levels of oxidized lipids and amino acids as well as lipid peroxidation indicate an oxidative stress due to ω 6 PUFA excess (Fig. 27.9).

Zhou et al. (2018) detected an additional mechanism of how low dietary ω 3/ ω 6 ratios can adversely affect fishes. Different dietary LCFAs influence the *fat/cd36* transcription in white muscles of grass carp. The FA translocase (FAT/CD36) is central in the transmembrane uptake of the LCFAs. mRNA levels of this translocase are highest in fishes fed groundnut (peanut, GO) diet (rich in 18:2 ω 6) and lowest in fishes fed linseed oil (LO) and FO diets rich in 18:3 ω 3 PUFAs (ALA, EPA, DHA). FAT/CD36-mediated FA transport into the skeletal muscle is critically coupled with muscle fuel selection and FA oxidation, indicating that an increase in FAT/CD36 stimulates fat lipolysis and oxidation.

With reference to LIN (18:2 ω 6) and LNA (=ALA, 18:3 ω 3), grass carp and Nile tilapia have different specific EFA requirements. Both species are categorized as primary consumers (Trushenski and Rombenso 2020). Jiao et al. (2020) compared activities of cellular FA uptake, β -oxidation, and esterification toward ^{14}C -labeled LIN and LNA in primary hepatocytes of grass carp (GCH) and Nile tilapia (NTH). GCH preferentially takes up LNA, while NTH prefers LIN. Both hepatocytes have higher β -oxidation activity to LIN than to LNA. LNA causes higher esterification than LIN in GCH does, but LIN and LNA cause similar esterification results in NTH

Fig. 27.8 Relationship between dietary $\omega 3/\omega 6$ ratio in the triglyceride fraction and fertilization rate in zebrafish eggs (Redrawn from Meinelt et al. (1999), with permission from Wiley)

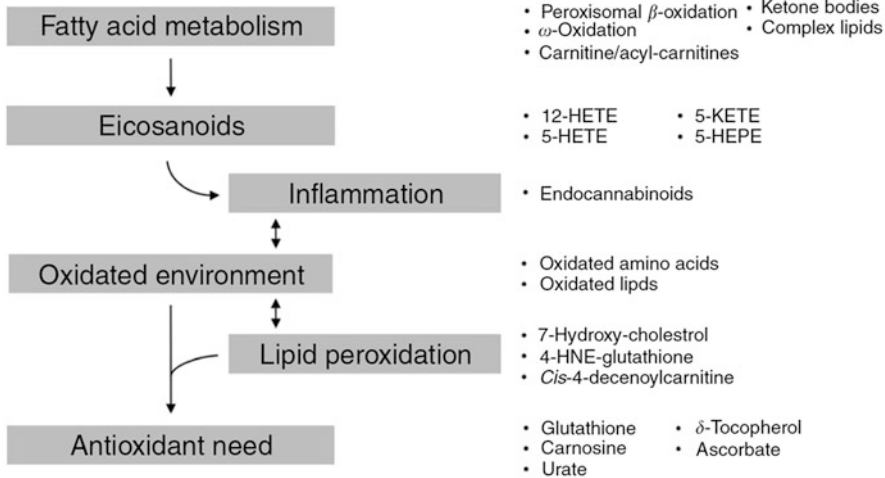
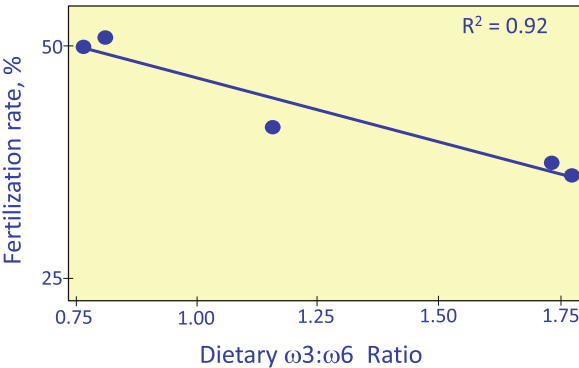
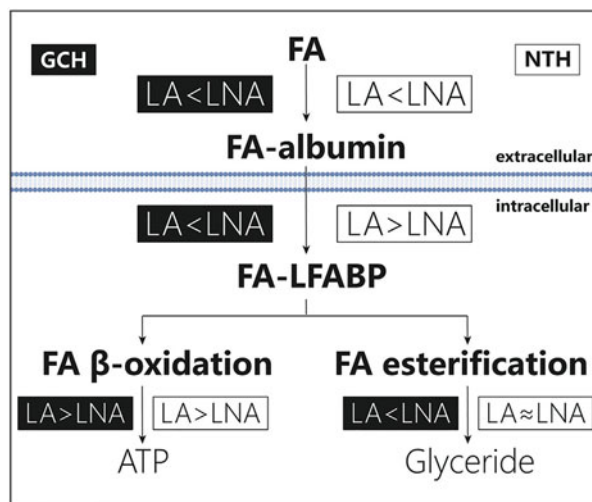


Fig. 27.9 High dietary arachidonic acid changes the metabolic fingerprint in zebrafish. Changes are characterized not only by a general change in lipid profiles and eicosanoids but also by changed metabolites indicating inflammation and lipid peroxidation as well as changes in the antioxidant status. Arrows indicate the suggestive physiological conditions in the fish. Indicative metabolites are given on the right side. **12-HETE** 12-hydroxyeicosatetraenoic acid; **5-KETE** 5-oxo-EETE, 5-ketoeicosatetraenoic acid (5-oxo-eicosatetraenoic acid); **5-HETE** 5-hydroxyeicosatetraenoic acid; **5-HEPE** 5-hydroxy-EPA; **4-HNE-glutathione** 4-hydroxy-nonenal-glutathione (From Adam et al. (2017), credit British Journal of Nutrition)

(Fig. 27.10). In sum, GCH have specific preference to take up and esterify LNA, while NTH preferentially take up LIN. These differences are in accordance with the specific EFA requirements of grass carp and Nile tilapia (Jiao et al. 2020).

Fig. 27.10 Cellular fatty acid metabolism patterns of different FAs in grass carp hepatocytes (GCH) and Nile tilapia hepatocytes (NTH) (From Jiao et al. (2020), with permission from Elsevier). **LA** linoleic acid (=LIN); **LNA** α -linolenic acid (=ALA); **LFABP** liver fatty acid binding protein



27.2.2.3 EPA, ARA, and DHA

Fatty acids are much more than pure fuel. A few recent examples are going to illustrate the far-reaching significance of PUFAs, such as ARA, EPA, or DHA. Nayak et al. (2017) fed diets supplemented with graded ARA to rabbitfish (*Siganus rivulatus*), a nominally herbivorous marine species. Fishes on moderate ARA doses (2.6% of total FAs) exhibit best growth and best survival during bacterial infection, partly due to induced increased levels of lysozyme and complement. Plasma lysozyme is a nonspecific immune component with mucolytic properties. Also sufficient dietary EPA and DHA supply is important for innate immunity: low levels or absence of these ω 3 PUFAs exerts immunosuppressive effects in Atlantic salmon (Arnemo et al. 2017). The metabolic conversion from EPA to DHA is governed by a combination of substrate availability (EPA) and feedback inhibition by the product (DHA), being especially relevant in the liver and intestine. In line with this, the stimulation of the hepatic ω 3 FA biosynthetic pathway at a transcriptional level was observed at low dietary levels of essential FA. This study demonstrates that the evaluation of growth and survival solely can underestimate the nutritional requirement of EPA and DHA. Essential FA deficiencies during early life stages may influence fish performance and their ability to deal with challenging stress conditions (Bou et al. 2017).

Recently, Wang et al. (2020b) identified underlying mechanisms of stimulated muscle growth in Wuchang bream: 0.8% dietary DHA increases muscle fiber density, the proportion of small-sized fiber, and sarcomere lengths. Gene transcription related to myogenesis and muscle growth (*myog*, *mrf4*) increase and gene transcription of myostatins (*mstna*, *mstnb*) is reduced. Myostatins act on muscle cells to inhibit muscle cell growth. These transcriptomic results show that DHA promotes white muscle hyperplasia and muscle fiber development.

Excess dietary DHA, however, can be detrimental; it can lead to increased plasma urea content and subcutaneous (mild) hemorrhaging as found in young barramundi (Glencross and Rutherford 2011). In Wuchang bream, underlying mechanisms comprise the following: WG of fish fed 1.6% DHA are lower than of those fed 0.2% DHA. *p53* transcription and protein expression are increased, while expression of downstream *cyclin D1* and *cyclin E1* is inhibited on 1.6% DHA diet (Wang et al. 2020a). This indicates that high levels of DHA activate the P53/cyclin pathway to induce cell cycle arrest.

In the southern flounder (*Paralichthys lethostigma*), an important food fish found along the northern Gulf of Mexico and mid-Atlantic coast of the USA, concentrations of DHA and ARA in head and body are correlated with concentrations of DHA and ARA in the diet (Oberg and Fuiman 2015). This translates into larval essential traits (visual responsiveness, turning rates during routine swimming). In addition, survival is related to dietary lipid content. The main feature of this study is the presence of DHA in almost every connection between the diet, FA pattern in tissues, and larval quality (Fig. 27.11). In contrast, ARA is present in the links between the diet and tissues, but it is not linked to any larval trait (Oberg and Fuiman 2015).

Noteworthy, EPA and DHA are anti-adipogenic and lipolytic factors in fish adipogenesis, whereas OA promotes lipid accumulation and adipogenesis (Salmerón 2018). Furthermore, OA improves growth of juvenile Nile tilapia but simultaneously stimulates the pro-inflammatory cytokine IL-1 β (Amornlerdpison et al. 2019)—the long-term effect, however, remains unclear. Further beneficial effects of dietary EPA, ARA, or DHA on life history traits are briefly listed in Table 27.4. It is obvious that growth and gonad development are the most often-studied traits. Noteworthy, in

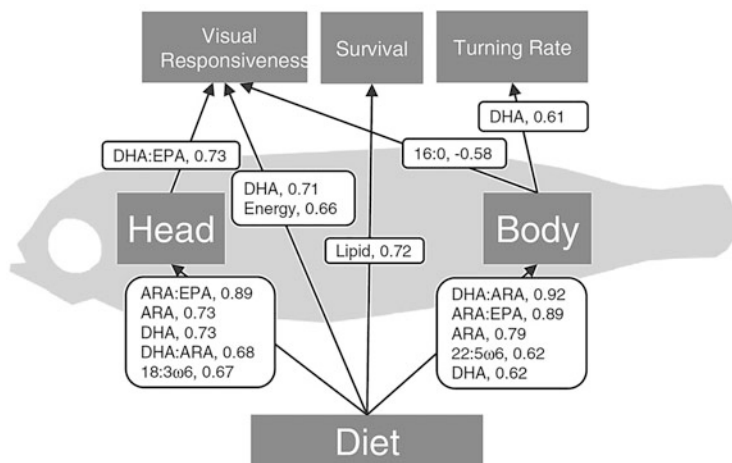


Fig. 27.11 Overview of linkages between diet, tissues, and larval quality traits in southern flounder (*Paralichthys lethostigma*). White boxes show correlations with fatty acids, crude lipid, and energy and their correlation coefficient (r) (From Oberg and Fuiman (2015), with permission from Elsevier)

Table 27.4 Effects of dietary EPA, ARA, or DHA on life history traits of selected farmed aquatic animals

Species, common name	PUFA, level (% dw)	Affected trait	References
Invertebrates			
<i>Crassostrea gigas</i> , Pacific oyster	ARA, 0, 0.25, 0.41 μg mL ⁻¹	Hemocytes↑, resistance against <i>Vibrio aestuarianus</i> ↑	Delaporte et al. (2006)
<i>Litopenaeus vannamei</i> , White leg shrimp	ARA, 0.15→2.1	Reproduction↑, larval survival↑	Xu et al. (2017b)
<i>Strongylocentrotus intermedius</i> , Sea urchin	ARA, 0→2.0	Optim. WG, gonad development @ 1.0	Zuo et al. (2018)
Fishes			
<i>Acipenser oxyrinchus</i> , Atlantic sturgeon	Artemia nauplii + PUFAs	Survival↑, WG↔	Kamaszewski et al. (2014)
<i>A. ruthenus</i> , Sterlet	Artemia nauplii + EPA21 + DHA14%tFAs	Survival↑, WG↔	Lundova et al. (2018)
<i>Anguilla japonica</i> , Japanese eel	ARA, 0→1.65	Respirat. burst↑; Optim. WG @ 0.92	Shahkar et al. (2016)
<i>Cynoglossus semilaevis</i> , Chinese tongue sole	ARA, 0→1.95	♂ gonad develop. ↑↑ ♀ gonad develop. ↑	Xu et al. (2017a)
<i>Dicentrarchus labrax</i> , European sea bass	ARA, 0→6	ARA ≤ 4 → pro-inflammatory genes↑ in DI	Rivero-Ramírez et al. (2020)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>E. lanceolatus</i> ♂, Hybrid grouper	ω3 PUFA, 0→2.5	Optim. WG↑, immunity↑, pathogen resistance↑ @ 1.5→1.7	An et al. (2020)
<i>Huso huso</i> , Beluga	Artemia nauplii + (EPA18% + DHA12%)	SGR↑, salinity/temperature stress resistance↔	Jalali et al. (2008)
<i>Lateolabrax japonicus</i> , Japanese sea bass	ARA, 0.1→2.1	@ 0.2→0.5: immunity↑, growth↑	Xu et al. (2010)
<i>Lutjanus malabaricus</i> , Malabar red snapper	ARA, 0.05→0.65	WG↔, muscle EPA↓, gill EPA↓	Chee et al. (2020)
<i>Megalobrama amblycephala</i> , Wuchang (blunt snout) bream	DHA, 0.8	Muscle fiber development↑	Wang et al. (2020b)

(continued)

Table 27.4 (continued)

Species, common name	PUFA, level (% dw)	Affected trait	References
<i>Morone saxatilis</i> , Striped bass	ARA, ~0.3→~0.8 in <i>Artemia</i>	Larval osmotic stress resistance↑	Harel et al. (2001)
<i>Rachycentron canadum</i> , Cobia	ARA, 0.5→1.9	Growth↑, ≥1.2→steatosis↑	Araújo et al. (2019)
<i>Salmo salar</i> , Atlantic salmon	EPA, DHA	EPA→DHA	Bou et al. (2017)
	DHA + EPA 0→1.4	Modulation of redox homeostasis and immunity	Xue et al. (2020)
<i>Seriola rivoliana</i> , Longfin yellowtail	DHA 0.3→3.2	Survival↑, stress resistance↑	Mesa-Rodriguez et al. (2018)
<i>Solea senegalensis</i> , Senegalese sole	ARA, 0.86 in <i>Artemia</i>	Proper skeletogenesis↑	Boglino et al. (2012)
<i>Sparus aurata</i> , Gilthead seabream	ARA/EPA/DHA ratio	Modulation of <i>elongases</i> expression; <i>Bacteroidetes</i> ↑, <i>Edw. Tarda</i> ↓	Magalhães et al. (2020a, 2020b)
<i>Tachysurus (Pelteobagrus) fulvidraco</i> , Yellow catfish	ARA, 0→1.3 (0.4→12.6 tFA)	Optim. WG, AOC @ 5.0→7.5 (tFA)	Ma et al. (2018)
<i>Trachinotus ovatus</i> , Golden pompano	DHA/EPA ratio	Optim. WG @ 1.4	Zhang et al. (2019)

↑ increase/lupregulation; ↔ no obvious effect; ↓ reduction/downregulation; WG weight gain; SGR specific growth rate; AOC antioxidant capacity; DI distal intestine; dw dry weight; tFAs total fatty acids

Chinese tongue sole, there occurs a gender-specific response upon dietary ARA supplementation.

27.2.2.4 The Janus Face of Dietary ARA

In tiger puffer, dietary ARA tends to induce dysfunction of the intestinal mucosal barrier and modulate the intestinal bacterial community (Yu et al. 2019). Even worse in Senegalese soles: high dietary ARA levels (1.2–1.7% dw) fed during early larval stages are mainly translated into a decrease in juvenile quality, since the animals exhibit a high incidence of pigmentary disorders (pseudo-albinism) and osteological anomalies related to eye migration and cranial remodeling during metamorphosis (Fig. 27.12) (Bogolino et al. 2014). Supplying high ARA amounts modifies the relative concentrations of prostaglandins that induced pigmentary disorders.

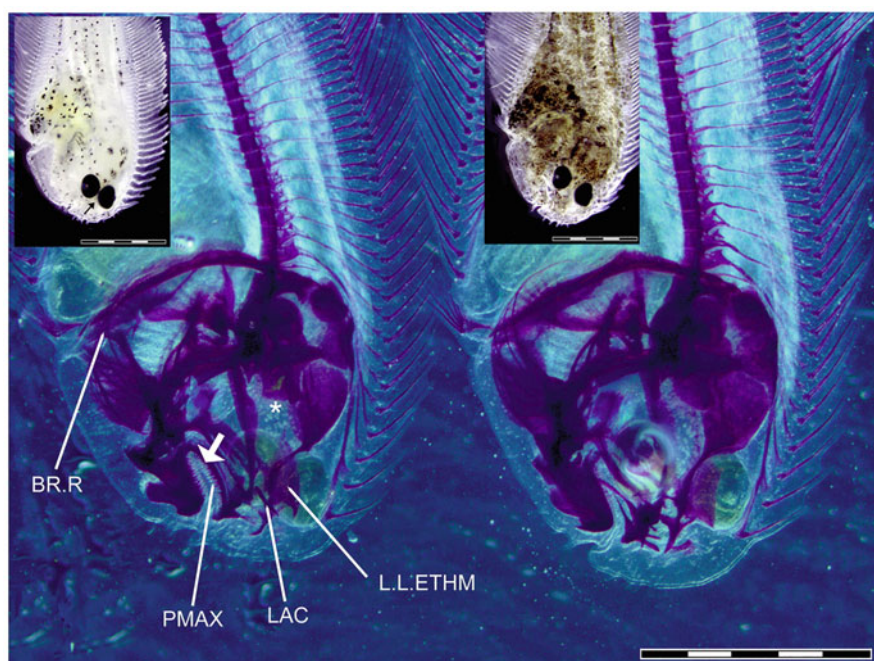


Fig. 27.12 Anterior ocular side of Senegalese sole (*Solea senegalensis*) early juveniles aged 50 days post-hatching. Inset images display a general view of a normally pigmented (right) and pseudo-albino (left) fish showing different pigmentary patterns and changes in head shape and in the interorbital distance (black arrow). Large images display cranial skeletal structures (alizarin red staining) in normally pigmented (right) and pseudo-albino (left) fish showing the most evident osteological differences between these fish. The asterisk indicates the underdevelopment of the sphenotic bone (SPOT) in pseudo-albino fish with regard to a normally pigmented specimen. Note the presence of teeth in right upper and lower jaws (white arrow) in the pseudo-albino specimen, as well as some skeletal disorders in order elements of the splanchnocranium and neurocranium. **BR.R** branchiostegal rays; **LAC** lacrimal; **L.L. ETHM** left lateral ethmoid; and **PMAX** premaxillary. Scale bar represents 2 mm (From Bogolino et al. (2014), with permission from Elsevier)

Overall, dietary ARA is Janus-faced. This statement finds confirmation. Koven et al. (2003) found that dietary ARA promotes survival in gilthead seabream encountering handling stress. In contrast, repetitive salinity change alters the nature of the stress response where dietary ARA appears to upregulate cortisol synthesis coinciding with reduced growth and increased mortality in *Sparus aurata* (Van Anholt et al. 2004).

However, there is considerable evidence that the importance of ARA as an EFA has been neglected in the past, in comparison to the $\omega 3$ PUFA, especially when considering the nutritional requirements of marine fishes (Bell and Sargent 2003). Dietary ARA reveals beneficial effects in several fish species: Tian et al. (2019) identified that dietary ARA decreases the expression of transcripts related to adipocyte development and chronic inflammation in the adipose tissue of juvenile grass carps. Moreover, ARA-mediated eicosanoids participate in the upregulation of immunity-related genes in the kidney (Tian et al. 2017). In the same line of evidence, Xu et al. (2018) showed that moderate levels of dietary ARA reduce lipid accumulation and inhibit cell cycle progression in the liver of Japanese sea bass (*Lateolabrax japonicus*).

Similarly, but on a low dietary ARA level, Torrecillas et al. (2017) found in European sea bass juveniles that ARA levels below 1% of total FAs reduce growth performance of juveniles. However, altered $\omega 3/\omega 6$ content affects its phagocytic capacity in relation to:

1. Variations on membrane fluidity, lipid raft formation, and cell signaling pathways due to changes in membrane phospholipid composition
2. Altered spectrum and amount of the prostaglandins produced, which in turn will affect not only the functionality of the head kidney leucocytes but also growth performance

Therefore, these results evidence the importance of an adequate supply of dietary $\omega 6$ LC-PUFA for European sea bass juveniles which maximizes the immune response.

Collecting further mosaic stones to complete the picture, also Norambuena et al. (2016) reported on an increased significance of dietary ARA in Atlantic salmon. The authors studied the impact of higher water temperatures which are encountered as a result of climate change and practice of farming of species in areas with environmental characteristics above the optimal physiological range of the fishes. This is, for instance, the case of Atlantic salmon farming in Australia during summer seasons. These fishes then show an increased dietary intake for ARA.

In addition, ARA supplementation in a plant protein-based diet fed to juvenile turbot improves growth and alleviates intestinal inflammation caused by dietary plant protein (Wei et al. 2021). Oyarzún-Salazar et al. (2021) found beneficial symptoms in the eurythermal sub-Antarctic Patagonian blenny (*Eleginops maclovinus*) reared at 25 °C. These authors, however, cannot judge whether the increased ARA levels in the muscles are the cause or the result of increased lethality of this fish at the supraoptimal temperature.

Despite the fact that the underlying mechanisms deserve future studies, supporting evidence of the significance of ARA is accumulating. Dietary ARA improves productive performance of striped bass (*Morone saxatilis*) juveniles under sub- to optimal temperatures (Araújo et al. 2021). Both ARA and DHA are needed at higher levels, especially when fishes are exposed to suboptimal temperatures: ARA is probably preferentially used to synthesize prostaglandins.

Transgenerational Effect

Recent studies show furthermore that dietary ARA has clear transgenerational effects: parental high dietary ARA levels modulate the hepatic transcriptome of adult zebrafish progeny (Adam et al. 2018). Volcano plots show a clear separation between the dietary groups in F_1 (Fig. 27.13). This is not as clear in the parental F_0 generation. It also shows a clear separation based on generation (F_0 vs. F_1). Twenty differentially expressed genes (DEGs) occur between control and high ARA in the F_0 generation (Fig. 27.13a) and 470 DEGs in F_1 generation (Fig. 27.13b). The parental diet modulates the transcription of a range of genes in the adult progeny connected to FA and retinoid metabolism, methionine cycle, transsulfuration pathway, and estrogen signaling. In a subsequent paper, Adam et al. (2019) proved that DNA methylation in zebrafish liver is at least one major mechanism underlying the transgenerational effect of dietary ARA. Whether these differences can be inherited to further generations is an area for further research.

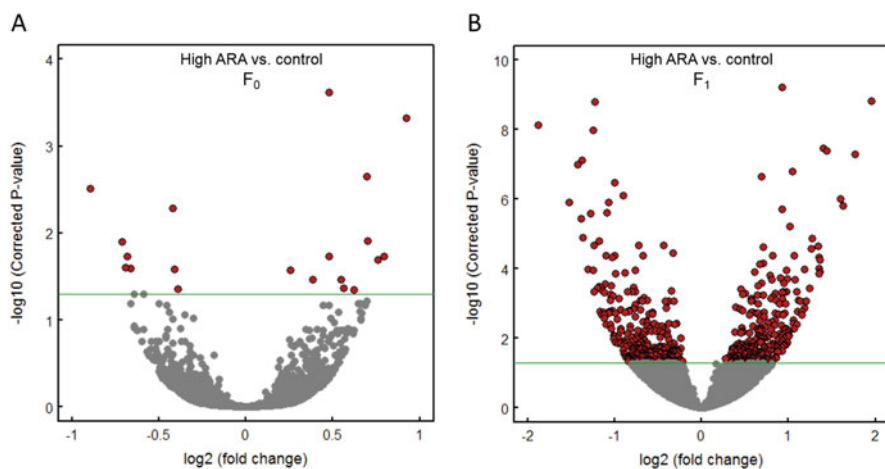


Fig. 27.13 Volcano plot of RNA-sequencing data from male livers in parental F_0 generation fed either a control or high ARA diet and their F_1 progeny where both groups received the control diet. Presented data represents overlapping genes from both RefSeq and Ensembl reference genome mapping (GRCz10). Red spots represent differentially expressed genes (DEGs) between control and high ARA group in F_0 (a) and F_1 (b) generation. The green line denoted the significance threshold (adjusted $P < 0.05$) for DEGs (From Adam et al. (2018), credit Public Library of Science)

27.3 Concluding Remarks

It is common knowledge that individual life history traits can be affected differently by different nutrients. However, information of how different chemical species of the same nutrient impact life history traits is rare. With EPA supporting egg production in *Daphnia*, Denoux et al. (2017) pointed out that the chemical speciation is an important issue to consider. This finding likely applies not only to PUFAs; rather, it should be taken as advice to focus future nutritional studies on chemical speciation of nutrients. Here, we may bet that a lot of the variability of nutritional studies with the same species and gross nutrient content derives from the neglect of chemical speciation of the nutrient.

Fortunately, epigenetics has finally moved into aquaculture and promises intriguing future results for sustainable management. The pioneering studies in zebrafish (Adam et al. 2018) and particularly in *S. aurata* (Perera et al. 2020) discovered that parental nutrition shapes the transcription of offspring’s genes central in lipid metabolism. This finding has potential to improve aquaculture practice in terms of producing fishes with desired lipid content and composition. Interesting questions emerge to be answered by studies to come:

- How many offspring generations can benefit from the designed parental nutrition by the shaped lipid metabolism?
- Which metabolic pathways in offspring can be further modulated by parental nutrition?
- Can this approach be transferred also to farmed invertebrates? Ecological knowledge of epigenetic heritage of stress resistance in cladocerans (Menzel et al. 2011; Suhett et al. 2011) encourages the transfer of this issue also to farmed animals.

Finally, it appears more appropriate to translate “Janus face” into the scientific term “hormesis” (Calabrese and Baldwin 2003). To low-dose dietary inclusion of, e.g., ARA and several other ingredients, hormesis appears to be the rule, rather than the exception to the rule, of dose–response (Calabrese 2020). Most of the beneficial effects of ARA referred to above have obviously been found in the low inclusion range and deserve further attention. Furthermore, the biomolecular mechanisms of hormesis are beginning to be understood (Celorio-Mancera et al. 2011; Steinberg et al. 2013; Rossnerova et al. 2020) and are worth being studied more intensively. This incentive includes that the dietary low inclusion levels need a much better fine-tuning than carried out in most current studies.

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Chapter 28

Biosynthesis of Polyunsaturated Fatty Acids—‘*Many Can, Some Can’t*’



Abstract Omega-3 (ω 3) long-chain polyunsaturated fatty acids (LC-PUFA) are well-known essential nutrients for vertebrates. A recent study identifies a high number of ω x desaturase sequences in a variety of invertebrates; consequently, the long-term consensus that solely marine microbes account for the ω 3 LC-PUFA production has to be revised by accepting that numerous abundant invertebrates have the autochthonous capacity to make significant contributions. Even many fish species appear capable of producing even DHA; it deserves further research of why the endogenous biosynthesis does not meet the corresponding demands. Moreover, it has to be evaluated if there are means to increase this biosynthesis by dietary or breeding means. The role of epigenetics in biosynthesis of LC-PUFAs is still in its infancy. However, emerging information on miRNAs indicates that they are central in regulating the biosynthesis of LC-PUFAs. The recent identification of miRNAs in the LC-PUFA biosynthesis is promising, and it can be expected that the catalog of miRNAs as well as target genes and pathway steps will be enlarged. Further epigenetic pathways will also move into the focus of scientific interest.

In the biosynthesis of complex lipids, the fatty acyl coenzyme A pool occupies a central position. Whether of dietary origin or produced de novo, all FAs form acyl-CoAs before being incorporated into membrane lipids. Essential polyunsaturated fatty acids (PUFAs) are produced by sequential microsomal desaturation and elongation of oleic acid (OA, 18:1 ω 9), linoleic acid (LIN—also LNA or LA, 18:2 ω 6), or α -linolenic acid (ALA, 18:3 ω 3), the parent FAs of the ω 9, ω 6, and ω 3 families (Bell et al. 1986) (\rightarrow Chap. 26, Fig. 26.1). As reviewed in previous chapters, the capability to synthesize long-chain PUFAs (LC-PUFAs) varies, due to the different feed source (Trushenski et al. 2020) and genetic background, and also between marine, freshwater, and migrating (anadromous, catadromous) fish species since the different habitats provide preys with different LC-PUFA patterns (Parzanini et al. 2020). Do similar differences also exist in the biosynthesis capability between herbivorous, carnivorous, and omnivorous species or between freshwater, brackish, and marine species? Recently, Monroig et al. (2018) and Kabeya et al. (2018a) reviewed a plethora of studies combining genomic and functional approaches which have illuminated vital aspects of PUFA desaturation and elongation with eventual impact

on nutritional strategies in farmed animals. These papers have the potential to shift current paradigms of PUFA biosynthesis and nutrition, respectively. Therefore, this chapter is going to highlight selected recent studies and compares fishes and invertebrates with emphasis on these reviews.

28.1 Invertebrates

In the freshwater crustacean *Daphnia pulex*, Schlechtriem et al. (2006) observed the conversion of C₁₈ FA precursors to eicosapentaenoic acid (EPA, 20:5 ω 3) and arachidonic acid (ARA, 20:4 ω 6) raised on PUFA-free diets pointing out a desaturase activity at the Δ^5 position, catalyzing the final step in the formation of EPA and ARA. This step can be attributed to FADS1 activity although the proofs of *fads1* or *fads2*¹ are yet lacking (Rivera-Pérez et al. 2020). In contrast, abundant *fads* mRNA are found in marine and brackish invertebrate species, such as the Chinese mitten crab (*Eriocheir sinensis*), Pacific whiteleg shrimp (*Litopenaeus vannamei*), and gazami crab (*Portunus trituberculatus*). In Porifera, only *fads2* is described in *Amphimedon queenslandica*, while only one *fads1* occurs in the genomes from ascidians (*Ciona intestinalis*) and cnidarians (*Nematostella vectensis*), as well as in nematodes and worms. However, from the cnidarian *Exaiptasia (Pallida) diaphana*, three copies of *fads1* are found (Rivera-Pérez et al. (2020) and references therein).

Monroig and Kabeya (2018) and Kabeya et al. (2018a) reported, in comprehensive papers, that (marine) invertebrates operate endogenous mechanisms for PUFA production different from that in vertebrates. These papers comprise methyl end (ω x, including Δ^6 and Δ^5) desaturases and show that invertebrates operate alternative and unusual pathways of PUFA biosynthesis involving gene families with complex phylogeny and functional diversity (Monroig and Kabeya 2018).

28.1.1 Methyl End (ω x) Desaturases in Invertebrates

Consensus was that marine microbes account for the production of ω 3 LC-PUFAs, given their possession of key enzymes including methyl end (or ω x) desaturases. As a kind of early hand waving indicating opposing phenomena, a number of LC-PUFA-rich copepods have been described as potential aquafeed: the marine cyclopoid *Acanthocyclops trajani* (Rahmati et al. 2020); the marine harpacticoids *Tigriopus californicus* (Kreeger et al. 1991), *Platychelipus littoralis* (Boyen et al.

¹*fads1*, *fads2*: fatty acid desaturase 1, encoding FADS1 (Δ^5 desaturase), and fatty acid desaturase 2, encoding FADS2 (Δ^6 desaturase). FADS2 is named so because it was thought to convert only ω 6 FAs but actually converts also some others. It is obligatory to build the longer chain ω 3 FAs from other simpler FAs (Guillou et al. 2003).

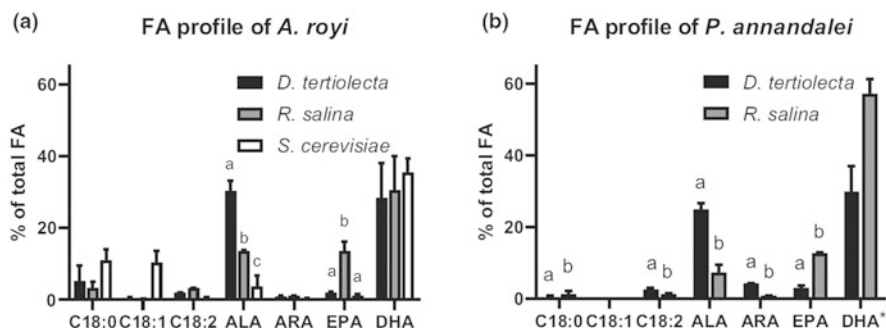


Fig. 28.1 Fatty acid profile of (a) *Apocyclops royi* fed *Dunaliella tertiolecta*, *Rhodomonas salina*, or *Saccharomyces cerevisiae* and (b) *Pseudodiaptomus annandalei* fed *D. tertiolecta* or *R. salina*. Samples of 40 individuals of *A. royi* and 20 individuals of *P. annandalei* of adult and advanced copepodites were collected in triplicates. The columns represent mean \pm S.D., $n = 3$. For (a) One-way ANOVA and Tukey's test were performed for comparisons, and for (b) t-tests were performed for all comparisons except for nonparametric data sets (*), for which Mann–Whitney U test was performed. Significant differences are denoted with letters ($P < 0.05$) (From Nielsen et al. (2021), with permission from Wiley)

2020), and *Microarthridion littorale* (De Troch et al. 2012); and the freshwater harpacticoid *Attheyella trispinosa* (Caramujo et al. 2008). More so-called self-enriching brackish planktonic copepods are described: *Pseudodiaptomus annandalei* (Rayner et al. 2017; Nielsen et al. 2020, 2021) and *Apocyclops royi* (Pan et al. 2017; Nielsen et al. 2020, 2021) are known as prominent feed for fish larvae (Doi et al. 1997; Liao et al. 2001). These copepods are rich in ω 3 LC-PUFAs and indicate the general potential of invertebrates to biosynthesize these essential FAs and provide sufficient resources for higher trophic levels. In fact, independent of LC-PUFA patterns in food microorganisms, these copepods biosynthesize high amounts of DHA (Fig. 28.1). From the food sources in this trial, only *Rhodomonas salina* contains detectable amounts of DHA (Nielsen et al. 2021).

A powerful innovation, Kabeya et al. (2018a) discovered 121 ω x desaturase sequences from 80 species within Cnidaria, Rotifera, Mollusca, Annelida, and Arthropoda (Fig. 28.2b). Horizontal gene transfer has contributed to this hitherto overlooked widespread distribution. Functional characterization of animal ω x desaturases provides evidence that multiple invertebrates have the ability to produce ω 3 PUFA de novo and further biosynthesize ω 3 LC-PUFAs. This finding represents a fundamental revision in our understanding of ω 3 LC-PUFA production in global food webs, by revealing that numerous widespread and abundant invertebrates have the endogenous capacity to make significant contributions in addition to that coming from marine microbes.

This also implies that this study contradicts the currently accepted paradigm (Pereira et al. 2003; Castro et al. 2016; Hixson and Arts 2016), because it shows unequivocally that desaturase activities are present in multiple metazoans, enabling them to endogenously produce ω 3 PUFA de novo (Fig. 28.2a). One of the ω x desaturases described in cnidarians, mollusks, annelids, and crustaceans is able to produce a variety of ω 3 LC-PUFA from their corresponding ω 6 precursors, in addition to 18:3 ω 3, confirming that these enzymes are true ω 3 desaturases with

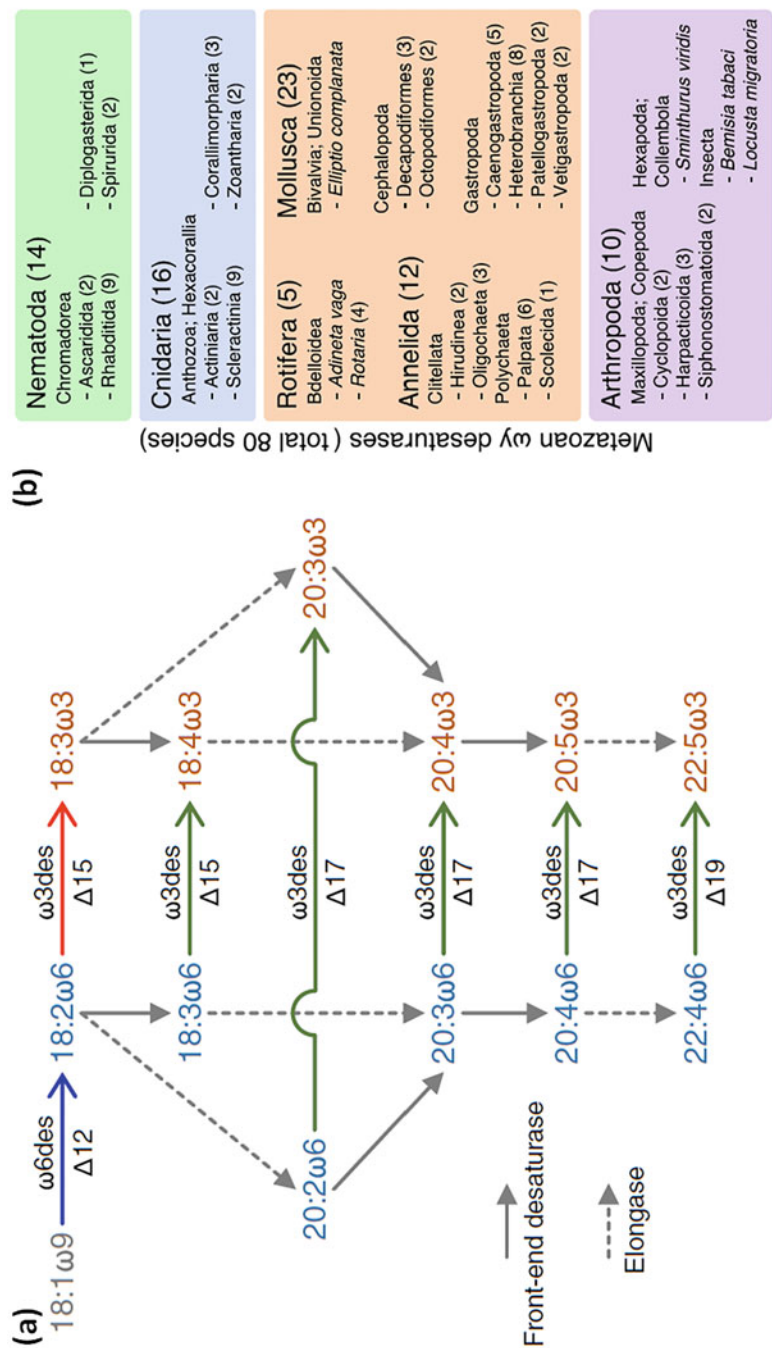


Fig. 28.2 (a) Functional characterization of metazoan ω desaturases. The de novo production of ω3 PUFA requires both ω6 (Δ¹²) (To recall: Δ indicates that the double bond is created at a fixed position from the carboxyl end of a fatty acid chain. ω counts the number of carbon atoms from the methyl end of the chain to the first double bond) (blue arrow) and ω3 (Δ¹⁵) desaturases (red arrow). Both LIN (18:2ω6) and ALA (18:3ω3) can be subsequently modified through the ω6

and $\omega 3$ long-chain PUFA biosynthesis pathways that proceed separately, at least in vertebrates, or can be interconnected by $\omega 3$ desaturases with Δ^{15} , Δ^{17} , or Δ^{19} activities (green arrows). (b) Summary of taxonomic groups highlighting metazoans in which ωx desaturases have been identified. Number of species is indicated in brackets (From Kabeya et al. (2018a), credit American Association for the Advancement of Science)

coexisting Δ^{15} , Δ^{17} , and Δ^{19} activities. This represents LC-PUFA synthesizing capabilities previously unknown outside microbes (Kabeya et al. 2018a).

Furthermore, the study shows that aquatic invertebrates now can be recognized as net producers of $\omega 3$ LC-PUFA, providing a much more widespread biosynthesis of these compounds in the marine environment than previously thought. Consequently, models that estimate global production of $\omega 3$ LC-PUFA (Budge et al. 2014; Hixson and Arts 2016), which are constructed on the assumption that single-cell microorganisms are the sole primary producers, need extensive supplementation or even revision (\rightarrow Chap. 30).

Although ωx desaturases are widespread among invertebrates, it is uncertain whether the bioconversion rates suffice to fully meet the physiological demands of all consumers of higher trophic levels. This concern gets support by previous studies reporting inconsistent results in invertebrates. A reevaluation seems inevitable. For instance, Goedkoop et al. (2007) found that larval *Chironomus riparius* can grow and develop well without dietary sources of EPA and ARA and with only very low levels of their precursors ALA and LIN, respectively. These results indicate that this species does not depend on dietary sources of EPA and ARA but apparently has the physiological capacity of synthesizing these FAs from shorter homologs. In *Sinonovacula constricta*, however, the first marine mollusk shown to possess all FAD and ELOVL activities required for LC-PUFA biosynthesis via the Sprecher² pathway (Sprecher et al. 1995; Ran et al. 2019), even the highly expressed FAD and ELOVL capabilities do not meet its ontogenetic requirement (Ran et al. 2018, 2020). Dietary DHA supply is still required.

$\omega 3$ LC-PUFA production by metazoans will undoubtedly be significant due to the abundance the animals have on the global scale. This emerging paradigm, however, requires future refinement studies. Nevertheless, it has already stimulated a number of studies which confirm the paradigm: in the sea urchin *Strongylocentrotus intermedius* (Wang et al. 2019), in the rotifer *Brachionus koreanus* (Lee et al. 2019), in the harpacticoid copepod *Tigriopus japonicus* (see below) (Lee et al. 2020b), in the Antarctic copepod *Tigriopus kingsejongensis* (Lee et al. 2020a), in the cephalopod *Octopus vulgaris* (Garrido et al. 2019b), or in the orange mud crab (*Scylla olivacea*) (Ting et al. 2020).

And Kabeya et al. (2020) continue their crusade for invertebrates as net LC-PUFA producers by demonstrating that the common ragworm (*Hediste (Nereis) diversicolor*) possesses two functional ωx enzymes with distinct desaturase capacities, $\omega x1$ and $\omega x2$. $\omega x1$ is an enzyme enabling de novo biosynthesis of C_{18} PUFA from OA and shows the ability to desaturate $\omega 9$ and $\omega 6$ substrates into $\omega 6$ and $\omega 3$ PUFA products, respectively. This points out that this enzyme operates $\omega 6$ and $\omega 3$ desaturase regioselectivities simultaneously. On the other hand, $\omega x2$ is an $\omega 3$ desaturase and enables the biosynthesis of the physiologically essential EPA and DHA. Taken together, this study illustrates that the LC-PUFA biosynthetic enzyme machinery of aquatic invertebrates is highly diverse.

²For more details, \rightarrow AAN III “Fish Oil Replacement—Terrestrial Oils.”

28.1.1.1 Dietary Poor Environment

Marine and freshwater harpacticoid copepods, living in dietary poor environments, are able to upgrade PUFAs in sufficient quantities. To demonstrate this capacity, Lee et al. (2020b) compared the harpacticoid *Tigriopus japonicus* with the planktonic copepod *Paracyclopina nana* with only limited capacity to biosynthesize LC-PUFAs (Figs. 28.3 and 28.4). Both copepods are fed four different microalgae (*Tetraselmis suecica*, *Isochrysis galbana*, *Chlorella* sp., and *Nannochloropsis oculata*). The FA profile of each algal species is depicted in Fig. 28.3; *Chlorella* lacks any LC-PUFA. Both copepods successfully synthesize various types of PUFAs. However, the class of FA conversion genes is different, and the transcript

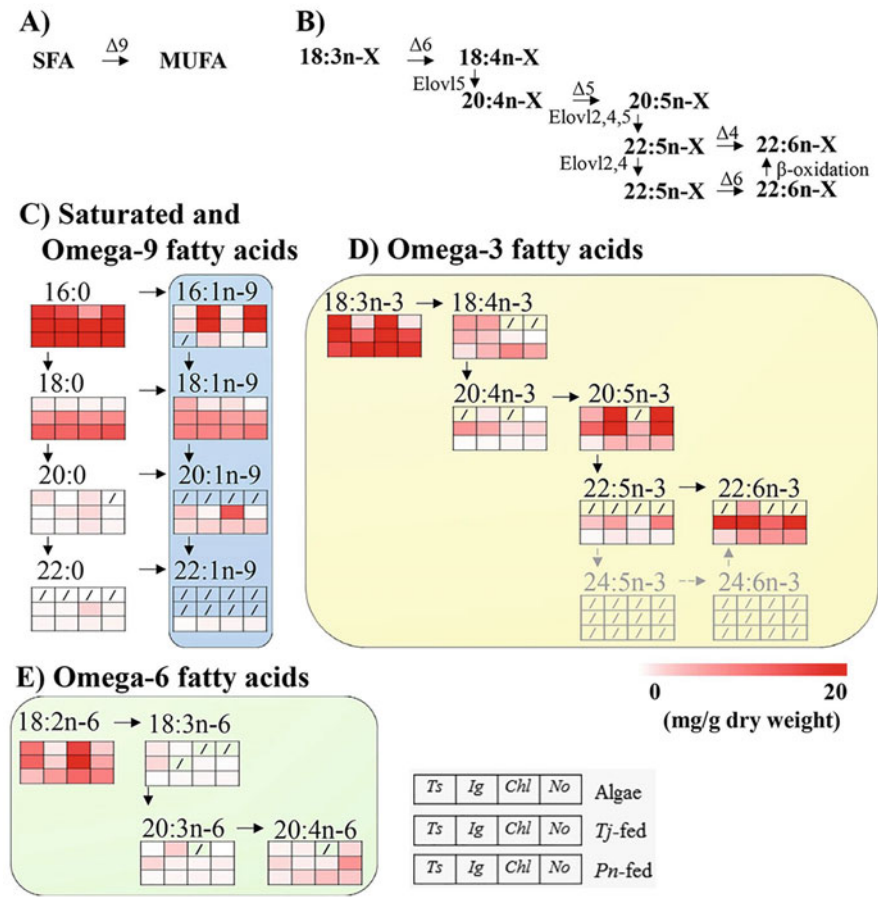


Fig. 28.3 (a) Biosynthetic pathways of monounsaturated fatty acid synthesis from saturated fatty acids. (b) Biosynthetic pathways of $\omega 3$ and $\omega 6$ fatty acids (modified from Monroig et al. (2013)). Single fatty acid profiles of (c) saturated and $\omega 9$ fatty acids, (d) $\omega 3$ fatty acids, and (e) $\omega 6$ fatty acids represented by a heat map. *Ts* *Tetraselmis suecica*; *Ig* *Isochrysis galbana*; *Chl* *Chlorella* sp.; *No* *Nannochloropsis oculata* (From Lee et al. (2020b), with permission from Elsevier)

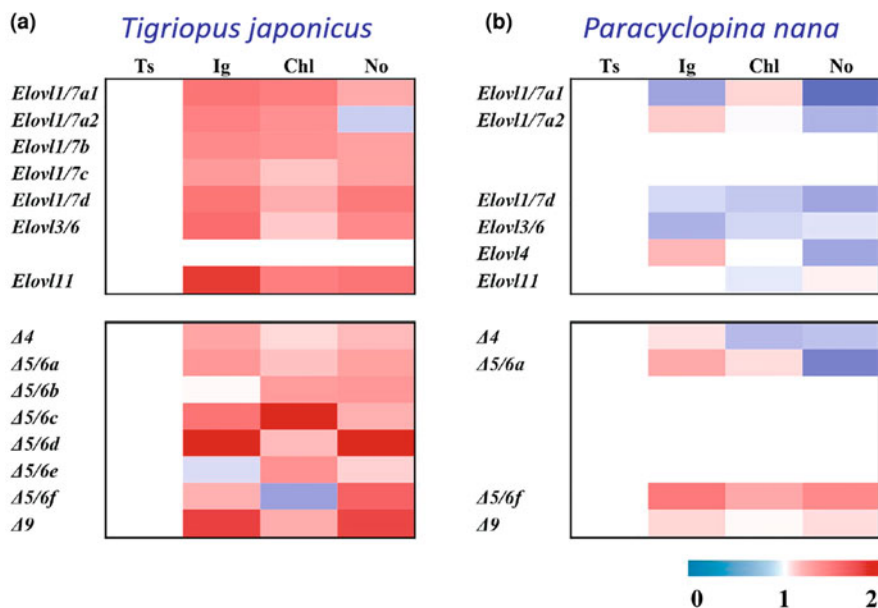


Fig. 28.4 Transcriptional profile of lipid metabolism-related genes in response to each microalga for 24 h as represented by a heat map: (a) *Tigriopus japonicus* and (b) *Paracyclops nana*. *Elovl* elongation of very-long-chain fatty acids; *Fads* fatty acid desaturase. For names, see Fig. 28.3 (From Lee et al. (2020b), with permission from Elsevier)

level shows different trends according to each microalgal species in the two copepods. Although dietary *Chlorella* does not provide ARA, EPA, or DHA, both copepods have these LC-PUFAs in their body lipids indicating that the animals are able to biosynthesize them. However, the content in *T. japonicus* is significantly higher than in the pelagic copepod. The improved biosynthesis capacity is based on the much higher diverse desaturation and elongation genes in *T. japonicus* compared to *P. nana* (Fig. 28.4). In *T. japonicus* and *P. nana*, seven (*elovl1/7a1*, *a2*, *b*, *c*, *d*, *elovl3/6*, and *elovl11*) and six (*elovl1/7a1*, *a2*, *d*, *elovl3/6*, *elovl4*, and *elovl11*) elongase of VLC-FAs (*elovl*) genes³ and eight ($\Delta^{5/6}a$, *b*, *c*, *d*, *e*, *f*, Δ^4 , and Δ^9) and only four ($\Delta^{5/6}a$, *f*, Δ^4 , and Δ^9) *fads* genes are identified, respectively (Fig. 28.4).

³*Elovl1* elongates saturated and monounsaturated C₂₀–C₂₆, *elovl2* C₂₀–C₂₂ PUFAs, *elovl3* saturated and unsaturated C₁₆–C₂₂, *elovl5* C₁₈–C₂₀ PUFAs, *elovl6* C₁₂–C₁₆, *elovl7* saturated and unsaturated C₁₆–C₂₂, *elovl4* synthesizes C_{≥26} (Monroig et al. 2010). Recent studies on invertebrate species have reported a variety of novel ELOVL classes. For example, two novel ELOVL classes (ELOVL A and B) were found in the order Actinaria by transcriptome assembly analysis of *Actinia tenebrosa*, *Telemactis* sp., and *Nemanthus annamensis* (Surm et al. 2018). Two novel ELOVL classes (ELOVL9 and 10) have also been identified in rotifers in the genus *Brachionus* (Lee et al. 2019). Phylogenetic analysis revealed a novel ELOVL class (ELOVL11) in *Tigriopus* (Lee et al. (2020a), references added). The exact function of ELOVL11 remains to be determined. Moreover, two *elovl8* genes have been identified in rabbitfish, which are unique to teleosts. Only ELOVL8B retains the ability to elongate C₁₈ and C₂₀ PUFAs to longer-chain FAs (Fig. 28.9) (Wen et al. 2020).

A proof of the hypothesis, the euryhaline rotifer *B. koreanus*, living in rather food-rich environments, behaves similar to *P. nana*: it is able to biosynthesize even DHA, however, only in very small traces (Lee et al. 2019).

28.1.2 Elongases in Invertebrates

Based on only a few studies with consistent results, it appears that the expression of FA elongases in invertebrates is nutritionally regulated (Table 28.1). These studies show that the elongase machinery is switched on, if the diet is devoid of LC-PUFAs, and vice versa, it is turned off, if the dietary supply suffices.

This impression of dietary regulation of the elongation pathway is consolidated by a recent study in orange mud crab (*Scylla olivacea*). Ting et al. (2020) functionally characterized two elongases. *Elov14* is expressed mainly in the hepatopancreas and gill tissues, while *elov16* predominates in digestive tissues. Collective data imply the contribution of these elongases in the elongation of C₁₈ PUFA to C₂₂ PUFA substrates during the limited dietary LC-PUFA intake. The uniqueness of *S. olivacea* ELOVL6 as a multifunctional elongase is sketched in Fig. 28.5.

In invertebrates, peculiar pathways of PUFA biosynthesis, such as the Δ⁸ pathway, have been described and even more can be expected when more invertebrate

Table 28.1 Response of elongase genes in selected aquatic invertebrates to LC-PUFAs provision, including newly identified elongase genes

Species	Elongase	Response	References
<i>Actinia tenebrosa</i>	NOVEL ELOVLA. ELOVLB		Surm et al. (2018)
<i>Apostichopus japonicus</i>	ELOVL5	LC-PUFAs↓→ <i>elov15</i> ↑	Zhao et al. (2019)
<i>Brachionus koreanus</i>	ELOVL2/5, ELOVL3/6, ELOVL9, ELOVL10	LC-PUFAs↓→ <i>elov1s</i> ↑ VLC-PUFA de novo	Lee et al. (2019)
<i>Nemanthus annamensis</i>	NOVEL ELOVLA. ELOVLB		Surm et al. (2018)
<i>Portunus trituberculatus</i>	ELOVL4	LC-PUFAs↑→ <i>elov14</i> ↓	Sun et al. (2020b)
<i>Scylla olivacea</i>	ELOVL4, ELOVL6	LC-PUFAs↑→ <i>elov14</i> ↓, <i>elov16</i> ↓	Ting et al. (2020)
<i>Scylla paramamosain</i>	ELOVL4-like	LC-PUFAs↑→ <i>elov14</i> ↓	Lin et al. (2018)
<i>Sinonovacula constricta</i>	ELOVL2/5, ELOVL4A, ELOVL4B, ELOVLC	LC-PUFAs↑→ <i>elov1s</i> (↓)	Ran et al. (2020)
<i>Strongylocentrotus intermedius</i>	ELOVL4, ELOVL5	LC-PUFAs↑→ <i>elov14</i> ↓, <i>elov15</i> ↓ during embryonic development	Ding et al. (2019)
<i>Telematactis</i> sp.	Novel ELOVLA. ELOVLB		Surm et al. (2018)

↑ upregulation; ↓ downregulation



Fig. 28.5 Proposed LC-PUFA biosynthetic pathway in *Scylla olivacea*. Enzymes in black and bold letters represent confirmed existing routes in the pathway, while enzymes in gray letters represent undiscovered enzymes (From Ting et al. (2020), with permission from the American Chemical Society)

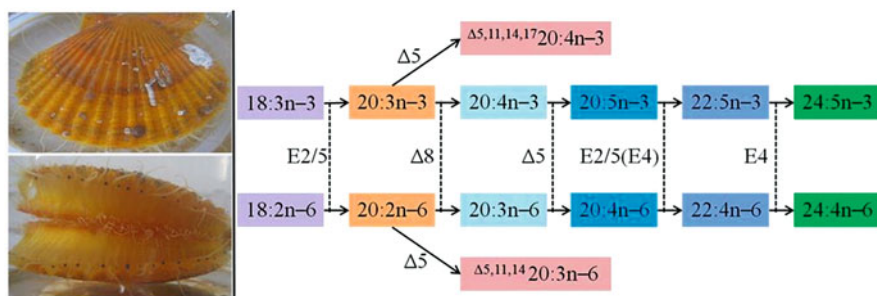


Fig. 28.6 Δ^8 pathway PUFA biosynthesis pathway exemplified in the noble scallop *Chlamys nobilis* (From Liu et al. (2014), credit American Chemical Society)

species will be studied. Biosynthesis of EPA and ARA from precursors adopts the major route of sequential Δ^6 -desaturation—elongation— Δ^5 -desaturation procedures. Alternatively, in the Δ^8 pathway, Δ^6 Fad is replaced by Δ^8 Fad. As a result, EPA and ARA are produced via sequential elongation— Δ^8 -desaturation— Δ^5 -desaturation steps (Fig. 28.6) (Park et al. 2009).

Mollusks possess a particular group of PUFAs called non-methylene-interrupted (NMI) FAs that can be biosynthesized endogenously. Monroig et al. (2013) reviewed that two distinct (ω x) desaturases account for the characteristic $\Delta^{5,9}$ unsaturation pattern of NMI FAs found in a wide range of mollusks. In noble scallop (*Chlamys nobilis*), Liu et al. (2014) showed that this species has the capability to endogenously produce EPA and ARA from PUFA precursors but limited ability to synthesize DHA from EPA. Preference of PUFA biosynthesis enzymes indicates that noble scallops rely on the dietary supply of ω 3 PUFA. In detail, a newly cloned elongase can elongate ARA and EPA to C_{22} and C_{24} , while the newly cloned scallop *fad* has Δ^8 desaturation activity and desaturates PUFA dietary 20:3 ω 3 and 20:2 ω 6 to 20:4 ω 3 and 20:3 ω 6, respectively (Fig. 28.6), providing the first compelling evidence that noble scallop can de novo biosynthesize EPA and ARA through the Δ^8 pathway. Also the purple sea urchin (*Paracentrotus lividus*) possesses desaturases that account for all the desaturation reactions required to biosynthesize EPA and ARA through the Δ^8 pathway (Kabeya et al. 2017b).

Zhukova (2019) described another quasi-endogenous source of unusual PUFAs, namely, the symbiosis with bacteria, exemplified in mollusks. Symbiotic associations between mollusks and microorganisms result in unique ecological strategies and increased metabolic diversity of the partners. Symbiotic microbes typically supply nutrients to hosts that provide shelter to the microbes. Bacteria are known to produce various odd and branched FAs (OBFAs) named “bacterial acids.” Accordingly, elevated concentrations of specific OBFAs, 16:1 ω 7 and 18:1 ω 7, coupled with a considerable reduction in ω 3 and ω 6 PUFAs produced by algae in lipids of the animals indicate a contribution of bacteria to the mollusks’ nutrition (Fig. 28.7). Symbiont-free mollusks (*Reata pulchella*, *Theora lubrica*) show the common LC-PUFA pattern with algae-derived ARA, EPA, and DHA dominating.

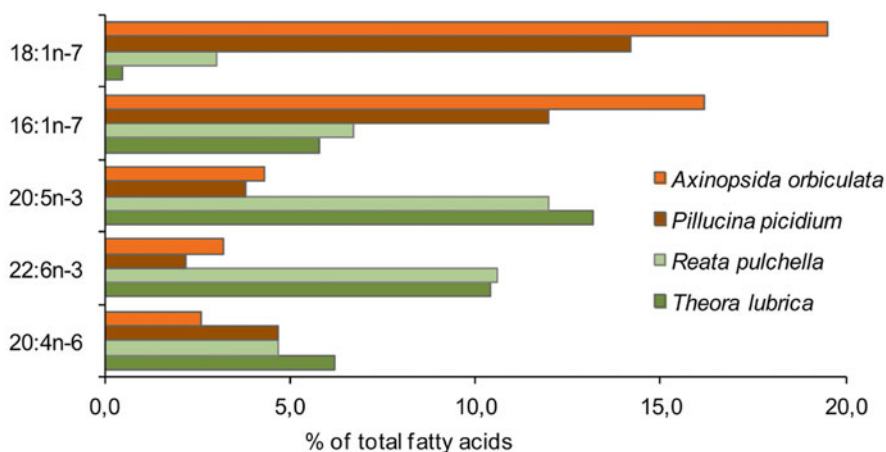


Fig. 28.7 Distribution of the most remarkable marker fatty acids of bacterial symbionts in the bivalve mollusks *Axinopsida orbiculata* and *Pillucina picidium*, containing sulfate-reducing symbiotic bacteria, and the symbiont-free bivalves, *Reata pulchella* and *Theora lubrica* (From Zhukova (2019), credit MDPI)

28.2 Fishes

Fish species differ in their capacity for endogenous synthesis of LC-PUFAs from dietary precursors (ALA, LIN). The potential of this biosynthesis is associated with the complement and function of *fads* and *elovl* genes it possesses. Teleosts possess only *fads2* genes in their genomes analyzed to date, and their number varies among lineages, from one to four paralogs (Castro et al. 2016). However, the only *fads1* reports for teleosts are from Japanese eel (*Anguilla japonica*) and Atlantic salmon (*Salmo salar*) (Rivera-Pérez et al. 2020). Very recently, a *fads6* gene has been identified in golden pompano (*Trachinotus ovatus*) (Zhu et al. 2019b) with $\Delta^4/\Delta^5/\Delta^8$ desaturase activity.

In contrast to freshwater fishes, many marine fishes lack the enzyme systems to elongate and desaturate C₁₈ FA or possess only a reduced capability (Ghioni et al. 1999). Consequently, marine fishes require EPA and DHA, whereas freshwater species have sufficient Δ^5 and Δ^6 desaturase and elongase activities to synthesize LC-PUFA. Thus, their requirements of PUFAs are met by ALA and LIN (Sargent et al. 2002). One reason behind this difference lies in their diet; marine food sources are richer in LC-PUFAs than freshwater sources. Already some eight decades ago, this phenomenon has been reported (Lovern 1935).

Supporting “classical” line of evidence, Li et al. (2008) showed that the euryhaline rabbitfish *Siganus canaliculatus* can convert LIN and ALA into LC-PUFA. Importantly, this activity is stronger in low than high salinities. Xie et al. (2015) confirmed these results by identifying *ex novo* production of LC-PUFA and expression of Δ^4 , Δ^6/Δ^5 *fad* and *elovl5* genes, pointing out that lower salinity enhances net accretion of LC-PUFA by improving *in vivo* desaturase and elongase activity and upregulating of hepatic Δ^4 , and Δ^6/Δ^5 *fads*.

In addition to the fact that the above dichotomy has been established based on a limited number of species, other confounding factors for LC-PUFA biosynthesis beyond trophic habit have been explored in recent years. Among them, the trophic level, the position of an organism within the food web, the “trophic ecology,” and diadromy have been also hypothesized as potential drivers modulating the range of desaturation and elongation capabilities in teleosts and thus their ability for LC-PUFA biosynthesis. It has become obvious that the capability for LC-PUFA biosynthesis in teleosts is more diverse than in other vertebrate groups and is possibly the result of a combination of factors that interact throughout the evolutionary history of each particular group or species (Fonseca-Madrigal et al. 2014). Recently, a meta-analysis by Trushenski and Rombenso (2020) claims that the trophic position of a fish species predicts the nutritional essentiality of PUFAs, rather than its habitat. However, Parzanini et al. (2020) reconfirmed that discrimination between freshwater and marine fish is feasible using FA profiles (\rightarrow Chap. 27); the found FA profiles are not necessarily completely of dietary or endogenous biosynthesis origin.

Noteworthy, also Li et al. (2017) confirmed the feedback between feed and expression of the genes and enzymes in large yellow croaker (*Larimichthys crocea*)

involved in the biosynthesis of very long unsaturated FAs: The higher the dietary doses of LC-PUFAs, the lower the expression of the corresponding genes, particularly *evolv4* and *evolv5*. This finding supports previous studies in striped snakehead (*Channa striata*) (Kuah et al. 2015), orange-spotted grouper (*Epinephelus coioides*) (2017), *Solea senegalensis* (Morais et al. 2015), *Cyprinus carpio* var. Jian (Ren et al. 2012), and nibe croaker (*Nibea mitsukurii*) (Yamamoto et al. 2010). Furthermore, functional studies also show that ELOVL4 and ELOVL5 of *Larimichthys crocea* have complementary functions since the latter can effectively elongate C₁₈ and C₂₀ PUFA substrates, while ELOVL4 is more effective in the elongation of C₂₀ and C₂₂ FA substrates (Li et al. 2017).

Combinations of certain desaturases and elongases enable several fish species to use vegetable oil (VO) for LC-PUFA biosynthesis. Oboh et al. (2016) found that the North African catfish (*Clarias gariepinus*) possesses *fads2* and *elovl2*. The two enzymes, and the previously reported ELOVL5, enable this catfish to carry out all desaturation and elongation reactions required for endogenous LC-PUFA synthesis from the C₁₈ precursors ALA and LIN, indicating that this species has the ability to effectively utilize VO rich in C₁₈ PUFA to satisfy essential LC-PUFA requirements. Continuing their catfish study, Oboh et al. (2017b) showed that this species possesses also two distinct *elovl4*-like elongases that participate in the biosynthesis of both very-long-chain saturated and polyunsaturated FAs.

In the same line of evidence, Giri et al. (2016) reported that also Atlantic salmon has the capability to biosynthesize DHA; however, the proper functioning of the enzyme systems is strongly dependent on micronutrients, such as Fe, Zn, Mg, and riboflavin, biotin, and niacin. Reduced dietary inclusion of these micronutrients significantly impairs the normal ω 3 LC-PUFA biosynthetic capabilities but not the transcription of corresponding genes. It can be predicted that the finding about this crucial role of micronutrients also applies to further marine fish species, which possess corresponding genes but lack enzyme activity.

Many studies have focused on FAD2 (Δ^6 Fad), since it is the rate-limiting enzyme involved in the PUFA biosynthesis from precursors: in Atlantic salmon (Xue et al. 2015), common carp (Ren et al. 2013), rainbow trout (Gregory et al. 2016), or channel catfish (Santerre et al. 2015). Vagner and Santigosa (2011) summarized that fish desaturases have preferences for ω 3 substrate. This is important in the context of the substitution of fish oils (FOs) with VOs in the diets of farmed fish. *Fads2* transcription and enzymatic activity are higher in fish fed VO diets (often rich in ALA) (Gregory et al. 2016) than in those fed FO diets, irrespective of their life cycle in seawater or freshwater. However, an excess of dietary ALA can block the *fads2* transcription (Bell et al. 1993; Izquierdo et al. 2008). For instance, in Nile tilapia, Chen et al. (2013) showed that dietary ALA above ~0.6% results in reduced growth performance.

C₁₈ FAs act as substrates for elongation. Collectively, previous studies have indicated that *fads* gene expression and enzymatic activity vary with dietary ALA/LIN (18:3 ω 3/18:2 ω 6) ratio (Xie et al. 2014, 2016). On high dietary ratios of ALA/LIN, upregulation of *fads2* transcription, for instance, is detected in *Siganus*

fuscescens, *Maccullochella peelii*, *Oncorhynchus mykiss*, and *Scatophagus argus* (Zhu et al. 2019a).

Unlike desaturases, there is a lack of data on the influence of dietary ALA/LIN ratios on the elongase expression. Xie et al. (2016) showed that the expression of *elovl4* and *elovl5* is significantly affected by dietary FA composition. In *Scatophagus argus*, the highest expression of mRNA occurs in liver on an ALA/LIN ratio of 1.7:1. In contrast, Zhu et al. (2019a) did not find any relationship between the expression of *elovl4a* and dietary FA composition in golden pompano (*Trachinotus ovatus*). Usually, the upregulation of at least one elongase gene in response to LC-PUFA-deficient diets can be expected. For instance, in *Solea senegalensis* and *Sparus aurata*, Torres et al. (2020a, b) detected that only *elovl4a*, but not *elovl5* and *elovl4b*, responds by upregulation if the diets are deficient in EPA and DHA. However, in a companion paper, the same laboratory found in Atlantic bluefin tuna (*Thunnus thynnus*) that the transcription of *elovl4* and *elovl5* decreases on an EPA- and DHA-poor diet (Betancor et al. 2020). This indicates that studies on the expression of FA elongases in response to dietary levels of LC-PUFA have yielded inconsistent results so far (Leaver et al. 2008; Tocher 2010; Monroig et al. 2018).

28.2.1 Herbivores

The rabbitfish *Siganus canaliculatus* is the first marine teleost proven to have the ability for biosynthesis of LC-PUFA from C₁₈ PUFA precursors. Two fatty acid desaturases 2 (Δ^4 FADS2 and Δ^6/Δ^5 FADS2) and two elongases (ELOVL4 and ELOVL5) are identified (Monroig et al. 2012; Garrido et al. 2019a). The activities of these enzymes enable this species to perform all elongation reactions required for the biosynthesis of the essential C_{20–22} LC-PUFA including EPA, ARA, and DHA, as well as the less common VLC-FAs (>C₂₄).

Until recently, the ontogenetic initiation of LC-PUFA biosynthesis in *S. canaliculatus* was unknown. Therefore, You et al. (2017) measured the gene expression of the four key enzymes for LC-PUFA biosynthesis (Δ^5/Δ^6 *fads*, Δ^4 *fads*; *elovl4*, *elovl5*) during the embryonic and unfed larval development as well as the FA composition of embryos. Maternal DHA is the primary FA utilized at the zygotic stages. The mRNA expression of the four key genes is found from 8 hpf onward, with a low level of *elovl5* mRNA contrary to the high levels of Δ^4 *fads* mRNA. Subsequently, the elevated expression of Δ^5/Δ^6 *fads* and two elongase genes occurs after hatching, and then a great increment in the expression of all four key genes is detected at 3–4 dph (Fig. 28.8). These findings indicate the production of DHA from EPA via the “ Δ^4 Fads” pathway (elongation of EPA to 22:5 ω 3 followed by direct Δ^4 desaturation to DHA) as well as the alternative “Sprecher’s shunt” (production of DHA from EPA via a C₂₄ intermediate) at the yolk sac larval stages. These two alternative pathways of DHA biosynthesis are widespread among teleost fish (Oboh et al. 2017a). Furthermore, the entire LC-PUFA biosynthetic pathway of converting

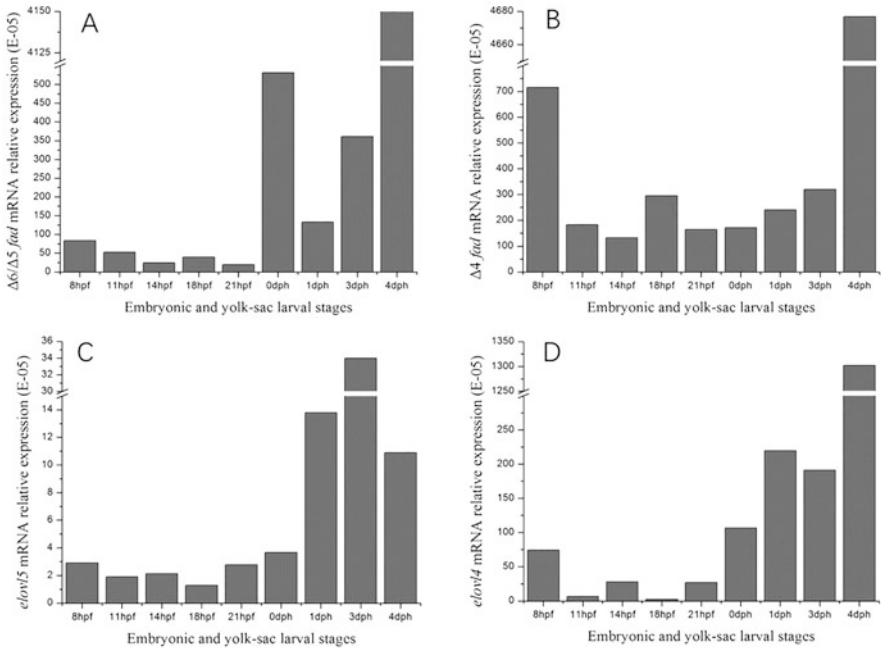


Fig. 28.8 Determination of the temporal expression patterns of Δ^6/Δ^5 *fad* (a), Δ^4 *fad* (b), *elovl5* (c), and *elovl4* (d) during embryonic and yolk sac larval development in *Siganus canaliculatus* by qPCR. At each stage, the relative mRNA levels of target genes were normalized with β -actin expression which was expanded to 10^5 -fold for comparison. **hpf** hours postfertilization; **dph** days post-hatching (From You et al. (2017); with permission from Elsevier)

C_{18} PUFA (ALA) precursors to DHA is activated just after the onset of first feeding by remarkably upregulating the expression of all four key genes.

Progress discovers increasing numbers of details about desaturases and elongases in rabbitfish. Wen et al. (2020) identified a novel *elovl4a*, which is primarily distributed in the brain, eye, and gonad. Nutritional and osmotic factors can regulate the transcription of *elovl4a* in the brain. In a companion paper, Li et al. (2020) identified additional *elovl8a* and *elovl8b* genes from rabbitfish. The *elovl8* genes are unique to teleosts. Rabbitfish ELOVL8B retains the ability to elongate C_{18} (18:2 ω 6, 18:3 ω 3, 18:4 ω 3) and C_{20} (20:4 ω 6, 20:5 ω 3) PUFAs to longer-chain FAs, whereas ELOVL8A has lost this ability. ELOVL8B is a new member of ELOVL protein family central in an alternative pathway for LC-PUFA biosynthesis (Fig. 28.9) (Wen et al. 2020).

28.2.1.1 Epigenetics

Epigenetics plays a crucial role in any biological process, and it is not astonishing that it is central also in PUFA biosynthesis; its exact role, however, is beginning to

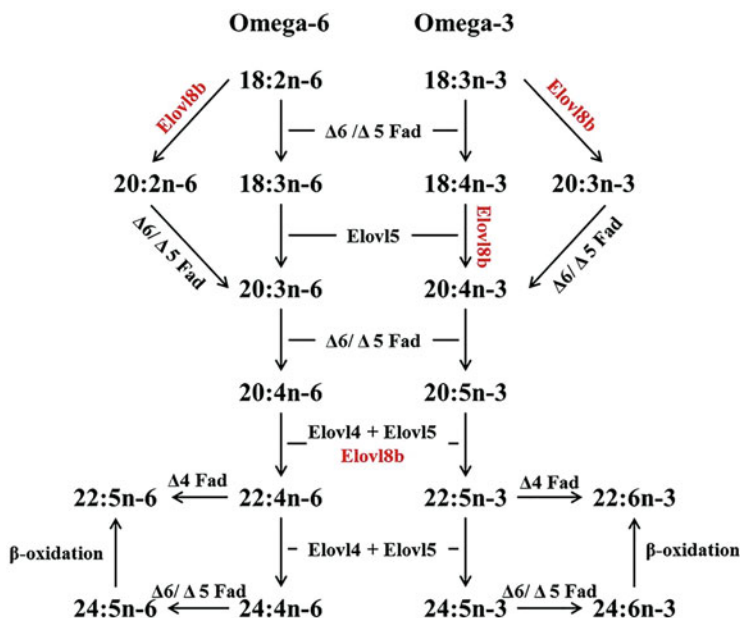


Fig. 28.9 The biosynthesis pathway of LC-PUFAs ($\leq C_{24}$) from linoleic (18:2 ω 6) and α -linolenic (18:3 ω 3) acids in rabbitfish. Enzymatic activities shown in the scheme are predicted from heterologous expression in *Saccharomyces cerevisiae* of the Δ^6/Δ^5 fatty acyl desaturase (Δ^6/Δ^5 fads), the Δ^4 fads (Li et al. 2010), the *elovl4* and *elovl5* elongases (Monroig et al. 2012), and *elovl8b* elongases (Li et al. 2020) (From Li et al. (2020), with permission from Elsevier, references added)

be understood, whereby *Siganus* is a preferred model genus with a current focus on microRNAs (miRNAs), particularly *miR-33*.

MiR-33 enhances LC-PUFA biosynthesis in *S. canaliculatus* by targeting *insig1*,⁴ which in turn upregulates *srebp1*⁵ (Fig. 28.10) (Sun et al. 2019). In companion studies, Chen et al. (2018b, 2019) identified that also *miR-24* and *miR-146a* are involved in this LC-PUFA biosynthetic pathway. In previous studies, also Zhang et al. (2014, 2016) found *miR-33* involved in the biosynthesis process along with *miR-17*. Both miRNAs target Δ^4 fads, a FADS different from that found by Sun et al. (2019). Moreover, based on the results from a PUFA supplementation assay, it appears that *miR-17* can act as switch to orchestrate the “ Δ^4 Fads” pathway in response to PUFA supplement in the rabbitfish liver. The picture becomes even

⁴Insulin-induced gene 1 plays an important role in the SREBP-mediated regulation of cholesterol biosynthesis: by binding to the sterol-sensing domain of SCAP (SREBP cleavage activating protein), it makes the SCAP/SREBP complex stay longer in the ER, thus prohibiting SCAP from carrying activated SREBP to the Golgi complex.

⁵Sterol regulatory element-binding proteins are transcription factors that bind to the sterol regulatory element DNA and are indirectly required for cholesterol biosynthesis and for uptake and fatty acid biosynthesis (Murray et al. 2009).

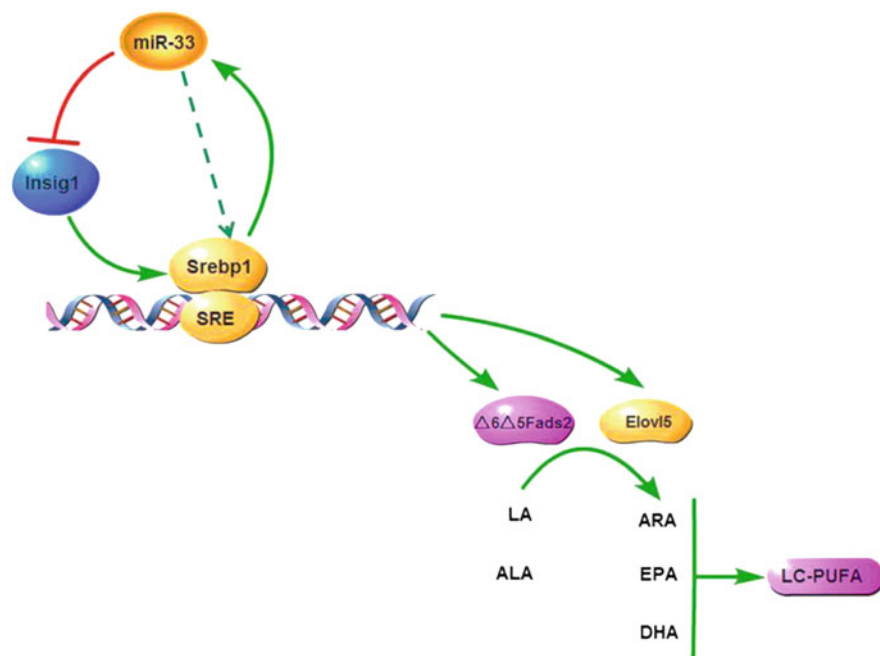


Fig. 28.10 The roles of *miR-33* in regulation of LC-PUFA biosynthesis. The putative mechanism is proposed for effects of *miR-33* overexpression on target genes, host genes, and LC-PUFA biosynthesis-related genes. Red arrows denote suppression; green arrows indicate promotion (From Sun et al. (2019), with permission from Springer Nature)

more complex: the *miR-15/16* cluster is involved in the regulation of LC-PUFA biosynthesis by targeting *ppary*. This cluster inhibits mRNA and protein levels of $\text{PPAR}\gamma$ and increases the mRNA levels of $\Delta^6\Delta^5\text{fads2}$, $\Delta^4\text{fads2}$, and *elovl5*, thereby increasing the production of LC-PUFAs (Fig. 28.11) (Sun et al. 2020a). $\text{PPAR}\gamma$ is a negative regulator of LC-PUFA biosynthesis in rabbitfish.

28.2.2 Omnivores

Only a few studies address endogenous PUFA biosynthesis in omnivorous fishes with *Oreochromis* as preferentially studied genus. However, the more species are going to be studied, the more intriguing results can be expected. For instance, Ferraz et al. (2019) discovered that a complete enzymatic capacity for LC-PUFA conversion of LIN and ALA into ARA, EPA, and DHA is present in the Amazonian *Colossoma macropomum*; the same seems to apply to common carp (Zhang et al. 2019).

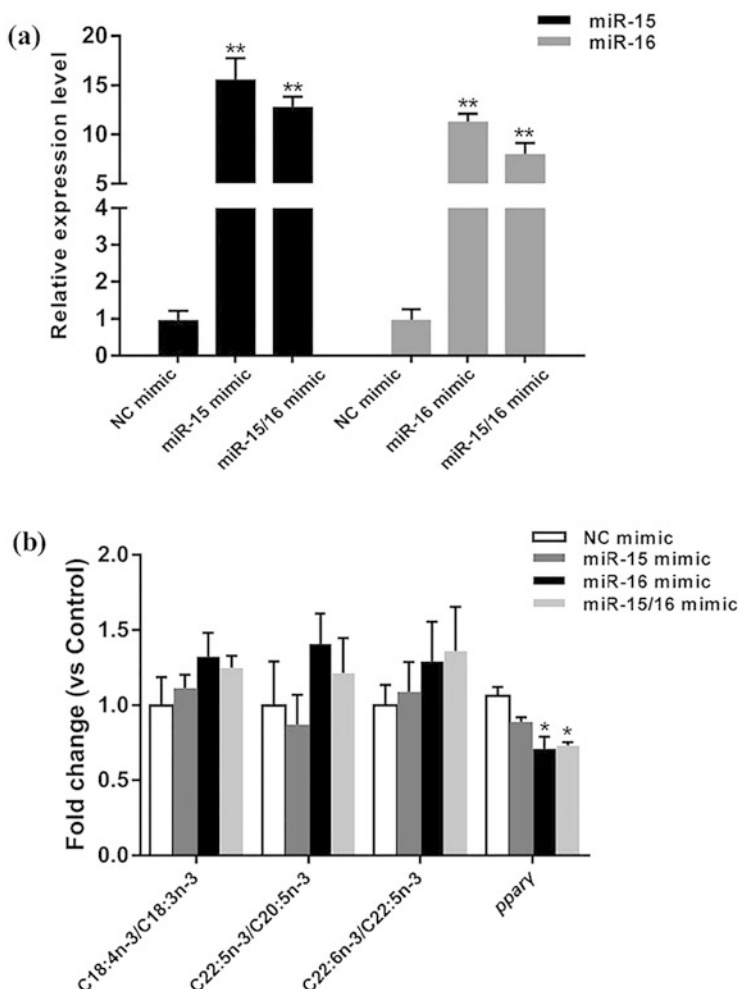


Fig. 28.11 Upregulation of *miR-15/16* cluster promoting LC-PUFA biosynthesis through inhibiting *ppary* in *Siganus canaliculatus* hepatocytes. **(a)** The expression of *miR-15* and *miR-16* mRNA was determined by qPCR. **(b)** The evaluation of $\Delta^6\Delta^5$ FADS2, ELOVL5, and Δ^4 FADS2 activity by desaturation and elongation indexes was performed in *miR-15/16* cluster overexpressed cells and the control cells. Additionally, the expression of *ppary* was also analyzed by qPCR. Data are means \pm SEM ($n = 6$). Asterisks represent significant differences (* $P < 0.05$, ** $P < 0.01$; ANOVA, Tukey’s test) (From Sun et al. (2020a), with permission from Springer Nature)

Nile tilapia feeds on plankton and aquatic plants. To identify its capacity of LC-PUFA biosynthesis, Karapanagiotidis et al. (2007) determined whether increasing dietary ALA contents in VO-based diets leads to elevated tissue deposition of DHA and showed that tilapia has only limited capacity to synthesize EPA and DHA from ALA. This finding is supported by Teoh and Ng (2016). Differently, Li et al. (2016) showed that dietary linseed oil (LO, rich in LIN and ALA) can partly replace

FO without affecting fish growth. The replacement results in an increase of DHA when diets are supplied with a high LO share, indicating that tilapia is able to elongate and desaturate EFA with relatively good efficiency. This finding is confirmed by a recent study in Nile tilapia juveniles identifying that DHA can be efficiently bioconverted from LIN; however, this biosynthetic pathway is substrate-limited. Therefore, this metabolic effort is insufficient to compensate for the significant reduced dietary intake of ω 3 LC-PUFA (Chen et al. 2018a).

28.2.3 Carnivores

Increasing numbers of reports on the activity of desaturases and elongases in carnivorous fishes are detecting a variety of pathways (Kabeya et al. 2017a, 2018b; Wang et al. 2020; Torres et al. 2020a; Xu et al. 2020) and tissues capable of endogenous synthesis of LC-PUFAs. Many fish species appear able to produce even DHA. It deserves further research of why the endogenous biosynthesis obviously is not sufficient to meet the requirement of the carnivores—probably because of lack of micronutrients as shown for Atlantic salmon by Giri et al. (2016).

28.2.3.1 Yellow Perch

In *Perca flavescens*, the predominant uptake of EFAs is via diet. Sawyer et al. (2016) constructed a process-based mass balance model that estimates concentrations of ω 3 PUFAs (ALA, EPA, DHA) and ω 6 PUFAs (LIN, ARA) from prey items. The model considers the processes of nutritional uptake, absorption, egestion, transformation (elongation and desaturation), and β -oxidation. EPA is taken up via ingestion at 94% and is almost sufficient to meet most of the demand; only minimal amounts of ALA are needed to be transformed to EPA to make up any shortfall. For EPA, the dominant loss mechanism is transformation to DHA (84%) (Fig. 28.12). The dominant DHA uptake pathway is transformation from EPA (87%) showing that the DHA dietary content is insufficient (Fig. 28.12). In sum, this model shows that transformation is critical for supplying the fish with adequate DHA, whereas sufficient ALA and EPA can be acquired through diet (Sawyer et al. 2016).

28.2.3.2 Salmonids

It is well established that freshwater and anadromous fish species, such as salmonids, have higher tolerances to dietary VO than marine fishes. Thus, for Atlantic salmon and rainbow trout, the total replacement of FO with a blend of VOs, poor in LC-PUFA does not necessarily result in diminished growth, feed conversion, or development of histopathology. For instance, Sanden et al. (2011) reported that

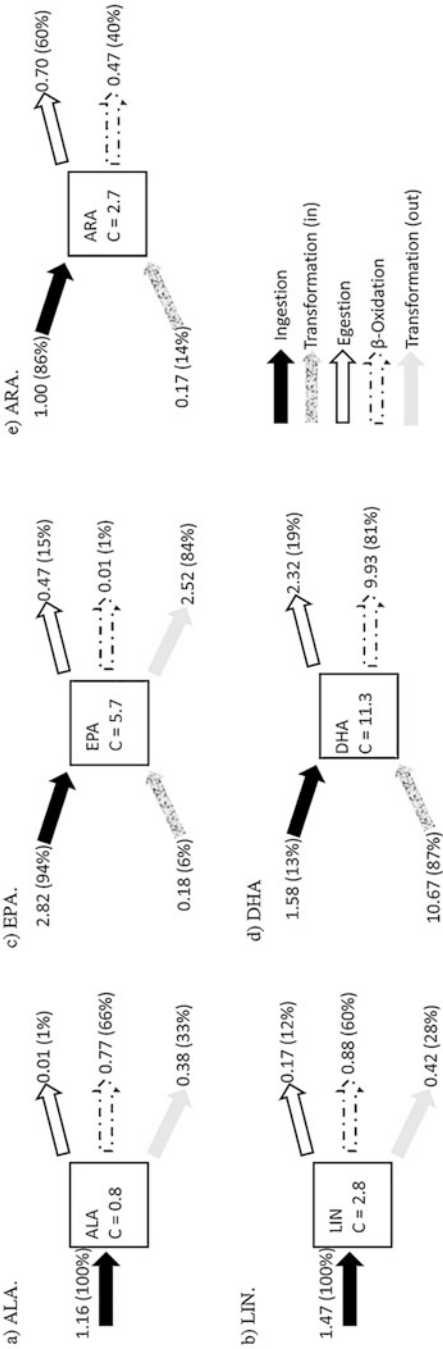


Fig. 28.12 Content (C; mg g^{-1} DW, numbers contained in boxes) and fluxes of inputs and losses (arrows; $\mu\text{mol d}^{-1}$ fish $^{-1}$ and percent contribution to total input and losses) for each PUFA estimated by the model for the application of the model to a yellow perch in the upper Bay of Quinte, Lake Ontario, Canada (From Sawyer et al. (2016), with permission from Elsevier)

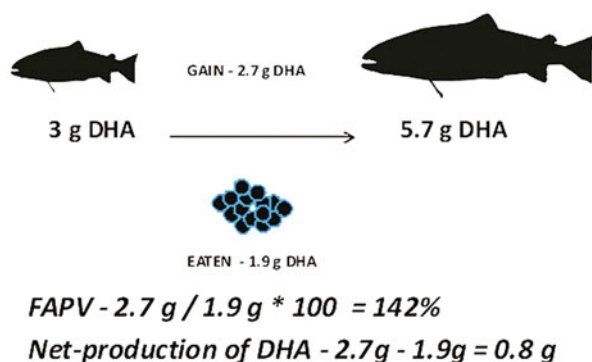


Fig. 28.13 Atlantic salmon as a net producer of long-chain $\omega 3$ fatty acids. This figure illustrates the trial for the high replacement diet and shows the content of DHA in whole fish before and after the feeding period. DHA gain is obtained by subtracting the DHA content in whole fish after the feeding period from the DHA content in whole fish before the feeding period. “DHA eaten” is calculated by using data on feed intake and content of DHA in the feed. The fatty acid productive value (FAPV) is calculated based on the ratio between “DHA gain” and “DHA eaten.” The net production of DHA is obtained when the DHA consumption is lower than the gain (From Sanden et al. (2011), with permission from the American Chemical Society)

Atlantic salmon can even be a net producer of DHA when dietary FO is replaced by VO, as balanced in Fig. 28.13.

Bell and Tocher (2009)⁶ summarized the regulation of PUFA biosynthesis in Atlantic salmon, one of the few species possessing *fads1*. Hepatic transcription of Δ^5 desaturase and elongase increases with increasing dietary LO. The expression of both Δ^5 desaturase and elongase is positively and negatively correlated with dietary ALA and $\omega 3$ PUFA, respectively. Feeding rapeseed oil (RO, rich in monounsaturated FAs), Δ^5 desaturase shows the greatest degree of regulation and is one of only three genes that are upregulated compared to fishes fed FO.

Also in Atlantic salmon, Hixson et al. (2017) showed that salmon fed high shares of ALA maintain DHA levels in muscle tissue and increase in $\omega 3$ and $\omega 6$ intermediary biosynthesis products in the muscle, pointing out endogenous production of DHA via dietary ALA. Significant correlations are observed among dietary and muscle tissue FA with *elovl2*, *fads2*, and *srebpl* transcripts, further supporting FA biosynthesis.

Another paper supports this finding. As mentioned in Chap. 26, LIN competes with ALA for LC-PUFA biosynthesis enzymes leading to the production of $\omega 6$ LC-PUFA, including ARA. Sprague et al. (2019) quantified the endogenous production of EPA and DHA from ALA in salmons fed from first feeding on diets that contain no EPA and DHA and determined the influence of dietary ALA:LIN ratios. With a dietary ALA:LIN ratio of 1, salmon fry/parr produce about 28 μg $\omega 3$ LC-PUFA per g fish per d, with a DHA:EPA ratio of 3.4. Production of $\omega 3$

⁶Extract taken with permission from Springer Nature.

LC-PUFA exceeds that of $\omega 6$ LC-PUFA by almost ninefold. This paper indicates also that increases in dietary LIN shift the LC-PUFA production in favor of ARA and disfavor of EPA and DHA.

Turchini and Francis (2009) showed that rainbow trouts fed LO actively bio-convert only small proportion of LIN and ALA up to DHA; the metabolic effort is insufficient to compensate for the reduced dietary intake of DHA in FO-based diets. A relatively large fraction of dietary ALA is used for energy production, but a larger part is simply deposited in the fish body. This finding supports, to some degree, an earlier paper by Wirth et al. (1997), who did not find any transformation of ALA into DHA in juvenile rainbow trouts. Recently, Ridwanudin et al. (2021) confirmed the hypothesis from Giri et al. (2016) in rainbow trouts. Although dietary nucleotides (uridine monophosphate and cytidine monophosphate) have no positive effect on growth, they modulate the FA composition of fishes by increasing muscular ARA and DHA contents particularly when it is supplemented in low fish oil-based diet.

28.3 Concluding Remarks

The discovery by Kabeya et al. (2018a) that the ωx pathway is widely distributed in a variety of invertebrates can have far-reaching consequences for the bottleneck of dietary LC-PUFA supplies. It can emerge as the long-term overlooked pathway of LC-PUFA provision by animals and can replace the assumption that only aquatic single-cell factories are responsible of LC-PUFA production for the entire food chain. This has to be taken as incentive that ecosystem studies with global efficiency and balance calculations of the ωx pathways are overdue. These studies have to answer the question: Can the biosynthesis of LC-PUFAs by invertebrates suffice the global requirements of the higher trophic levels of the food chain?

Increasing numbers of studies are detecting a variety of pathways and tissues capable of endogenous synthesis of LC-PUFAs. Even novel *fads2* paralogs and *fads* genes with variable desaturation capability are identified. The same applies to elongase genes which open even new biosynthesis pathways. Further discoveries can be expected when the catalog of studied species is expanded. Many fish species appear able to produce even DHA—however, obviously not in sufficient quantities. It deserves further research of why the endogenous biosynthesis meets the corresponding demands only in very few cases. Moreover, future issues are: Can the biosynthesis be increased by adding inorganic and organic micronutrients (Giri et al. 2016; Ridwanudin et al. 2021) or pre- and probiotics, by nutritional programming, selective breeding, or biomolecular techniques, such as CRISPR-Cas-based genome editing (Datsomor et al. 2019)?

Recent studies in *Daphnia* show that several genes are found, involved in PUFA metabolism, but with yet unknown function (Windisch and Fink 2018; Fink and Windisch 2019). Although *Daphnia* is a keystone invertebrate in freshwater pelagic systems, it is not the only prey item, particularly for usually benthivorous fishes,

such as common carps or breams. This indicates that it is worth to recheck the (recycled?) hypothesis that freshwater invertebrates are not able to biosynthesis essential LC-PUFA. In other words, has this hypothesis to be replaced by the recently emerged one by Kabeya et al. (2018a) stating that genes for de novo biosynthesis of ω 3 PUFA are widespread in freshwater, terrestrial, and mainly marine invertebrates?

The role of epigenetics in biosynthesis of LC-PUFAs is still in its infancy. However, emerging information on the controlling role of miRNAs indicates that they are central in regulating the biosynthesis of LC-PUFAs. So far, this comprises only the endogenous pathway of PUFA biosynthesis, disregarding the diet-mediated silencing/activating of PUFA synthesis genes. Dietary supplementation with methyl donors, for instance, affects the one-carbon metabolism with consequences for the methylation of DNA or histones. What can be expected as eventual corresponding feedback in the LC-PUFA biosynthesis?

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Chapter 29

LC-PUFAs in Reproduction and Behavior—‘*Good Cop–Bad Cop?*’



Abstract Steroidogenic enzymes, egg quality, hatching and fertilization rates, and early larval survival over successive spawning seasons are positively correlated with increased levels of $\omega 3$ LC-PUFAs (EPA, DHA) and ARA. Excess levels of dietary DHA, EPA, and, particularly, ARA can adversely affect the quality of eggs and larvae; it can reduce survival and lower fecundity in a variety of fish species. Optimal dietary supply of EPA and DHA induces proper behavior in fishes and aquatic invertebrates; deficient nutrition can lead to misbehavior bearing the risk of being predated with eventual population extinction under natural conditions. In fishes, dietary EPA and DHA deficiencies can delay the functional development of brain and vision. There are first indications that LC-PUFAs (particularly ARA) interfere within the one-carbon metabolism and modulate offspring DNA methylation. This finding can serve as signal for extensive studies of LC-PUFAs and epigenetics in aquatic animals.

Polyunsaturated fatty acids (PUFAs) are essential for life history traits and Darwinian fitness. PUFA supplements (particularly $\omega 3$ PUFAs) are promoted for general health reasons. PUFAs serve as precursors to various signal molecules central in reproduction and behavior. $\omega 6$ as well as $\omega 3$ C_{20} -PUFAs can influence reproductive processes through a variety of mechanisms. They provide the precursors for prostaglandin synthesis (\rightarrow Chap. 27, Box: Eicosanoids) and can modulate the expression patterns of many key enzymes involved in both prostaglandin and steroid metabolism. They are essential components of all cell membranes. The proportions of different PUFAs in tissues of the reproductive tract reflect dietary consumption. Spermatozoa require a high PUFA content to provide the plasma membrane with the fluidity essential at fertilization.

In mammals, both $\omega 3$ and $\omega 6$ PUFA supplements have been fed to various species before collecting oocytes for in vitro fertilization. Positive, negative, and no effects on subsequent embryo development have been reported. When PUFAs are added directly to oocyte maturation medium, high doses of linoleic acid (LIN, 18:2 $\omega 6$) are consistently deleterious, while α -linolenic acid (ALA, 18:3 $\omega 3$) is associated with beneficial outcomes (Wathes et al. 2013).

In case of aquatic animals, is the state of knowledge superior to that of mammals? To answer this question, this chapter selects some recent examples and shows also which information ecological studies of aquatic animals have in store.

29.1 Reproduction

Regulation of gonadal steroidogenesis occurs mainly through gonadotropins: follicle-stimulating hormone and luteinizing hormone. This regulation is related to the expression of an array of genes that encodes steroidogenic enzymes, which ensure the ability of cells to produce steroid hormones from cholesterol and its derivatives. Transcription of several genes is influenced by dietary PUFA composition, in particular, by the $\omega 6/\omega 3$ PUFA (LA[LIN]/ALA) ratio. Corresponding dietary ratios of 20.1, 4.5, 3.9, and 0.7 are prepared by increasing the linseed oil at the expense of corn oil and fed to Nile tilapia (Fig. 29.1) (Orlando et al. 2020).

The first step for biosynthesis of steroid hormones is the conversion of cholesterol to pregnenolone through the side chain cleavage enzyme, encoded by *cyp11a*. Then, pregnenolone is converted to E_2 by the action of many steroidogenic enzymes encoded by a number of genes, including *hsd3 β 1*, *cyp17*, and *cyp19a1a* (Orlando et al. 2020). In this study, experimental diets differentially regulated gene expression of these key genes. Expression levels of *cyp11a* and *hsd3 β 1* are not influenced by the diets. However, higher levels of *cyp17* are observed in fishes fed LIN-rich diets (Fig. 29.1). This study indicates that dietary LIN/ALA ratio for tilapia should be 3.9

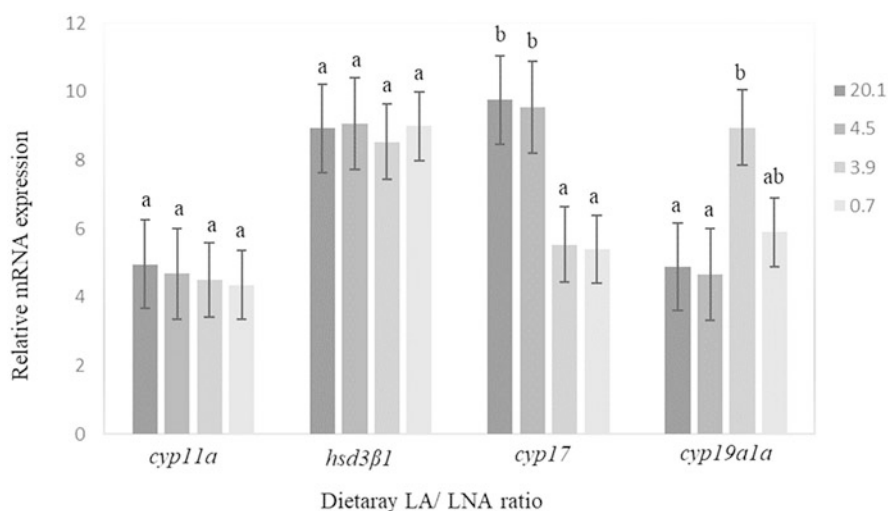


Fig. 29.1 Gene expression of key protein related to steroidogenesis in the gonads of female Nile tilapia fed different LA[LIN]/ALA diets. Steroidogenic enzymes: *cyp11a*, *hsd3 β 1*, *cyp17*, and *cyp19a1a*. (*cyp11a* (cholesterol side chain cleavage enzyme) is a mitochondrial enzyme that catalyzes conversion of cholesterol to pregnenolone. *hsd3 β 1* (3 β -hydroxysteroid dehydrogenase) catalyzes the biosynthesis of the steroid progesterone from pregnenolone. *cyp17* (17 α -hydroxylase; cytochrome P450 17A1) has both 17 α -hydroxylase and 17,20-lyase activities and is a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens. *cyp19a1a* (ovarian aromatase) drives the transformation of androgens to estrogens (Clelland and Peng 2009; Guiguen et al. 2010)) Values are means \pm SEM (From Orlando et al. (2020), with permission from Elsevier)

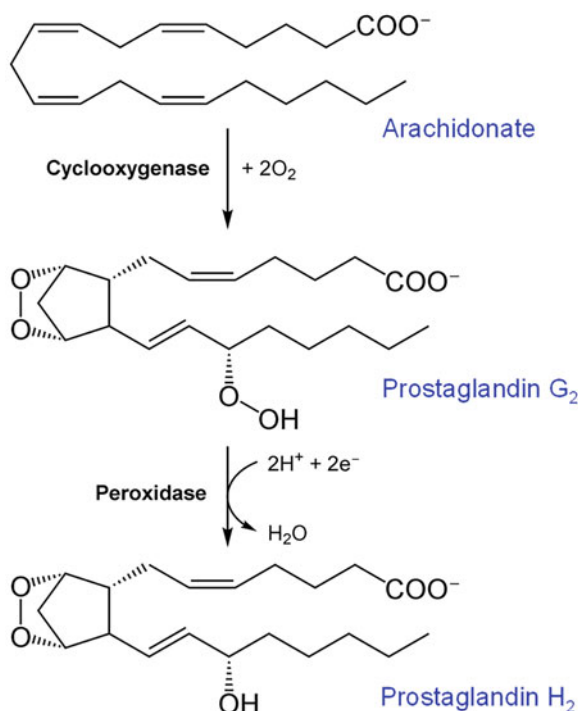
to achieve highest expression of ovarian aromatase and optimal spawning performance (Orlando et al. 2020).

Research has shown that the egg quality, including fertilization and hatching rates, and early larval survival over successive spawning seasons (Bruce et al. 1999) are positively correlated with elevated levels of dietary $\omega 3$ PUFAs (EPA, DHA) and ARA. This is found, e.g., in gilthead seabream, Atlantic cod, European seabass, or crescent sweetlips (*Plectorhinchus cinctus*) (Li et al. 2005), as well as zebrafish. Egg FA compositions can be modulated by broodstock diets in various species, such as gilthead seabream, European seabass, striped jack, Atlantic cod, yellowtail, Eurasian perch, Nile tilapia, yellowfin seabream (*Acanthopagrus latus*) (Zakeri et al. 2011), and crescent sweetlips with the aim of producing high-quality eggs and larvae with EFA contents optimized to give the developing embryos and larvae the best chance of success at a time of increased EFA requirement (Tocher 2010).

29.1.1 Arachidonic Acid

Increased attention is being paid to the effects of dietary arachidonic acid (ARA) on reproduction and larval recruitment of fishes and invertebrates. ARA is a major precursor in the synthesis of certain prostaglandins (Fig. 29.2), central in

Fig. 29.2 Biosynthesis of prostaglandin G_2 and H_2 from arachidonic acid (credit Yikrazuul, Wikimedia)



reproduction. The name prostaglandin derives from the prostate gland, since the compounds were first isolated from seminal fluid (Goldblatt 1935). Prostaglandins are a subclass of eicosanoids and, as such, physiologically active lipid compounds; they are tissue hormones and differ from endocrine hormones in that they are not produced at a specific site (gland) but in many places throughout the body. Eicosanoids from ARA are considered pro-inflammatory (Kuehl and Egan 1980), whereas those from ω 3 PUFAs are weaker analogs and less inflammatory than eicosanoids from ARA, inactive, or even anti-inflammatory (→Chap. 27, Box: Eicosanoids). All prostaglandins contain 20 carbon atoms, including a 5-carbon ring.

Using a metabolomic approach, Xue et al. (2015) were one of the first to uncover that epigenetics is central in ARA metabolism. DNA demethylation upregulates the expression of key enzymes in ARA metabolism and increases the content of certain prostaglandins. Furthermore, Li et al. (2018) showed that ARA and LIN metabolisms are associated with serum homocysteine (central in one-carbon metabolism) levels. Taken together, these findings raise the question of how methyl donor-rich diets impacting the one-carbon metabolism interfere within the prostaglandin biosynthesis pathway.

Cyclooxygenase (COX, officially known as prostaglandin-endoperoxide synthase) is an enzyme that is responsible for formation of oxygenated products from ARA, namely, prostanoids, including thromboxane and prostaglandins, such as prostacyclin. A member of this enzyme family is known as prostaglandin G/H synthase, catalyzing the conversion from ARA to prostaglandin H_2 via a short-living prostaglandin G_2 intermediate (Fig. 29.2).

In Pacific white shrimp, Xu et al. (2017) detected that ARA in diets for early maturation stages enhances the final reproductive performances. Low dietary ARA (4.7% of total FAs), but not high dietary ARA (12.4%), increases spawning performance and fecundity of female shrimps, diameter of fertilized eggs, and metamorphosis rate of nauplii. The hatching rate ranks as follows: control < high ARA < low ARA. Furthermore, appropriate maternal dietary ARA supplementation improves even low-salinity tolerance of nauplii and zoea larvae.

Appropriate doses of dietary ARA improve the egg quality also of the Japanese eel (Furuita et al. 2007) (Table 29.1). In contrast to his East-Asian relative, however, the European eel does not respond to dietary ARA; instead, ω 3 PUFAs stimulate ovarian development. da Silva et al. (2016) assume that it is likely that elevated dietary ω 3 PUFAs contribute to the progression of ovarian maturation of this eel. This may be unique to European eels, since in other freshwater and marine species, ARA seems to stimulate oocyte maturation, while EPA actually inhibits ARA-induced steroidogenesis. A more comprehensive survey of effects of dietary ARA on maturation, reproduction, and larval performance in aquatic animals is presented in Table 29.1. It becomes obvious that most, but not all, fishes respond positively to dietary ARA amendment. For instance, larval viability of the Guinean fingerfish (*Monodactylus sebae*) or common sole (*Solea solea*) is clearly reduced. Furthermore, results in *Lutjanus campechanus* and *Sparus aurata* are not consistent and need clarification.

Table 29.1 Reported effects of dietary arachidonic acid on maturation, spawning, reproduction, and offspring survival in selected aquatic animals

Species	Affected trait (% dry matter)	References
Invertebrates		
<i>Crassostrea corteziensis</i>	0→9: 1.0→oocyte production↑	Hurtado et al. (2009)
<i>Diadema setosum</i>	0.03→0.37; max larval survival @ 0.25; 0.37: growth↓	Nhan et al. (2020)
<i>Litopenaeus stylirostris</i>	Biofloc 0.07: larval survival↑	Cardona et al. (2016)
<i>L. vannamei</i>	0.8; 3.2: immunity (↑)	Aguilar et al. (2012)
	0→2.1: spawning rate↑, fecundity↑	Xu et al. (2017)
<i>Macrobrachium nipponense</i>	0.86: non-specific immunity↑; >1.5: <i>Aer. hydrophila</i> resistance↓	Ding et al. (2018)
<i>M. rosenbergii</i>	1.0,2.0: egg clutch weight↑, fecundity↑, hatching rate↑, number of larvae↑, reproductive effort↑	Kangpanich et al. (2016)
<i>Penaeus monodon</i>	2.5: sperm quality↔	Meunpol et al. (2005)
	0→0.8: fecundity↑, egg production↑	Coman et al. (2011)
<i>Strongylocentrotus intermedius</i>	1.0: growth↑, gonad development↑, larval performance↑	Zuo et al. (2018)
Fishes		
<i>Anguilla japonica</i>	~0.7: egg quality↑	Furuita et al. (2007)
<i>A. anguilla</i>	2.2, 2.9: ovarian development ↔	da Silva et al. (2016)
<i>Cynoglossus semilaevis</i>	~1: fecundity↑, fertilization rate↑, larval survival↑	Liang et al. (2014)
<i>Gibelion (Catla) catla</i>	0.2→3.6: gonadal maturation↑	Nandi et al. (2001)
<i>Centropristis striata</i>	2.3: fertilization success↑	Seaborn et al. (2009)
	0→0.6: larval growth and survival↑	Rezek et al. (2010)
<i>Cynoglossus semilaevis</i>	0→2.9: larval growth and survival↑ @ 1.0→1.4	Yuan et al. (2015)
<i>Danio rerio</i>	0.2→2.1: F ₁ genes of β-oxidation↑, DNA methylation↑	Adam et al. (2018, 2019)
<i>Dicentrarchus labrax</i>	0.4→4.6; @ 1.4: spawning↑, egg quality↑	Bruce et al. (1999)
	<0.01→0.04: egg quality↑	Furuita et al. (2000)
	0.3→1.2: larval handling stress resistance↑	Atalah et al. (2011b)
	0.5→4.0: prostaglandin↑	Torrecillas et al. (2017)

(continued)

Table 29.1 (continued)

Species	Affected trait (% dry matter)	References
<i>Gadus morhua</i>	0.3: fertilization↑, larval viability↑	Røjbek et al. (2014)
<i>Hippoglossus hippoglossus</i>	0.04→0.2: spawning performance↑, egg and larval quality↑	Mazorra et al. (2003)
<i>H. reidi</i>	Male 0.02→0.04: juveniles: growth↑, snout distortion↓	Otero-Ferrer et al. (2016)
<i>Lutjanus campechanus</i>	0.1, 0.8: fertilization↔, reproduction↔	Papanikos et al. (2008)
<i>Monodactylus sebae</i>	0.8: spawning frequency↑, egg output↑, larval viability↓	Ohs et al. (2013)
<i>Monopterus albus</i>	2.8→6.8: growth↓, reproduction↓	Zhou et al. (2011)
<i>Oreochromis niloticus</i>	0→0.4: reproduction↑	Ng and Wang (2011)
<i>Oryzias latipes</i>	2: egg quality ↑	Kowalska and Kowalski (2014)
<i>Paralichthys olivaceus</i>	0.1→1.2: spawning quality↑, reproduction↑	Furuita et al. (2003)
<i>Perca fluviatilis</i>	1.0→3.0: eggs↑, larval quality↑, follicle maturation↑	Henrotte et al. (2010, 2011)
<i>Rachycentron canadum</i>	0→1.9: juvenile growth↑, prostaglandin (↑)	Araújo et al. (2019)
<i>Sander lucioperca</i>	3.0: embryo survival↓	Ljubobratović et al. (2020)
<i>Scophthalmus maximus</i>	0→0.1: juvenile growth↑	Castell et al. (1994)
<i>Seriola dorsalis</i>	1.1→3.5: egg quality↑	Stuart et al. (2018)
<i>Solea senegalensis</i>	0.1→0.8: ♂♂ steroid production↑	Norambuena et al. (2013)
<i>S. solea</i>	0→2.6: hatching rate↓, embryonic survival↓	Parma et al. (2015)
<i>Sparus aurata</i>	0.4→0.74: viable eggs↑	Scabini et al. (2011)
	0→1.27: larval survival↔	Atalah et al. (2011a)
	0.1→0.5: larval survival↑, larval osmotic and handling stress resistance↑	Koven et al. (2001, 2003)
<i>Trichopodus trichopterus</i>	0→2.12: fecundity↑, yolk sac diameter↑, hatching rate↑; optim @ 1.6–2.12	Masoudi Asil et al. (2017)

(continued)

Table 29.1 (continued)

Species	Affected trait (% dry matter)	References
<i>Xiphophorus helleri</i>	0.5→1.7: offspring number↑	Ling et al. (2006)

↑ support/increase, ↔ indifferent/no obvious effect, ↓ adverse effect/reduction. Contrasting the two *Danio rerio* papers above which show the significance of ARA in the female reproduction process, Nowosad et al. (2017) and Martins et al. (2020) reported that supplementing the diet of spawners with PUFA (especially with DHA) improves both the egg quality and the larvae growth parameters. Therefore, it may be concluded that reproductive functions of the cyprinid family appear to require both ω6 and ω3 PUFAs (Takeuchi 1996; Meinelt et al. 1999, 2000; Jaya-Ram et al. 2008)

By adjusting dietary ARA and EPA, studies of different teleost species were able to improve the reproductive performance of captive broodstock (Bruce et al. 1999; Bell and Sargent 2003; Furuita et al. 2003). Henrotte et al. (2010) found in Eurasian perch (*Perca fluviatilis*) that adjusting dietary ARA and EPA to even levels improves the reproductive success. With respect to Eurasian perch’s genetic relatedness to pikeperch (*Sander lucioperca*), it is hypothesized that similar beneficial effects of ARA-EPA increase on egg quality will be visible in pikeperch as well (Ljubobratović et al. 2020). However, contrary to this assumption, increasing the dietary ARA level to EPA level adversely alters reproductive performance and reduces even survival of embryos. However, the authors did not use graded dietary supplementation, and the applied dose exceeds those of most of the other studies in which beneficial effects are observed (Table 29.1); the authors may have overlooked the beneficial (hormetic) dose range. Therefore, it cannot be excluded that the described reduced larval survival is the result of excess rather than appropriate ARA doses. Nevertheless, this paper is a clear incentive for identifying adverse effects of high dietary ARA doses and underlying mechanisms more carefully.

Some more details: Masoudi Asil et al. (2017) found that the widely distributed ornamental *Trichopodus trichopterus* needs at least 1% ARA in its maturation diet to improve reproductive performance. The best efficiency is achieved with 2% ARA, based on fecundity, yolk sac diameter, and hatching rate. This is consistent with the findings in Atlantic halibut (*Hippoglossus hippoglossus*), in which a dietary level of 1.8% ARA (as % of TFA) exerts beneficial effects on spawning performance and egg and larval quality (Mazorra et al. 2003). With increased levels of dietary ARA, spawning and egg quality is improved also in European seabass and Japanese flounder (Furuita et al. 2000) (Table 29.1). Somewhat more frugal is the European cod: Røjbek et al. (2014) found that eggs from broodstock fed a diet with a 0.3% ARA level excel with increased fertilization and hatching success (Table 29.1).

In tongue sole (*Cynoglossus semilaevis*), 1.1–1.4% dietary ARA is beneficial to larval growth. This regulation is due to altering the transcription of *cyclooxygenase-2* and *5-lipoxygenase* (Yuan et al. 2015). COX-2 takes part in the conversion of ARA to prostaglandin H₂ (Fig. 29.2), and 5-lipoxygenase catalyzes the dioxygenation of

ARA and, therefore, transforms it into leukotrienes as well as a wide range of other biologically active products.

Consistently, Nayak et al. (2018) reported that dietary supplementation with $\omega 6$ LC-PUFA-rich algae modulates zebrafish immune function and improves resistance to streptococcal infection. Furthermore, dihomo- γ -linolenic acid (DGLA (eicosatrienoic acid), 20:3 $\omega 6$) is almost as effective as ARA.

Several studies have shown that excessive levels of dietary DHA, EPA, and ARA can adversely affect the quality of eggs and larvae and juvenile pigmentation and, furthermore, reduce survival and lower fecundity in a large variety of fishes. This is documented in brill (*Scophthalmus rhombus*) (Hachero-Cruzado et al. 2012), Nile tilapia (Santiago and Reyes 1993), Japanese (olive) flounder (Furuita et al. 2000), crescent sweetlips (*Plectorhinchus cinctus*) (Li et al. 2005), Japanese eel (Furuita et al. 2007), and gourami (*Trichopodus trichopterus*) (Seifi Berenjestanaki et al. 2014). Several studies attribute this adverse effect particularly to ARA, for instance, in cobia (*Rachycentron canadum*) (Nguyen et al. 2010), Japanese flounder (Furuita et al. 2007), gilthead seabream (Koven et al. 2003), yellowtail flounder (*Limanda ferruginea*) (Copeman et al. 2002), turbot (Estévez et al. 1999), or swordtail (Ling et al. 2006).

Egg development and larval survival of the common sole are adversely affected by dietary ARA and DHA. This result indicates the need for a specific broodstock feed, at least for this species (Parma et al. 2015). However, the authors also assume that unusually high temperatures during spawning and embryonic development may have caused this adverse effect of dietary PUFAs. One may continue that the PUFAs are no longer needed as membrane constituents to keep them flexible; rather, they are now metabolized as natural xenobiotics (→Steinberg 2012).

29.2 Behavior

Optimal dietary supply of LC-PUFAs, particularly EPA and DHA, induces proper behavior in fishes and aquatic invertebrates in nature, and vice versa, deficient LC-PUFA nutrition can lead not only to skeletal deformities (Roo et al. 2009), reduced immunocompetence (Xu et al. 2016), brain malformation (Masuda et al. 1999), or increased lethality (Turkmen et al. 2017) but also to misbehavior that, in turn, bears the risk of being predated with eventual extinction of the population.

It is well understood that *Daphnia* avoids being predated by vertical migration to deep, cold water refuges (Lampert 1989). To maintain membrane viscosity, adaptation to low temperatures in poikilotherms depends on essential dietary lipids. Consequently, Brzeziński and von Elert (2015) hypothesized that limitation by essential lipids affects habitat preference and predator avoidance behavior in this cladoceran. The authors used cholesterol- or EPA-enriched green alga *Scenedesmus obliquus* and cyanobacterium *Synechococcus elongatus*. In an artificial stratified water column, the authors tested whether the absence of these dietary lipids constrains predator avoidance (habitat preferences) in *Daphnia*. The authors studied pond-dwelling *D. magna* and lake-dwelling *D. longispina* both capable of

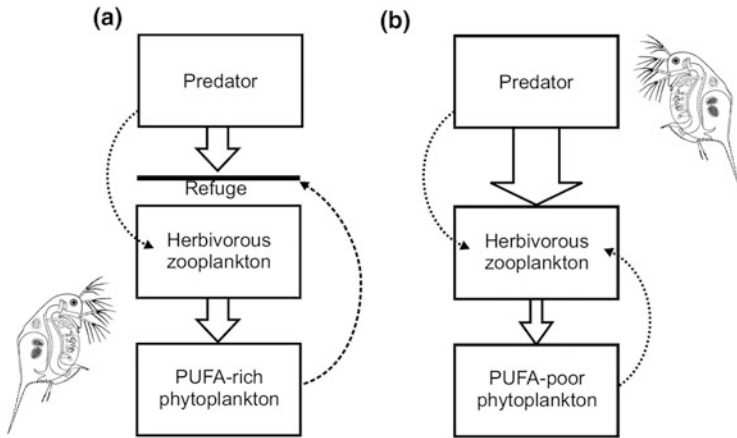


Fig. 29.3 Limited availability of polyunsaturated fatty acids (PUFAs) affects the food chain topology. Predators affect herbivorous prey directly (causing mortality, broad arrow) and indirectly (by inducing changes in life history, behavior, etc., dashed line). **(a)** Under non-limiting, PUFA-rich conditions, the direct effects are mitigated due to a habitat shift of the prey. **(b)** Under PUFA-poor conditions, the antipredator refuge cannot be accessed, exposing herbivorous prey to direct effects of predation. Hence, predator–prey interactions depend on the biochemical quality of phytoplankton (From Brzeziński and von Elert (2015), with permission from Springer Nature; drawing of *Daphnia* credit wpcipart)

performing vertical migration. They found that the capability of avoiding fish predation through migration to the deeper and colder environment is suppressed, unless the diet is supplemented with appropriate EPA (Fig. 29.3) but not with cholesterol. Thus, their ability to access a predator-free refuge and avoid predator encounters depends upon food quality that facilitates membrane homeoviscous adaptation. Interestingly, Hahn and von Elert (2020) subsequently showed that EPA limitation in *Daphnia* performing vertical migration is more severe for maternal than for offspring generations, since migrating females alter the allocation of FAs to the offspring in a manner, which is beneficial to the offspring which may be capable of regaining the full amplitude of vertical migration.

These *Daphnia* papers show that the indirect effects of malnutrition can be as significant as direct effects in terms of population persistence. How this effect can translate into the nutrition of fishes and farmed crustaceans deserves future studies. Nevertheless, this means that often applied gross life history traits, such as growth, survival, or ontogenetic development, have to be supplemented by more subtle ones, such as Darwinian fitness, maintenance of populations, or success of offspring generations.

As marine counterparts, nauplii of the tropical calanoid copepod *Pseudodiaptomus annandalei* were tested for their response to food deprivation. Nauplii starvation leads to significant depletion of ARA, EPA, and DHA. The starvation period reduces the nauplii escape performance (Fig. 29.4) making them

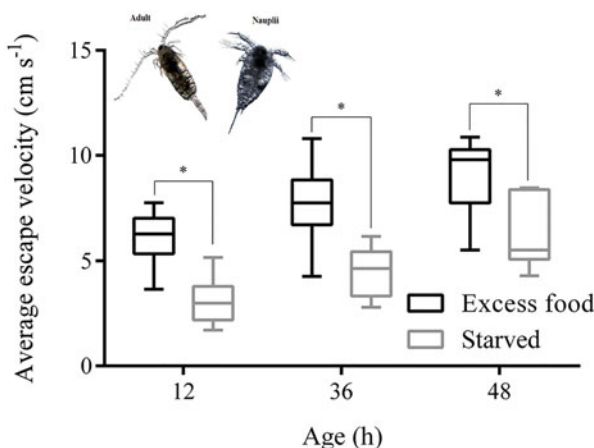


Fig. 29.4 Average escape velocity of three nauplii batches of the calanoid copepod *Pseudodiaptomus annandalei* before and after 48 h starvation when provoked by suction flow simulating a predator. The copepod received the algae *Tetraselmis chuii* prior to starvation. The escape velocity was calculated from 24 escape events from each of the six treatments for a total of 144 escape reactions. Different lowercase letters represent significant differences ($P < 0.05$ ANOVA) between all treatments (From Hansen et al. (2020), with permission from Elsevier)

more vulnerable to increased capture rate by predators, such as fish larvae (Hansen et al. 2020). Hence, the trade-off to being physiologically capable of tolerating starvation can be an increased loss of individuals due to predation.

Comparable to the *Daphnia* studies, several papers report adverse bottom-up effects in fishes. Masuda et al. (1998) found that proper DHA supply is central in the ontogeny of the schooling behavior in yellowtail (*Seriola quinqueradiata*). Bell et al. (1995) pioneered by relating DHA deficiency to impaired prey capture ability in juvenile herring (*Clupea harengus*): The animals have reduced vision at low light intensities. Comparably in gilthead seabream larvae, Benítez-Santana et al. (2007) detected that dietary $\omega 3$ PUFA deficiency induces reduced visual responses. A reduction in the dietary EFA content by enrichment with vegetable oils affects larval behavior, reduces cruise swimming speed, and especially delays the appearance of the response to visual stimulus. This indicates a delay in the functional development of brain and vision combined with reduced EPA and DHA contents in eyes and brains.

Similar to gilthead seabream, also post-larvae of Senegalese sole behave strongly modulated by their diets (Ibarra-Zatarain et al. 2015). The authors evaluated the influence of different vegetable oils (linseed, soybean, and olive oils) and fish oil. *Artemia* enriched with soybean emulsion has the most elevated contents of total $\omega 6$ PUFAs, mainly LIN (18:2 $\omega 6$). ARA levels are relatively stable among the dietary treatments and range from 1.2 (olive diet) to 1.5 (fish and soybean diets). The content of $\omega 3$ PUFA is highest in *Artemia* enriched with linseed emulsion, mainly due to high ALA (18:3 $\omega 3$) contents. Finally, levels of $\omega 3$ LC-PUFA, EPA, and DHA are

highest in *Artemia* enriched with the fish emulsion. Larvae fed with fish oil emulsion are significantly larger and, in an individual-based test, presented higher total activity time and total distance moved than larvae from the other dietary treatments. This study shows that (I) Senegalese soles display a defined proactive–reactive behavior from early ontogenesis and (II) dietary FA composition influences the proactive–reactive behavioral dimension of stress coping style of the larvae.

More FA-specifically, Benítez-Santana et al. (2014) showed that DHA, but not EPA, enhances escape behavior in *Sparus aurata*. This behavior is controlled by so-called Mauthner cells: a higher neural activity is found in these cells in larvae fed elevated dietary DHA contents. Mauthner cells are a pair of big and easily identifiable neurons (one for each half of the body) located in the hindbrain in fishes that are responsible for a very fast escape reflex.

A comparable trial with similar results was carried out with pikeperch larvae and fry fed low levels of DHA (Lund et al. 2014). The larvae tend toward delayed escape responses and significantly slower peak acceleration rates during escape responses following a stimulus. This effect consists up to 90 days after the dietary treatment is terminated, demonstrating a long-term effect of early (mal)-nutritional history. The anxious behavioral profile of the fry low in DHA lends support to long-term central effects, such as a brain developmental pattern, being the cause of these behavioral effects.

Similar to vertical migrating *Daphnia* and escaping *Pseudodiaptomus* nauplii, recent research shows that changes in the EFA composition of the diet of the marine fishes, red drum (*Sciaenops ocellatus*), and gilthead seabream (*Sparus aurata*) quickly translate into changed EFA patterns of their eggs (Harel et al. 1994; Fuiman and Faulk 2013). In red drum, Fuiman and Ojanguren (2011) identified a transgenerational effect of LC-PUFAs: the FA content of eggs determines the antipredator performance of the larvae (Fig. 29.5). Visual response distance is most strongly and negatively related to 18:1 ω 7. This indicates that the higher the 18:1 ω 7 content of the eggs, the shorter the flight distance of the larvae and the higher the risk of being predated. On the other hand, elevated ARA and ALA (18:3 ω 3) contents of eggs increase the flight distance and the visual duration of the flight effect of the larvae. Individually or collectively, both traits have the potential to improve the recruitment success and to maintain populations. The authors assume that also DHA may be important for normal functioning of marine fish larvae; its significance is studied in subsequent trials.

As underlying regulatory mechanism of the DHA effect, Fuiman and Perez (2015) identified metabolic programming. This phenomenon occurs when variations in nutrition during a specific developmental window result in long-term metabolic effects (\rightarrow AAN III “Nutritional Programming”). Fuiman and Perez show that maternal provisioning of eggs with DHA varies with DHA content of the maternal diet. When offspring are reared on a DHA-replete diet, whole-body DHA content of offspring depends on the amount of DHA that is in the egg. They further demonstrate that whole-body DHA content is correlated with traits related to offspring fitness (escape responses, routine swimming, growth, and survival)—the more DHA, the fitter the offspring.

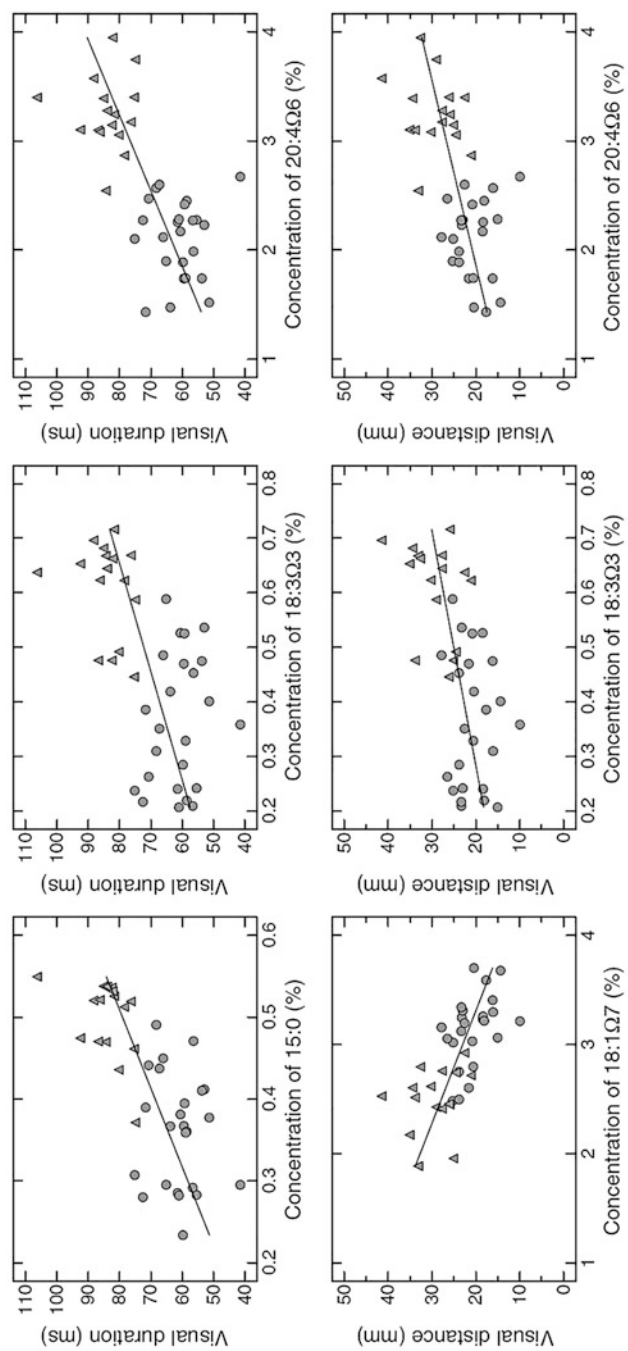


Fig. 29.5 Relationships between selected visual response performance traits of red drum (*Sciaenops ocellatus*) larvae and concentration of fatty acids in eggs. Each point represents the mean performance value for $n = 5\text{--}17$ (average $n = 10.9$) larvae plotted against the concentration of a selected fatty acid in the batch of eggs that produced those larvae. Symbols identify years (circle, 2005; triangle, 2006). All regressions are statistically significant ($P < 0.05$) (From Fuiman and Ojanguren (2011), with permission from Elsevier)

29.3 Concluding Remarks

There is no real “bad cop”! The PUFAs ARA, EPA, and DHA are essential nutrients, however, only in a certain range of dietary supplementation. Excess doses of these PUFAs exert adverse effects in the consumers and even in succeeding generations. This “Janus face” applies particularly to ARA, whereby the catalog of adverse effects and underlying pathways deserve much more attention and documentation. In mammals, for instance, it has been discovered that dietary excess of ARA can favor obesity with subsequent secondary diseases (Pisani et al. 2014).

To recall the Janus face of ARA, the pro-inflammation effect is well understood and common textbook knowledge (Kuehl and Egan 1980; Murray et al. 2009). However, also anti-inflammatory metabolites are produced with ARA as educt. For instance, Gundala and Das (2019) reported that ARA treatment leads to an increase in the formation of lipoxin A4, a potent anti-inflammatory metabolite. This issue deserves a closer look also in the nutritional business of aquatic animals. One prerequisite is a finer-tuned gradation of the dietary ARA content.

Even more intriguingly, transgenerational effects of ARA nutrition have recently been discovered, such as metabolic programming and the fact that high parental dietary ARA modulates DNA methylation patterns in progeny zebrafish liver. However, the effects of nutritionally induced DNA methylation modulation at specific CpG *loci* in a transgenerational context and the extent of epigenetic gene regulation need to be verified by further studies (Adam et al. 2019). And vice versa, ARA and LIN metabolisms are associated with serum homocysteine contents indicating that these nutrients can impact the one-carbon metabolism. It appears worthwhile studying, which condition favors the one or the other pathway.

Last, but not least, the comprehensive depiction of the ecological *Daphnia* example points out that appropriate nutrition with LC-PUFAs can exert beneficial indirect effects which wait to be identified and evaluated also in farmed aquatic animals. With respect to aquaculture, one more aspect deserves consideration: the controlling role of the intestinal microbiota. In AAN I (The Intestinal Microbiota, Steinberg (2018)), we have reviewed papers pointing out the outstanding role of probiotics in changing a timid behavior of zebrafish into a more valiant one (Davis et al. 2016a, b). It is worth testing whether the combination of appropriate PUFA provisioning plus probiotics can further improve the performance of farmed aquatic animals.

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Chapter 30

Trophic Transfer of PUFAs—‘Vital Ones Reach Top Predators’



Abstract Central of the trophic transfer is its efficiency, the ratio between production of one trophic level and that of the previous one. Long-chain polyunsaturated fatty acids (LC-PUFAs), particularly docosahexaenoic acid (DHA), are preferentially retained by higher trophic levels in disfavor of nonessential FAs and bulk carbon. Traditional beliefs have to be revised: Autochthonous LC-PUFA sources prevail even in lotic headwater food webs. The hypothesis that (marine) single-cell factories, sensitive to global warming, are the sole source of $\omega 3$ LC-PUFAs is being challenged by the identification of the widespread occurrence of ωx genes in animals and by finding potentially additional sources.

Fatty acid (FA) patterns in animals and the requirements of C_{18} PUFAs or LC-PUFAs are telltale; they provide insight in storage and feeding habits of organisms, characterizing trophic relationships in food webs. For instance, ratios between certain FAs can be used as trophic indices (Cripps and Atkinson 2000; Graeve and Greenacre 2020; Trushenski and Rombenso 2020; Parzanini et al. 2020). These biomarker-based approaches have in common that information on diet composition and/or trophic habit is integrated over a time scale of several weeks to months.

This kind of bioindication is going to be exemplified with one marine and one freshwater food chain study. In the Arctic Ocean, two hyperiid amphipods, *Themisto libellula* and *T. abyssorum* (Fig. 30.1), are important components of pelagic ecosystems. They represent a substantial food source for marine vertebrates and are a key link between zooplankton secondary production and higher trophic levels (Auel et al. 2002). Both species are carnivorous and prey on mesozooplankton; however, their prey animals are not identical, although both species co-occur sympatrically in large areas of the Arctic Ocean and adjacent seas. *T. libellula* is characterized by higher ratios of PUFA/SFA than *T. abyssorum* (Fig. 30.1).

However, clear differentiation is only feasible on the level of individual FAs. The FA patterns are dominated by the long-chain PUFA moieties 20:5 $\omega 3$ (eicosapentaenoic acid, EPA) and 22:6 $\omega 3$ (docosahexaenoic acid, DHA), short-chain saturated compounds (16:0 and 14:0), and monounsaturated FAs (MUFAs) of varying length, i.e., 16:1 $\omega 7$, 18:1 $\omega 9$, 20:1 $\omega 9$, and 22:1 $\omega 11$. High amounts of 20:1 $\omega 9$ and 22:1 $\omega 11$ in *T. libellula* indicate predation on herbivorous *Calanus*

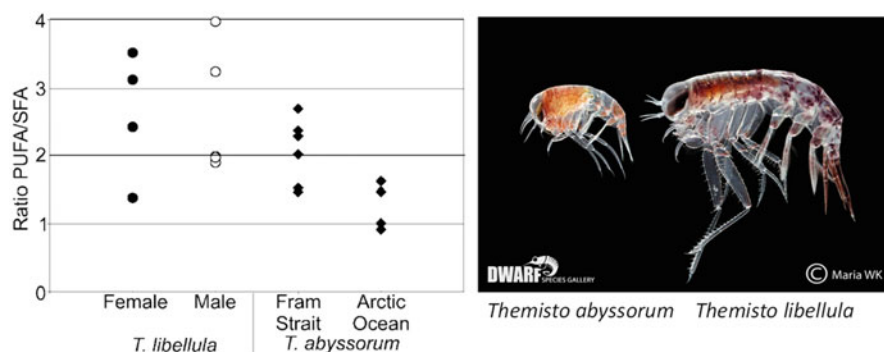


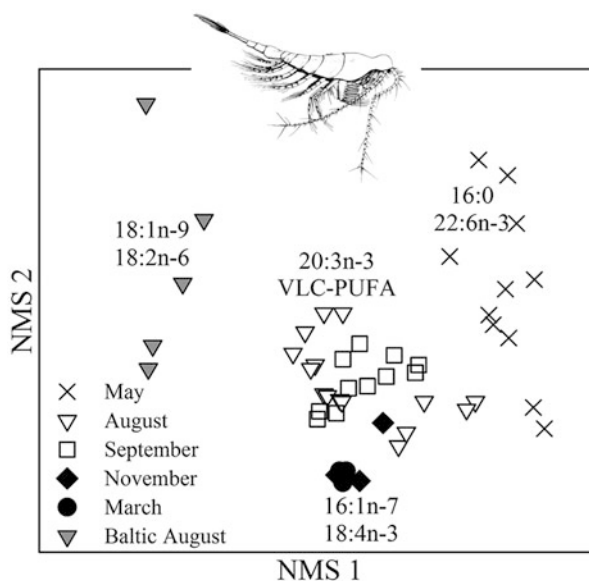
Fig. 30.1 Fatty acid biomarker ratio: polyunsaturated versus saturated fatty acids (PUFA/SFA) in *Themisto libellula* and *T. abyssorum* (From Auel et al. (2002), with permission from Springer Nature; image credit Maria Włodarska-Kowalczyk, Sopot, Poland)

copepodids, and elevated levels of EPA and 16:1 ω 7 in this amphipod point out a close connection with ice-algal production. In contrast, *T. abyssorum* is characterized by lower amounts of EPA, and its biomarker ratios indicate a higher trophic level. This observation complies with the subarctic-boreal origin of *T. abyssorum* and its occurrence in deeper layers of the Arctic Ocean, where it feeds on omnivorous and/or carnivorous prey (Auel et al. 2002).

In large boreal lakes, Hiltunen et al. (2014) investigated the trophodynamics of the glacial-relict copepod *Limnocalanus macrurus* in terms of seasonal variation in the composition of FAs, wax esters, and sterols. *L. macrurus* is a calanoid copepod of marine origin that has adapted to living in freshwater environment. Consequently, lake copepods are compared with Baltic ones. Vast wax ester reserves of *Limnocalanus* are accumulated during a period of only 2 months and comprise also MUFAs, PUFAs, and saturated fatty alcohols. In winter, the mobilization of wax esters is selective, and the proportion of long-chain polyunsaturated wax esters decline first. PUFAs account for >50% of all FAs throughout the year. The most abundant PUFAs are always DHA (Fig. 30.2), EPA, and 18:3 ω 3 (α -linolenic acid, ALA). ALA is the most important FA associated with the accumulation of lipid reserves in *Limnocalanus*. It is common in lipids of many phytoplankton taxa and therefore always present in the food sources of *Limnocalanus* during summer.

These examples point out the question of how do species-specific requirements of essential FAs (EFAs) translate into the ecosystem scale. How does the varying trophic transfer of EFAs impact Darwinian fitness and warrant the persistence of populations of the higher trophic levels? And how do PUFA compositions of prey communities structure the predator community? Does the hypothesis of Kainz et al. (2017) that PUFAs in fishes increase with total lipids irrespective of feeding sources and trophic position apply generally or does it hold true only for specific cases? Several ecological papers address these questions, and there is a potential of scientific inspiration for or cross-fertilization of the highly artificial systems of aquaculture. On the other hand, Trushenski and Rombenso (2020) recently revised a classical paradigm and show that the demand of fishes for LC-PUFAs appears to

Fig. 30.2 Nonmetric multidimensional scaling plot of the total fatty acid composition (mol %) of *Limnocalanus macrurus* in different seasons and habitats. The fatty acids that were responsible for separation of seasons and habitats according to SIMPER are shown, e.g., *Limnocalanus* had more 16:0 and 22:6n-3 in May than in other seasons and more 18:1n-9 and 18:2n-6 in the Baltic Sea than in lakes (From Hiltunen et al. (2014), with permission from Springer Nature; image credit FAO)



be a function of trophic levels. Fishes occupying low trophic levels are capable of de novo LC-PUFA synthesis and require only C_{18} PUFAs in their diet, whereas those occupying high trophic levels are unable to synthesize LC-PUFAs from C_{18} precursors and therefore require a dietary source of intact LC-PUFA (for details and pros and cons, →Chap. 27).

30.1 Trophic Transfer

One of the core features of the trophic transfer is its efficiency, which is defined as the ratio between production of one trophic level and that of the previous one. To demonstrate this, Gladyshev et al. (2011) studied the transfer efficiency of essential PUFAs from producers to consumers in a eutrophic reservoir and evaluated whether the essential compounds are transferred through trophic chains with the same efficiency as bulk carbon. They found that essential PUFAs are transferred from the producers to the primary consumers with about twice higher efficiency than bulk carbon. In contrast, C_{16} PUFAs, which are synthesized exclusively by phytoplankton, but are not essential for animals, have significantly lower transfer efficiencies than both bulk carbon and essential PUFAs. This example demonstrates an increase in food quality for high trophic levels by different transfer efficiencies of essential and nonessential nutrients.

Comparing transfer efficiencies of individual LC-PUFAs, Helenius et al. (2019) found that the assimilation efficiencies of EPA and DHA in the copepod *Calanus*

finmarchicus fed the cryptophyte *Rhodomonas salina* range from 5% to 15%, remaining slightly higher on average for DHA than EPA. This is reflected in the more efficient trophic transfer of DHA (up to 28%), compared to 13% for EPA, and confirms the general finding of Gladyshev et al. (2011).

Hartwich et al. (2013) investigated the role of PUFAs during the oligotrophication of a large, deep European lake (Lake Constance). Multiple regression analysis indicates a shift from a simultaneous limitation by food quantity (in terms of carbon) and quality (i.e., ALA) during the mesotrophic phase to a complex multiple nutrient limitation mediated by food quantity, phosphorus, and particularly ω 3 FAs in the following oligotrophic phase. Hartwich et al. (2013) also showed that *Daphnia* and copepods follow different strategies of PUFA accumulation during the oligotrophication phase. *Daphnia* adjusts its PUFA accumulation to the seasonally changing dietary supply in order to cover its rather constant physiological demand. Copepods adjust their PUFA accumulation to their seasonally changing physiological requirements associated with their life history strategy. Particular, the active overwintering implies a high demand of PUFAs to maintain membrane fluidity of the copepods (=homeoviscous adaptation).

A marine counterpart, Whitefield et al. (2018) examined differences in reproductive fitness of deposit-feeding sea cucumber (*Apostichopus* [*Parastichopus*] *californicus*) females on detritus of either the green alga *Tetraselmis* sp. or the diatom *Thalassiosira* sp., which differ in FA composition. Females on *Tetraselmis* diet have higher fecundity, but their larvae show reduced survival relative to females feeding the diatom *Thalassiosira* sp. Differences are observed in the content of EPA, ARA (arachidonic acid, 20:4 ω 6), and DHA in eggs and gonads from females fed the two diets. Diet-related variations in fecundity and egg quality directly affect the recruitment success in *A. californicus* and indicate that reproductive strategies alter under different feed conditions. If feeding conditions are favorable to planktotrophic larvae, then production of a larger number of eggs with lower energy density (*Tetraselmis* – feed) may be acceptable, whereas *Thalassiosira* feed favors maternal investment in a smaller number of better-provisioned eggs.

30.2 Trophic Upgrade and Trophic Dynamics

Heterotrophic protists are central in the transfer of energy and FAs in any aquatic food chain, since they are the most important grazers of bacteria and picoplanktonic photoautotrophic microorganisms. They can feed on a wide range of particle sizes ranging from viruses to particles several times larger than they are. However, there is one major constraint: Marine ciliates are incapable of synthesizing sterols and PUFAs (Klein Breteler et al. 2004). Deficiency of sterols seems to make bacterivorous ciliates unsuitable as a single food for marine copepods. Apart from energy, ciliates seem to contribute little nutritive value to the diet of higher trophic levels, and this may limit secondary production during periods of low algal abundance.

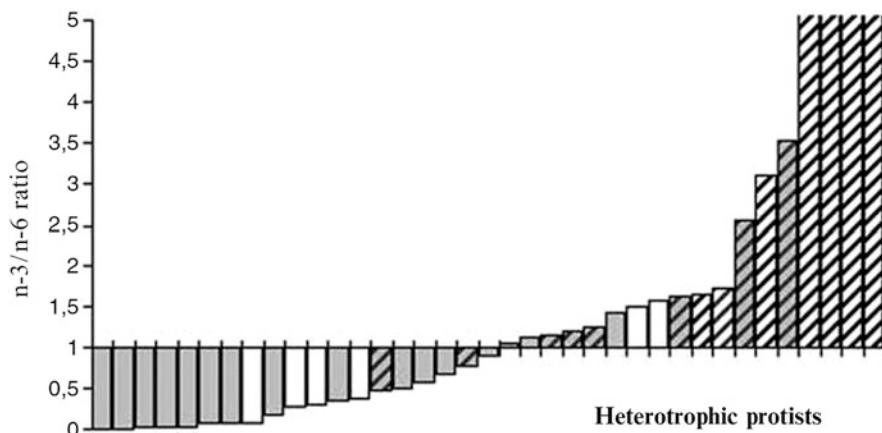
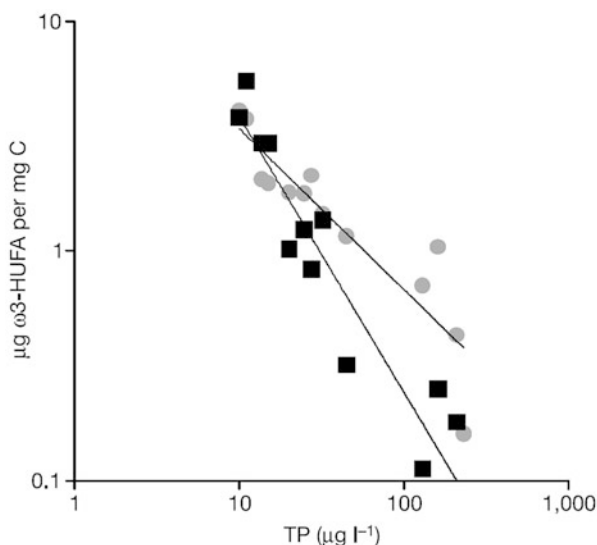


Fig. 30.3 $\omega 3/\omega 6$ ratio of freshwater (gray), marine (white), algivorous (striped), and bacterivorous (non-striped) heterotrophic protists (From Desvillettes and Bec (2009), with permission from Springer Nature)

Two main factors affect the PUFA composition of heterotrophic protists: their habitats (marine vs. freshwater) and their diet (bacterivorous vs. algivorous) (Desvillettes and Bec 2009). The PUFA composition of freshwater ciliates is dominated largely by $\omega 6$ compounds. They contain mainly C_{18} PUFAs, but some genera can contain significant amounts of ARA. In contrast, marine ciliates contain high levels of $\omega 3$ PUFAs. A similar pattern exists for freshwater and marine heterotrophic flagellates: Marine bacterivorous flagellates efficiently produce $\omega 3$ PUFAs, whereas freshwater bacterivorous heterotrophic protists are characterized by low $\omega 3/\omega 6$ ratios. The $\omega 3/\omega 6$ ratios are higher for marine algivorous heterotrophic protists than for freshwater heterotrophic protists. Dietary influence on lipids in heterotrophic protists has been recognized for a long time with recent studies clearly showing that both marine and freshwater algivorous heterotrophic protists exhibit higher levels of $\omega 3$ PUFAs than bacterivorous ones (Fig. 30.3) (Desvillettes and Bec 2009). The high $\omega 3$ contents of algivorous heterotrophic protists are associated with accumulation of dietary PUFAs. Such accumulation tends to be species-specific as different heterotrophic protists feeding the same diet exhibit differences in their PUFA composition (Boëchat and Adrian 2005). Furthermore, accumulation of dietary PUFA by heterotrophic protists is compound-specific (Bec et al. 2003; Broglio et al. 2003) and, thus, comparable to the *C. finmarchicus* example above.

Macrophyte-dominated littoral freshwaters are home to highly diverse cladoceran (*Eurycerus*, *Daphnia*, *Ceriodaphnia*, *Simocephalus*, and others) communities, because they provide a variety of food sources, such as bioseston and epiphyton. These sources clearly differ in FA quality and quantity. Epiphyton is of higher food quality than seston, as studied in a macrophyte-rich backwater system (Masclaux et al. 2014). A resource partitioning occurs among the four cladocerans. While *Eurycerus* feeds mostly on epiphyton, *Daphnia* prefers phytoplankton, and

Fig. 30.4 Regressions between lake total phosphorus (TP) concentration and sestonic EPA and DHA. Gray circles indicate EPA (20:5 ω 3); black squares indicate DHA (22:6 ω 3) (From Müller-Navarra et al. (2004), with permission from Springer Nature)



Ceriodaphnia and *Simocephalus* are able to forage on sestonic and epiphytic resources. The complementarity of the resources used is one of the factors supporting the high diversity of microcrustaceans in these habitats. Moreover, this high diversity and the complementarity of species using the resources thus lead to a highly efficient use of resources and to a high transfer of essential compounds to secondary consumers (Masclaux et al. 2014).

It is well accepted that, due to eutrophication, the efficiency with which biomass and energy are transferred through the food web and sustain the productivity of higher trophic levels (such as fishes) declines with system productivity (Carpenter and Kitchell 1984; McQueen et al. 1986). More specifically, Müller-Navarra et al. (2004) showed that several ω 3 PUFAs are correlated to the trophic status of the lake in summer seston. With increasing total phosphorus (P) content in the water, the ω 3 PUFAs, EPA, DHA (Fig. 30.4), and octadecatetraenoic acid decrease but not ALA. This indicates that a decreasing efficiency in energy transfer with increasing lake productivity can be explained by differences in ω 3 PUFA-associated food quality at the (micro)plant–animal interface.

Subsequently, several intriguing and comprehensive studies on FA trophic transfer have been carried out in boreal and subarctic lakes in recent years. These studies have the potential to serve as role models for detailed studies in farmed fishes and invertebrates. Therefore, a closer look on these studies is promising, especially since boreal and subarctic lakes comprise the most numerous of all lake types on Earth (Wetzel 2001).

High ω 3/ ω 6 ratios show the autochthonous contribution to aquatic food webs. Low ω 3/ ω 6 ratios (<1) are indicative of a high contribution of allochthonous food sources. In their field study, Lau et al. (2013) analyzed the FA composition of the

benthic generalist *Asellus aquaticus* collected from boreal lakes, ponds, and streams across gradients in ambient nutrient levels. The ratios of EPA to total FAs, EPA/ ω 3, and ω 3/ ω 6 of *Asellus* increase up to four times with increasing nutrient enrichment and decreasing humic matter content of the water. This is likely because dietary ω 3 FAs are more prevalent in benthic habitats of eutrophic than oligotrophic systems. Growth response and PUFA accumulation of *Asellus* differ between seasons: *Asellus* optimizes PUFA accumulation in spring but somatic growth in autumn, pointing out the significance of spring accumulation for reproduction. An increase in nutrients will release benthic consumers from growth limitation and favor more efficient trophic transfer.

In their next field study, Lau et al. (2014) tested the hypothesis that autochthonous primary production is the main driver for consumer production, despite being limited by light availability and low nutrient supplies, and greater supply of allochthonous carbon. Therefore, the authors sampled pelagic and benthic consumer taxa representing different trophic levels from dystrophic lakes: isopod *Asellus aquaticus*, megalopteran *Sialis lutaria*, dipteran *Chaoborus flavicans*, and European perch *Perca fluviatilis*. FA analyses show that the relative ω 3 FA and PUFA concentrations increase with trophic level (*Asellus* < *Sialis* and *Chaoborus* < *Perca*). In addition, PUFA and ω 3 FA (with EPA) concentrations increase in consumers with the autochthonous contribution. This paper indicates that consumers in dystrophic lakes predominantly rely on energy from autotrophs and that their PUFA concentrations depend on the relative contribution of the autochthonous resources.

Also for *Daphnia*, it has been shown that a high ω 3/ ω 6 ratio is beneficial for growth and reproduction (Taipale et al. 2015). Vice versa, high dietary ω 6 FA contents decrease somatic growth and offspring production (Peltomaa et al. 2017). As underlying mechanism, Peltomaa et al. (2017) hypothesize that excess consumption of ω 6 FAs reduces the conversion of C₁₈ ω 3 FA, such as ALA, to EPA and DHA, since ω 6 and ω 3 FAs compete for the same LC-PUFA biosynthesis enzymes (→ Chap. 26).

The general preference for ω 3 FAs translates to fishes, piscivorous birds, and other higher trophic levels. Strandberg et al. (2015) presented evidence that selective trophic transfer of PUFA in large lakes results in high proportions of ω 3-PUFAs, particularly DHA, in vendace. In general, the proportion of C₁₈ PUFAs decline in the food chain, while C₂₀ and C₂₂ PUFAs increase (Fig. 30.5). The reasons for the trophic enrichment of C₂₀ and C₂₂ PUFA are selective incorporation and mobilization of PUFAs and likely also bioconversion of C₁₈ PUFAs to C₂₀ and C₂₂ PUFAs (Watanabe et al. 1989; Bell et al. 2007). Calanoid copepods as well as mysids, which are abundant in the studied oligo-mesotrophic large lakes (Rahkola-Sorsa 2008), likely account for the effective trophic transfer of DHA to vendace.

Similarly, Sushchik et al. (2017) proved that the trophic level determines FA content and composition of roach, bream, perch, and pike from Krasnoyarsk Reservoir (Siberia, Russia), thereby verifying the hypothesis of Trushenski et al. (2020) with field studies. There are higher percentages and contents of DHA in fishes of the higher trophic level, perch and pike, than in roach and bream. This indicates a higher trophic transfer efficiency (=selective accumulation) of this PUFA in food chains. In

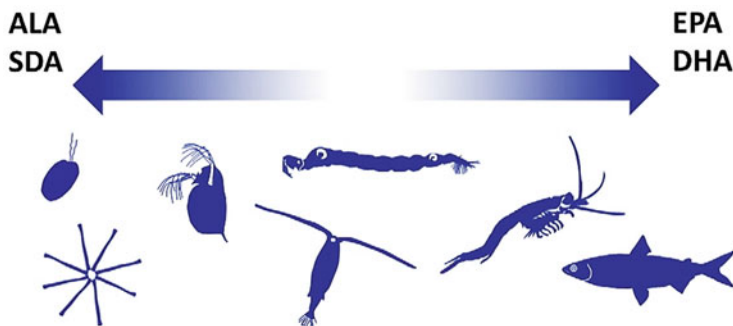


Fig. 30.5 Increase of long-chain PUFAs (EPA, DHA) and concomitant decrease of C_{18} FAs in the food chain in a boreal lake. (From Strandberg et al. (2015), with permission from Elsevier). Displayed organisms from left to right: cryptomonad, *Asterionella* (diatom), *Daphnia*, *Limnocalanus* (copepod), *Chaoborus*, *Mysis* shrimp, vendace. **ALA** α -linolenic acid; **SDA** stearidonic acid; **EPA** eicosapentaenoic acid; **DHA** docosahexaenoic acid

contrast, percentages and contents of EPA increase in fishes of the lower trophic level, roach, and bream.

A significant trophic upgrade of FAs occurs in marine copepods. De Troch et al. (2012), Cnudde et al. (2013), and Werbrouck et al. (2017) reported that several benthic harpacticoid copepods, such as *Microarthridion littorale*, *Delavalia palustris*, *Alteutha* spp., *Harpacticus* sp., or *Tigriopus californicus*, apparently develop the ability to elongate FAs and to exploit niches with poor-quality food. Moreover, by improving the quality of the food they graze upon, in terms of EPA and DHA contents, harpacticoid copepods upgrade the nutritive value of food available to the higher trophic levels in marine food webs (also →Chaps. 26–29).

In Chap. 31, the trophic upgrading of sterols by parasitic chytrids will be presented in detail. This role is not restricted to sterols; recently, Rasconi et al. (2020) confirmed that this trophic upgrade applies also to FAs. The authors show that zoospores of even the cyanobacterium (*Planktothrix rubescens*)-parasite system tend to be enriched in EPA and are significantly enriched in SDA and ARA.

Another, probably in its significance also underrated, pathway of PUFA provisioning via parasites has been described by McKee et al. (2020): Parasite infectious stages (*cercariae*) provide EFAs and lipid-rich resources to freshwater consumers from benthic hosts (Fig. 30.6).

30.2.1 Paradigm Shift: Headwater Food Webs

The well-accepted, highly educational River Continuum Concept (RCC) implies that consumers in headwater streams have greater dietary access to terrestrial basal resources in form of leaf litter (Vannote et al. 1980). This is thought to be also valid for PUFAs. In two recent studies, however, this paradigm is being challenged.

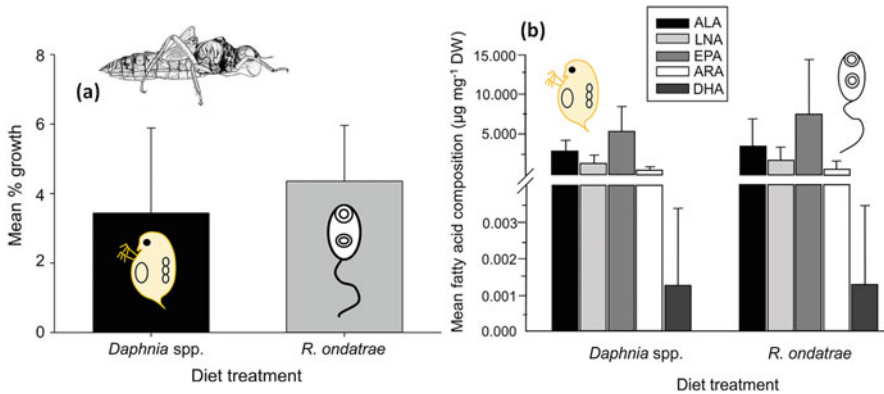


Fig. 30.6 (a) Mean (+ SD) % growth (head width change) over 5 weeks for dragonfly (*Leucorrhinia intacta*) naiads ($n = 39$) fed mass equivalents of two different preys (*Daphnia* spp. or *Ribeiroia ondatrae* cercariae). No significant difference was found between the two diet treatments. (b) Mean \pm SD absolute essential fatty acid (ALA, LNA (LIN), EPA, ARA, and DHA) composition of *Leucorrhinia intacta* naiads ($n = 39$) fed mass equivalents of two different preys (*Daphnia* spp. or *R. ondatrae* cercariae) (From McKee et al. (2020), with permission from Springer Nature. Inserted drawing of a *Leucorrhinia* exuvia from Hovmöller and Johansson (2004), with permission from Elsevier)

Based on two hypotheses, Fujibayashi et al. (2019) investigated the dietary EFA origin for the predatory white-spotted charr (*Salvelinus leucomaenis*) in four Japanese headwater streams and detected the following: (1) the dietary allochthonous contribution for fishes in headwater stream food webs positively relates with canopy cover, and (2) EFAs originate from autochthonous organic matter regardless of canopy cover. The results indicate that, in fact, autochthonous organic matters are the main dietary origin of EFAs regardless of canopy cover (Fig. 30.7). This is indicated by the bulk stable carbon and nitrogen isotope ratios of *S. leucomaenis*, which are the most enriched among all samples and close to those of epilithic biofilms. Epilithic biofilms are the only source of DHA, the LC-PUFA highly prominent in the charr.

In a large-scale field study, Ebm et al. (2021) provide supporting evidence that algal PUFAs nurture fishes in headwater stream habitats. Contrary to the RCC implications, algae rather than terrestrial sources supply aquatic vertebrates with PUFA. Algal PUFAs that are first incorporated by benthic invertebrates serve as precursors for critical PUFAs required for fish organs, in particular for DHA in fish brains and eyes (Fig. 30.8). In detail, the PUFA composition of muscle, liver, brain, and eye samples in sedentary (European bullhead) and potentially migratory (brown trout and rainbow trout) fishes differs from that of their potential food sources (macroinvertebrates, epilithon, and fresh and conditioned terrestrial leaves) (Fig. 30.8). Epilithon and fresh and conditioned leaves in streams differ from each other in their PUFA composition. Fresh leaves contain predominantly the medium-chain PUFA ALA and LIN (LA), whereas conditioned leaves additionally contain traces of SDA and EPA. Compared to leaves, epilithon and benthic invertebrates

Fig. 30.7 Biplot for stable isotope ratios of bulk carbon and nitrogen of basal organic carbon sources, *Salvelinus leucomaenis*, and surface baits from *S. leucomaenis* stomachs in Babame, 2018. Error bars represent standard deviation (From Fujibayashi et al. (2019); credit MDPI Basel; image credit Brevoort (1856))

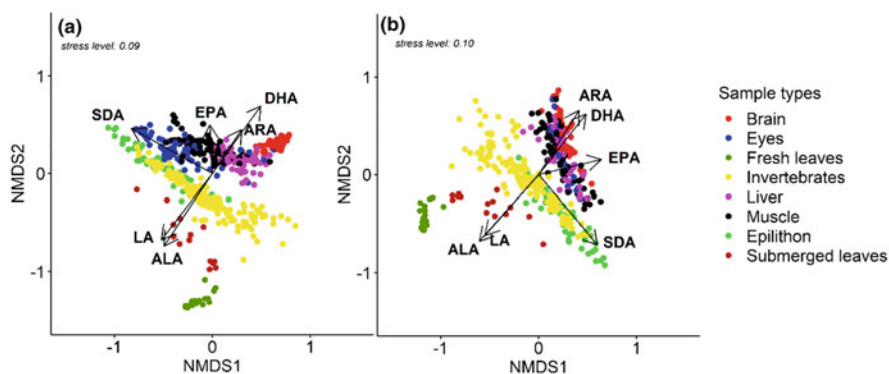
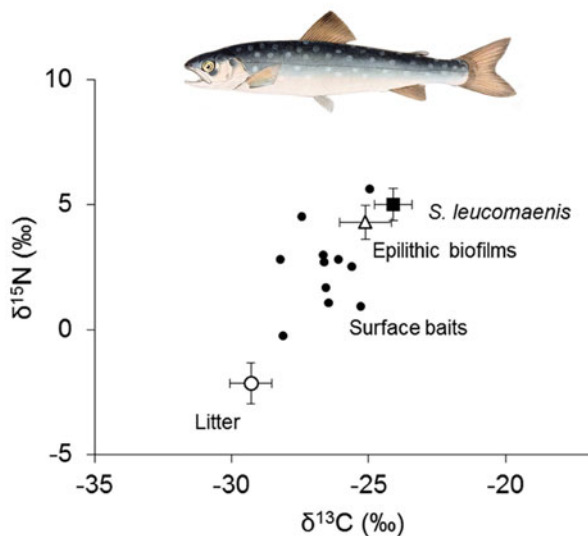


Fig. 30.8 Nonmetric multidimensional scaling of arcsine-square-root-transformed PUFA (% of total FAME) of basal resources, benthic invertebrates, and (a) salmonids (*Salmo trutta fario*, *Oncorhynchus mykiss*) and (b) European bullhead (*Cottus gobio*). ALA α -linolenic acid; SDA stearidonic acid; EPA eicosapentaenoic acid; DHA docosahexaenoic acid; LA (LIN) linoleic acid; ARA arachidonic acid (From Ebm et al. (2021), credit Springer Nature)

contain high levels of EPA and traces of DHA. Fishes differ mostly from their potential resources (macroinvertebrates, epilithon, and fresh and conditioned leaves) by their high DHA content—as seen also in the Japanese charr example above. The PUFA composition of consumers (benthic macroinvertebrates and fish) is more similar to autochthonous (algal) than to allochthonous terrestrial sources (fresh and conditioned leaves). This field study demonstrates the nutritional importance of algal PUFAs in aquatic consumers in headwater regions. Furthermore, both studies may

serve as basis for future validating studies and as start of a slight revision of the classical RCC.¹

These studies confirm previous ones by the same group: Brett et al. (2017) reported that, although shredders obtain most of their carbon by leaf consumption, they acquire and selectively retain their physiologically important ω 3 PUFA from high-quality algae. In a companion paper, Guo et al. (2017) reported that stream food webs are highly enriched in EPA and DHA, with algae richer in EPA in temperate than in subtropical rivers. Consequently, stream invertebrates from both temperate and subtropical rivers preferentially retain EPA from algal sources. Contrastingly, DHA is selectively and highly retained in all fish taxa. Particularly, freshwater salmonids—as white-spotted charr—have a very high relative DHA content, which indicates that they preferentially retain this PUFA or biosynthesize it from EPA (→Chap. 28). The PUFA profiles of other fish taxa reflect their dietary PUFA supply more closely.

30.3 Man-Made Trophic Downgrading?

Expanding horizons to the real world and combining this nutritional aspect to ongoing environmental deterioration, Hixson and Arts (2016) point out that global warming will significantly change FA profiles in primary producers serving as dietary source for higher trophic levels. The authors conducted an exploratory data synthesis with several hundred FA profiles from six major groups of marine and freshwater phytoplankton algae. Temperature is strongly correlated with a decrease in the proportion of ω 3 LC-PUFA and an increase in ω 6 FA and saturated FA (SFA) (Fig. 30.9). Hixson and Arts predict that global ω 3 LC-PUFA production will be reduced by 8.2% for EPA and 27.8% for DHA with an increase in water temperature of 2.5 °C. Using a previously published estimate of the global production of EPA by diatoms, which contribute to most of the world's supply of EPA, the authors predict a loss of 14.2 Mt. of EPA annually as a result of ocean warming. Reduced production of these EFA, as a consequence of climate warming, will adversely affect species that depend on these compounds for optimum physiological function. Such profound changes in the biochemical composition of phytoplankton cell membranes can lead to cascading effects throughout the world's ecosystems (Hixson and Arts 2016). Very recently, Colombo et al. (2020) expanded this prediction to DHA: Global warming will reduce also the *de novo* synthesis of DHA by algae. However, in contrast to these alarming papers, Marmillot et al. (2020) conclude in a comprehensive study of Arctic and Subarctic waters that the production of PUFA-rich phytoplankton is likely to persist in the near future. Challenging alike, Jin et al. (2020) show, yet only in a limited number of diatoms, that long-term exposure to increasing temperature can offset predicted losses in marine food quality (PUFAs) caused by

¹My students liked this wonderful educational concept very much—thank you, Robin & Co!

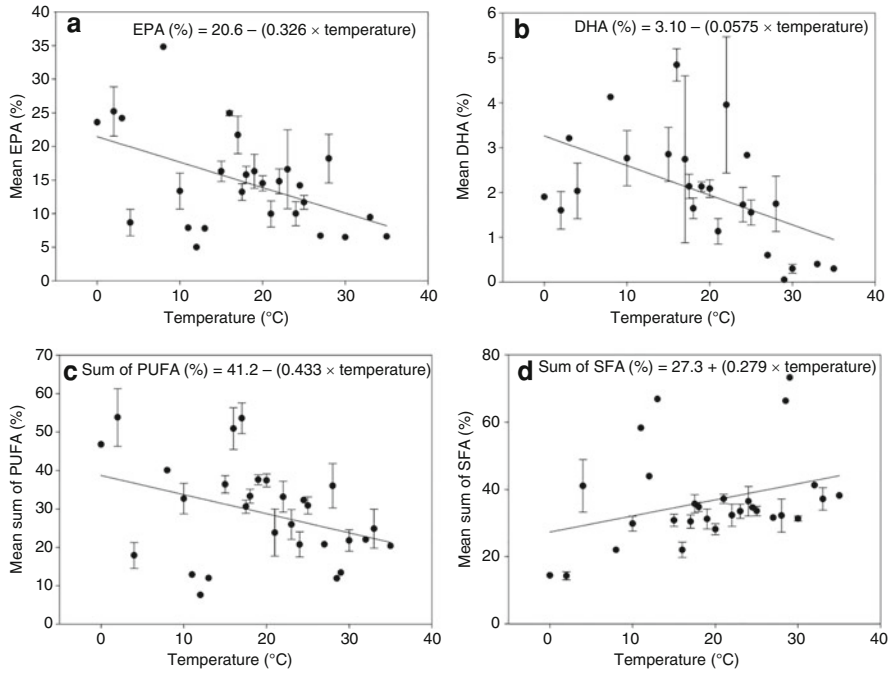


Fig. 30.9 Linear regressions showing the relationship between temperature and diatom fatty acids (mean \pm SE): (a) EPA, (b) DHA, (c) sum of PUFA, and (d) sum of SFA ($P < 0.01$) (From Hixson and Arts (2016), with permission from Wiley)

ocean warming. If this applies to phytoplankton algae on a broader scale, it would be challenging to understand the underlying modes of action.

A comparable loss of EPA and DHA contents in feeds, as described for ocean warming, is suspected to happen in freshwaters. Taipale et al. (2016) reported that eutrophication (increase of nutrients) and brownification (increase of terrestrial humic substances due to global warming and reduced acid rain deposition) in boreal and subarctic lakes have an adverse impact on the trophic transfer of EPA and DHA. Both impacts change the phytoplankton EPA and DHA contents. These changes can be traced to the EPA and DHA content of piscivorous fishes (shown in European perch). In particular, the EPA and DHA contents of phytoplankton biomass are significantly lower in eutrophic lakes than in the oligo-mesotrophic or dystrophic ones. Concomitantly, a strong correlation is found between the DHA content in the muscle of piscivorous perch and phytoplankton DHA content as well with the contribution of DHA-synthesizing phytoplankton taxa (dinoflagellates and chrysophytes). Accordingly, the EPA + DHA content of perch muscle decreases with increasing total phosphorus and dissolved organic carbon concentration in the lakes (Fig. 30.10). Although eutrophication generally increases biomass production

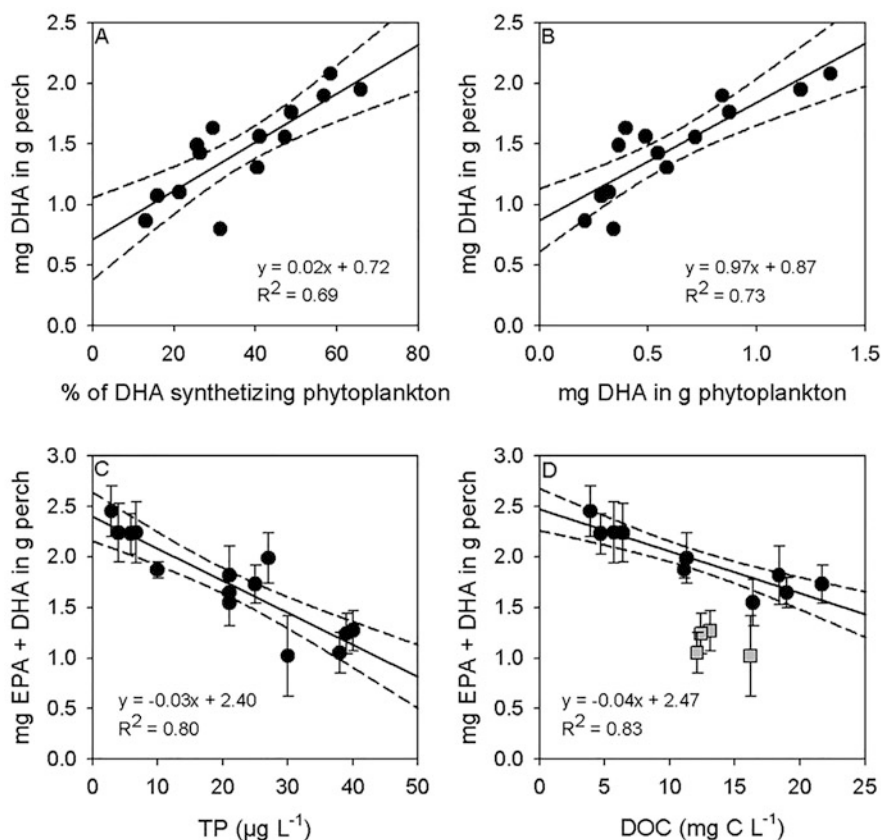


Fig. 30.10 (a) DHA content (mg DHA in g muscle) of perch (total length > 23 cm) muscle related to the proportion of DHA synthesizing phytoplankton (cryptophytes, dinoflagellates, chrysophytes, euglenoids) and (b) DHA content of phytoplankton (mg DHA in g wet weight) in the lakes. (c, d) EPA and DHA content in perch muscle related to total phosphorus (TP) and dissolved organic carbon (DOC) concentration of the lakes. The regression between perch EPA + DHA content and lake DOC concentration does not include the lakes with TP > 30 $\mu\text{g L}^{-1}$ (marked as “gray squares”). Regression equations with R^2 and lines with 95% confidence limits are also shown. All linear regressions were significant ($P < 0.05$) (From Taipale et al. (2016), credit Elsevier)

across different trophic levels, the high proportion of low-quality primary producers (cyanobacteria) reduces EPA + DHA content in the food web up to predatory fish.

Eutrophication comprises nutrient enrichment. In freshwaters, most attention has been paid to phosphorus as initial factor of freshwater eutrophication (Likens et al. 1971; Schindler 1974). However, Trommer et al. (2019) recently showed that also nitrogen enrichment leads to changing FA composition of phytoplankton and adversely affects zooplankton in a natural lake community. Increasing nitrogen reduces EPA, but not ALA, content in the seston (sum of suspended organic particles). Reduction of all PUFAs in the seston reduces growth of *Daphnia*, as

prominent keystone grazer in the planktonic food chain. However, an N-related reduction in secondary production can, as mentioned several times, affect higher trophic levels, such as fishes.

The catalog of marine and freshwater disaster scenarios can easily be enlarged. However, before doing so, we have to answer the question of how realistic these scenarios really are. Did the studies, particularly the marine ones, really consider all ecological and biomolecular information currently available?

30.4 Concluding Remarks

The above predictions of man-made trophic downgrading sound real and dramatically alarming. They hold true, if planktonic single-cell primary producers are the only source of LC-PUFAs and if they have not the capability of acclimation or epigenetic-mediated adaptation. However, this assumption has recently been challenged. The discovery of ω x genes in a variety of (marine) invertebrates shows that ω 3 LC-PUFA production by metazoans will undoubtedly be a significant contribution to the dietary ω 3 LC-PUFA pool (Kabeya et al. 2018), although balances on ecosystem scales are yet missing.

Very likely, higher trophic levels can even evade phytoplankton sources, use their foraging plasticity, and use another food source. It is worth to intensify corresponding studies in marine and freshwater invertebrates and food chains. Furthermore, it can be hypothesized that the nutrient-specific foraging, described for bulk macronutrients (Mayntz et al. 2005, 2009), also applies to qualitative composition. Empirical studies are worth being carried out.

Another potential source of PUFAs has been identified by McKee et al. (2020), namely, parasite infectious stages from benthic hosts can provide LC-PUFAs to pelagic freshwater consumers. However, whether this finding turns out either as nice textbook supplementation or as significant, but overlooked, ecosystem source of LC-PUFAs for higher trophic levels deserves future attention.

Referring to harpacticoid copepods, this chapter pointed out that invertebrates living in a dietary poor environment are able to sufficiently upgrade PUFA (also →Chap. 28). Since long, it is well understood that such harpacticoids, e.g., *Tigriopus californicus*, are crucial in fish larvae nutrition (Kreeger et al. 1991). So-called self-enriching planktonic copepods *Pseudodiaptomus annandalei* and *Apocyclops royi* from brackish habitats are also well-known excellent feed for fish larvae since long (Doi et al. 1997; Liao et al. 2001). Obviously, these biosynthetic properties have been considered an exception to the general rule but not the rule itself; rather, as it now turns out, it is the rule. The inventory of marine invertebrates appears to be more advanced than that of freshwater invertebrates; this backlog has to be caught up, and the existing list of freshwater invertebrates able to biosynthesize LC-PUFAs has to be updated and enlarged.

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Chapter 31

Sterols, Phospholipids, and Wax Esters— *‘Stay Healthy, Avoid Cholesterol’*



Abstract Due to beneficial side effects of dietary cholesterol and phytosterols, these lipidic compounds need reconsideration as feedstuff for fishes. Studies point out that their controlling function appears to be more complex than previously anticipated. Since cholesterol cannot be biosynthesized *de novo*, it is an essential nutrient to crustaceans, mollusks, and likely other invertebrates. In fishes, stimulated LC-PUFA biosynthesis by dietary cholesterol occurs, and cholesterol is considered a hidden treasure of fish nutrition. However, deficient as well excess dietary cholesterol levels lead to adverse effects, such as inflammation and reduced antibacterial peptide production. Adverse effects of high-cholesterol diets can be reduced after supplementation with probiotics. The replacement of cholesterol by phytosterols is documented in several studies, but the information is not yet consistent. Appropriate dietary phospholipids strengthen the immune and antioxidant defense systems and tight junction barrier in fishes. Except for Atlantic salmon, the nutritional role of wax esters in other lipid species remains obscure. A few studies point out a significant role of intestinal microbiota and epigenetics in the nutrition with sterols, phospholipids, and wax esters.

Like leftovers of a sumptuous meal, this chapter comprises specific, less well-studied, but still significant, forms of dietary lipids and their effects, namely, cholesterol and phytosterols, selected phospholipids, and wax esters. Except for cholesterol, these dietary lipidic compounds are not well understood as nutritional compounds of aquatic animals. Moreover, many intriguing papers originate from ecological studies and have the potential of cross-fertilizing aquaculture science and practice—and vice versa: Innovative biomolecular studies have been carried out with selected farmed animals and are seeking their counterparts in aquatic ecology.

31.1 Sterols

Dietary sterols are absorbed in the intestine and transported to the liver for metabolism. Mammals and zebrafish share similar sterol uptake and excretion molecules in the intestinal epithelial cells as depicted in Fig. 31.1 (Takase and Ushio 2018). Dietary

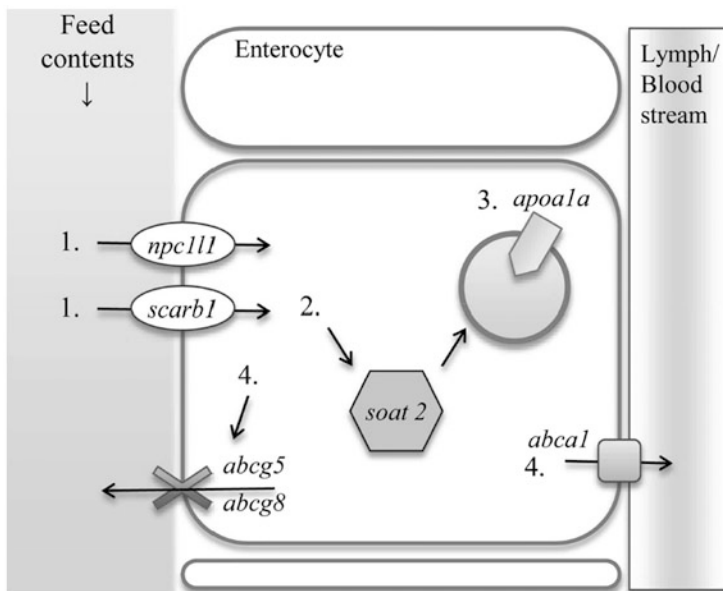


Fig. 31.1 Diagram of sterol uptake and excretion genes. Sterols enter the small intestinal epithelial cells via Niemann–Pick C1-like protein 1 (1. *npc1l1*) and scavenger receptor class B, member 1 (1. *scarb1*), and are esterified with fatty acid by O-acyltransferase 2 (2. *soat2*). Esterified sterols are encapsulated with apolipoprotein A-I (3. *apo1a*). Excessive sterols are excreted out of the cells via ATP-binding cassette subfamily G, members 5 and 8 (4. *abcg5/abcg8*) and ATP-binding cassette, subfamily A, member 1 (4. *abca1*). Nomenclature in the parentheses represents gene name. Lipoprotein particles are excreted from intestinal epithelial cells to lymph in mammals, whereas to portal vein in fish (From Takase and Ushio (2018), credit MDPI, Basel)

sterols enter the small intestinal epithelial cells through a Niemann–Pick C1-like protein 1 (NPC1L1) and scavenger receptor class B, member 1 (SCARB1). Free sterols are then subjected to esterification by sterol O-acyltransferase 2 (SOAT2). Esterified sterols are encapsulated into lipoprotein particles and transported out of the epithelial cells. Apolipoprotein A-I (APOA-I) is responsible for the formation of pre-high-density lipoprotein (HDL) particles in humans, which then is associated with sterol transportation from the small intestine to the liver. Excessive sterols are excreted out of the cells through the ATP-binding cassette subfamily G, members 5 and 8 (ABCG5, ABCG8), back into the lumen in mammals. Another ATP-binding cassette, subfamily A, member 1 (ABCA1), also exports sterols from the small intestinal epithelial cells into the circulation to contribute to the formation of HDL particles (Fig. 31.1) (Takase and Ushio (2018) and references therein).

31.1.1 Cholesterol

Cholesterol (Fig. 31.2) is an essential structural component of cell membranes. It is central in maintaining both membrane structural integrity and fluidity. Its synthesis is

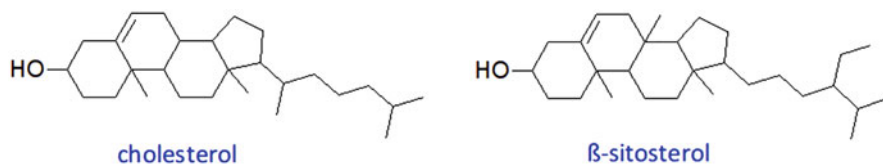


Fig. 31.2 Structures of cholesterol and β -sitosterol, a major phytosterol

an expensive process for cells in terms of energy. This pathway takes place in cytoplasm. Liver and intestines are major contributors to endogenous production. Acetyl-CoA units are joined to form a 30-carbon compound, and then three carbons are removed to produce cholesterol which has 27 carbon atoms (Kumari 2018). Cholesterol is considered a nonessential dietary compound—except of crustaceans and mollusks, which are incapable of *de novo* synthesis of cholesterol (van den Oord 1964; Zandee 1966; Teshima and Kanazawa 1971; Kumar et al. 2018).

In 2016, the US Department of Agriculture Dietary Guidelines Advisory Committee recommended that Americans eat as little cholesterol as possible.¹ Does this recommendation also apply to aquatic animals?

31.1.1.1 Invertebrates with Emphasis on Crustaceans

Increasing research addresses the potential regulatory effects of dietary cholesterol in aquatic invertebrates. To assess dietary demand of cholesterol, many papers use life history traits, such as growth or molting frequencies in crustaceans. In brief, dietary amendments actually improve growth performance. Furthermore, a recent biomolecular study of a *Macrobrachium* sp. identifies a plethora of responsive genes and identified several major pathways (Gu et al. 2017a), and the *Daphnia* example is going to show beneficial transgenerational effects of dietary cholesterol.

Cholesterol is required for growth, development, and reproduction (Table 31.1). To explore the potential of sterol limitation in growth of copepods (*Acartia hudsonica*, *A. tonsa*, *Calanus finmarchicus*), Hassett (2004) fed adult individuals diatoms with and without cholesterol supplementation. Nearly threefold increased egg production rates occur in *C. finmarchicus* after feeding the supplemented diet. Even egg hatch rates benefit from dietary cholesterol: *A. hudsonica* hatches at 91% when fed *Thalassiosira weissflogii* supplemented with cholesterol and only at 40% on unsupplemented diatoms. In a companion trial, Crockett and Hassett (2005) confirmed that cholesterol-enriched diet enhances the egg production also in *A. hudsonica*.

Dietary cholesterol supplementation follows an optimum curve. Niu et al. (2012) showed in *Litopenaeus vannamei* that the level of dietary cholesterol should be

¹<https://www.choosemyplate.gov/2015-2020-dietary-guidelines-answers-your-questions>

Table 31.1 Effects of dietary cholesterol in juvenile/postlarval crustaceans (other than *Daphnia*)

Species	Cholesterol level, %	Observed trait	References
<i>Artemesia longinaris</i> , Argentine stiletto shrimp	0.5	Growth↔, survival↑	Petriella et al. (1984)
<i>Carcinus maenas</i> , Shore crab	1.4→2.1	Growth/survival↑	Ponat and Adelung (1980)
<i>Cherax quadricarinatus</i> , Red claw crayfish	0.5		Hernández et al. (2004)
<i>Euphausia superba</i> , Antarctic krill	69→83% body content of total sterols		Ericson et al. (2019)
<i>Homarus americanus</i> , American lobster	0.5	Growth/survival↑	Castell et al. (1975)
<i>Litopenaeus vannamei</i> , Pacific white prawn	0.05→0.5		Gong et al. (2000)
	In SBM-based diet: 0.2→0.4	Growth↑	Duerr and Walsh (1996)
	0.2		Zhang et al. (2019)
	0→0.4 + phospholipid 1.1→2.1	Resistance against <i>vibrio alginolyticus</i> ↑	Yan et al. (2020)
<i>Macrobrachium amazonicum</i> , Amazon river prawn	2.0	Morphotypic differentiation↑, WG↔	Ruiz et al. (2019)
<i>Macrobrachium nipponense</i> , oriental river prawn	0.9	Growth↑	Gu et al. (2017b)
<i>Macrobrachium rosenbergii</i> , Giant river prawn	≤0.6	Growth/survival↑	D’Abramo and Sheen (1994)
<i>Marsupenaeus japonicus</i> , Kuruma prawn	0.1, 0.5		Teshima et al. (1997)
<i>Penaeus monodon</i> , Black tiger prawn	0.2→1.0		Smith (2001)
	0, 1.0 + astaxanthin	Resistance against air exposure↑	Niu et al. (2014)
<i>Penaeus penicillatus</i> , Redtail prawn	0.5→1.0	Growth/survival↑	In Kumar et al. (2018)
<i>Portunus (pelagicus) armatus</i> , Blue swimmer crab	1.0	Growth↑	Noordin et al. (2020)
<i>Portunus trituberculatus</i> , Swimming crab	0.6	Growth↑, molting frequency↑	Sun et al. (2017)
	0.8		Han et al. (2018)
<i>Procambarus clarkii</i> , Red swamp crayfish	0.5		Tian et al. (2020)
<i>Scylla paramamosain</i> , Green mud crab	0.4→1.6	Growth↑, immunity↔	Zheng et al. (2018)
<i>Scylla serrata</i> , Mangrove crab, megalopa	0.14→1.0	Survival (↑)	Holme et al. (2006)

↑ support/increase; ↔ no apparent effect; WG weight gain; SBM soybean meal

strictly controlled and not exceed approximately 1.0%. Rather, excess cholesterol is detrimental for larval shrimp growth but obviously not for survival.

Feeding oriental river prawn (*Macrobrachium nipponense*) graded cholesterol diets, Gu et al. (2017a) presented the first biomolecular study in a farmed crustacean. Among the identified pathways are metabolic and biosynthetic pathways, fatty acid (FA) metabolism and biosynthesis, and immune-related pathways (Fig. 31.3). This paper confirms that cholesterol is an essential nutrient for *M. nipponense* and can be considered an incentive for future studies.

In aquatic mollusks, too, cholesterol is an essential nutrient. Furthermore, also the capacity for bioconversion of sterols, de novo, is generally low or absent. Therefore, the sterols in microalgae are particularly important for the nutritional value of bivalve mollusks. Sterols are key factors affecting growth and development of bivalves, especially gonads and reproduction (Soudant et al. 2000; Gatenby et al. 2003; Delgado et al. 2004; Giner et al. 2016). Studies also show that cholesterol is the most abundant sterol in sea scallops, Pacific oysters, or Philippine clams (Kraffe et al. 2008; Le Grand et al. 2011). When cholesterol is deficient, even dietary plant sterol can promote growth of bivalves (Cheng et al. 2020).

Trophic Upgrade

Intriguing evidence of beneficial effects of dietary cholesterol and other sterols comes from work with the ecological model cladoceran *Daphnia*. Here, sterols are often provided only after food algae have been parasitized. Parasitic chytrids are the dominant parasites in aquatic ecosystems. They have a free-living zoosporic stage, during which they actively search for new hosts and which are excellent food for zooplankton in terms of size, shape, and nutritional quality (high content of C₁₈-MUFAs and sterols, particularly cholesterol and its precursors (sitosterol (Fig. 31.2) and stigmasterol)). In the field, densities of chytrids can range from 10¹ to 10⁹ spores L⁻¹. When large inedible phytoplankton species are infected by chytrids, nutrients within host cells are transferred to zooplankton via zoospores (Kagami et al. 2014). This “mycloop” may be significant in shaping aquatic food chains.

In addition to parasitic chytrids, saprotrophic chytrids play an important role. The massive deposition of pollen during the pollination of anemophilous tree species can have a strong effect on secondary production in freshwaters. Masclaux et al. (2011) showed that the such pollen cannot support zooplankton growth directly because of their refractory walls. Nevertheless, damage of the pollen grain wall by chytrid fungi creates a microbial food web transferring and upgrading pollen nutrients to metazoan grazers (Fig. 31.4).

Recent lipid analyses uncover an important facet of the mycloop (Gerphagnon et al. 2019). The PUFA composition of chytrid parasites generally reflects that of their phytoplankton hosts; however, they produce also sterols de novo that are absent in their phytoplankton host. They upgrade the biochemical quality of host-derived organic matter, even from cyanobacteria (Fig. 31.5).

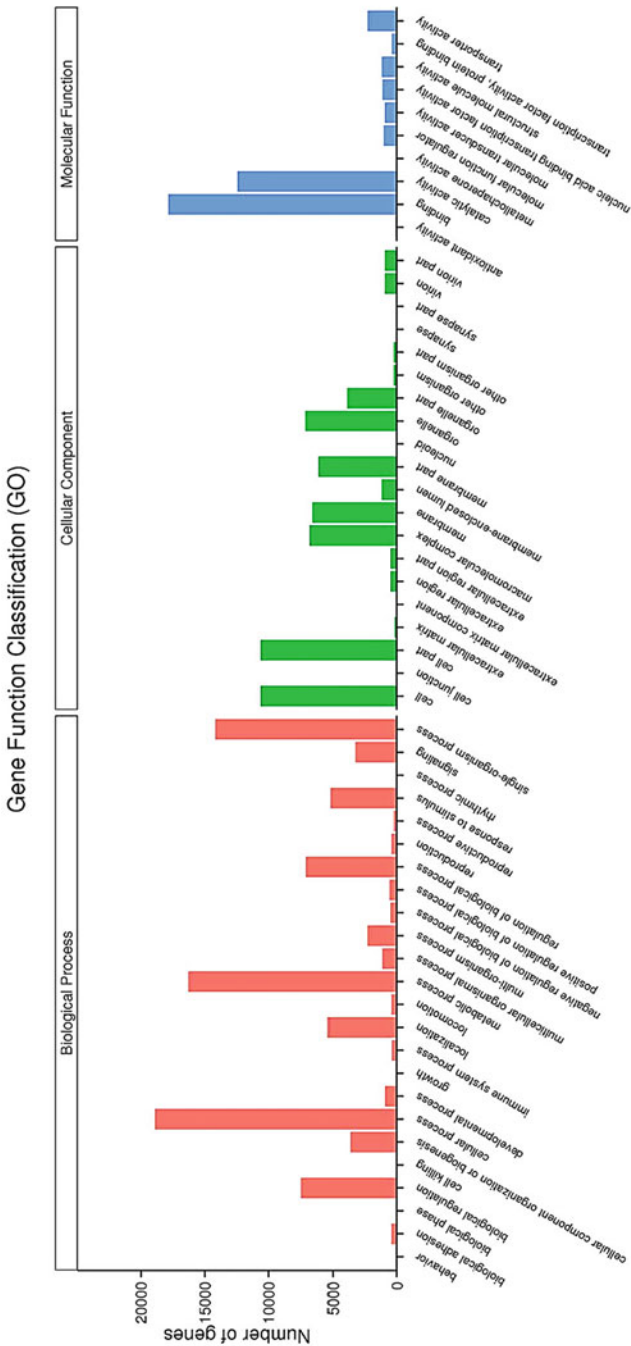


Fig. 31.3 Histogram of gene ontology (GO) classifications of unigenes in the oriental river prawn (*Macrobrachium nipponense*) fed dietary cholesterol (From Gu et al. (2017a), with permission from Elsevier)

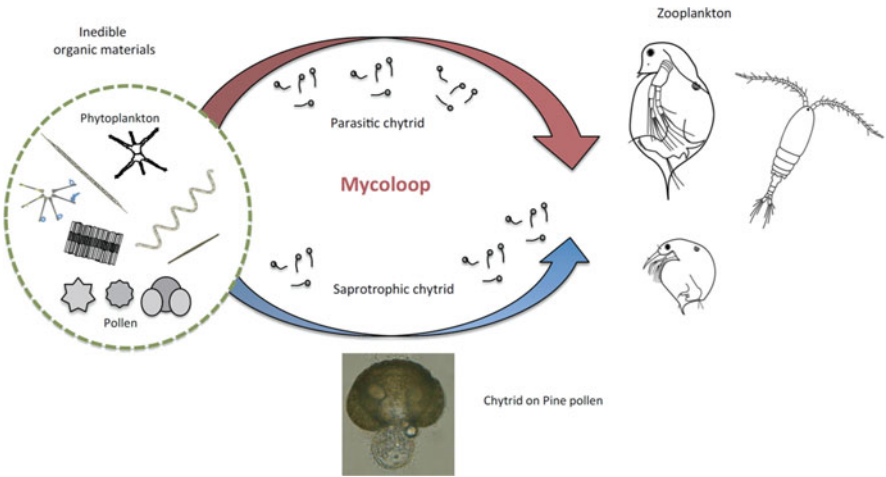


Fig. 31.4 Possible mycloops in freshwater and marine environments. Saprotrophic chytrids play important roles in aquatic food webs, by decomposing inedible organic material such as pollens. Zoospores released from pollen may be consumed by zooplankton, functioning as another “mycloop.” In addition to chytrids, other zoosporic fungi, or fungal-like protists, such as Cryptomycota and Labyrinthulomycota, can infect phytoplankton or consume large inedible organic material, which may be grazed by zooplankton in freshwater and marine environments (From Kagami et al. (2014), credit Frontiers Media)

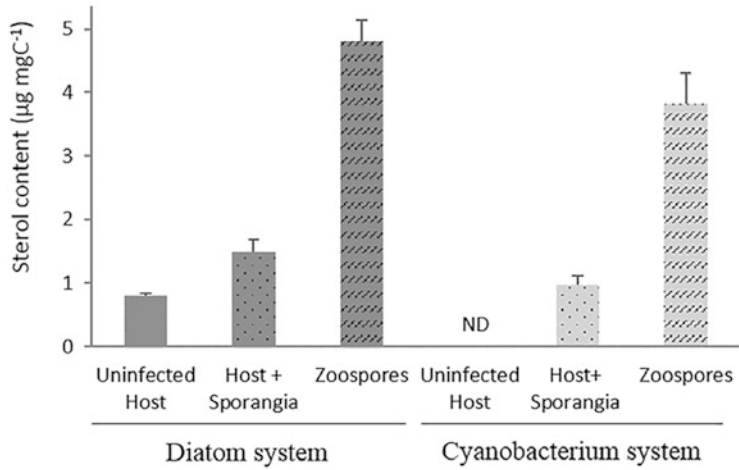


Fig. 31.5 Sterol content of different fractions in a diatom and a cyanobacterium system. Values means + SD. ND Not detected (From Gerphagnon et al. (2019), with permission from Wiley)

Transgenerational Effects

Dietary cholesterol does not only beneficially affect maternal generations but translates also into life history traits of filial generations. In two *Daphnia* spp., Martin-

Creuzburg et al. (2005) showed that somatic and population growth rates, maternal weights, numbers of viable offspring, and survival rates are reduced with diminishing availability of cholesterol. Sterol-limited growth leads to reduced energy transfer at the plant–animal interface and, therefore, to a decoupling of primary and secondary production. Subsequently, Martin-Creuzburg and von Elert (2009) reported in detail that the somatic growth of *D. magna* feeding on the cyanobacterium *Synechococcus elongatus* is primarily constrained by the absence of sterols, whereas egg production is primarily limited by the absence of LC-PUFAs.

31.1.1.2 Fishes

Based on the ecophysiological experience above and on fish oil replacement studies in fishes, Norambuena et al. (2013) posed the challenging question whether cholesterol is a hidden treasure of fish nutrition. As all vertebrates, teleosts are able of synthesizing cholesterol and, as such, have no apparent dietary demand for it. The authors determined in rainbow trouts whether dietary cholesterol fortification in a vegetable oil-based diet can manifest any effects on growth and feed utilization, as well as in vivo FA metabolism. No effects occur on growth and feed efficiency; however, in fishes fed cholesterol-supplemented dietary, no endogenous biosynthesis of cholesterol and a remarkably decreased apparent in vivo FA β -oxidation are recorded, while in control fishes, cholesterol is abundantly biosynthesized with increased apparent in vivo FA β -oxidation. Only minor effects are observed in the activity of stearoyl-CoA desaturase (Δ^9 FA desaturase), but a significant increase is observed for both the transcription rate in the liver and the apparent in vivo activity of the FA Δ^6 desaturase (bioconversion of 18:2 ω 6 [LIN] to 18:3 ω 3 [ALA] and 24:5 ω 3 to 24:6 ω 3 [DHA]) and elongase, with increasing dietary cholesterol. This is indicative of a stimulated LC-PUFA biosynthesis due to dietary cholesterol.

Noteworthy, this process is epigenetically controlled: Zhu et al. (2018) pointed out the potential that some, in the blood circulating, miRNAs are reflective of hepatic cholesterol metabolism. In particular, circulating *miR-33a*, *miR-122*, *miR-128*, and *miR-223* are in the focus of interest (for details, see below).

This biomolecular information is replenished by Deng et al. (2013), who reported that dietary cholesterol supplementation in the range between 0.9 and 1.2% improves the antioxidant capacity, nonspecific immune response (phagocytosis, Fig. 31.6a), and disease resistance (against *Aer. hydrophila*) of rainbow trouts on soybean meal-based diets (Fig. 31.6b). Excess cholesterol (1.5%), however, does not elicit further immune response or disease resistance.

Consequently, cholesterol is garnering additional interest from fish nutritionists as amendment of plant protein- and plant oil-based diets. Improved growth performance by dietary cholesterol supplementation is found also in juvenile turbot (*Scophthalmus maximus*) (Yun et al. 2011). Feeding graded cholesterol to juvenile yellowtail kingfish (*Seriola lalandi*), Guerra-Olvera and Viana (2015) identified that this lipid, formulated with vegetable ingredients, enhances growth with optimal contents between 2.9 and 4.1 g kg⁻¹ of crude fat. Deficient as well as excess supplies

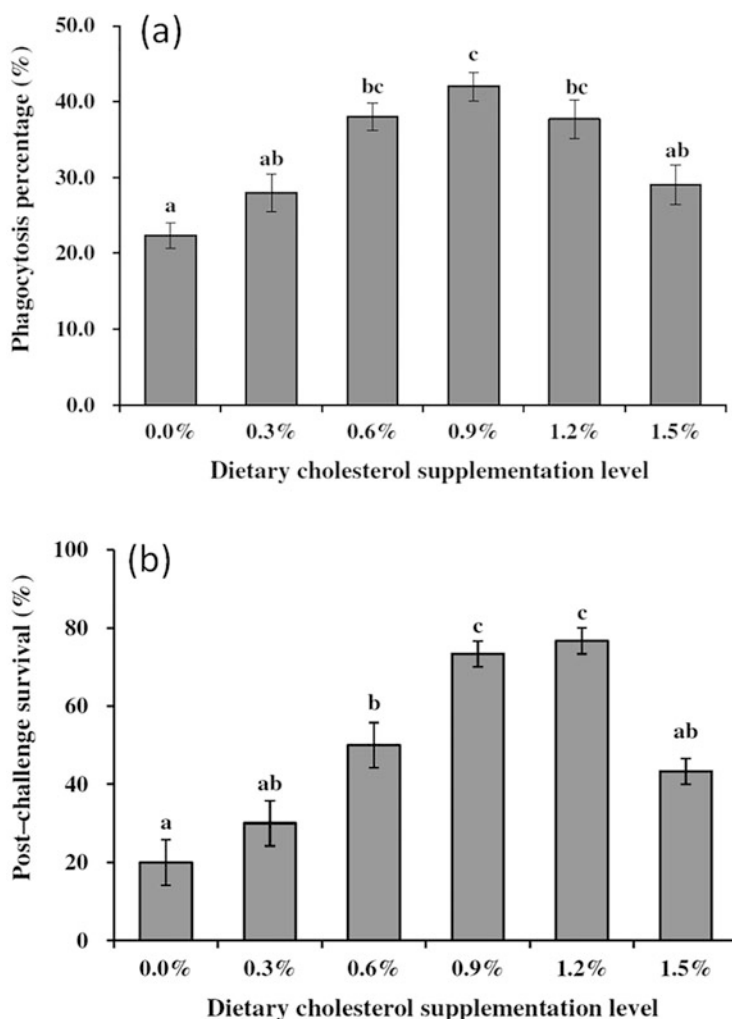


Fig. 31.6 Effects of dietary supplemented graded levels of cholesterol on phagocytosis of head kidney (a) and on post-challenge survival (b) of rainbow trout. Data are expressed as mean \pm SEM. Bars assigned different superscripts were significantly different ($P < 0.05$) (From Deng et al. (2013), with permission from Elsevier)

cause adverse effects. In fact, Progatezky et al. (2014) found pro-inflammatory effects of ingested dietary excess cholesterol in zebrafish via transcription of *il-1 β* in intestine with subsequent inflammation.

Wang et al. (2018) presented clear biomolecular evidence that even deficient dietary cholesterol adversely impacts immune organs (head kidney, spleen, and skin) in young grass carps and leads to skin hemorrhage and lesion as well as increased mortality after *Aer. hydrophila* challenge (Fig. 31.7). Low levels of dietary

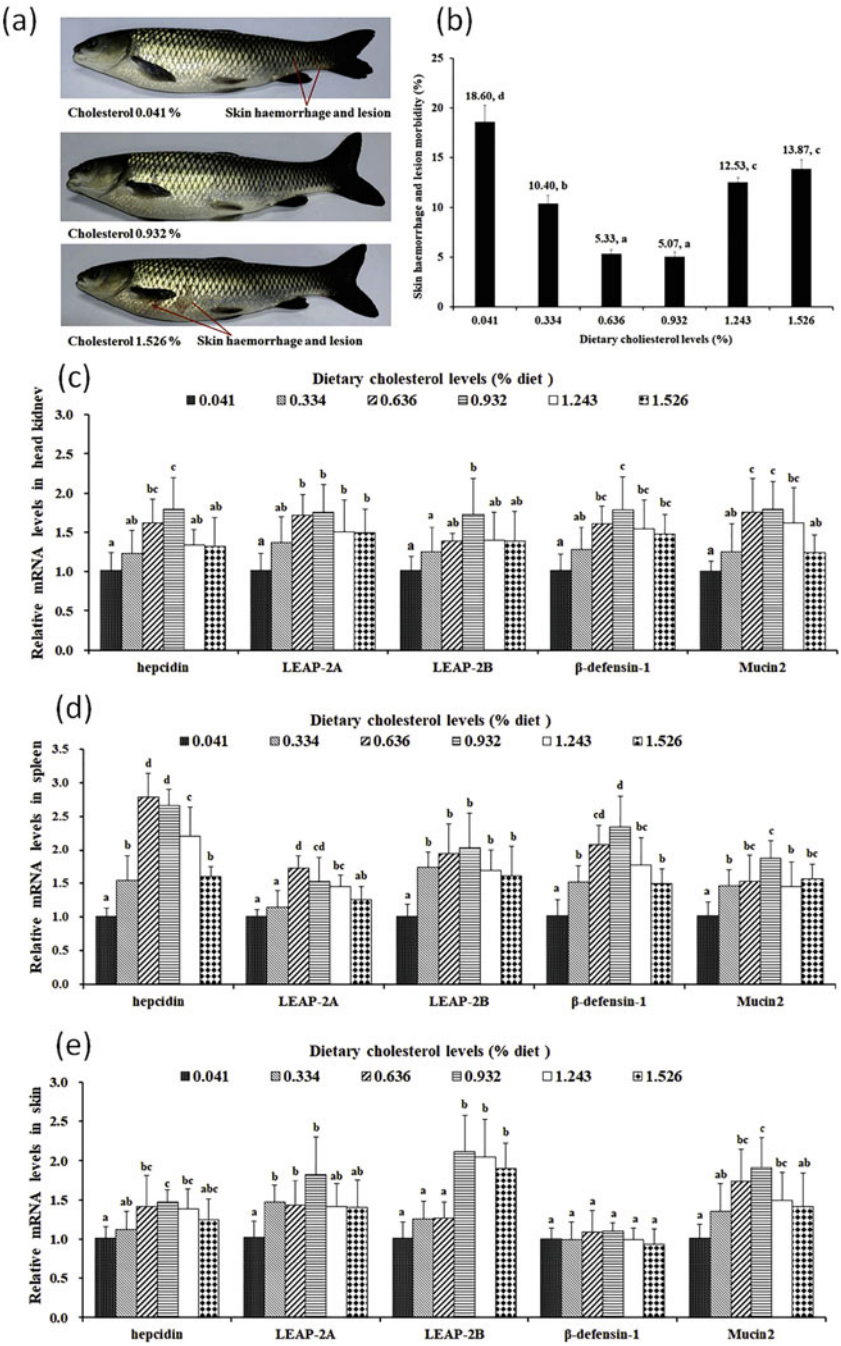


Fig. 31.7 Skin hemorrhage and lesions in young *Ctenopharyngodon idella* fed diets with graded levels of cholesterol for 60 days and then infected with *Aeromonas hydrophila* for 14 days. (a) Low or excess levels of dietary cholesterol lead to obvious skin hemorrhage and lesions. (b) Skin hemorrhage and lesion morbidity in young individuals. Relative mRNA levels of *hepcidin*, *leap*-

cholesterol impair the immunity by downregulating *β -defensin-1* in head kidney and spleen and by upregulating pro-inflammatory cytokines (*il-1 β* , *il-12p40*) in head kidney.

The optimal dietary cholesterol level for young grass carp, estimated as ~0.8%, upregulates *hepcidin* and *mucin2*, *leap-2a*, and *β -defensin-1* in the head kidney. Except for *β -defensin-1* in the skin, these defense genes are also upregulated in spleen and skin (Fig. 31.7). Hecpudin is a key regulator of the entry of iron into the circulation with antimicrobial property, and β -defensin is an antimicrobial epithelial peptide; LEAPs are liver-expressed antimicrobial peptides, and mucins comprise a family of high molecular weight, heavily glycosylated proteins, which are key components in most gel-like secretions and serve functions from lubrication to cell signaling—they form chemical defense barriers.

It deserves future research whether dietary excess cholesterol can be controlled by epigenetic pathways as described in humans (Hennessy et al. 2019) or by adding appropriate probiotics as shown in zebrafish (Lim et al. 2017). With regard to human nutrition, Lim et al. (2017) carried one of the few studies on the cholesterol–gut microbe–host axis and assessed the cholesterol-lowering effect of two probiotic bacteria. The observed cholesterol-lowering effect is accompanied by downregulation of *npc1l1* in the intestines, upregulation of *abca1* in the livers, and downregulation of *appa* in the brain (for functions, →Fig. 31.1). Most interestingly, probiotic-fed zebrafishes exhibit improved spatial learning and memory.

Epigenetic Regulation

Recent papers describe the action of microRNAs in cholesterol metabolism. To identify the role of miRNA in the adaptation to plant-based diet, Zhu et al. (2019, 2020) used two lines of rainbow trout: the selected line has an improved adaptation to plant-based diets, and the nonselected line serves as control. Of various miRNAs, only the expression of hepatic *miR-33a* is elevated in control trouts fed plant-based diet (Fig. 31.8). *miR-33a* targets genes encoding proteins involved in cholesterol catabolism, particularly SREBPs (Zhu et al. 2020). SREBP-2 and *miR-33a* synergistically enhance the expression of cholesterol synthesis genes.

By exploring the mechanism by which miRNAs mediate high fat-induced changes of cholesterol metabolism, Chen et al. (2020) found an additional epigenetic pathway in yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*). *miR-144* mediates cholesterol metabolism via direct regulation of the hepatic transcription factor C/EBP α . This transcription factor belongs to the CCAAT/enhancer-binding protein



Fig. 31.7 (continued) 2a, *leap-2b*, *β -defensin-1*, *mucin2* in (c) head kidney, (d) spleen, and (e) skin of young individuals. Data are expressed as means of three replicates with five fish each + SD. Different letters indicate significant differences ($P < 0.05$) (From Wang et al. (2018), with permission from Elsevier)

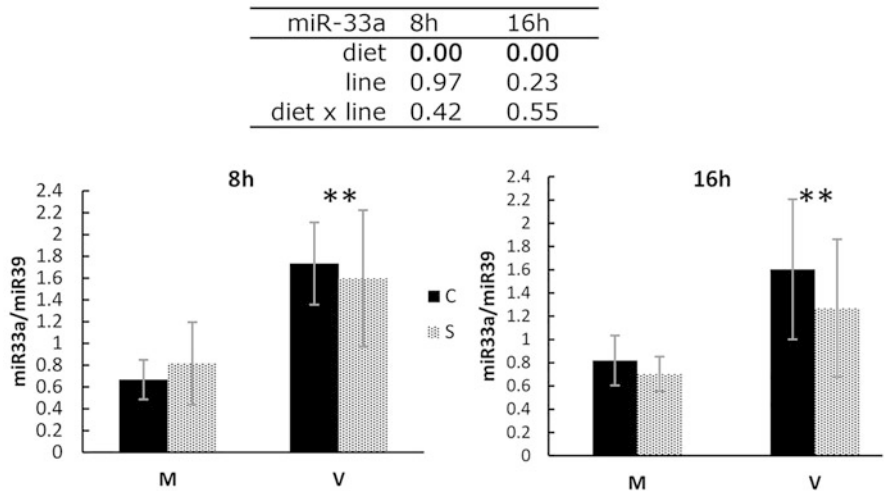


Fig. 31.8 Expression of *miR-33a* involved in cholesterol metabolism at 8 h and 16 h, in the liver of rainbow trout from selected (S) line and control (C) line fed the marine (M) diet and (V) vegetable diet. The selected line was selected for better survival and growth performance with a 100% plant-based diet during three generations. The control line was the common trout without any artificial selection. Expression values are normalized by spike *miR-39*. Values are means \pm SD ($n = 6$). (Results of two-way analysis of variance are indicated with the P value.) Two stars represent $P < 0.01$ for diets/lines, respectively (From Zhu et al. (2019), with permission from Elsevier)

(C/EBPs) family of six transcription factors. C/EBP α is required for the maintenance of energy homeostasis, lipid storage, and adipogenesis (Rosen et al. 2002).

31.1.2 Phytosterols

Phytosterols are phytosteroids similar to cholesterol. More than 200 phytosterols and related compounds have been identified (Moreau et al. 2002). Several studies evaluate whether also dietary phytosterols can be nutritionally utilized by aquatic animals and, thus, replace cholesterol.

31.1.2.1 Invertebrates

Intriguing studies have been conducted again with planktonic cladocerans. The microcrustaceans can utilize phytosterols, which are bioconverted to cholesterol to meet the requirement for somatic growth and reproduction (Martin-Creuzburg et al. 2009). However, not all phytosterols are equally well suited for bioconversion; the cholesterol threshold concentration of sterol-limited growth of *Daphnia magna* is 8.9 $\mu\text{g mg}^{-1}$ C, and added phytosterols are required between 3.5 and 34.3 $\mu\text{g mg}^{-1}$

C (Martin-Creuzburg et al. 2014). Noteworthy, the phytosterols fucosterol, brassicasterol, ergosterol, and stigmasterol support *Daphnia* somatic growth more efficiently and in lower amounts than cholesterol itself (Martin-Creuzburg et al. 2014).

Based on phytosterol concentrations in over 9000 field samples of boreal and subarctic lakes, Peltomaa et al. (2017) calculated that in 70% of the lakes, somatic growth of *Daphnia* is not limited by phytosterols. Nevertheless, *Daphnia* development can occasionally be sterol-limited. This is most common in cyanobacterial dominated lakes, obviously with low parasitic pressure by chytrids.

Marine and saline lake crustaceans, too, can bioconvert phytosterols into cholesterol as shown in a pioneering paper in *Marsupenaeus japonicus* and *Artemia salina* (Teshima 1971). In penaeid crustaceans, dietary phytosterols are not as effective in growth promotion as cholesterol (D'Abramo 1998). However, corresponding reports are inconsistent, since *L. vannamei* on a diet with 0.1% of phytosterols grows as well as those fed cholesterol (Morris et al. 2011). Ju et al. (2010) showed that a dietary phytosterols mix (including β -sitosterol) does not affect growth in this shrimp. This contradiction can be taken as need of further in-depth studies (\rightarrow Chap. 40). In larvae and juveniles of penaeids as well as in freshwater *Macrobrachium rosenbergii*, the demand is age-dependent. In the latter, a blend of various dietary phytosterols is equally effective as cholesterol in satisfying the dietary sterol requirement of juveniles (D'Abramo 1998).

Microalgae that constitute the main dietary source of suspension-feeding bivalves contain an array of phytosterols, in most cases lacking cholesterol. Giner et al. (2016) reported that the rapid growth of bivalves on microalgal diets with no cholesterol is based on the fact that phytosterols can satisfy the dietary requirement for cholesterol through metabolic conversion. For instance, the scallops under study efficiently dealkylate sterols to cholesterol. Furthermore, the observed formation of $\Delta^{5,7}$ sterols (provitamin D) from Δ^5 sterols represents the likely initiation of steroid hormone biosynthesis (Giner et al. 2016).

31.1.2.2 Fishes

Replacing dietary fishmeal and fish oil with plant ingredients decreases dietary cholesterol by introducing phytosterols. Obviously, a single meal containing phytosterols does not yet affect the uptake or tissue distribution of cholesterol as documented in zebrafish (van Spankeren et al. 2019). However, what happens on the long-term?

Actually, long-term provision of high levels of dietary phytosterols affects lipid metabolism and increases liver and plasma triacylglycerols (TAG) as found in Atlantic salmon (Liland et al. 2013). Fishes fed low-cholesterol diets exhibit an increased expression of genes encoding proteins involved in cholesterol uptake and synthesis. The expression of these genes is, however, partially inhibited by rapeseed oil, likely due to the high dietary phytosterol content. Taken together, adverse effect of dietary phytosterols on cholesterol uptake and metabolism has clearly been demonstrated and has to be taken into account when determining optimal dietary

cholesterol for Atlantic salmon and other fishes (Sissener et al. 2018). A reevaluation of the hypothesis that dietary phytosterols lead to development of fatty liver in Atlantic salmon revealed that phytosterols are not the cause of a fatty liver development (Sissener et al. 2017).

In addition to cholesterol mimic, phytosterols can stimulate the immune system by mimicking pathogen attack (Costas et al. 2014). In gilthead seabream, most important changes are related to innate immunity, namely, upregulation of interleukin 10. Usually, *Il-10* is upregulated following lipopolysaccharide stimulation and bacterial or parasitic infections, indicating a central role in anti-inflammatory responses. Therefore, the authors recommend that the utilization of phytosterols should be done only moderately to avoid chronic inflammation.

Surprising results were reported by Takase and Ushio (2018), who studied changes in intestinal gene expression of zebrafish upon β -sitosterol (Fig. 31.1) administration. β -sitosterol upregulates the expression of genes for sterol uptake to intestinal epithelial cells and further transportation to hepatocytes. Obviously, fishes, at least omnivorous ones, actively uptake β -sitosterol for utilization and eventual excretion indicated by upregulation of several membrane-bound transporter genes from the ATP-binding cassette (ABC) subfamilies G and A. However, which kind of utilization takes place in the fish remains to be evaluated.

31.2 Phospholipids

Phospholipids (PLs) are major components of all cell membranes and consist of two hydrophobic FA “tails” and a hydrophilic “head” containing phosphate. The two components are joined together by a glycerol molecule (glycerophospholipid) or by a sphingoid base² (sphingophospholipid).

The major classifications of PLs are (Molina-Poveda 2016):

1. Phosphatidylcholine (PC): phosphatidic acid linked to choline (lecithin), with respect to potential feed sources, egg yolk, soybeans, grains, wheat germ, fish, legumes, yeast, and peanuts.
2. Phosphatidylethanolamine (PE): phosphatidic acid linked to ethanolamine (cephalin), in chicken eggs and plant tissues.
3. Phosphatidylinositol (PI): phosphatidic acid linked to inositol, in plant tissues.
4. Phosphatidylserine (PS): phosphatidic acid linked to serine, in plant leaves, while not common in animal tissues.
5. Phosphatidylglycerol (PG): phosphatidic acid linked to glycerol, in bacterial and animal membranes and plant leaves.
6. Diphosphatidylglycerol (DPG): two PGs connect to a glycerol backbone (cardiolipin), in bacteria, animal, plant, and fungi membranes.

²Set of aliphatic amino alcohols.

31.2.1 Invertebrates

31.2.1.1 Farmed Invertebrates

Phospholipids provided in different dietary forms have been shown to be required for growth and survival of juveniles and larvae of shrimps. In most studies, lecithin derived from soybeans is the phospholipid source. The apparent absence of such a demand in adults indicates that the requirement is age-specific and is based on an insufficient rate of synthesis to meet the demands of the comparatively rapid growth rates characteristic of early life stages (NRC 2011). The PL requirement of farmed shrimps ranges from 0.5–1.0% (kuruma prawn larvae) to 1.0–1.5% (tiger shrimp, Pacific white shrimp juveniles), 3.0% (kuruma prawn juveniles), and 4.0% (juvenile *Portunus trituberculatus* (Sun et al. 2017; Han et al. 2018)). Other studies report a requirement of 6.5% dietary de-oiled soybean lecithin for Pacific white shrimp juveniles (Coutteau et al. 1996) (→Chap. 40).

In contrast to other invertebrates and fishes, larval and juvenile kuruma prawns require dietary sterols and phospholipids as indispensable nutrients. Metabolic studies in juveniles indicate that dietary PLs take part in the transport of dietary lipids, especially cholesterol, through the hemolymph in their body (Teshima 1998).

As with the fish studies, some shortcomings exist in accurately determining the specific demands, because dietary sources of PLs used in investigations often are not based on consistent compositions. Not all phospholipids are equivalent. For juvenile American lobsters (*Homarus americanus*), PC is found to be the active compound that reduces mortality when compared to PE (ovine source) and PI (soybean source) (NRC 2011). Another example: a level of 1.0% of pure PC or PI, extracted from bonito eggs and soybean, respectively, is found to be most effective in growth and survival of larval *M. japonicus*. Pure PC derived from chicken eggs and pure PE derived from bonito eggs and ovine brain do not have equivalent effects (Michael et al. 2007). Similarly, Li et al. (2014) found that egg yolk lecithin promotes growth of juvenile swimming crabs (*P. trituberculatus*) better than soybean lecithin, and the latter contributes to better FA profiles in muscle and hepatopancreas than phosphatidylcholine (Wang et al. 2016). Studying *L. vannamei*, papers show that the addition of a few percent dietary soybean lecithin, in addition to the other marine oils from squid and fish, reduces the requirements of LC-PUFA from fish oil (Sánchez et al. 2014) and improves survival of juveniles (Hu et al. 2011).

It is well understood that broodstock nutrition affects egg quality, embryonic development, and postlarval quality in crustaceans (Cavalli et al. 2000). The beneficial effect of dietary soybean lecithin (SL) on ovarian development and reproductive performance applies to several freshwater and marine crustacean broodstocks, including *M. rosenbergii* (Cavalli et al. 2000), *Eriocheir sinensis* (Wu et al. 2010), *P. monodon* (Millamena et al. 1986), *L. vannamei* (Cahu et al. 1994), and *M. japonicus* (Alava et al. 1993). Wang et al. (2013) expanded this catalog by adding *Cherax quadricarinatus* and show that the transcription of the *vitellogenin* (*vgt*) gene in the hepatopancreas peaks in individuals fed the 2% SL diet, with a lower expression observed at lower or higher SL percentages (Fig. 31.9a).

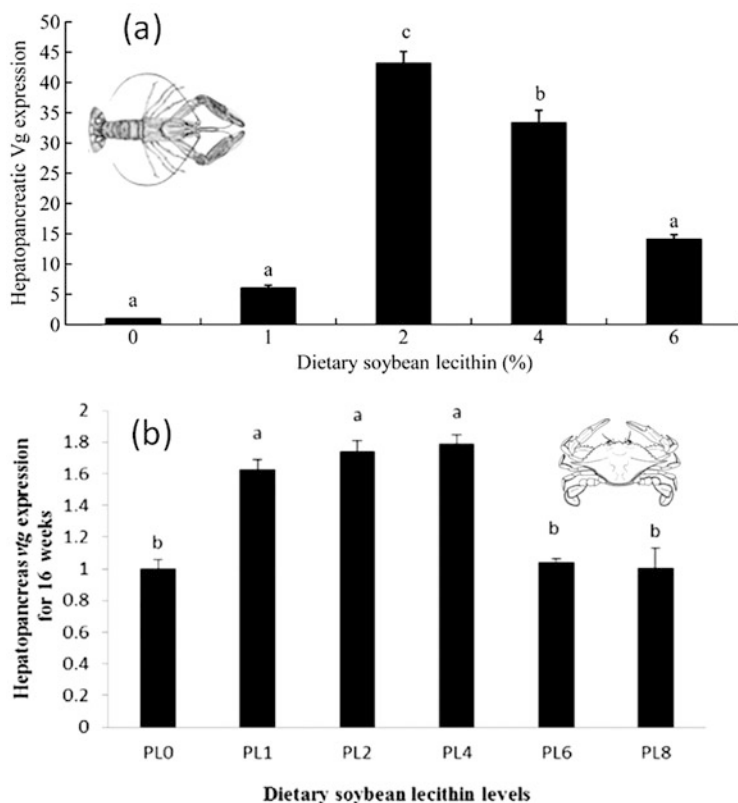
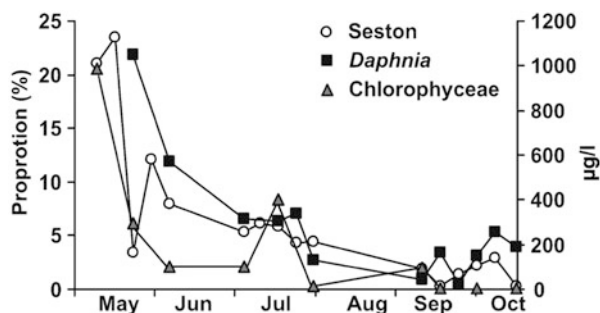


Fig. 31.9 *Vitellogenin* (*vtg*) mRNA expression in the hepatopancreas of female *Cherax quadricarinatus* (a) and *Portunus trituberculatus* (b) fed experimental diets with graded lecithin content. Statistical significance is represented by different letters ($P < 0.05$) (a) from Wang et al. (2013) and (b) from Ding et al. (2017), with permission from Wiley and Elsevier; images credit FAO)

Similarly, Ding et al. (2017) showed in *P. trituberculatus* that dietary lecithin promotes the *vtg* transcription (Fig. 31.9b). Furthermore, the dietary PL inclusion increases progesterone and 17 β -estradiol levels in the serum after feeding the diets for 16 weeks. Overall, dietary lecithin promotes ovary development and oocyte maturation indicated by at least three biomolecular and biochemical markers. Dietary 2–4% lecithin appears to suffice in decapod crustaceans.

Beneficial effects of dietary lecithin are not restricted to crustaceans. González-Durán et al. (2008) reported that dietary soy lecithin enhances the production and incorporation of arachidonic acid in shell and gonad of the sea urchin *Strongylocentrotus droebachiensis* as well as growth and production of *Lytechinus variegatus* juveniles and gonadal development in adults (Gibbs et al. 2009, 2015). In the same line of evidence, in common octopus (*Octopus vulgaris*) and cuttlefish (*Sepia lycidas*), marine lecithin has a beneficial effect on paralarval and juvenile growth (Garrido et al. 2016).

Fig. 31.10 The contribution of 18:3 ω 3 PLFA typical of Chlorophyceae as a proportion of total PLFAs in both seston (mean value of 0–1.2 m) and *Daphnia*, together with the abundance of Chlorophyceae ($\mu\text{g L}^{-1}$) (From Taipale et al. (2009); with permission from Wiley)



31.2.1.2 Zooplankton

On the ecosystem scale, Taipale et al. (2009) showed that the phospholipid FA (PLFA) composition of freshwater zooplankton, like *Daphnia*, corresponds rather well to that of their in situ diet of phytoplankton and bacteria with a lag period of around 1 week in the case of adult animals. The PLFAs of seston reveal the dominant available food sources, and relating these to the *Daphnia* PLFA profile provides insights into the seasonal changes in *Daphnia* diet (Fig. 31.10). This indicates that PLFA profiles can be used estimating the importance of phytoplankton and bacteria in zooplankton diets in freshwater systems. This is obviously the only available trophodynamics study with reference to PLFAs.

31.2.2 Fishes

Phospholipids are essential ingredients in fish larval and juvenile diets and improve growth and survival of larvae, enhance feed intake and utilization, increase stress resistance, and improve skeletal development (Martins et al. 2020). Cahu et al. (2003) found a clear dose–effect relationship feeding larval sea bass (*Dicentrarchus labrax*) graded phospholipid diets.

Also Uyan et al. (2009) reported that juvenile amberjack (*Seriola dumerili*) increases feed intake and shows improved growth fed non-fishmeal diets, if appropriate PL levels are supplied. Comparisons and determination of absolute dietary requirements, however, are hampered by the use of a wide range of PL preparations that vary greatly both in PL content and composition. As the majority of studies have used crude mixed PL preparations, it is difficult to elucidate which specific PL class imparts beneficial effects. Based on the few studies in which single pure PL species have been used, the rank order for efficacy appears to be phosphatidylcholine > phosphatidylinositol > phosphatidylethanolamine > phosphatidylserine (Tocher et al. 2008).

Dietary requirements vary between 0% and 14%. Some figures are as follows: white sturgeon (0%), carp (2%), ayu (3%), stellate sturgeon (3–7%, Jafari et al.

(2018)), pikeperch (9%), rainbow trout (14%), turbot (2%), red seabream (5%), knife jaw (3–7.4%), Japanese flounder (7%), or gilthead seabream (>9%), followed by the highest reported value for European sea bass at 12% of the diet. In juveniles, the PL requirements range from 1.5% (striped jack), 2–3% (European sea bass), 4–6% (Atlantic salmon), 7% (Japanese flounder), up to 14% (rainbow trout) (NRC 2011). Well within this range, Adel et al. (2017) found that 2–3% lecithin inclusion in diets enhances activities of digestive enzymes, antioxidant enzymes, antibacterial factors, and mucosal immune parameters in common carp.

Soybean lecithin (SBL) can replace fish oil. In carnivorous silvery-black porgy (*Sparidentex hasta*) juveniles, Pagheh et al. (2019) substituted up to 67% fish oil with SBL without reduction in weight gain and feed intake. However, increasing dietary SBL leads to a significant decrease in muscle EPA and DHA, whereas the concentrations of different PL classes increase.

Qualitative requirements indicate that PC may be more important for growth and PI more for development. The mechanism of apparent essentiality of PLs in larval and early juvenile fish is not fully clear. It is hypothesized that PL biosynthesis pathways are inefficient or not fully developed in larvae of a number of fish species. In larvae fed diets rich in TAG, lack of sufficient dietary PL limits lipoprotein synthesis in enterocytes, leading to impaired transport of lipid (energy supply) nutrients to tissues. Dietary PL may provide intact glycerophosphobase backbones, which bypasses this limitation. Thus, growth stimulation appears to be due to improved transport, assimilation, and utilization of dietary lipids (Tocher et al. 2008).

Importantly, dietary excess levels of PLs can induce skeletal maldevelopment (Villeneuve et al. 2006). PLs containing high levels of DHA and EPA induce cephalic (Fig. 31.11) and vertebral column deformities adversely affecting fish

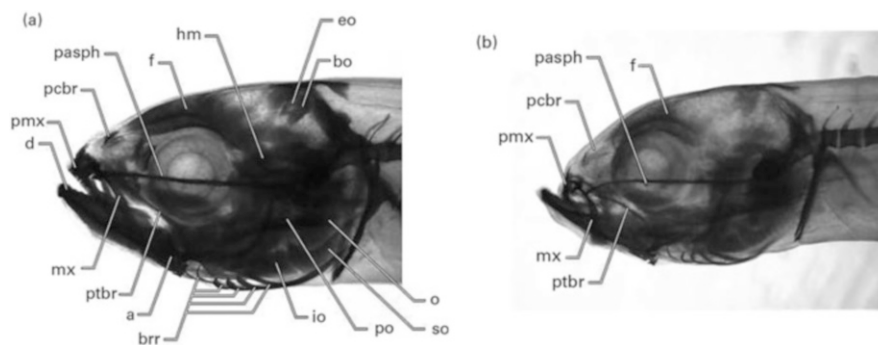


Fig. 31.11 Dietary-induced malformations in the cranium of European sea bass larvae (age 40 d post-hatching). (a) Normal larvae. (b) Deformed larvae (abnormal structures are indicated). Specimens were stained with Alizarin red to reveal calcified structures. **a** angular, **brr** branchiostegal rays, **bo** basioccipital, **d** dentary, **eo** exoccipital, **f** frontal, **hm** hyomandibula, **io** interoperculum, **mx** maxilla, **o** operculum, **pasph** parasphenoid, **pcbr** lamina paracerebralis, **pmx** premaxilla, **po** preoperculum, **ptbr** pterygoid process, **so** suboperculum (From Villeneuve et al. (2006), with permission from the Cambridge University Press)

growth and survival with a downregulation of *retinoid X receptor α* (*rxra*), *retinoic acid receptor α* , *retinoic acid receptor γ* , and *bone morphogenetic protein-4*. Already 5% induces skeletal deformities, while 1.5–2.5% dry matter supplied by PL PUFAs improves growth, survival, and skeletal development (Cahu et al. 2009).

Further studies highlight the significance of PLs for immunity of larvae and juveniles. In juvenile grass carp, Feng et al. (2016) discovered that optimal PL supplementation improves the complete immune and antioxidative machineries as well as the tight junction barrier:

1. Activity of lysozyme, acid phosphatase, content of complement component 3 (C3), and transcription of *liver expressed antimicrobial peptide 1* (*leap-1*) and *leap-2* mRNA.
2. Transcription of *interleukin 10*, *transforming growth factor $\beta 1$* , *inhibitor factor $\kappa B\alpha$* (*ikba*), and *target of rapamycin* (*tor*).
3. Activities of anti-superoxide anion (ASA), anti-hydroxyl radical (AHR), copper/zinc superoxide dismutase (SOD1), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR).
4. Glutathione content and mRNA levels of *sod1*, *cat*, *gpx*, *gr*, and *NF-E2-related factor 2* (*nrf2*) genes.
5. Transcription of *occludin*, *claudin b*, *claudin c*, *claudin 12*, and *zonula occludens 1*.

Moreover, appropriate dietary PL supplementation decreases:

1. *Tumor necrosis factor- α* , *interleukin-1 β* , *nuclear factor- κB p65* (*nf- κb p65*), *ikb kinase- β* (*ikkb*), and *ikb kinase- γ* (*ikky*) mRNA expression.
2. Malondialdehyde (MDA), protein carbonyl (PC), and reactive oxygen species (ROS) content and the transcription of *kelch-like-ECH-associated protein 1a* (*keap1a*) and *keap1b*.
3. Transcription of *myosin light chain kinase* (*mlck*) and *p38 mitogen-activated protein kinase* (*p38 mapk*).

In yellow croaker (*Larimichthys crocea*), the beneficial effects of dietary PLs on survival and growth performance are restricted to the larval stage (Feng et al. 2017). Beneficial effects of dietary PLs on survival and growth performance of large yellow croaker take place, inter alia, via reduction of lipid deposition in the liver of juveniles through utilizing more diacylglycerol for PC synthesis, rather than triglyceride synthesis.

An intriguing plasticity of PL utilization occurs in Senegal sole larvae (Hachero-Cruzado et al. 2020). While a low supply of PL delays growth, the larvae maintain viability and competence to trigger a compensatory growth when a shift in the diet occurs without affecting metamorphosis success or survival rates. The lipid accumulation in the intestine and the lack of efficient lipid mobilization mechanisms in spite of a coordinated activation of the gene expression related to PL intermediate synthesis, PL remodeling, and several apolipoprotein transcripts are mainly responsible for the negative effects on growth. Particularly, apolipoprotein *apob2* is downregulated in larvae fed with low PL indicating that PL levels positively regulate

apob2 expression in the intestine of sole larvae. Supportingly, Daprà et al. (2011) found reduced *apob* expression in rainbow trout fry fed a PL-free diet compared to those fed PL-supplemented diets. Therefore, apolipoprotein B is likely to chylomicron³ biosynthesis; it is an important structural component of chylomicrons (Iqbal and Hussain 2009). The lack of full functionality for PL biosynthesis pathways limits the lipid dietary uptake in these early sole larvae (Hachero-Cruzado et al. 2020).

31.3 Wax Esters

Some zooplankton species, such as the well-known North Atlantic calanoid copepod, *Calanus finmarchicus*, serve as $\omega 3$ FA-rich diets for wild and farmed salmon and contain high amounts of wax esters instead of TAG (Fig. 31.12). Wax esters are esters of a LC-FA and a monohydric LC fatty alcohol (Fig. 31.12).

Although these esters are considered difficult to digest by many fishes, e.g., Atlantic halibut (Colombo-Hixson et al. 2011), a diet rich in wax esters derived from calanoid copepods, and thus high in monoenoic LC fatty alcohols such as 20:1 ω 9 (gondoic acid), has no obvious physiologically detrimental effects in rainbow trouts (Oxley et al. 2005). This notion can be generalized for salmonids (Bogevik et al. 2008): It is well understood that juvenile Atlantic salmon feed on these zooplankton species. This indicates a capacity to utilize these lipids. Salmon have several adaptations to digest diets rich in wax esters. These include increased feed conversion, higher production of bile, and higher activity of lipolytic enzymes in the midgut. The Atlantic salmon feeds and grows on diets with a medium amount of wax esters (30% of the lipid) with results comparable to individuals maintained on fish oil diets. Higher wax ester levels (50% of the lipid), however, cause poorer lipid digestibility and growth, so that optimal utilization of wax esters in Atlantic salmon is closer to 30% than 50% of the dietary lipids (Bogevik 2011). The incorporation of LC-fatty alcohols from wax esters occurs more slowly and is stronger temperature-dependent than that of fish oils.

The release of sufficient amounts of bile and enzymes into the intestines of fish is crucial for optimal digestion (Tocher 2003). Bogevik et al. (2009) and Oxley et al. (2009) elucidated the potential of salmon to adapt the digestive capacity following wax ester feeding and found that the gallbladder volume increases upon wax ester feeding, which agrees well with previous reports from rainbow trout (Tocher and Sargent 1984). Correspondingly, Minghetti et al. (2010) detected two enzymes in Atlantic salmon responsible for the effective utilization of wax esters, namely, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). ADH3 is

³Chylomicrons are lipoprotein particles that consist of triglycerides, phospholipids, cholesterol, and proteins. They transport dietary lipids from the intestines to other locations in the body (Nelson and Cox 2005).

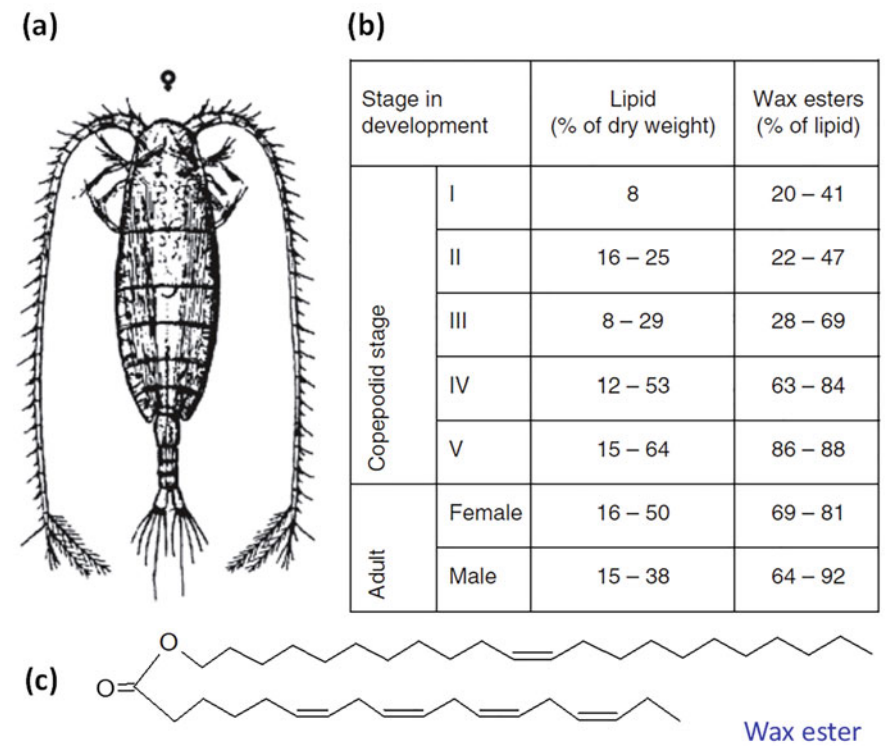


Fig. 31.12 *Calanus finmarchicus*: (a) morphology (adapted from Sars 1903) and (b) variation in lipid and wax ester level among different stages caught in the North Atlantic (From Bogevik (2011), with permission from Wiley). (c) Wax ester (22:1 alcohol and 18:4 fatty acid, a typical storage lipid of marine zooplankton (Lee et al. 2006))

capable of oxidizing LC-fatty alcohol, and ALDH3a2 subsequently oxidizes LC-fatty aldehyde to the corresponding FA.

In larval fish nutrition, however, waxes have not yet been considered, despite the fact that they are found in a wide range of marine copepods and transferred through the food chain to fishes, accumulate in eggs, and become energy/nutrient sources in several species at the larval stage. In some fishes, waxes constitute up to 70% of egg dry matter (or 91–97% of neutral lipid content). Previous reviews of the role of lipids in larval fish nutrition do not mention the presence, function, or importance of waxes. It remains unknown why the deposition of wax esters is favored over TAGs in body and eggs in some fishes, such as mullet (*Mugil*), striped bass (*Morone*), perch (*Perca*), burbot (*Lota lota*), gourami (*Trichogaster*), coelacanth (*Latimeria*), capelin (*Mallotus*), or turbot (*Scophthalmus*). It is assumed that these lipids (with comparable degrees of unsaturation) have similar densities, caloric value, and compressibility. They differ though in their respective coefficients of thermal expansion, a predominance of extracellular versus intracellular location and rate of mobilization (Dabrowski et al. (2015) and references therein).

Furthermore, the acquisition and function of waxes in selected freshwater fishes remains a mystery, as the deposited wax esters are not of food origin like in marine fishes, their synthesis pathways are little studied, and their utilization in larval stages is little known (Dabrowski et al. 2015). For yellow perch, the authors hypothesize that a *de novo* synthesis of wax esters takes place in the ovaries and serves as nutrients for the development of embryos and larvae.

31.4 Concluding Remarks

Due to surprising side effects of dietary cholesterol and phytosterols, these lipidic compounds need reconsideration as feedstuff for fishes. Studies point out that their controlling function appears to be more complex than previously anticipated. For instance, appropriate nutrition with purified phytosterol improves innate immunity. Further unexpected effects of dietary cholesterol can be expected. Therefore, a hologenomic approach à la Limborg et al. (2018) is necessary to shed more light onto this issues, particularly because appropriate probiotics alleviate adverse effects of high-cholesterol diets.

Epigenetics, in form of miRNAs targeting lipid metabolizing genes, has made its way into cholesterol homeostasis in fishes; farmed invertebrates and dietary lipidic compounds, other than cholesterol, however, are still undiscovered in this respect. Furthermore, dietary compounds affecting the one-carbon metabolism and therefore the potential methylation of DNA and histones deserve future attention. Nevertheless, already current knowledge of corresponding epigenetics appear that promising that new perspectives of the role of miRNAs in fish selective breeding are convincingly discussed (Zhu et al. 2019) and await empirical testing. And, as in many previous chapters, the question remains unanswered: What is the contribution of the gut microbiota in the reported results? Can the effect of dietary sterols be improved if the gut microbiota composition is optimized, for instance, by coadministering of suitable probiotics? Encouraging results on this issue have been provided by Lim et al. (2017) who showed that the gut–brain axis can be modulated via lactic acid bacteria-mediated changes of the cholesterol content.

The above description of the mycoloop as trophic upgrade of low-quality food (Frenken et al. (2018, 2020)) provokes the general question of whether its conceptualization can be extended to aquaculture. In particular, can the quality of autotrophic bioflocs (→AAN III “Fishmeal Replacement—Bioflocs”) be improved by introducing parasitic chytrids? Can planktonic and benthic filamentous cyanobacteria be entrained into the food chain by introduction of chytrids enhancing cyanobacterial fragmentation? And, lastly, if chytrids are already part of the bioflocs, are there means to increase their share by manipulating environmental conditions? An ecology-based improvement of aquafeed will increase consumers’ acceptance of improved aquatic farmed animals over biotechnologically improved ones—at least in Europe.

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Chapter 32

Vitamin A—‘Does It Keep the Veterinarian Away?’



Abstract With retinol (vitamin A, VA), this chapter starts the series of contributions about vitamins in the nutrition of aquatic animals. It focuses on functional studies of VA, on biochemical and biomolecular levels, on mechanistic details of hypo- and hypervitaminosis, and on cross talk between vitamins and other nutrients. Retinoid metabolism involves many different forms, including retinyl esters, retinol, retinal, retinoic acid (RA), and oxidized and conjugated metabolites of both retinol and RA. RA is the main active metabolite of VA and necessary during somatic, nervous, immunity, and gonadal development. VA deficiency is well documented. The few available broodstock studies indicate that this life stage has increased VA requirement. Cross talks between RA and vitamin D interfere within bone formation and calcification. Disruption of retinoic signaling via excessive or deficient RA can cause teratogenic effects. Excess VA supply during embryonic development can lead to impairment of immunity or skeletal malformation via interactions with *hox* and *dlx* genes. Deficient VA supply impairs immunity, disturbs intestinal cell-to-cell integrity, and promotes apoptosis. Currently, the role of intestinal microbes and epigenetics appears to be almost overlooked, although intriguing mammalian studies point out retinoic acid as one major epigenetic regulator.

A vitamin is a vital organic nutrient that an animal requires in limited amounts for normal growth, immunity, reproduction, health, and general maintenance. The organism cannot synthesize the compound in sufficient quantities, and it must be obtained through diets. Vitamins can be divided into two main groups: fat-soluble (vitamins A, D, E, and K) and water-soluble ones (vitamin B complex, choline, and vitamin C).

Vitamin A (VA) is a group of unsaturated nutritional organic compounds that includes retinol, retinal, retinoic acid (RA) (Fig. 32.1), and several carotenoids as provitamin A. There are approximately 600 different carotenoids known to exist, but only about 50 of these have provitamin A activity with β -carotene as most potent retinol precursor (Fernández and Gisbert 2011).

NRC (2011), Molina-Poveda (2016), and Hernandez and Hardy (2020) provide comprehensive surveys of vitamin requirements of aquatic animals. This chapter will therefore focus on interesting functional studies of VA, mainly on biochemical and biomolecular levels, on mechanistic details of hypo- and hypervitaminosis, and on

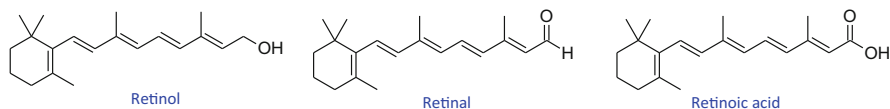


Fig. 32.1 Structures of retinol (vitamin A, VA), all *trans* retinal (VA aldehyde), and all *trans* retinoic acid (VA acid, RA)

cross talks between vitamins themselves and vitamins and other nutrients where applicable.

Retinoids are required for maintaining many essential physiological processes, including normal growth and development, normal vision, healthy immune system, normal reproduction, healthy skin, and barrier functions. In mammals, an excess of 500 genes is regulated by RA. The body must acquire VA from the diet in order to maintain these essential physiological processes. Retinoid metabolism involves different forms, including retinyl esters, retinol, retinal, RA (Fig. 32.1), and oxidized and conjugated metabolites of both retinol and RA. In zebrafish embryos, RA signaling is necessary during the pre-segmentation stages to pattern the anterior–posterior axis of the central nervous system and to induce a pectoral fin bud (Grandel et al. 2002; Retnoaji et al. 2014). In response to treatments with exogenous RA, an early RAR/RXR signaling system upregulates enzymes for the degradation and downregulates enzymes for the synthesis of RA, protecting the zebrafish embryo against RA teratology (Oliveira et al. 2013).

Retinoid metabolism involves many specific carrier proteins and enzymes as well as other proteins, which are involved in mediating also triglyceride and/or cholesterol metabolism (D’Ambrosio et al. 2011). Therefore, a cross talk with other lipophilic vitamins, such as vitamin D (VD), and lipophilic nutrients can be expected and will be discussed below.

Vertebrates have a complex and tightly controlled metabolism for retinoids and their precursors, the carotenoids (Fig. 32.2). The retinoid signaling pathway (RSP) is highly conserved through evolution (Fernández et al. 2018). Consequently, the following RSP description is based mainly on studies with mammals (D’Ambrosio et al. 2011) and yet identified differences in fishes.

For optimal retinoid absorption, fat must be consumed along with the ingested retinoid in order to facilitate its entry into enterocytes from the lumen through chylomicron formation. Carotenoids, like β -carotene, but also lutein and lycopene, are taken up through a process involving scavenger receptor class B member 1 (SR-B1), cluster determinant 36 (CD36), or Niemann–Pick C1-like 1 (NPC1L1, \rightarrow Chap. 31) (Fig. 32.2). β -carotene can be incorporated intact and unmodified into nascent chylomicrons and then transported by the bloodstream or can be converted to retinal by a central cleavage catalyzed by β -carotene 15,15’oxygenase 1 (BCO1) in the enterocytes that converts one molecule of β -carotene into two molecules of retinal. The control of the expression of *bco1* at promoter level by the RA-inducible homeobox transcription factor ISX can represent the first negative feedback regulation step on RSP, which might also explain the large individual variability in intestinal β -carotene conversion (Fig. 32.2). In addition, ISX also regulates *sr-b1*

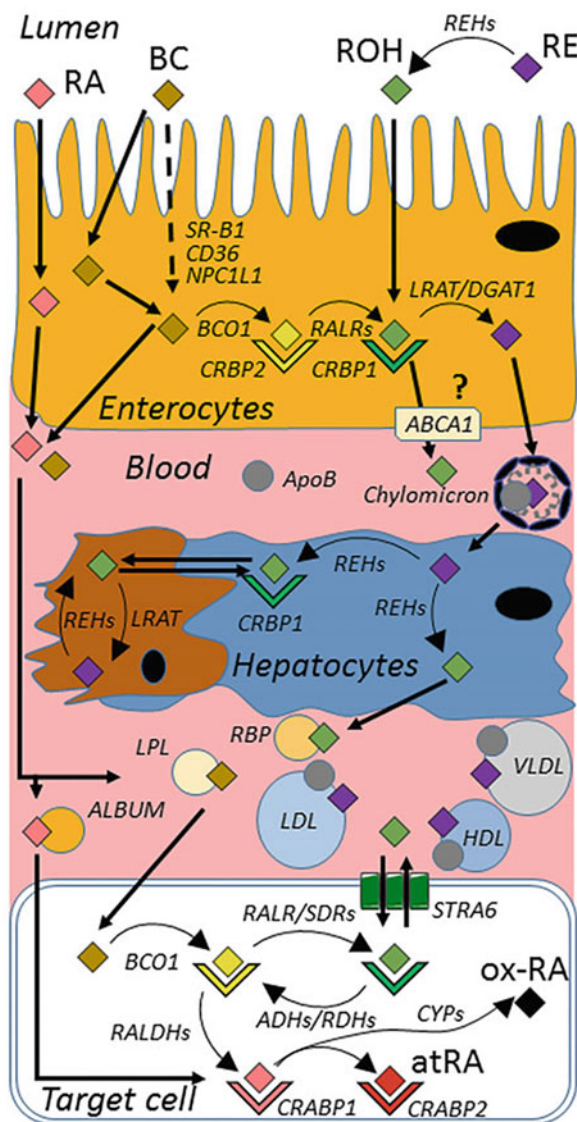


Fig. 32.2 Overview of the metabolism of vitamin A (VA) and the retinoid signaling pathway (RSP) from absorption to target cells. *ABCA1* ATP binding cassette A1 [Apolipoproteins are proteins that bind lipids to form lipoproteins. They transport lipids (and fat-soluble vitamins) in the blood, cerebrospinal fluid, and lymph (Nelson and Cox 2005, →Chap. 31)]; *ADHs* alcohol dehydrogenases; *ALBUM* albumin; *ApoB* apolipoprotein B; *atRA*, all-*trans* retinoic acid; *BC* carotenoids including mainly β -carotene but also lutein and lycopene; *BCO1* β -carotene-15,150-oxygenase 1; *CD36* cluster determinant 36; *CRBP1* cellular retinol-binding protein 1; *CRBP2* cellular retinol-binding protein 2; *CRABP1* cellular retinoic acid-binding protein 1; *CRABP2* cellular retinoic acid-binding protein 2; *CYPs*, cytochromes P450; *DGAT1* diacylglycerol acyltransferase 1; *HDL* high-density lipoprotein; *LDL* low density lipoprotein; *LPL* lipoprotein lipase; *LRAT* lecithin/retinol acyltransferase; *NPC1L1* Niemann-Pick C1-like 1; *RA* retinoic acid; *RALDHs*, retinaldehyde dehydrogenases; *RALRs*, retinal reductases; *RBP* retinol-binding protein;

expression in a RA-dependent manner, meaning that the intestinal absorption of carotenoids is tightly regulated by a negative feedback loop, directly depending on the organism’s needs for VA. Independent of this regulatory step, the produced retinal must bind to cellular retinol-binding protein 2 (CRBP2) and then undergo reduction to retinol through short-chain dehydrogenase/reductase (SDR) enzymes or retinal reductases (RALR). Retinol can be also directly incorporated by enterocytes from retinyl esters hydrolyzed in the intestine by pancreatic triglyceride lipase (PTL), pancreatic lipase related protein 2 (PLRP2), or retinyl ester hydrolases (REHs). Retinol formed from dietary carotenoids or directly absorbed from the intestine binds to CRBP2 and is esterified to retinyl ester (RE). The resulting RE, as the dietary carotenoid that has not undergone conversion to retinoid, is then packed into nascent chylomicrons, which are secreted into the lymphatic system. Retinol is also assumed to be secreted involving ATP binding cassette A1 (ABCA1) (Fernández et al. (2018)¹ and references therein).

In human monocytes, immunomodulatory effects of VA through epigenetic changes are identified. Several pathways are described: (1) RA blocks the DNA methylation machinery and leads to transcriptional inhibition or DNA hypomethylation. (2) RA also alters the ten-eleven translocation (TET) (→Chap. 18) activity through conversion of 5hmC to 5mc so that gene transcription proceeds. (3) RA can also act as an inducer for some miRNA by modulating the epigenome. Moreover, VA blocks histone deacetylases and simultaneously activates histone acetyltransferases leading to acetylation of chromatin structure and resulting in activation of gene expression (Nur et al. 2021). Equivalent information for aquatic animals is not yet available.

32.1 Functional Studies

32.1.1 Invertebrates

Dietary carotenoids are the sole biological precursors of retinoids in marine invertebrates (Villanueva et al. 2009). After conversion to retinoids, they are involved in the activation of hormonal nuclear receptors (Dall 1995).

Fig. 32.2 (continued) RDHs retinol dehydrogenases; **RE** retinyl ester; **REHs** retinyl ester hydrolases, pancreatic triglyceride lipases and pancreatic lipase related protein 2; **ROH** retinol; **SDRs** short-chain dehydrogenase/reductases; **SR-BI** scavenger receptor class B member 1; **STRA6** stimulated by RA gene 6 protein homolog; **ox-RA** oxidized retinoic acid metabolite; **VLDL** very low density lipoprotein (From Fernández et al. (2018), with permission from Springer Nature)

¹Extract taken with permission from Springer Nature.

Retinoids play a prominent role in embryonic and gonadal development and differentiation of various cell types (Linán-Cabello et al. 2002). These authors compiled the recommended supplementation levels of VA in shrimp feeds as 15,000 IU kg⁻¹ (VA palmitate) for *Marsupenaeus japonicus* and 4800 IU kg⁻¹ (VA acetate) or 16,000 IU kg⁻¹ (VA palmitate)² for *Litopenaeus vannamei*. Hernandez et al. (2009) found that 7500 IU VA kg⁻¹ (VA palmitate) meets the dietary demands of *M. japonicus*; the minimal dietary VA concentration in growing *Penaeus monodon* is 2511 retinol equivalents kg⁻¹ (~8400 IU kg⁻¹, VA acetate) (Shiau and Chen 2000).

In *Fenneropenaeus chinensis*, elevated levels of VA in broodstock diet have beneficial effects on fecundity and larval quality (Mengqing et al. 2004). The survival from nauplii 2 to zoea 1, the survival of the mysis stage, and the survival of postlarvae are higher on VA-rich than on VA-poor diets. Furthermore, increased numbers of shrimps on elevated VA diets survive hypoosmotic stress. Dietary 60 mg g⁻¹ VA acetate improves fecundity and larval quality; the effects of higher levels of VA acetate, however, still need to be evaluated for determining the dietary optimum for *F. chinensis* broodstock.

Fernández-Gimenez et al. (2016) summarized VA deficiency-caused changes of the midgut gland of Argentine shrimps, especially *Pleoticus muelleri*. The severe changes include shrinking of cells and desquamation of apical borders, cellular death, tissue disorganization, and cell lysis. These histological results agree well with earlier studies in *P. monodon* (Reddy et al. 1999), reporting disrupted basal membranes and cells fed VA-deficient diets.

32.1.2 Fishes

Hernandez and Hardy (2020) provide an updated survey of VA requirement. The growth-based estimates range from 0.3–0.6 mg kg⁻¹ diet (channel catfish) to 31 mg kg⁻¹ (European sea bass), equaling 1000–2000 IU kg⁻¹ diet to ~100,000 IU kg⁻¹ diet. In addition, the NRC (2011) compilation presents a long list of historical VA deficiencies, with skeletal deformities and pathological eye modifications as major symptoms.

In terms of VA functioning, one of the best studied species is Senegalese sole (*Solea senegalensis*): Fernández and Gisbert (2011) sketched the function of VA as morphogenetic nutrients. There are two primary active forms of retinoids:

- (i) Retinal (Fig. 32.1), used as the chromophore of rhodopsin in the eye
- (ii) RA, the main active metabolite of VA

²Vitamin A: 1 IU (international unit) = 0.3 microgram (μg) as retinol, = 0.6 microgram (μg) as β-carotene (<https://dietarysupplementdatabase.usda.nih.gov/conversions.html>, accessed August, 2017).

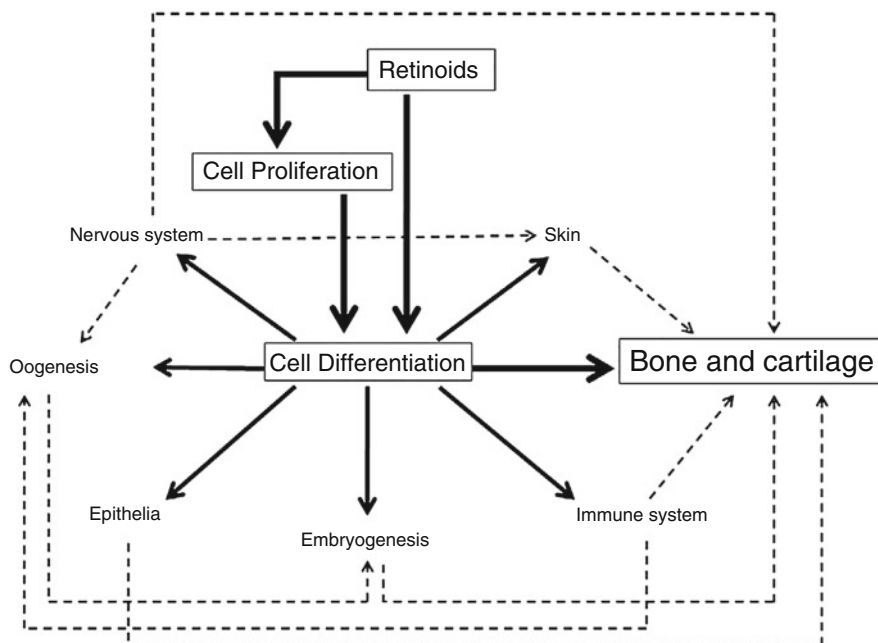


Fig. 32.3 Retinoid schematic pathways controlling cell proliferation and differentiation, which regulate the development of different vertebrate tissues and systems. Solid arrows denote direct regulation of retinoids, whereas dashed arrows indicate possible interactions between different tissues and systems with bone development (From Fernández and Gisbert (2011), with permission from Elsevier)

RA binds to nuclear RA receptors (retinoic acid receptors and retinoid X receptors, RARs and RXRs, respectively) and, through them, regulates gene expression, cellular differentiation, and proliferation processes, determining body pattern, growth, and the development of skeletal structures as well as the nervous and immune systems (Fig. 32.3) (Fernández and Gisbert 2011).

From available dietary VA studies in flatfishes, Fernández and Gisbert (2011) and Georga et al. (2011) draw the following conclusions:

- (i) VA imbalances have important effects on skeletogenesis, and these effects depend on the species (ontogenetic development and physiology), developmental stage (larva, juvenile, or broodstock), dietary VA chemical species, and rearing conditions.
- (ii) Broodstock VA requirements are still unknown for most flatfish species.
- (iii) Larvae have different requirements: less than 44,666 and 50,000 IU kg⁻¹ for Senegalese sole and Japanese flounder larvae, respectively.
- (iv) Dietary VA effects on juvenile skeletogenesis seem to be lower than during larval development; the optimum is 8000–9000 IU kg⁻¹ based on growth performance.

The latter laboratory published several insightful studies on the Senegalese sole with particular reference to expression profiles of genes involved in VA metabolism (*crbp2* cellular retinol-binding protein II; *rbp* retinol-binding protein; *crabp1* cellular retinoic acid-binding protein I) (Darias et al. 2012). The amount of VA absorbed by larvae is controlled at the intestinal level by *crbp2*. The expression of this gene decreases with increasing larval age, thus preventing excessive accumulation of this vitamin in target tissues and adverse hypervitaminosis effects.

In detail, exogenous RA modulates the expression of genes involved in the synthesis (*rdh10a* and *aldh1a2*), degradation (*cyp16a1*), and cellular transport (*crbp1*) of retinoids to establish a negative feedback regulatory mechanism to balance endogenous RA levels in Senegalese sole larvae (Boglino et al. 2017). Moreover, RA modulates the expression of its own RAR and RXR receptors and several other genes involved in the hypothalamus–pituitary–thyroid (*thrb* thyroid receptor β) and GH–IGF axes to establish a cross-communication necessary to regulate cellular functions. Via VD, RXR is also involved in lipid metabolism (\rightarrow Chap. 35).

Although the impact of dietary VA is well known, the underlying signaling pathways remain to be better understood. Dietary VA levels provided to Senegalese sole at different developmental stages indicate that the earlier the nutritional imbalance applies, the higher the adverse effect on skeletogenesis (Fernández et al. 2017). Furthermore, VA effects seem to be, at least partially, due to impaired metabolism and signaling of thyroid hormones as shown by the perturbed development of thyroid follicles, thyroid hormone compartmentalization in thyroid follicles, and whole-body expression of *thyroid receptors* (*trs*). These results indicate a dose-dependent interaction between VA and thyroid hormones.

Darias et al. (2012) proposed the following VA pathway: VA is ingested by the larvae through enriched *Artemia* in the forms of retinyl palmitate, retinol, and RA in lower proportion. In the enterocytes, retinol is transformed into retinyl esters and transported to the liver by chylomicrons (Fig. 32.4). Cellular-binding protein 2 (CRBP2) facilitates intestinal VA transport and metabolism. VA in the liver is stored mainly in lipid droplets in hepatocytes (\rightarrow Chap. 24) and released into the blood as needed, with *rbp* expressed in the liver playing a key role in the mobilization of VA from this organ to target tissues elsewhere (Fig. 32.4). In the target cells, most likely RBP transfers retinol from plasma to the cytoplasmic cellular retinol-binding proteins (CRBPs). The increase in *rbp* expression found between the completion of metamorphosis and at post-metamorphic stages indicates that during this period, there is an increased demand for VA (ossification of the skeleton, transition of juvenile–adult physiology, and phenotype). Inside the cells, retinol is transformed into the active metabolite, RA, by a precise and complex regulation. CRABP1 binds RA and is responsible for maintaining adequate concentrations of free RA (Fig. 32.4).

RA is able to interact with specific transcription factors in the nucleus, such as RAR, RXR, and PPAR (peroxisome proliferator-activated receptor), after which these nuclear receptors bind to the RA responsive elements (RAREs) of specific genes to modulate their expression (Fig. 32.4). These genes are involved in diverse

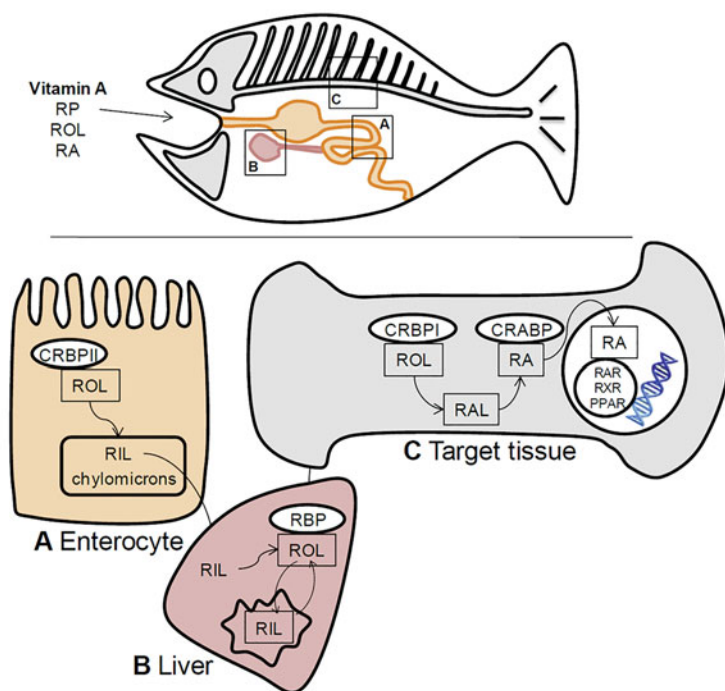


Fig. 32.4 Cartoon showing the theoretical metabolism of vitamin A in fish based on the existent knowledge for other vertebrates. Vitamin A digestion, absorption by the intestinal enterocytes (a), transport to the liver (b), and delivering to the target tissues (i.e., bone, c) are represented. **CRABP** cellular retinoic acid-binding protein; **CRBP1** cellular retinol-binding protein I; **CRBP2** cellular retinol-binding protein II; **PPAR** peroxisome proliferator-activated receptor; **RA** retinoic acid; **RAR** retinoic acid receptor; **RBP** retinol-binding protein; **RIL** retinyl; **ROL** retinol; **RP** retinyl palmitate, **RXR** retinoic X receptor (From Darias et al. (2012), with permission from Elsevier)

processes, including VA metabolism, but also in lipid metabolism (by activation of *ppar*, *fabp* [fatty acid-binding protein]) or skeletogenesis through the activation of osteocalcin via VD receptors (Darias et al. 2012).

In addition to the function of VA during the ontogenetic development, it enhances the immunocompetence of early juveniles (Fernández et al. 2015). After an induced bacterial infection with a 50% lethal dose of *Photobacterium damsela* ssp. *damsela*, Senegal sole juveniles on elevated doses of dietary VA increase their resistance to this challenge via differential expression of genes involved in the complement system (→Chap. 2).

Studies on the regulation of lipid metabolism by VA itself are limited. VA feeding can promote lipase activity and decrease lipid deposition in the livers of Senegalese sole (Darias et al. 2012) and gilthead seabream larvae (Fernández and Gisbert 2011). Yang et al. (2017) determined effects of graded VA levels on lipid metabolism enzyme activities and *fatty acid synthase* (*fas*) and *hepatic lipase* (*hl*) mRNA expression levels in the livers of juvenile orange-spotted grouper (*Epinephelus*

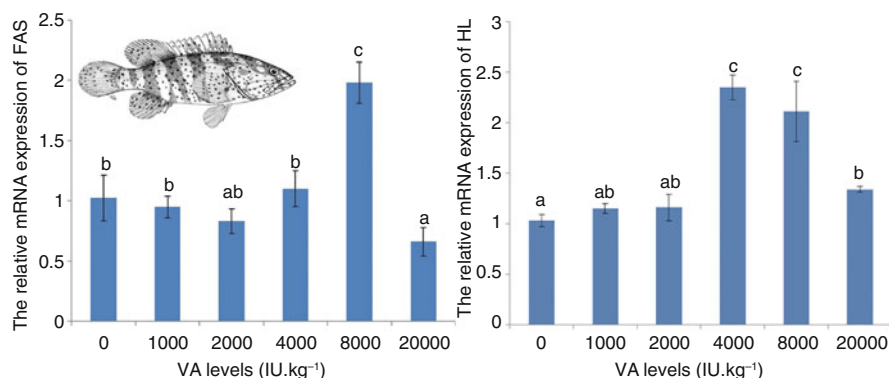


Fig. 32.5 Expression profiles of fatty acid synthase (*fas*) and hepatic lipase (*hl*) in the liver of juvenile *Epinephelus coioides* with graded dietary vitamin A levels (From Yang et al. (2017), with permission from Elsevier; image credit FAO)

coioides) (Fig. 32.5). Hepatic lipase catalyzes the hydrolysis of triacylglyceride and converts intermediate-density lipoprotein (IDL) to low-density lipoprotein (LDL). In *E. coioides* juveniles, dietary VA levels between 4000 and 8000 IU·kg⁻¹ promote lipolytic enzyme activity and the expression of lipid decomposition genes in the liver, indicated *fas* and *hl* transcription.

32.1.2.1 Broodstock Demands

Compared to juveniles, broodstock individuals have an increased dietary demand of VA. Hernandez and Hardy (2020)³ provided the following survey: rainbow trout requires levels of 60,000–200,000 IU kg⁻¹ to sustain reproductive performance. Japanese flounder increases the production of normal larvae when the broodstock is fed 50,000 IU kg⁻¹. In Japanese eel, dietary inclusion of VA improves larval survival if the fertilized eggs contain 50 µg g⁻¹ VA. However, reduced survival rates are observed at higher dietary VA doses.

A comparable phenomenon is found in rainbow trouts. Excess VA levels (700,000 IU kg⁻¹) in the broodstock diet induce high embryonic mortalities. Despite the importance of VA in reproduction, egg production, egg hatchability, and early larval survival and development, the dietary requirement for VA has not been determined for broodstock of any fish species. It is not known if requirements reported for juvenile fish differ from those of maturing fish (Hernandez and Hardy 2020). For effects of larvae fed VA excess, see below.

³Extract taken with permission from Wiley.

32.2 Cross Talk Between Vitamin A and Vitamin D

The fat-soluble VA and VD are essential nutrients in the diet of vertebrates. They are metabolized to bioactive RA and calcitriol (CTR, $1,25(\text{OH})_2\text{D}_3$, →Chap. 35). RA and CTR, involved in bone metabolism, require fine-tuned regulation of their synthesis and breakdown.

Antagonism of VA and VD is described, for instance, in Atlantic salmon. RA injection decreases plasma CTR levels, while CTR injection does not affect RA metabolites but lowers CTR in plasma (Ørnsrud et al. 2009). Lowered plasma CTR correlates with decreased bone *matrix Gla⁴ protein (mnp)* and *collagen 1 alpha2 chain (colla2)* transcription in all groups and with decreased *alkaline phosphatase (alp)* in CTR-injected fish. RA-treated salmon has enhanced *alp* transcription, irrespective of reduced plasma CTR. The transcription of the branchial *epithelial Ca channel (ecac)* and the unidirectional intestinal influx of Ca are stimulated following RA–CTR treatment. Plasma Ca, Mg, and P are not affected by any treatment. The results point out a cross talk of RA with the VD endocrine system in Atlantic salmon. Enhanced Ca flux and osteogenesis (*alp* transcription) in the RA-treated fishes and inhibition of *mnp* transcription reveal unprecedented disturbance of Ca physiology in hypervitaminosis A. This study indicates a dual effect of RA on bones. While RA inhibits matrix formation, it also appears to activate genes involved in matrix mineralization. Suppression of plasma CTR levels can partly explain the effects of VA on the bone. The authors recommend that dietary intake of VA should not exceed $37 \text{ mg retinol kg}^{-1} \text{ body weight day}^{-1}$ in Atlantic salmon postsmolts (Ørnsrud et al. 2013).

In addition to interaction with lipophilic vitamins, Lie et al. (2016) described a cross talk between VA and arachidonic acid (ARA). This cross talk alters also the skeletal mineralization in Atlantic cod larvae without any effects on the transcriptional level. Larvae fed high ARA + VA diets are shorter and have less mineralized bones than control animals.

32.3 Hypo- and Hypervitaminosis A

Disruption of retinoic signaling via excessive or deficient RA can cause teratogenic effects on developing embryos. Olive flounder on excess VA *palmitate results in reduced growth and high incidence of bone deformities* (Dedi et al. 1995). In most teleosts, cartilage components in the pharyngeal skeleton, neurocranium, and pectoral fin develop from embryonic to early larval stage before first feeding (Suzuki et al. 2000). The development of these skeletal components is susceptible to the

⁴Gla is the uncommon amino acid γ -carboxyglutamic acid introduced into proteins by a posttranslational carboxylation of glutamic acid residues. This modification introduces an affinity for Ca ions.

effects of maternal factors stored in eggs. RA controls gene expression essential for pharyngeal and pectoral fin development. In pioneering papers, Alexandre et al. (1996) and Ellies et al. (1997) showed in zebrafish and Suzuki et al. (2000) in Japanese flounder that exogenous RA (as low as 10^{-7} M) exerts strong teratogenic effects toward the pharyngeal skeleton.

As one major biomolecular pathway of the craniofacial cartilage dysmorphogenesis, the interaction with the *hox* gene cluster is discussed. *Hox* genes are involved in patterning the anterior–posterior axis, and RA is known to regulate the *hox* transcription by binding to regulatory elements found within the *hox* clusters. Alexandre et al. (1996) identified that overexpression of *hoxa-1* following injection of RA causes abnormal growth of the brain. Moreover, inadequate dietary retinol levels alter morphogenesis through the modulation of *hox* gene expression for the pelvic fin (Mazurais et al. 2009).

Related to the *hox* cluster pathway are *dlx* genes. Ellies et al. (1997) found that the craniofacial cartilage dysmorphogenesis coincides with a loss of *dlx* gene expression in RA-treated zebrafish embryos, central in craniofacial development and well preserved across species (Stock et al. 1996).

Atlantic salmon fry were reared on a fishmeal-based diet with increasing levels of VA (6, 122, and 938 mg kg⁻¹ retinol in dry feed) from start feeding (Ørnsrud et al. 2002). Signs of VA stress, such as reduced fat stores, liver size, and growth, are found in individuals receiving 122 or 938 mg kg⁻¹. Signs of VA toxicity, such as increased mortality, abnormal vertebral growth, and reduced growth, occur in groups receiving the highest dose.

Optimal requirement of dietary VA in juvenile Wuchang brems (*Megalobrama amblycephala*) is 3900 IU kg⁻¹. Both excess and deficiency of VA reduce white blood cell count, red blood cell count, hemoglobin content, and complement 3 and complement 4 contents and increase blood alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities (Liu et al. 2016). These elevated activities indicate damage to the liver (Davis 2004). Furthermore, non-optimal VA diets enhance the mortality after pathogen infections.

In compliance with these results, but on much higher VA levels, Hilton (1983) reported that major signs of hypervitaminosis A in rainbow trout are growth depression; increased mortalities; abnormal and necrotic anal, caudal, pectoral, and pelvic fins; and pale-yellow, fragile livers. The maximum tolerable level of VA is approximately 904,000 IU kg⁻¹ diet, and levels above 2,704,000 IU kg⁻¹ diet are toxic. Hypervitaminosis A in trout also results in spinal deformities. Furthermore, liver iron levels decline with increasing levels of dietary and liver VA, indicating an additional adverse effect of VA in trout.

VA deficiency decreases growth in young grass carps and their resistance to enteritis; moreover, intestinal immune functions are compromised (Zhang et al. 2017). A companion paper shows that VA deficiency impairs also the intestinal physical barrier and cell apoptosis and disrupts the cell-cellular tight junction complex in this species (Jiang et al. 2019) with an optimum VA level at 2622 IU/kg diet (Jiang et al. 2019). In a subsequent paper, Jiang et al. (2020) added that dietary VA deficiency impairs also immune functions and structural integrity in the

systemic immune organs, head kidney, and spleen. It causes histopathological changes such as blood vessel wall incassating and endothelial cell swelling and defluxion in head kidney and spleen.

Adding further symptoms of hypovitaminosis to the catalog of adverse effects, Huang et al. (2018) found in silver sillago (*Sillago sihama*) hemorrhages, erosion, increased mortality, and sluggish movement. Moreover, an optimum curve for the intestinal Na^+/K^+ ATPase occurs depending on the dietary VA supply. This enzyme reflects the status of nutrients absorbed through epithelial cells (Shao et al. 2016), due to its important role in keeping balance of Na^+ and K^+ across biological membranes (Clausen et al. 2017). Therefore, decreased Na^+/K^+ ATPase activity reduces the intestinal absorptive capability. The activity of Na^+/K^+ ATPase decreases also at excess VA levels and has its apparent maximum at 2202 IU kg^{-1} diet (Huang et al. 2018). In good accordance, Shao et al. (2016) reported that on-growing gibel carps on excess dietary VA of 5751 IU kg^{-1} show reduced Na^+/K^+ ATPase activity.

Most intriguingly, excess RA induces fusion of centra by degenerating intervertebral ligament cells in Japanese flounder (Wu et al. 2016). Larvae were exposed to RA for 3 days at mid-metamorphosis, a critical stage for vertebral deformity. Intervertebral ligament, which is known to form intervertebral joints in cooperation with the notochord, is severely degenerated by RA excess, leading to fusion of centra. During further development of the adult, growth of centra is severely suppressed in an anterior–posterior direction in RA-treated fish, and the notochord tissue is lost from fused centra, resulting in complete loss of intervertebral joints and fusion of centra (Fig. 32.6). The cumulative effect of these modifications is a truncated body form.

Similar results are reported for gilthead seabream juveniles (Fernández et al. 2011). These findings clearly indicate that maintaining RA at appropriate levels is essential for the functioning of intervertebral ligament tissues. In addition to damages in the vertebral column, Bailey et al. (2016) found that VA excess alters the social behavior and some aspects of the motor function in exposed zebrafish, caused by disruptions of RA receptor signaling during early development.

32.4 Concluding Remarks

Although an entire catalog of adverse effects of dietary excess VA is already available, the underlying biomolecular modes of action are elucidated only in a few cases and very recently. The same applies to interactions with other vitamins and lipophilic nutrients. This situation points out the urgent need to underpin phenotypic findings by biomolecular studies. If biomolecular methods have been applied to verify supposed pathways, it does often not overcome the state of using the expression of selected genes as sophisticated biomonitor. Holistic perspectives and comprehensive empirical approaches are found only in a very limited number of papers.

In the same line of concern, the role of intestinal microbes and epigenetics appears to be almost overlooked. In general, vitamin A is not well understood as

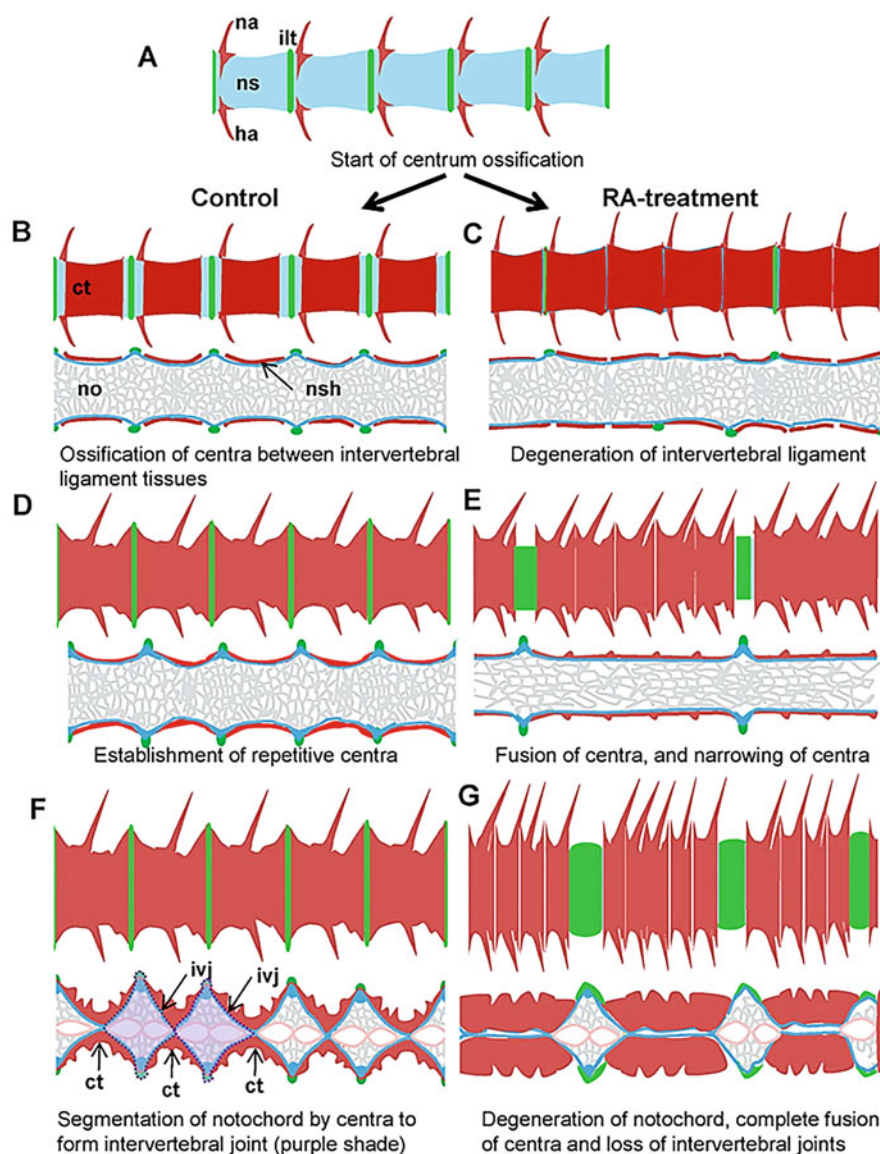


Fig. 32.6 Scheme of vertebral column development and progress of deformity in the Japanese flounder. (a) Vertebral column in mid-metamorphic stage (29 dpf), start of retinoic acid (RA) exposure. (b, d, f) Control group. (c, e, g) RA group. In (b–g), upper is lateral view and lower is a dissection in sagittal direction. **ct** centrum; **ha** hemal arch; **ilt** intervertebral ligament tissue; **ivj** intervertebral joint; **na** neural arch; **no** notochord; **ns** notochord sheath (From Wu et al. (2016), with permission from Wiley)

classical epigenetic substrate in aquatic animals; however, information from human nutrition and medicine indicates its involvement in diverse epigenetic pathways. For instance, RA can modulate epigenetic patterns by altering the levels of SAM and S-adenosylhomocysteine (SAH) or directing the enzymes that catalyze DNA methylation and histone modifications (→Chap. 33) (Park et al. 2012). Actually, VA is now considered a major regulator of epigenetic mechanisms in terms of DNA, RNA, and histone modifications along with expression of noncoding RNAs (Nur et al. 2021). Supportingly, Heidor et al. (2019) found that VA can induce cell differentiation and apoptosis of neoplastic and preneoplastic cells⁵ via modulated gene transcription. VA acts through nuclear receptors that are tightly regulated by histone modifications, such as acetylation, and DNA methylation.

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⁵A neoplasm is an abnormal mass of tissue that forms when cells grow and divide more than they should or do not die when they should. Neoplasms may be benign (not cancer) or malignant (cancer) (National Cancer Institute, National Institutes of Health, USA).

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Chapter 33

Vitamin B Complex—‘Do These Compounds Keep Veterinarians Away?’



Abstract The vitamin B complex comprises hydro- as well as lipophilic compounds and, at least, the lipophilic vitamins exhibit adverse effects if supplied in excess. It is obvious that vitamins are required not only for somatic and neural development and for growth, reproduction, and progeny survival but also for health, immune response, and pathogen and parasite resistance. With grass carp juveniles, the immune response is currently studied as detailed as possible with additional emphasis on deficient and excess supplies. This approach can serve as role model for other responses traits and other nutrients. Studies of hypervitaminosis of hydrophilic vitamins are rare in aquatic animals. However, documented adverse hypervitaminosis effects of hydrophilic vitamin C (→Chap. 34) may serve as an incentive for studying potential hypervitaminosis effects of hydrophilic compounds of the vitamin B complex more carefully. Although several compounds of this vitamin complex are known educts in the 1C metabolism, epigenetic pathways in vitamin requirement and metabolism as well as involvement of the gut microbiome need increased scientific attention.

Among all vitamins, the vitamin B complex deserves particular attention because several compounds take part in the one-carbon (1C) metabolism and can act as a source and coenzymes of epigenetic modifications (Fig. 33.1). Vitamin B₉, vitamin B₁₂, vitamin B₆, vitamin B₂, choline, and betaine (oxidation product of choline) take part in DNA methylation via regulating the universal methyl donor S-adenosylmethionine (SAM) and methyltransferase inhibitor S-adenosylhomocysteine. Other nutrients such as retinoic acid (vitamin A) can modulate epigenetic patterns by altering the levels of SAM and S-adenosylhomocysteine (SAH) or directing the enzymes that catalyze DNA methylation and histone (DNA-binding protein) modifications (Park et al. 2012) (→Chap. 32).

DNA methylation is currently the best-understood epigenetics mechanism, which entails the addition of a methyl group at cytosine–guanine dinucleotides (CpG). Methionine (Met) is converted to SAM. Folate-derived methyl groups are utilized for remethylation of homocysteine to produce Met. Choline provides methyl groups for the folate-independent homocysteine remethylation reaction. After transferring the methyl group, SAM is converted to SAH, an inhibitor of methyltransferases

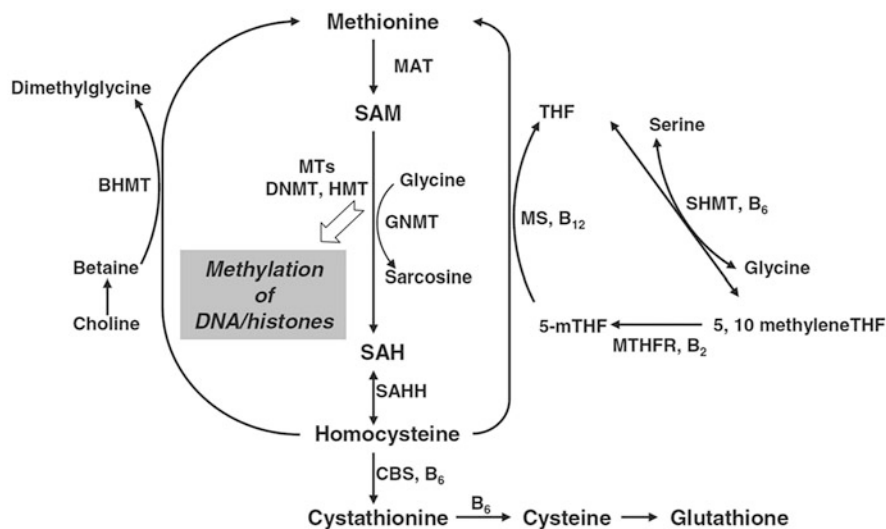


Fig. 33.1 One-carbon (1C) metabolism. *S*-adenosylmethionine (SAM) is the unique methyl donor for many biological methylation reactions including DNA and histone methylation. *S*-adenosylhomocysteine (SAH) is an inhibitor of methyltransferases such as DNA methyltransferases (DNMT) and histone methyltransferases. In 1C metabolism vitamins B₂, B₆, B₁₂, and folate are coenzymes, while methionine, choline, betaine, and serine are methyl donors. THF, tetrahydrofolate; 5-mTHF, 5-methyltetrahydrofolate; MT, methyltransferases; HMT, histone methyltransferases; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; SHMT, serine hydroxymethyltransferase; GNMT, glycine *N*-methyltransferase; CBS, cystathionine-β-synthase; MAT, methionine adenosyltransferase; SAHH, *S*-adenosylhomocysteine hydrolase; BHMT, betaine homocysteine methyltransferase; B₂, vitamin B₂; B₆, vitamin B₆; B₁₂, vitamin B₁₂. (From Park et al. (2012), with permission from the Cambridge University Press)

(Fig. 33.1) (Park et al. 2012). Epigenetic regulation comprises the modulation of major nutrients: For instance, Espe et al. (2020) showed that diets with high contents of methyl donors lead to reduced triacylglycerol (TAG) accumulation in liver cells in Atlantic salmon by reduced transcription of TAG biosynthesis genes.

The vitamin B complex comprises eight water-soluble vitamins. Although these compounds share similar names, they are chemically distinct vitamins. Each B vitamin is either a coenzyme for key metabolic processes or a precursor needed to make one. Dai and Koh (2015) pointed out possible protective roles of B vitamins, particularly, B₂, B₆, B₉, and B₁₂, in bone physiology and general health, neurological functioning, and healthy aging as described for humans (Kennedy 2016).

As often shown for nutrients, also the supply of vitamins follows optimum curves: Atlantic salmon fed a nutrient mix of Met, vitamin B₁₂, folic acid, and vitamin B₆ improves growth and reduces liver size, but when in excess, growth is reduced (Espe et al. 2019). Table 33.1 shows the ranges of individual requirements; practical details can be found in the reviews by Hansen et al. (2015), Molina-Poveda (2016), or Hemre et al. (2016). The latter authors provide also B vitamin recommendations in Atlantic salmon fed plant-based diets and summarize that biotin

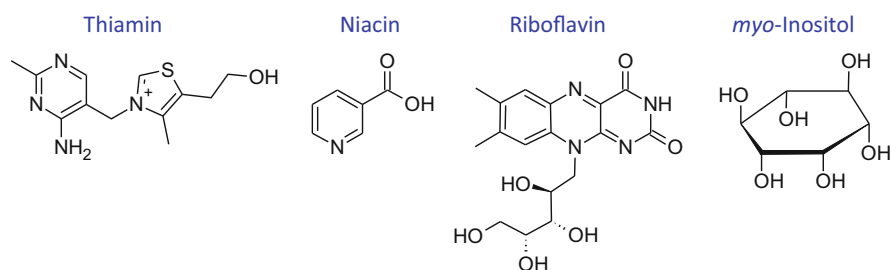
Table 33.1 Ranges of vitamin B, myo-inositol, and choline demands of invertebrates and fishes (Hansen et al. 2013, 2015; Hemre et al. 2016; Molina-Poveda 2016)

			Demand, mg kg ^{−1} diet	References
Echinoderms				
B ₂	<i>Apostichopus japonicus</i>		10–18	Okorie et al. (2011)
Mollusks				
B ₁	<i>Haliotis discus hannai</i>		60	Zhu et al. (2002)
B ₅			25	Zhu et al. (2004)
B ₆			23–40	Mai et al. (2007)
B ₇			0.67–0.70	Wu et al. (2002)
B ₉			2.6–5.3	Miao et al. (2013)
Shrimps	Demand, mg kg^{−1} diet			
	Minimal		Maximal	
B ₁	13— <i>Penaeus monodon</i>		120— <i>Marsupenaeus japonicus</i>	
B ₂	22.5— <i>P. monodon</i>		80— <i>M. japonicus</i>	
B ₃	7.2— <i>P. monodon</i>		400— <i>M. japonicus</i>	
B ₅	101— <i>P. monodon</i>		750— <i>Fenneropenaeus indicus</i>	
B ₆	72— <i>P. monodon</i>		200— <i>F. indicus</i>	
B ₇	0.4— <i>Fenneropenaeus chinensis</i>		2.4— <i>P. monodon</i>	
B ₉	0.5— <i>Macrobrachium rosenbergii</i> (Asaikkutti et al. 2016)		5— <i>F. chinensis</i>	
B ₁₂	0.01— <i>F. chinensis</i>		0.2— <i>P. monodon</i>	
myo- inositol	2000— <i>Fenneropenaeus merguiensis</i>		3400— <i>P. monodon</i>	
Choline	600— <i>M. japonicus</i>		6200–7840 lipid-dependent <i>P. monodon</i> (Shiau and Cho 2002)	
Fishes	Demand, mg kg^{−1} diet		Atlantic salmon	
	Minimal	Maximal	NRC (2011) recommendation	Addition to plant- based diets (Hemre et al. 2016)
B ₁	0.5— <i>Cyprinus carpio</i> , <i>Epinephelus coioides</i>	21— <i>Schizothorax prenanti</i> (Xiang et al. 2016)	3.4	3.4
B ₂	2.7— <i>Oncorhynchus mykiss</i>	25—Pacific salmon	4–7	10–12
B ₃	1— <i>O. mykiss</i>	200—Pacific salmon	1–10	65
B ₅	7— <i>O. mykiss</i>	50— <i>C. carpio</i>	20	22
B ₆	1— <i>O. mykiss</i>	20—Pacific salmon	2–16	10
B ₇	0.046— <i>Lateolabrax japonicus</i>	3.2— <i>Liza parsia</i>	0.25	0.25
B ₉	0.08— <i>Epinephelus malabaricus</i>	30–50— <i>Mugil cephalus</i>	1–2	3.3

(continued)

Table 33.1 (continued)

			Demand, mg kg ⁻¹ diet	References
B ₁₂	0.005— <i>Danio rerio</i> 0.015—Pacific salmon	0.094— <i>Ctenopharyngodon idella</i>	0.02	0.17
myo-inositol	94— <i>Oplegnathus fasciatus</i>	617— <i>Paralichthys olivaceus</i>		Not determined
Choline	50— <i>O. mykiss</i>	3000— <i>C. idella</i>		Not determined

**Fig. 33.2** Structures of thiamin, niacin, riboflavin, and *myo*-inositol (MI)

(vitamin B₇) and thiamine (vitamin B₁) levels are sufficient in plant-based diet, as no addition beyond the feed ingredients seems to be necessary. The other B-vitamins should be added at or above the NRC (2011) recommendations for salmonids to optimize growth and hinder change in liver lipid deposition (Table 33.1).

33.1 Vitamin B₁: Thiamin

Thiamin (Fig. 33.2) consists of substituted pyrimidine and thiazole rings linked by a methylene bridge. It exists mainly in various interconvertible phosphorylated forms, chiefly thiamin pyrophosphate (TPP). TPP functions as a coenzyme in the metabolism of carbohydrates and branched-chain amino acids (AAs). It is involved in two main types of metabolic reactions: decarboxylation of α -keto acids (e.g., pyruvate, α -ketoglutarate, and branched-chain keto acids) and transketolation (e.g., among hexose and pentose phosphates) (NRC 1998).

In salmonids, thiamin deficiency leads to the so-called M74 disease in Baltic Sea fishes, early mortality syndrome (EMS) in Laurentian Great Lakes fishes, and the Cayuga syndrome in the Finger Lakes (New York State) fishes. Such maternally transmitted thiamin deficiency manifests as abnormal swimming behavior, general weakness, and poor response to physical stimuli, darker skin pigmentation, whitened liver, pale gills, reduced tissue glycogen levels, extremely low thiamin levels (6% of healthy fry values), and necrotic changes in the brain areas (Waagbø 2010). Affected

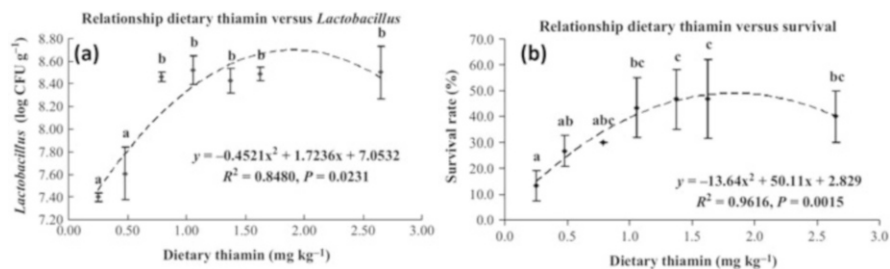


Fig. 33.3 Relationships between dietary thiamin (mg kg⁻¹) and *Lactobacillus* counts (a) and survival rate (b) in juvenile *Cyprinus carpio* var. Jian after *Aeromonas hydrophila* challenge after the feeding trial. Different superscripts indicate significant differences ($P < 0.05$). (From Feng et al. (2011), with permission from Wiley)

fry shows signs of oxidative stress based on altered expression of many redox-sensitive genes (Vuori and Nikinmaa 2007). The oxidative stress can be transmitted from parents to eggs. Hitherto, factors causing oxidative stresses for Baltic salmon populations remain elusive; they may be related to changes in the food web, possibly increase of possibly toxic phytoplankton due to eutrophication or salinity changes. Actually, experiments in Jian carps prove that thiamin strengthens antioxidant defense indicated by reduced lipid peroxidation and protein oxidation (Li et al. 2014). However, oxidative stress is not a specific reaction to environmental toxins; rather, respiratory (oxidative) burst is the first endogenous defense line against any kind of particulate and dissolved invaders (Steinberg 2012). It is therefore likely that there are more specific response reactions to be discovered.

In Jian carps, optimal thiamin supply beneficially affects cellular immunity including leucocyte phagocytic activity, humoral defense, serum immunoglobulin M (IgM) content, and intestinal *Lactobacillus* growth (Feng et al. 2011). Intestinal *Lactobacillus* counts increase, and maximum values are obtained at ~1.7 mg thiamin kg⁻¹ diet (Fig. 33.3a). After challenge with *Aeromonas hydrophila*, the survival of carps increases with rising dietary thiamin levels until the optimum is reached (Fig. 33.3b). In *Channa punctata* fingerlings, Zehra and Khan (2018c) determined the thiamin requirement as 2.3–2.6 mg kg⁻¹, well in the range recommended by NRC (2011).

33.2 Vitamin B₂: Riboflavin

Riboflavin (Fig. 33.2) is a water-soluble, yellow, fluorescent compound and functions as coenzyme in numerous redox reactions. It is a precursor of cofactors, which are needed for flavoprotein enzyme reactions, including activation of other vitamins (NRC 1998) and takes part in the 1C metabolism (Fig. 33.1). Riboflavin-deficient diets lead to oxidative stress, indicated by lipid peroxidation (Huang et al. 2010). Furthermore, they cause cataract and photophobia and result in short body vertebrae

in fishes (Waagbø 2010). Only a few nutritional studies are available and briefly listed in Table 33.1. In Jian carp, 5 mg riboflavin kg^{-1} diet improves growth performance and intestinal enzyme activities (Li et al. 2010); and that 10 mg riboflavin kg^{-1} diet mitigates the effects of multiple stressors (arsenic, high temperature, and bacterial challenged) in striped catfish (*Pangasianodon hypophthalmus*) (Kumar 2021).

33.3 Vitamin B₃: Niacin

Niacin (Fig. 33.2) functions as cosubstrate/coenzyme for the transfer of the hydride ion with numerous dehydrogenases (NRC 1998). In mammals, niacin can be converted to nicotinamide adenine dinucleotide, which is a substrate for poly (ADP-ribosylation) of histones and other DNA-binding proteins (Oommen et al. 2005), thereby being central in epigenetic pathways.

Niacin deficiency results in extremely high mortality of penaeid shrimps. Prominent signs of deficiency are anorexia and aversion to feed. In some shrimps, prolonged deficiency causes blackening of gills and death. Appropriate diet with 250 mg niacin kg^{-1} produces high survival and growth, whereas dietary excess results in retarded growth and lowered survival (Boonyaratpalin 1998).

In *C. punctata* fingerlings, Zehra and Khan (2019b) determined the niacin demand as 40–50 mg kg^{-1} . Moreover, Li et al. (2016a) and Feng et al. (2016) traced the effect of niacin deficiency in juvenile grass carps back to the biomolecular level: It decreases the digestion and absorption capacities via reducing digestive and brush border enzyme activities and downregulating the enzyme gene transcriptions related to the TOR¹ pathway of hepatopancreas and intestine. Furthermore, deficient diet impairs the intestinal mucosal immune function as well as the gill immunity and antioxidant capacity and diminishes its tight junction proteins.

Occasionally, also the amide of niacin is successfully applied as dietary supplement. It improves growth, intermediary metabolism, and glucose homeostasis of Wuchang bream fed high-carbohydrate diets (Shi et al. 2020a).

33.4 Vitamin B₅: Pantothenic Acid

Pantothenic acid (PA, Fig. 33.4) is vital to the synthesis and maintenance of coenzyme A (CoA), a cofactor and acyl group carrier for many enzymatic processes. It is a component of the FA synthase complex. Most tissues transport PA into cells

¹Target of Rapamycin, a nutrient-sensitive protein kinase controlling protein synthesis, cell growth, cell proliferation, cell motility, cell survival, autophagy, transcription, and aging (→Box: Yin and Yang of Energy Regulation, Chap. 5).

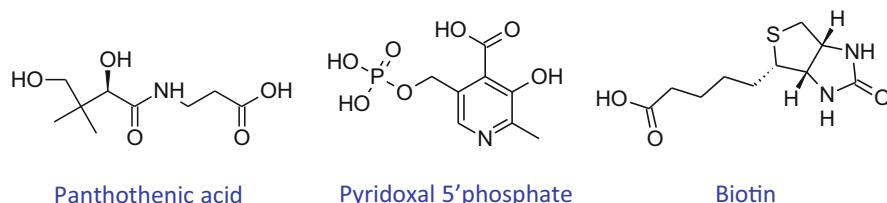


Fig. 33.4 Structures of pantothenic acid, pyridoxal 5'phosphate, and biotin

for the synthesis of CoA. Furthermore, CoA is involved in the synthesis of AAs, FAs, ketone bodies, cholesterol, phospholipids, steroid hormones, neurotransmitters (such as acetylcholine), and antibodies (NRC 1998).

Enhanced serum complement activity in red seabream, when fed elevated PA doses (Yano et al. 1988). Furthermore, PA is essential in promoting growth, enhancing intestinal enzyme activities, and increasing protein and lipid deposition in fishes (Wen et al. 2009). Signs of PA deficiency are hemorrhages, sluggishness, high mortality, anemia, and severe hyperplasia of the epithelial cells of gill lamellae (Raggi et al. 2016).

In *C. punctata* fingerlings, dietary ~ 35 mg kg⁻¹ PA is optimal (Zehra and Khan 2018a). Similar to niacin malnutrition, dietary PA deficiency as well as excess depress growth, intestinal and gill mucosal immune, and physical functions in grass carps again by regulating the TOR signaling pathway (Li et al. 2015). In juvenile Wuchang bream, Qian et al. (2015) found that PA deficiency causes oxidative stress and intestinal hypofunction. Furthermore, the expression of genes involved in liver FA synthesis increases with increasing dietary PA levels, resulting in an enhanced hepatic synthesis of saturated FAs and increased muscle content of polyunsaturated FAs (PUFAs).

33.5 Vitamin B₆: Pyridoxine, Pyridoxal, Pyridoxamine

The active form of VB₆, pyridoxal 5'phosphate (Fig. 33.4), functions as a coenzyme in the metabolism of AAs, glycogen, and sphingoid bases (substrates of neurotransmitter biosynthesis) (NRC 1998). Akhtar and Ciji (2020) emphasized the diverse roles of this vitamin in different metabolic pathways in fishes, ranging from DNA synthesis and repair to antioxidant defense, stress mitigation, immunomodulation, and thermal tolerance. However, determinations of the optimal demand in many aquaculture species are still missing; the same applies to establishing VB₆ broodstock demands (Akhtar and Ciji 2020).

Pyridoxine deficiency symptoms such as anorexia, poor survival, convulsion, helically swimming, and hyperirritability are documented, for instance, in grouper (*Epinephelus coioides*). This fish also shows low blood hemoglobin levels, liver glutamic-pyruvic transaminase, and superoxide dismutase activities (Huang et al.

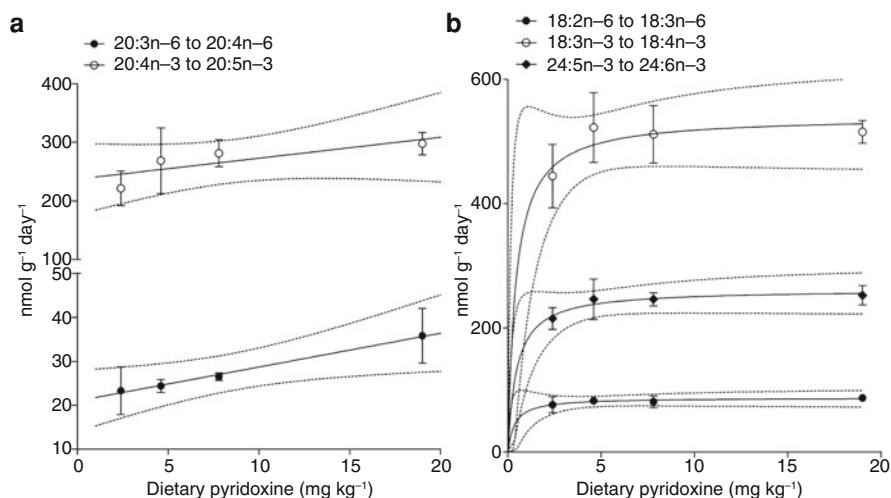


Fig. 33.5 (a) Apparent in vivo elongase and (b) desaturase activities in rainbow trout fed graded dietary pyridoxine contents. Elongase acting on $\omega 6$ PUFA 18:3 $\omega 6$ and 20:4 $\omega 6$; and desaturase acting on 18:3 $\omega 6$, 18:3 $\omega 3$, and 24:5 $\omega 3$. (From Senadheera et al. (2012), with permission from the American Chemical Society)

2005). For *Labeo rohita*, Akhtar et al. (2012) recommended a dietary pyridoxine supplementation of 100 mg kg⁻¹ diet that augments growth and combats minor stress symptoms in fingerlings reared at high water temperatures. Zehra and Khan (2018b) determined a pyridoxine requirement in juvenile *C. punctata* between 7.6 and 10.4 mg kg⁻¹ of dry diet. Moreover, Feng et al. (2010) reported that pyridoxine enhances the immune response of juvenile Jian carps, whereby improvement of the intestinal microbiome may be one underlying mechanism, although the *Lactobacillus* counts remained unchanged.

Supplemental pyridoxine stimulates $\omega 3$ long-chain PUFA (LC-PUFA) biosynthesis in rainbow trout fed vegetable oil-based diets (Senadheera et al. 2012). It stimulates the activity of FA elongase as well as Δ^6 and Δ^5 desaturases. The elongase activity is shown with C₁₈ and C₂₀ precursors (Fig. 33.5a) and the desaturase activity with C₁₈ and C₂₄ substrates (Fig. 33.5b), respectively. These activities, however, are insufficient to compensate for a diet lacking LC-PUFA but do highlight potential strategies to maximize this activity in cultured fishes, when fish oil is replaced with vegetable oils.

Zheng et al. (2017) identified impairment in young grass carps by pyridoxine deficiency and drafted its biomolecular immunomodulation. Compared to the optimal dietary pyridoxine level (~5.0 mg kg⁻¹, (Zheng et al. 2020)), pyridoxine deficiency:

1. Reduces innate and adaptive immune components including lysozyme (LYZ) and acid phosphatase (ACP) activities, C3, C4, and IgM contents and downregulates

the transcription of antimicrobial peptides *hepcidin* (=leap-1), *leap-2A*, *leap-2B*,² and *β-defensin-1* as well as the mucus forming *mucin2*

2. Aggravates inflammatory responses via downregulation of anti-inflammatory cytokines *tgf-β1*, *tgf-β2*, *il-4/13A*, *il-10*, and *il-11* and upregulation of pro-inflammatory cytokines *tnf-α*, *il-1β*, *il-6*, and *il-8*

Another dysfunction of pyridoxine deficiency can even be lethal, namely, the disturbance of the intestinal physical barrier function since it does protect no longer from pathogen invasion (Wu et al. 2018). This deficiency:

1. Induces oxidative damage indicated by lipid peroxidation and protein oxidation as well as reduced activities and transcription of antioxidant enzymes.
2. Induces apoptosis via p38 mitogen-activated protein kinase signaling.
3. Disrupts tight junctions indicated by downregulating a series of transmembrane proteins.

Only a few papers address the involvement of gut microbiota in pyridoxine effects. In golden pompano, the relative abundance of the parasitic *Tenericutes* and potential pathogenic *Bacteroidetes* decrease, while *Proteobacteria* and *Firmicutes* with nonpathogenic species improve on dietary VB₆ up to 7.5 mg kg⁻¹ (Huang et al. 2019). Metabolites of the altered microflora, such as short-chain FAs, which could beneficially affect the host, are not yet recorded.

33.6 Vitamin B₇: Biotin

Biotin (Fig. 33.4) plays a key role in the metabolism of lipids, proteins, and carbohydrates. It is a coenzyme to four carboxylases: acetyl CoA carboxylase, which is involved in the synthesis of FAs from acetate; pyruvate CoA carboxylase, essential in gluconeogenesis; β-methylcrotonyl CoA carboxylase, involved in leucine metabolism; and propionyl CoA carboxylase, central in the metabolism of energy, AAs, and cholesterol (NRC 1998). Furthermore, covalent attachment of biotin to histones plays a role in gene silencing and in cellular response to DNA damage (Oommen et al. 2005).

Xu et al. (2017) observed no effects of biotin on growth, body parameter, and whole-body composition in juvenile Wuchang bream fed 5% or 11% lipid diets. In contrast, appropriate biotin diets increase antioxidant capacity in Jian carps (Feng et al. 2014). Furthermore, dietary biotin plays a role in hepatopancreas FA synthesis and expression of β-oxidation-related genes and promotes hepatopancreas PUFA synthesis. Some figures of juvenile demands are as follows: *Gibelion (Catla) catla* 0.41–0.87 mg kg⁻¹ diet (Khan and Khan 2019), *C. punctata* 0.44 mg kg⁻¹ (Zehra

²Liver-expressed antimicrobial peptides

and Khan 2019a), and *Larimichthys crocea* only $>0.039 \text{ mg kg}^{-1}$ (Zhang et al. 2017).

Interestingly, Yossa et al. (2015b) reported a biotin-modulated reproductive success in zebra fish. In the presence of biotin-sufficient males, biotin-sufficient females spawn more eggs (222 eggs) than those of biotin-deficient females (19 eggs). The same pattern is observed with biotin-deficient males (8 vs. 2 eggs). Likewise, biotin-sufficient males generate a higher percentage of fertilized eggs (90% vs. 42%), hatching rate (62% vs. 27%), and larval survival (98% vs. 37%) than that of biotin-deficient males. Therefore, the biotin status of males is of high significance for successful breeding in zebra fish—and most likely also in other fishes.

McMahon (2002) considered the biotin synthesis by gut microflora as significant source for higher organisms. Actually, Yossa et al. (2011) provided clear evidence that the intestinal microbial synthesis is a significant source of biotin to zebrafish: Fishes fed antibiotic-supplemented diet show reduced growth, health, and feed utilization. Previously, endogenous supply of biotin occurs in channel catfish (Robinson and Lovell 1978) and ayu sweetfish (*Plecoglossus altivelis*) (Sugita et al. 1992). Whereas the supply in channel catfish is obviously not sufficient to meet the demand, the sweetfish harbors biotin-producing bacteria as a major part of the microbial community, and biotin-consuming bacteria constitute only a minor part of the enteric microbial community. Obviously, no other studies have yet considered this pathway as biotin source in aquatic animals. Instead, Zhao et al. (2012) chose the reciprocal approach and studied the impact of dietary biotin on the intestinal microflora. Potentially pathogenic *Aeromonas* and *Escherichia coli* decrease with increasing dietary biotin up to 0.15 mg kg^{-1} diet, while beneficial *Lactobacillus* and *Bacillus* significantly increase with dietary biotin levels up to 0.05 and 0.15 mg kg^{-1} diet, respectively.

In on-growing grass carps, biotin deficiency (below 0.2 mg kg^{-1} diet):

1. Impairs the nonspecific and specific immune responses by decreased levels of immune components, antimicrobial peptides, and IgM as well as mRNA levels of antimicrobial peptides;
2. Triggers the inflammatory response by upregulating mRNA levels of pro-inflammatory cytokines IL-1 β , TNF- α , IFN- γ 2, IL-6, IL-8, IL-12p40, IL-15, and IL-17D and downregulating the mRNA levels of anti-inflammatory cytokines IL-10, IL-11, IL-4/13A, and TGF- β 1 in head kidney and spleen (He et al. 2020).

Studies of hypervitaminosis of water-soluble vitamins, including biotin, are lacking. Since biotin is not retained in the body, the maximum tolerable limit or toxicity of this vitamin is considered being of no concern for aquatic animals (Yossa et al. 2015a), especially since poultry and swine studies indicate that they can tolerate 4 to 10 times the amount of optimal dietary requirement without adverse effects.

33.7 Vitamin B₉: Folic Acid

Folic acid (VB₉, also vitamin B_c, vitamin M, folacin, or pteroyl-L-glutamate, Fig. 33.6) acts as co-enzyme in the form of tetrahydrofolate (THF), which is involved in transfer of 1C units (Fig. 33.1) and in the metabolism of nucleic acids and AAs. THF takes part in pyrimidine nucleotide synthesis; it is needed for normal cell division (NRC 1998). VB₉ deficiencies adversely influence embryonic development by affecting cell proliferation and survival, which depends on continuous DNA synthesis, as well as embryonic development and growth through DNA methylation (Mæland et al. 2003). Dietary VB₉ supplementation of aquatic animals is well documented.

VB₉ requirement (in mg kg⁻¹) covers a wide range (Table 33.1): *G. catla* 0.22–0.56 (Khan and Khan 2020), *Acipenser baerii* 1.82–4.41 (Jamalzad Falah et al. 2020), *Sparus aurata* 2.0 (Amri et al. 2020), and *Mugil cephalus* even 30–50 (Badran and Ali 2021).

Anemia in VB₉-deficient (<1.0 mg kg⁻¹) channel catfish is characterized by pale livers, spleens, gills and kidneys, and malformations of blood cells, such as macrocytosis, binucleated erythrocytes, “spectacle” cells or increased numbers of hemocytoblasts. Furthermore, Duncan and Lovell (1994a) showed that appropriate dietary VB₉ reduces mortalities in individuals challenged with *Edwardsiella ictaluri*. Worth mentioning, there is an interaction between VB₉ and ascorbic acid (VC): When diets contain more than the minimal VC requirement for growth, VB₉ concentrations of 0.4 mg kg⁻¹ appear adequate. However, if dietary VC is marginal, a 10 times higher amount of VB₉ is required (Duncan and Lovell 1994b). In *Channa punctata* juveniles and young grass carps, optimal dietary VB₉ supply is

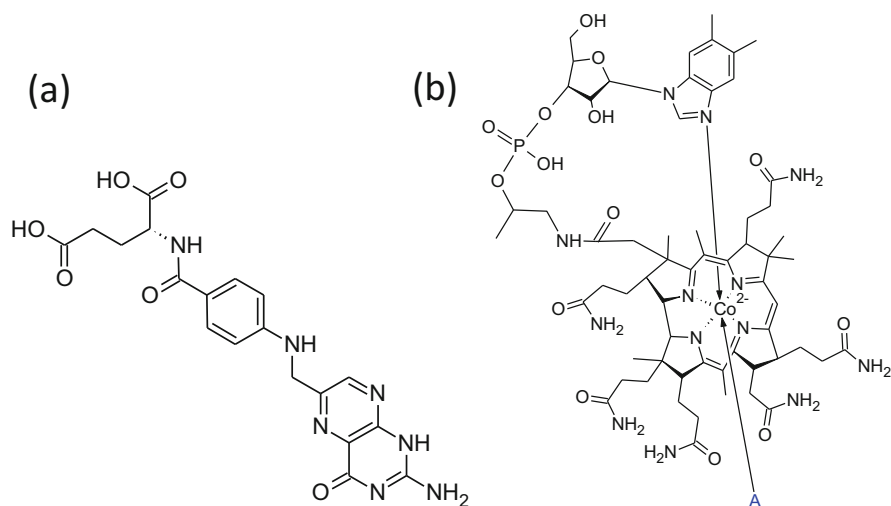


Fig. 33.6 Structures of (a) folic acid and (b) cobalamin, vitamin B₁₂. A = deoxyadenosine in deoxyadenosylcobalamin, A = CH₃ in methylcobalamin, A = C ≡ N in cyanocobalamin

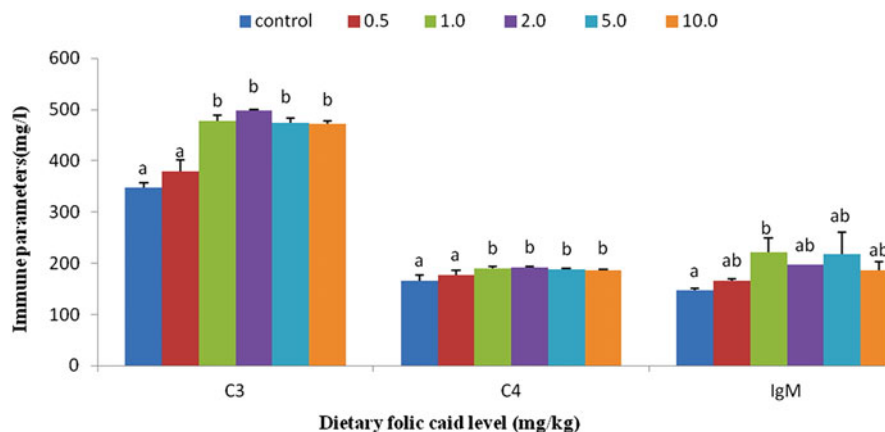


Fig. 33.7 Complement 3 (C3), complement 4 (C4), and immunoglobulin M (IgM) contents in serum of Wuchang bream fingerlings fed with dietary folic acid for 10 weeks. Different superscripts denote significant differences ($P < 0.05$) between groups; values are mean + SE. (From Sesay et al. (2016), with permission from Elsevier)

0.74–1.15 mg kg⁻¹ (Zehra and Khan 2020) and ~1.9 mg kg⁻¹ (Shi et al. 2020b), respectively.

In young grass carps, the effect of dietary VB₉ deficiency on retarding growth can be attributed to reduced immunity and barrier functions in the gills (Shi et al. 2015). Furthermore, VB₉ deficiency decreases the antioxidant response indicated by reduced Cu/Zn superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activities and gene transcription as well as *nf-e2-related factor 2* gene expression in fish gills. The latter is a pivotal transcription factor that induces the transcription of antioxidant enzymes gene (Jiang et al. 2014).

In the same line of evidence, Sesay et al. (2016) found that dietary VB₉, particularly between 1.0 and 2.0 mg kg⁻¹ diet, improves growth performance, immune parameters (particularly C3 and IgM, Fig. 33.7), and digestive as well as antioxidant enzyme activities in Wuchang bream fingerlings. In addition, appropriate dietary VB₉ (≤ 5 mg kg⁻¹) enhances acute high-temperature resistance (Sesay et al. 2017).

It has often been reported that the levels of dietary VC and vitamin E required to enhance immune response in fishes are much higher than those required for growth. For instance, grouper 6×, Japanese seabass 10×, turbot 50×, Atlantic salmon 138×, and channel catfish 200× of vitamin C; grouper 4×, Atlantic salmon 5×, rainbow trout 6×, and gilthead seabream 12× of vitamin E are required in diets for enhancing immune response (Lin et al. (2011) and references therein). In contrast to the different requirements of these vitamins, Lin et al. (2011) proved in young grouper that the dietary amount of VB₉ needed to stimulate its immune responses and to maximize the growth are equal.

In invertebrates, too, appropriate VB₉ supplies increase antioxidant capacity and improve immunity and disease resistance. Wei et al. (2016) challenged young

Chinese mitten crabs with *A. hydrophila*. The cumulative mortality is lowest in crabs on 2.0 mg dietary VB₉ and highest in the VB₉-free controls. In terms of growth, antioxidant status, and improved survival, a similar dietary VB₉ value (0.5–2.0 mg kg⁻¹) applies to giant river prawn (Asaikkutti et al. 2016).

33.8 Vitamin B₁₂: Cobalamin

Vitamin B₁₂ (cobalamin, Fig. 33.6) is a complex organometallic cofactor associated with three subfamilies of enzymes: the adenosylcobalamin-dependent isomerases, the methylcobalamin-dependent methyltransferases, and the dehalogenases (Banerjee and Ragsdale 2003). In the 1C metabolism (Fig. 33.1), it functions as coenzyme for a critical methyl transfer that converts homocysteine to Met and for a separate reaction that converts L-methylmalonyl coenzyme A (CoA) to succinyl-CoA. Cobalamin is involved in the cellular metabolism of carbohydrates, proteins, and lipids. It is essential in the production of blood cells in bone marrow, for nerve sheaths and proteins and improved immunity (NRC 1998). In Chinese mitten crab, medium dietary doses of VB₁₂ (0.2 mg kg⁻¹) increase survival after infection with *A. hydrophila* (Wei et al. 2014).

As shown in Wuchang bream, VB₁₂ is essential to maintain normal growth and physiological functions. Supplementation of 0.12 mg kg⁻¹ VB₁₂ boosts intestinal digestive capability and hepatic *insulin-like growth factor-I* expression (Li et al. 2016b). This figure complies well the dietary cyanocobalamin demand of *C. punctata* juveniles: 0.07–0.15 mg kg⁻¹ diet (Zehra and Khan 2019b).

The production of vitamins by fish gut microbes has occasionally been reported. *Cetobacterium somerae* is known to produce abundant amounts of VB₁₂, and this organism is commonly present in the gastrointestinal tract of a number of fish species. Consequently, various fishes, in which the bacterium is present in the gastrointestinal tract, such as Nile tilapia (Lovell 1982) and common carp, have no dietary VB₁₂ requirement. Species in which the bacteria are not commonly present, such as channel catfish or Japanese eel, have a clear demand for dietary VB₁₂ (Merrifield and Rodiles 2015). For rainbow trout, Sugita et al. (1991) showed that the required VB₁₂ is mainly supplied by *Aeromonas* spp. (including *A. hydrophila*), *Enterobacteriaceae*, *Moraxella*, and *Acinetobacter*, along with *Bacteroides* type A, *Bacteroidaceae*, and *Clostridium*.

33.9 Inositol

Inositol or cyclohexane-1,2,3,4,5,6-hexol is a sixfold alcohol (polyol). It exists in nine possible stereoisomers, of which the most widely occurring one in nature is *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, or *myo*-inositol (MI, Fig. 33.2). MI is central as structural basis for inositol phosphates, secondary messengers in eukaryotic cells. In

addition, inositol serves as an important component of the structural lipid phosphatidylinositol (PI, →Chap. 31) and its various phosphates, the phosphatidylinositol phosphate (PIP) lipids.

MI has once been considered a member of the vitamin B complex (formerly vitamin B₈); however, because it can be synthesized from glucose by many aquatic animals, e.g., sunshine bass (*Morone chrysops* × *M. saxatilis*) (Deng et al. 2002), channel catfish (Burtle and Lovell 1989), Nile tilapia (Peres et al. 2004), or the abalone, *Haliotis discus hannai* (Mai et al. 2001), it is no longer considered essential. For inositol-requiring fish species, Molina-Poveda (2016) reported a span from 94 (parrot fish *Oplegnathus fasciatus*) to 617 mg kg⁻¹ diet (olive flounder *Paralichthys olivaceus*). Well in this range, Zehra and Khan (2019c) determined an inositol requirement in *Channa punctata* juveniles between 375 and 505 mg kg⁻¹.

Shrimps require higher MI amounts than fishes, ranging from 2000 mg kg⁻¹ dry diet in *Fenneropenaeus merguensis* (Aquacop 1978) and 2700 mg kg⁻¹ in *Litopenaeus vannamei* (Chen et al. 2018) to 3400 mg kg⁻¹ diet in *P. monodon* (Shiau and Su 2004). Michael and Koshio (2008) documented a sparing effect in kuruma shrimp (*Marsupenaeus japonicus*) juveniles: Supplementation of 0.12% dietary choline can compensate the needed phospholipids when fed MI-deficient diets.

Dietary MI deficiency leads to damage of the physical barrier function in all intestinal segments (Li et al. 2017). Vice versa, sufficient dietary MI supply upregulates the transcription of cell cycle proteins (*cyclin b*, *cyclin d*, *cyclin e*) and the *E2F transcription factor 4* (*e2f4*) (Fig. 33.8). The same genes plus *nrf2* in the head kidney and spleen are involved in increased immunity of juvenile Jiang carps following an infection with *A. hydrophila* (Jiang et al. 2016). MI deficiency results in insufficient activation of antioxidant enzyme transcripts to control ROS production following the pathogen challenge. Thus, MI deficiency causes an oxidative damage to the head kidney and spleen and further depresses immunity of these important immune organs. Furthermore, MI deficiency cannot promote the proliferation

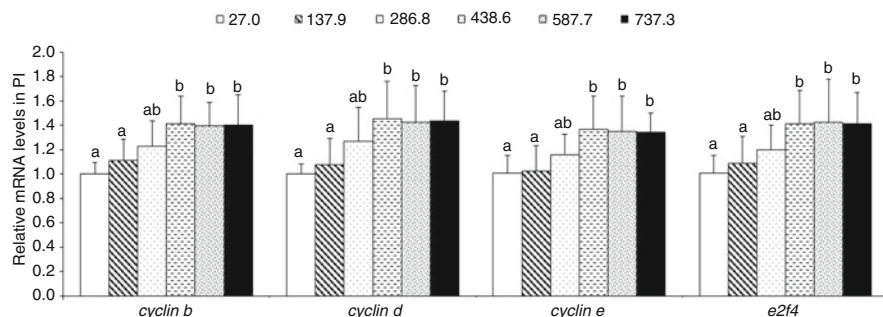


Fig. 33.8 Relative levels of *cyclin b*, *cyclin d*, *cyclin e*, and *e2f4* in the proximal intestine (PI) of young grass carps fed diets containing graded levels of *myo*-inositol. Columns with different letters are significantly different ($P < 0.05$). (From Li et al. (2017), with permission from Elsevier)

signaling-associated *e2f4/cyclin* transcription and thus cannot repair the damaged head kidney and spleen tissues, which further depresses the immunity of these organs.

Life history traits can improve on sufficient MI diets. Cui et al. (2020) identified enhanced salinity tolerance in turbot via the cytokine-cytokine receptor interaction and Jak-STAT signaling pathway (Cui and Ma 2020). This pathway transmits information from extracellular chemical signals to regulate growth, survival, differentiation, phagocytosis, and pathogen resistance (Murray 2007).

Moreover, optimal dietary MI improves not only the digestive organ development and osmotic stress resistance, digestive and absorptive capacity, but also intestinal microbiota as shown in juvenile Jian carps (Jiang et al. 2009). This indicates that fish immunity receives indirect support by optimal dietary MI supply via host microbiome. Intestinal *A. hydrophila* and *E. coli* counts decrease with increasing levels of dietary MI up to ~230 and ~690 mg kg⁻¹ diet, respectively, while *Lactobacillus* increases with rising MI levels up to 990 mg MI kg⁻¹ diet.

With 300 to ~420 mg kg⁻¹ diet, the optimal MI supply for juvenile grass carps lies in the range of Jian carps and improves their immunity (Li et al. 2018). The described pathway is related to NF-κB and TOR signaling in the intestinal immune barrier function in all three intestinal segments (Fig. 33.9). Vice versa, dietary MI deficiency damages the intestinal immune barrier function via:

1. Reducing the resistance to pathogens by decreasing innate immunity: lysozyme and acid phosphatase activities, and complement 3 (C3), complement 4 (C4), and IgM contents, as well as the downregulation of gene encoding antibacterial peptides *leap-2a*, *leap-2b*, *hepcidin*, *β-defensin-1*, as well as *mucin2*.

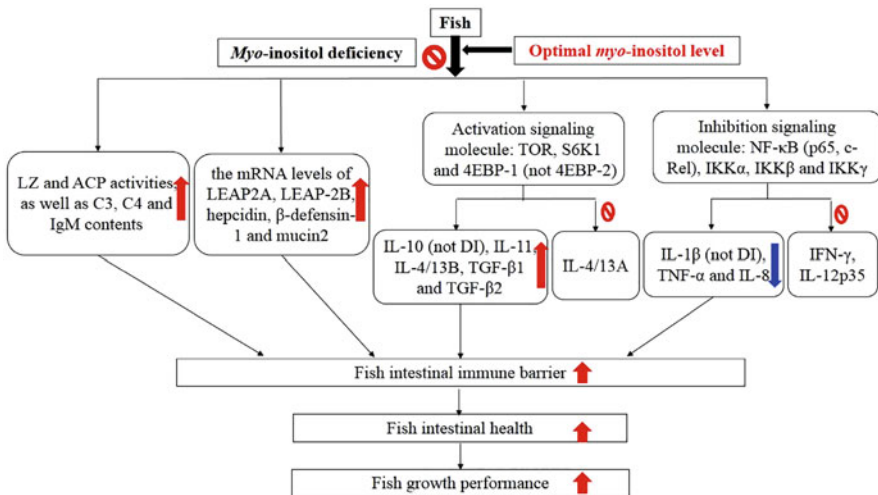


Fig. 33.9 The potential action pathways of dietary *myo*-inositol regulated the intestinal health in young grass carps. (From Li et al. (2018), with permission from Elsevier)

2. Aggravating inflammation by upregulating pro-inflammatory cytokines *il-1 β* , *tnf- α* , and *il-8* (part of the NF- κ B³ signaling pathway).
3. Aggravating inflammation by downregulating anti-inflammatory cytokines *il-10*, *il-11*, *il-4/13b*, *tgf- β 1*, and *tgf- β 2* (part of the TOR signaling pathway).

Invertebrates, too, benefit from dietary *myo*-inositol. Recently, Bu et al. (2020) showed that the exogenous MI is important to achieve improvement of antioxidative status and nonspecific immunity in Chinese mitten crab. Dietary MI supplementation in the range of 1.6–1.7 g kg⁻¹ is suitable. Transcriptomics reveals that dietary MI reduces hepatic TAG accumulation through inhibition of lipid synthesis-related genes (*srebp1*, Δ^9 *fad*, *elovl6*, *fas*) and promoting a lipid export-related gene (*microsomal triglyceride transfer protein*). The reported beneficial effect of MI in this crab complies well with previous studies of growth improvement and increase of stress resistance, found in giant tiger prawn (Shiau and Su 2004), Pacific white shrimp (Chen et al. 2018, 2019), or kuruma shrimp (Kanazawa et al. 1976).

33.10 Choline

Choline (Fig. 33.10) is a quaternary ammonium salt and basic constituent of lecithin (→Chap. 23) which is present in many plants and animal organs. It functions as a precursor for acetylcholine, phospholipids, and the methyl donor betaine (the oxidation product of choline). Choline cannot be synthesized in animals (Lykidis 2007); rather, it is an essential dietary component important for structural integrity of cell membranes, C1 metabolism (Fig. 33.1) (Richard et al. 2011), cholinergic neurotransmission, transmembrane signaling, and lipid and cholesterol transport and metabolism (NRC 1998). Choline deficiency leads to oxidative damage. In grass carps, it causes opposing changes in the mRNA levels of intestinal versus hepatopancreatic *gpx4*, *mgst3*, *gst-theta*, *gst-mu*, *gst-kappa*, *mgst1*, and *mgst2* (Wu et al. 2017b). These genes encode enzymes involved the glutathione redox homeostasis.

In kuruma shrimp, Michael et al. (2006) identified that dietary choline can supply postlarvae and juveniles with the needed methyl group when fed Met-deficient diets. Later, Michael and Koshio (2016) reported that choline chloride, at a concentration of 0.6 g kg⁻¹ diet, acts as protector against osmotic stress in postlarvae. There is an obvious interaction between choline and lipid requirement in shrimps, since lipid-rich diets require elevated dietary choline contents (Shiau and Cho 2002); a mechanistic explanation for this phenomenon is missing so far.

³The nuclear factor- κ B (NF- κ B) represents a family of transcription factors that are normally kept inactive in the cytoplasm. In response to multiple stimuli such as bacterial or viral products, or various types of stress, NF- κ B is activated, enters the nucleus, and activates transcription of a variety of genes participating in the immune and inflammatory response, cell adhesion, growth control, and protection against apoptosis (Israel 2010).

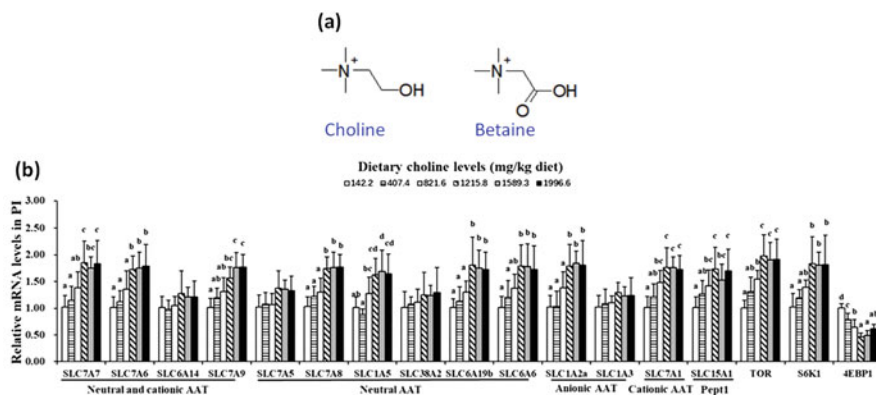


Fig. 33.10 (a) Structure of choline and betaine. (b) Relative expression of amino acid transporters (AAT) and peptide transporter 1 in the proximal intestine of juvenile grass carp fed diets containing different levels of choline for 70 days. Data are means + SD of six fish in each group. Different letters indicate significant differences ($P < 0.05$). (From Yuan et al. (2020b), with permission from Elsevier)

In juvenile *Acipenser baerii*, Yazdani Sadati et al. (2014) determined the minimal dietary choline demand at 1.5 g kg^{-1} for growth. A similar value is found in grass carp ($\sim 1.3 \text{ g kg}^{-1}$ (Yuan et al. 2020b)). With $7.5\text{--}8.5$ and $1.1\text{--}1.6 \text{ g kg}^{-1}$ diet, the microcladoceran *Moina macrocopa* (D'Abramo and Baum 1981) and the grouper *Epinephelus coioides* (Qin et al. 2017) have relatively high and relatively low choline requirements, respectively. Medium requirement (3 g kg^{-1}) is found in juvenile yellowtail kingfish (*Seriola lalandi*) (Liu et al. 2019). Furthermore, Cleveland et al. (2020) supplemented rainbow trout broodstock diets with 0.8% choline and show that, in three out of six strains, offspring growth is improved.

Pathways of dietary choline deficiency are identified again in juvenile grass carps (Yuan et al. 2020b). It decreases growth and damages the AA absorption capacity (Fig. 33.10). In particular, choline deficiency reduces the contents of free neutral AAs including Met, Trp, Thr, Ile, Leu, Val, Gly, Pro, Ser, Cys, and Tau in all intestinal segments attributable to decreased mRNA levels of the corresponding AA and peptide transporters. Moreover, choline insufficiency depresses the *tor* signaling molecule mRNA levels and TOR protein abundance in the fish gut. AA transporters and peptide transporter 1 are regulated by mTOR through phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1) and downregulation of *eukaryotic initiation factor 4E binding protein 1* (4E-BP1).⁴

As to be expected, the choline requirement follows optimum curves, so in kuruma shrimp (Fig. 33.11), *Moina macrocopa*, or gibel carp (*Carassius gibelio*). Adverse effects of dietary excess choline are common. High dietary choline up to 4430 mg kg^{-1} decreases protein productive value in the study of juvenile gibel

⁴Member of a family of translation repressor proteins and a well-known substrate of mechanistic target of rapamycin (mTOR) signaling pathway (Qin et al. 2016)

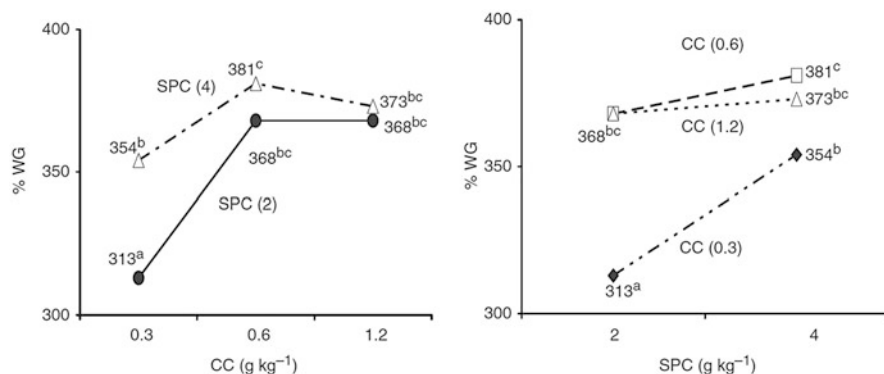


Fig. 33.11 Effect of different dietary choline chloride (CC) and soybean phosphatidylcholine (SPCh) levels on percent weight gain (%WG) of juvenile *Marsupenaeus japonicus*. (From Michael et al. (2005), with permission from Wiley)

carps (Duan et al. 2012). Protein productive value has been reported to be stable when dietary choline is higher than the optimal level (Michael et al. 2006; El-Husseiny et al. 2008). Therefore, it becomes obvious that there is no common tendency of choline requirement between shrimps and fishes and further investigations are needed to investigate the adverse impact of high dietary choline on protein utilization.

Using weight gain (WG) as response variables, Michael et al. (2005) found that there are different effects and interactions of choline chloride (CC) and phosphatidylcholine (PC)—the water- and fat-soluble sources of choline—on the performances of kuruma shrimp juveniles (Fig. 33.11). Both choline sources (CC and soybean phosphatidylcholine, SPCh) show significant interactions: A beneficial effect of CC can be observed with increasing levels, however, at the low level of SPCh and vice versa. The magnitude of the beneficial effect of CC changes with the different dietary levels of SPCh. Moreover, the inclusion of higher levels of SPCh (4 g kg⁻¹, Fig. 33.11a) or CC (0.6 and 1.2 g kg⁻¹, Fig. 33.11b) diminishes the effect of the other source. The optimum growth is obtained when the diet contains 0.6 g CC and 2 g SPCh kg⁻¹ diet. Further increases in either CC or SPCh do not enhance growth. The authors assume that this phenomenon reflects the substantial role of these two compounds as structural components of cells rather than as coenzymes. Furthermore, the authors hypothesize that kuruma shrimps can utilize CC for synthesis of PC under the shortage of necessary PC possibly in the cytosine diphosphate-choline pathway.

To some extent, this study complies with an earlier one by Gong et al. (2003) who found a convertibility of choline in *L. vannamei* juveniles: Lecithin can effectively provide choline. Conversely, the synthesis of phospholipids from choline does not meet the requirement for phospholipids, which may be one reason why dietary choline appears unable to substitute for lecithin. In contrast to the shrimps, juvenile

Atlantic salmon can obviously convert choline into phospholipids, particularly at low Met levels (Espe et al. 2015).

Supporting the optimal dietary requirement paradigm, Wu et al. (2016, 2017a) identified adverse effects in juvenile Jian carps on the biomolecular level. Dietary choline deficiency and excess induce intestinal inflammation and alteration of intestinal tight junction protein transcription. Furthermore, the transcription of *leap-2A*, *leap-2B*, *hepcidin*, and *defensin-3* in the entire intestine is reduced by choline deficiency indicating an impairment of the innate immunity in juveniles.

Furthermore, Wu et al. (2016) identified that dietary choline excess (1.8 g kg⁻¹ diet):

1. Depresses intestinal antimicrobial defense by decreasing intestinal lysozyme and ACP activities, IgM content, and mRNA levels of antimicrobial peptides.
2. Induces leucocyte infiltration and inflammation in the intestine associated with upregulated mRNA levels of pro-inflammatory cytokines and downregulated mRNA levels of anti-inflammatory cytokines and signaling molecules.
3. Impairs intestinal physical barrier by decreasing the mRNA levels of tight junctions and signaling molecule p38MAPK in the mid and distal intestine. p38MAPK are a class of mitogen-activated protein kinases that are responsive to stress stimuli, such as cytokines, heat shock, and osmotic shock, and are involved in cell differentiation, apoptosis, and autophagy (Segalés et al. 2016).

Similarly, Zhao et al. (2016) reported that optimal dietary choline regulates antibacterial activity, inflammatory response, and barrier function in the gills of grass carps. Optimal dietary choline improves the integrity of fish gills by decreasing the oxidative damage via increases of glutathione content and Cu/Zn-SOD, CAT, GPx, GST, and GR activities and corresponding mRNA levels. The dietary choline requirement for young grass carps lies between 1.2 and 1.6 g kg⁻¹ diet—well in the range given in Table 33.1.

Recently, Yuan et al. (2020a) contributed some more mosaic stones to the growing picture of beneficial effects of dietary choline. Optimal choline supplementation (1) reduces the incidence of rotten gill and protects gill morphology; (2) increases the contents of choline, acetylcholine, betaine, and phosphatidylcholine in the gills; and (3) reduces the degree of DNA fragments.

Also recently, Ismail et al. (2020) found that the supplementation of betaine to plant-based diets improves growth performance in Nile tilapia. Furthermore, the addition of betaine increases the intestinal absorption capacity, blood health, and *igf-1* expression and reduces lipogenesis through markedly decrease of *fas* and lipoprotein lipase (*lpl*) mRNA expression in Nile tilapia. This agrees well with findings in Wuchang bream (Wang et al. 2019), in which betaine supplementation decreases liver lipid accumulation caused by high-carbohydrate diet. The beneficial effect takes place through improvement of the gut microbial community, trimethylamine formation (indicated by the expression of associated microbial genes and bacterial taxa), the formation of trimethylamine-*N*-oxide, and bile acid metabolism.

Invertebrates, too, benefit from dietary betaine. *Macrobrachium rosenbergii* responds to dietary betaine supplementation (0.5% betaine) with increased growth,

elevated $\omega 3$ LC-PUFA content, elevated antioxidant activity, and strengthened innate immunity (Dong et al. 2020). Consequently, the survival of individuals on 0.5% betaine diet increased after challenge with *Vibrio cholerae*.

33.11 Concluding Remarks

The vitamin B complex comprises a variety of hydro- as well as lipophilic compounds, and, at least, the lipophilic vitamins exhibit adverse effects if supplied in excess. It is obvious that vitamins are required not only for development and growth, reproduction, and progeny survival but also for health, immune response, and pathogen and parasite resistance. Currently, with grass carp juveniles, the immune response is studied as detailed as possible. This approach can serve as role model for other responses traits and in other fish species.

Although several vitamins of the B complex take part in the 1C metabolisms, the epigenetics of dietary vitamin B effects did not yet find sufficient attention in nutritional studies. Almost the same applies to significance and role of the intestinal microbiota in biosynthesis or modification of vitamin B compounds. Both issues do not deserve this stepmotherly treatment – the current attention is below the standard of other nutrients and below livestock studies. Usually, intestinal biosynthesis by microflora seems to be insufficient to meet the actual requirement (Mai et al. 2001; Lee et al. 2008); however, only a few reports are available on feeding trials to improve this situation by providing pre- or probiotics.

The vitamin B requirements appear to be species-specific so that one dietary formulation cannot suffice all the various demands, especially since, for a given species, several of these compounds possess beneficial and adverse dose ranges close together. With studies of dietary excess vitamins affecting immune response, underlying mechanisms are beginning to be well understood and, thus, providing part of a basis for dietary remedies. Concerning hydrophilic vitamins, the classical paradigm that excess doses are excreted appears still applicable. However, in the light of recently discovered adverse effects of excess vitamin C (Ristow et al. 2009), this paradigm has to be reconsidered and reevaluated.

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Chapter 34

Vitamin C—‘*An Apple a Day Keeps the Veterinarian Away*’



Abstract The beneficial effect of vitamin C is almost universally recognized. This appreciation applies also to aquafeed with beneficial effects well documented in many studies on VC demand. VC is a naturally occurring sugar acid with antioxidant properties acting effectively as scavenger against a broad spectrum of reactive oxygen and nitrogen species. Beside this spontaneous action, VC takes part in a plethora of regulatory pathways including spermatogenesis, innate immunity, or pathogen and stress resistance. In addition to its role as gene regulator, recent advances disclose a previously unknown function of VC, namely, epigenetic regulation conserved in organisms from yeast to mammals—likely also in aquatic invertebrates and fishes. Systematic studies, however, are lacking. In VC requiring fishes, hypovitaminosis C is well documented: Hemorrhages, scoliosis, or broken backbone syndromes are common. Hypovitaminosis targets a complex network that depresses gill physical as well as immune barriers. In contrast, hypervitaminosis C is not well understood; it deserves futures attention, since there are a few indications that this hypervitaminosis does exist and exerts adverse effects. VC has the potential to modulate the intestinal microflora in favor of beneficial microorganisms; however, also this aspect lacks systematic studies. Furthermore, the feedback of (modulated) intestinal microflora on the vitamin supply of wild and farmed aquatic animals is not yet addressed.

Since its discovery in the late 1920s, probably no other chemical has ever been as celebrated as ascorbic acid (vitamin C, VC, Fig. 34.1). The beneficial effect of VC is almost universally recognized. VC is a naturally occurring sugar acid with antioxidant properties acting effectively as scavenger against a broad spectrum of reactive oxygen species (ROS) such as peroxy and hydroxyl radicals ($\cdot\text{OH}$), superoxide ($\cdot\text{O}_2^-$), singlet oxygen ($^1\text{O}_2$), and hydrogen superoxide (H_2O_2) (Fig. 34.1). Furthermore, it provides protection against oxidative damage through vitamin E (VE)-dependent neutralization of lipid hydroperoxyl radicals, thereby preventing lipid peroxidation (LPO) (Buettner 1993), and through protecting proteins from alkylation by products of electrophilic LPO (Ching et al. 2015). Overall, its role is protection of cells from auto-oxidation, particularly in the case of oxidative (respiratory) burst of macrophages.

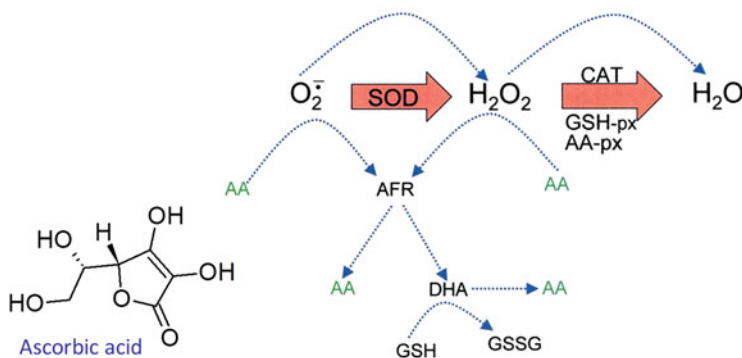


Fig. 34.1 Ascorbic acid (AA, structure) and its role in quenching of reactive oxygen species (e.g., $\cdot O_2^-$, H_2O_2). Blue dotted lines indicate nonenzymatic reactions. (From Arrigoni and De Tullio (2002), with permission from Elsevier). SOD, superoxide dismutase; CAT, catalase; GSH-px, glutathione peroxidase; AA-px, ascorbate peroxidase (only in plants); AFR, ascorbate free radical; DHA, dehydroascorbic acid; GSH, glutathione; GSSG, glutathione disulfide

VC itself is very unstable in feeds because of the sensitivity to heat and light. More stable but still bioavailable derivatives of VC comprise L-ascorbyl-2-sulfate (C2PP), L-ascorbyl-2-monophosphate-Mg (C2MP-Mg), L-ascorbyl-2-monophosphate-Ca (C2MP-Ca), L-ascorbyl-2-monophosphate-Na/Mg (C2MP-Na/Mg), L-ascorbyl-2-polyphosphate (C2PP), and ascorbate-2-glucose (C2D) (Dawood and Koshio 2018). The derivatives possess different bioavailability measured as biopotency. For instance, in tiger shrimp (*Penaeus monodon*) and Malabar grouper (*Epinephelus malabaricus*), the biopotency of these compounds are C2MP-Mg (100%), C2MP-Na (84%), C2PP (64%), C2S (25%) (Shiau 2001) and C2MP-Na (100%), C2PP (84%), C2MP-Mg (46%), and C2S (32%) (Lin and Shiau 2004, 2005b), respectively.

Moreover, VC supports growth, development, reproduction, wound healing, stress resistance, and lipid metabolism through its action on the transcription of, at least, carnitine synthesis and acetyl CoA carboxylase (Han et al. 2019) (Fig. 34.6). VC plays a central role also in immune response and resistance to infectious diseases (Sahoo 2007). It acts as cofactor in many reactions involving hydroxylating enzymes; for instance, collagen synthesis is catalyzed by VC-dependent hydroxylases. These tissues become therefore damaged, if the formation of collagen is impaired by insufficient VC levels in the body. VC deficiency also reduces complement activity in fishes (\rightarrow Box: Complement System, Chap. 2). In addition, VC is involved in stress response; thus, VC requirement increases in stressful situations. It can compensate for stress-induced downregulation of the immune system. VC is also involved in tyrosine metabolism and interacts with several elements of nutritional significance such as Se. It reduces the toxicity of metals (Cd, Ni, or Pb) (Verlhac Trichet et al. 2015). Furthermore, VC is crucial in iodine uptake by thyroid tissues with prolonged VC deficiency resulting in hypo-functioning of the thyroid gland (Agrawal and Mahajan 1981).

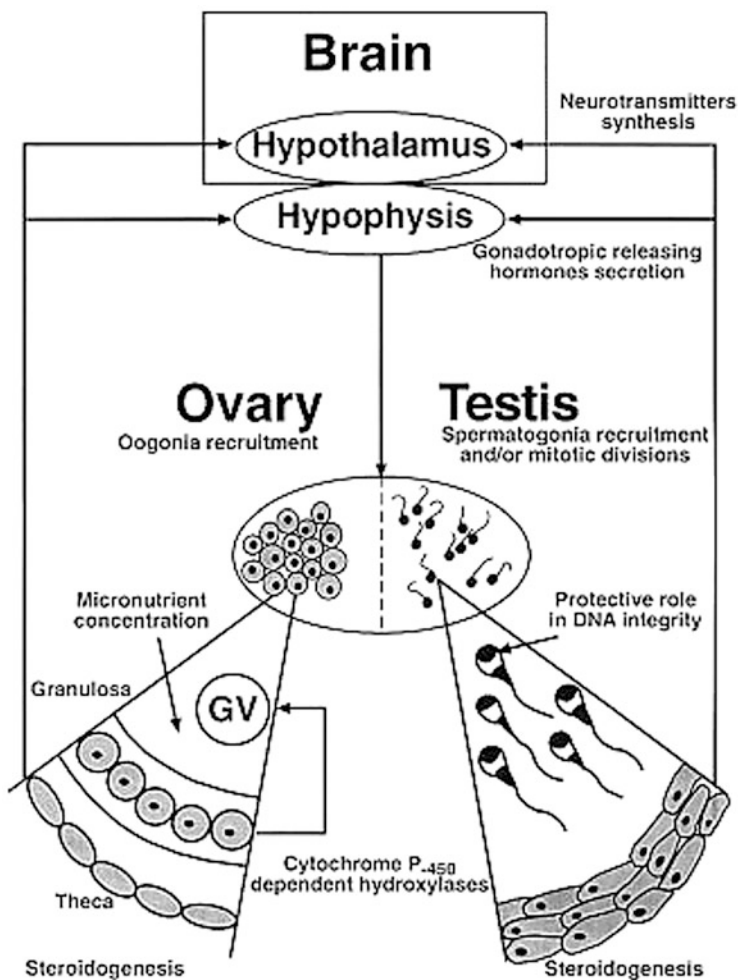


Fig. 34.2 Identified sites of the reproductive system of fish, which may be used to determine the most sensitive components of the role of ascorbic acid. (From Dabrowski and Ciereszko (2001), with permission from Wiley)

VC is central in reproduction (Fig. 34.2). It is well understood that reproduction increases the maternal demand for VC. Soliman et al. (1986) carried out an interesting experiment in which Mozambique tilapia broodstock were fed a diet supplemented with VC and their progeny were fed unsupplemented diet. Nevertheless, this fry shows improved performance, in terms of growth, food utilization, and survival, indicating that broodstock VC supplementation results in VC transfer via eggs to newly hatched fry, thus preventing VC deficiency during early life stages. More examples of VC effects on reproductive traits are listed in Table 34.1.

Table 34.1 Typical effects of dietary vitamin C on life history traits in selected aquatic animals, with special reference to immunity and stress tolerance

Species	Life history trait	Dietary vitamin C supply, mg kg ⁻¹	References
Invertebrates			
<i>Apostichopus japonicus</i>	Growth↑ Antioxidant status↑	600 – 1500 600 – 4700	Ren et al. (2016)
<i>Enteractopus megalocyathus</i>	Paralarval growth↑, paralarval survival↑	50 mg L ⁻¹	Hernández et al. (2019)
<i>Haliotis discus hannai</i>	Hepatopancreatic antioxidant genes↑	70	Wu et al. (2014)
<i>Jasus edwardsii</i>	Fecundity↔, VC content of eggs and embryos↑	240	Smith et al. (2008)
<i>Litopenaeus stylirostris</i>	Collagen metabolism↑, wound repair↑	nd	Lightner et al. (1979)
<i>L. vannamei</i>	Oxidative burst↑, phenoloxidase↑	200 + 1000 β-glucan	Wu et al. (2016)
	Broodstock maturation↑	3000	Sangha et al. (2000)
	Salinity stress tolerance↑ Growth↑	306 – 320	Chen et al. (2017)
	Growth↑ Anoxia tolerance↑	191 360	Niu et al. (2009)
	Ammonia resistance↑	Enriched <i>Artemia</i>	Wang et al. (2006)
	Female survival↑ Fecundity, reproduction↔	400 – 1200	Du et al. (2004)
<i>Macrobrachium malcolmsonii</i>	Growth↑ (Fig. 34.8) Growth↓	100 200–400	Asaikkutti et al. (2016)
<i>M. rosenbergii</i>	Stress resistance↑ (chelate claw ablation)	1200–2400	Manush et al. (2005)
	Growth↑, survival↑	104	D'Abramo et al. (1994)
	Growth↑	120	He and Lawrence (1993)
	Fecundity↑, hatching success↑	60 + E 300	Cavalli et al. (2003)
<i>Marsupenaeus japonicus</i>	Growth↑	92	Moe et al. (2005)
	Hatching rate↑ Metamorphosis rates nauplii → zoea I↑	500	Nguyen et al. (2012)
	Ovary development↑	500 – 1000	Alava et al. (1993)
<i>Pacifastacus leniusculus</i>	Growth↑	0 – 800 Optim @ 200	Celada et al. (2013)

(continued)

Table 34.1 (continued)

Species	Life history trait	Dietary vitamin C supply, mg kg ⁻¹	References
<i>Penaeus californiensis</i>	Collagen metabolism↑, wound repair↑	n.d.	Lightner et al. (1979)
<i>P. monodon</i>	Growth↑	200	Shiau and Jan (1992)
<i>Procambarus clarkii</i>	Growth↑, immunity↑, antioxidant capacity↑	0 – 320	Kong et al. (2021)
Fishes			
<i>Acipenser baerii</i>	Immune response↑	500	Xie et al. (2006)
<i>A. ruthenus</i> × <i>A. baerii</i>	Growth↔, collagen in tissues↑	<1000	Gy. Papp et al. (1999)
<i>Anabas testudineus</i>	Larval growth↑, larval survival↑	PUFA & VC enriched <i>Moina micrura</i>	Singh et al. (2019)
<i>Anguilla japonica</i>	Bactericidal activity↑, serum protein↑	27 – 645	Ren et al. (2005)
	Immunity↔, bactericidal activity↔	32 762	Ren et al. (2007)
	Immunity↑, bactericidal activity↑		
<i>Arapaima gigas</i>	Weight gain↔, white blood cell count↑	500; 800; 1200	de Andrade et al. (2007)
<i>Carassius gibelio</i> var. CAS III	Lysozyme↑ @100 T-AOC↑ @220	0→750	Shao et al. (2018)
<i>Chanos chanos</i>	Spawn↑, egg viability↑, hatching rate↑, survival↑	1000	Emata et al. (2000)
<i>Cirrhinus mrigala</i>	Scoliosis↑, mortality↑	Deficient diet	Mahajan and Agrawal (1980)
	<i>Aeromonas</i> resistance↑	1000	Sobhana et al. (2002)
<i>Clarias batrachus</i>	Growth↑, survival↑, immunity↑ Sod↑	500 2000	Kumari and Sahoo (2005)
<i>C. gariepinus</i>	Wound healing↑	600 – 7000	Erazo-Pagador and Din (2001)
	Xenobiotic stress↓	1000	Datta and Kaviraj (2003)
<i>Coptodon (tilapia) zillii</i>	Growth↑	3000	Anadu et al. (1990)
<i>Ctenopharyngodon idella</i>	Gill rot morbidity↓	156	Xu et al. (2016)
	Hormetic response of fatty acid biosynthesis (Fig. 34.4)	~20–620	Han et al. (2019)

(continued)

Table 34.1 (continued)

Species	Life history trait	Dietary vitamin C supply, mg kg ⁻¹	References
	<i>Aeromonas hydrophila</i> resistance↑	320	Han et al. (2020)
<i>Cyprinus carpio</i>	Growth↔, survival↔	Enriched <i>Artemia</i>	Sandor et al. (2018)
	Growth↑, stress response↑	50	Imanpoor et al. (2017)
<i>C. carpio</i> var. Jian	Growth↑, intestinal <i>E. coli</i> ↓, <i>Lactobacillus</i> ↑, <i>Bacillus</i> ↑	40.9	Liu et al. (2011)
<i>Epinephelus coioides</i>	HSP70↑, mucus secretion↑	750	In Koshio (2007)
<i>E. malabaricus</i>	Growth↑ Innate immunity↑, survival↑	45.3 >6 × 45.3	Lin and Shiau (2004, 2005a)
<i>Heterobranchus longifilis</i>	Growth↑, survival↑, hematocrit↑, hemoglobin↑	50	Ibiyo et al. (2006)
<i>Heteropneustes fossilis</i>	Xenobiotic stress resistance↔	500	Saha and Kaviraj (2009, 2013)
	Xenobiotic stress resistance↑	1000	
<i>Ictalurus punctatus</i>	Growth↔, survival↔	183	Launer et al. (1978)
	<i>Edwardsiella ictaluri</i> survival↓, antibody production↑, complement activity↑	3000	Li and Lovell (1985)
	<i>Edw. ictaluri</i> resistance↔	82; 2071	Li et al. (1993)
	<i>Edw. ictaluri</i> resistance↑	25 – 50	Li et al. (1998)
		3000	Lim et al. (2000)
	Serum protein↑, SOD↑, phagocytosis↔	100; 2000	Yildirim-Aksoy et al. (2008)
<i>Labeo bata</i>	Immunity↑, <i>Edw. ictaluri</i> resistance↑	100; 200; 500	Sahoo et al. (2016)
<i>L. rohita</i>	Respiratory burst↑	10	Nayak et al. (2007)
	Aflatoxin resistance↑, <i>Aer. Hydrophila</i> resistance↑	500	Sahoo and Mukherjee (2003)
	Phagocytosis↑ Growth↑, interleukin production↑, serum bactericidal activity↑	100 200 500	Misra et al. (2007)
<i>Larimichthys crocea</i>	Immune response↑, disease resistance↑	12 – 490	Ai et al. (2006)

(continued)

Table 34.1 (continued)

Species	Life history trait	Dietary vitamin C supply, mg kg ⁻¹	References
<i>Lateolabrax japonicus</i>	Growth↑, immunity↑	12 – 490	Ai et al. (2004)
<i>Leiocassis longirostris</i>	Ammonia resistance↑, growth↑	38; 364; 630	Liu et al. (2008)
<i>Mayaheros urophthalmus</i>	Growth↑ Health↑	40 110	Chávez de Martínez (1990)
<i>Megalobrama amblycephala</i>	pH-stress resistance↑	135–252	Wan et al. (2014)
	Growth↑, non-specific immunity↑, heat stress resistance↑	700	Ming et al. (2012)
<i>Micropterus salmoides</i>	Growth↑, non-specific immunity↑, survival after pathogen infection↑	0→175, optim. 125 – 150	Yusuf et al. (2020)
<i>Monopterus albus</i>	Immunity↑	2200	Tan et al. (2007)
	Resistance against <i>Aer. hydrophila</i> ↑	0→280, optim. @ 80	Hu et al. (2020)
<i>Morone chrysops</i> × <i>M. saxatilis</i>	Resistance against <i>Streptococcus iniae</i> infection↔	2500	Sealey and Gatlin (2002a)
<i>Mugil cephalus</i>	Growth↑, survival↔	45; 82	Swain et al. (2016)
<i>Notemigonus crysoleucas</i>	Survival↑	20	Chen et al. (2003)
	Survival upon heat stress↑	40	
	Complement↑, disease resistance↑	219	
<i>Oplegnathus fasciatus</i>	Growth under low oxygen stress↑	3000	Kato et al. (1992)
<i>Oncorhynchus mykiss</i>	Sperm motility↑	300	Canyurt and Akhan (2008)
	Sperm quality↑	440	Ciereszko and Dabrowski (1995)
	Egg quality↑, hatching rate↑	300	Dabrowski and Blom (1994)
	Lymphocyte proliferation↑	18 – 1760	Hardie et al. (1993)
	Resistance against IHN ^a virus↔	5000	Ishikawa et al. (2013)
	Pathogen resistance↑	5 – 10 × level for growth	Navarre and Halver (1989)

(continued)

Table 34.1 (continued)

Species	Life history trait	Dietary vitamin C supply, mg kg ⁻¹	References
	Hatching success↑	115	Sandnes et al. (1984)
	Complement↑, macrophage oxidative burst↑	1000; 4000	Verlhac et al. (1996)
	Anemia↑, estradiol-17β↓, vitellogenin↓	Deficient diet	Waagbø et al. (1989)
	Survival↑	2000	Wahli et al. (1998)
<i>O. nerka</i>	Resistance against IHN virus↔	5000	Ishikawa et al. (2013)
<i>Oreochromis niloticus</i>	Growth↑, hematocrit↑, innate immunity↑, <i>Aer. hydrophila</i> resistance↑	500	Ibrahim et al. (2010)
	Resistance against <i>Aer. hydrophila</i> ↑, various stressors↑	600 + 0.1% β-glucan	Barros et al. (2014)
	Growth↑, resistance against <i>Edwardsiella tarda</i> ↔	2000	Kim et al. (2003)
	Survival after <i>Str. iniae</i> infection↔	100; 2000	Lim et al. (2010)
	Innate immunity↑, survival after <i>Streptococcus agalactiae</i> infection↑	0; 400; 600	El Basuini et al. (2021)
<i>Oreochromis</i> 'hybrids'	Macrophage immunity↑ @ each exposure	30; 300; 1000; 3000; 5000	Hung et al. (2007)
<i>Pagrus major</i>	Bactericidal activity↑, lysozyme activity↑	>107	Ren et al. (2008)
	Growth↑, immune response↑, hyposalinity resistance↑	800; 1000; 1200	El Basuini et al. (2017)
<i>Pampus argenteus</i>	Growth↑, stress resistance↑	450	Peng et al. (2013)
<i>Paralichthys olivaceus</i>	Survival after freshwater exposure↑	10	In Koshio (2007)
<i>Piaractus mesopotamicus</i>	Protection of erythrocytes↑	500	Garcia et al. (2007)
<i>Platichthys stellatus</i>	Antioxidant potential↑, innate immunity↑	0; 50; 100; 200; 400; 600; 800	Yu et al. (2020)
<i>Poecilia reticulata</i>	Resistance against osmotic stress↑	1000 – 2000	Lim et al. (2002)
<i>Rutilus caspicus</i>	Skin mucus antimicrobial activity↑, growth↑	1000; 1500; 2000	Roosta et al. (2014)
<i>Salmo caspius</i>	Growth↑, nonspecific immunity↑	10; 44; 89; 189; 384	Arab and Islami (2015)
<i>S. salar</i>	Immunity during stress↔	82; 440; 3170	Thompson et al. (1993)

(continued)

Table 34.1 (continued)

Species	Life history trait	Dietary vitamin C supply, mg kg ⁻¹	References
	Resistance against <i>A. salmonicida</i> ↑	2750	Hardie et al. (1991)
	Antibody production↔	500; 5000	Sandnes et al. (1990)
	Short-term resistance against <i>A. salmonicida</i> ↔	150; 1000	Lygren et al. (1999)
	Resistance against <i>A. salmonicida</i> ↑	4000	Waagbø et al. (1993)
	Complement↑, <i>A. salmonicida</i> resistance↑	2750	Hardie et al. (1991)
	Antibody↑	2980	Erdal et al. (1991)
<i>Scophthalmus maximus</i>	Non-specific immunity↔	800 – 1200	Roberts et al. (1995)
<i>Sebastes schlegelii</i>	Growth↑, nonspecific immunity↑	50; 100; 200; 400	Kim and Kang (2015)
<i>Seriola quinqueradiata</i>	Survival after air or low salinity exposure↑	390	In Koshio (2007)
<i>Sillago sihama</i>	Growth↑, hepatic immunity↑, antioxidant capacity↑	100–145	Huang et al. (2020)
<i>Sparus aurata</i>	Non-specific immunity↑, respiratory burst↑	3000	Ortuño et al. (1999, 2001)
	Hypoxia resistance(↔)	10; 25; 50	Henrique et al. (2002)
	Stress resistance↑	158	Montero et al. (1999)
	Growth↑, immunity↑	115 – 103	Liang et al. (2017)
<i>Tachysurus (Pelteobagrus) fulvidraco</i>	Growth↑	29	Eo and Lee (2008)
	Nonspecific immunity↑	82	
<i>Takifugu rubripes</i>	Growth↑, antioxidant status↑, innate immunity↑	49; 96; 190; 382; 780	Cheng et al. (2017)
	Apoptosis↓, DNA damage @ hypothermal stress		Cheng et al. (2018)
<i>Tor putitora</i>	Serum lysozyme↑	0→300	Khan et al. (2019)

nd, not determined; ↑, support; ↔, indifferent or ineffective; ↓, reduction, retardation; T-AOC, total antioxidant capacity

^aInfectious hematopoietic necrosis

The notice of the eminent role of VC in fish reproduction, however, is preceded by French papers reporting VC significance in shrimps and crabs (*Palaemon serratus*, *Cancer pagurus*) (Guary et al. 1975; Guary and Guary 1975) but recognized only lately by the scientific community. Later, several benefits, such as improved growth, survival, fertility, immunity, stress response, and reduction of skeletal deformities in teleosts, have been attributed to VC supplementation (Sandnes 1991). Moreover, Dabrowski and Ciereszko (1996) identified that VC has beneficial effects on sperms of rainbow trout males: VC protects against male infertility. The underlying mechanism appears prevention of oxidative damage to sperm cell DNA and thereby maintenance of genetic integrity. VC deficiency is associated with high shares of abnormal offspring (Ciereszko and Dabrowski 2000).

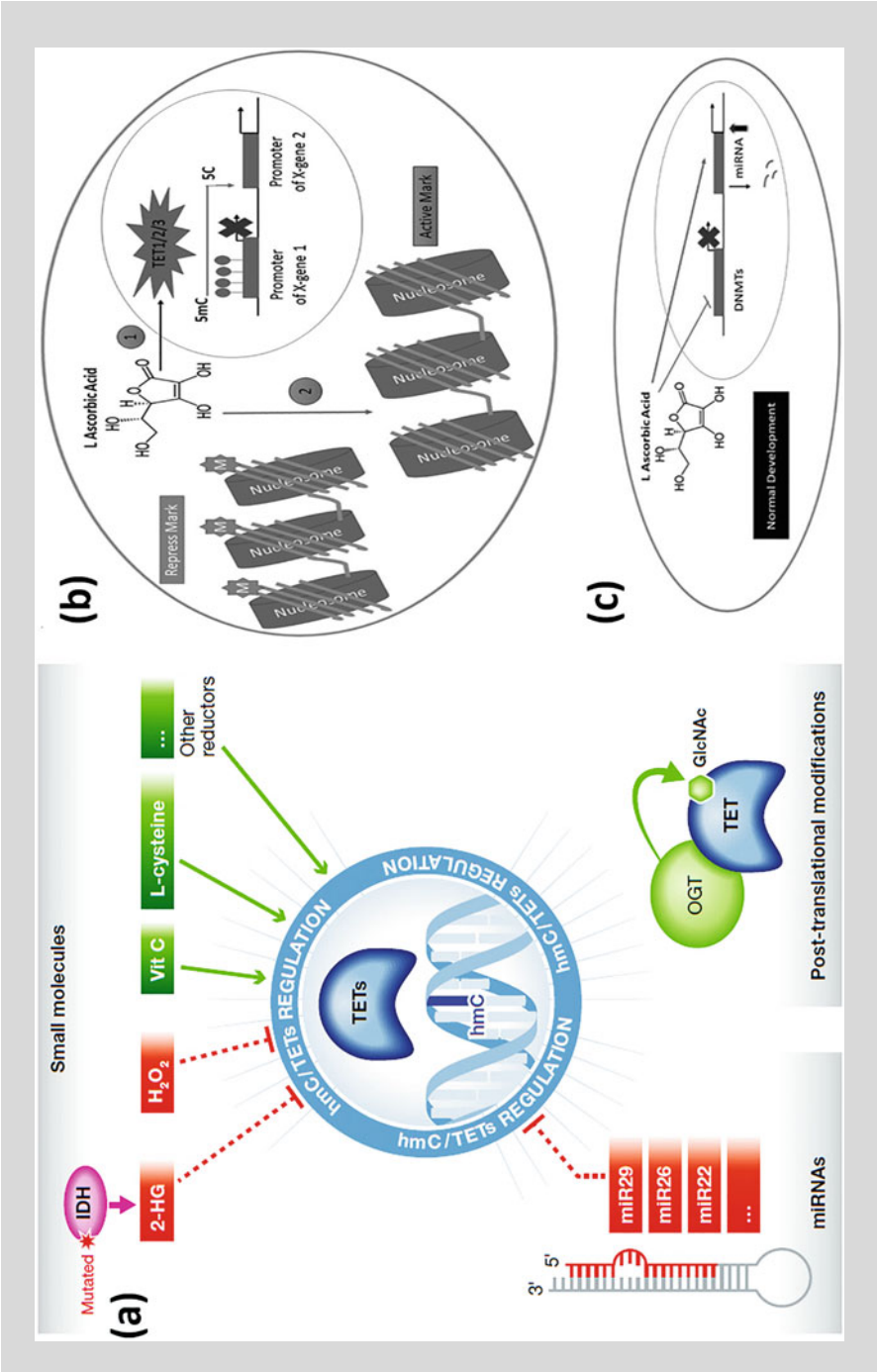
Studies are emerging which identify the involvement of VC in the regulation of transcription and stabilization of specific mRNAs in fishes, again demonstrating that VC is more than just a pure antioxidant. Jiménez-Fernández et al. (2015) reported that VC modifies the transcript levels of 16 genes involved not only in antioxidant defense (*gpx1*) but also in tissue structure (*colla1*, *colla2*, and *colla3*), stress response (*hsc70-1*, *hsc70-2*, *hsc70-3*, *gr1*, *gr2*, *pomcb*), glycolysis (*gapdh1*, *pkm*), osmoregulation (*softer*, *nkcc2*, *nkcc1*), and pigmentation (*mcl1r*) of Senegalese sole.

Box: Vitamin C and the Epigenome

In addition to its role as gene regulator, recent advances disclose a previously unknown function of VC, namely, the epigenetic regulation in mammals (Chung et al. 2010; Camarena and Wang 2016). Under physiological pH conditions, VC exists predominantly as ascorbate anion and is a major regulator of the methylation-demethylation cycle: Ascorbate acts as a cofactor for methylcytosine dioxygenases responsible for DNA demethylation and likely as cofactor for histone demethylases (Young et al. 2015). Thus, by primarily participating in demethylation of both DNA and histones, ascorbate appears to be a mediator of the interface between the genome and the environment.

Methylation of the fifth carbon of cytosine was the first epigenetic modification to be discovered in DNA. Recently, three new DNA modifications have become known: hydroxymethylcytosine, formylcytosine, and carboxylcytosine, all generated by oxidation of methylcytosine by ten-eleven translocation (TET) enzymes. These modifications can initiate full DNA demethylation, but they are also likely to participate in epigenetic signaling per se—like also methylcytosine (Delatte et al. 2014). The position of TET enzymes in the epigenetic machinery is sketched in Box Fig. 1a.

(continued)



(continued)

Box Fig. 1 (a) Regulation of TETs/methylcytosine oxidation. TET proteins can be regulated at multiple levels, all having a potential impact on global or local methylcytosine oxidation. Small molecules such as 2-hydroxyglutarate (2-HG) or hydrogen peroxide (H_2O_2) inhibit the catalytic activity of TETs while others, such as vitamin C, enhance their activity. Similarly, small microRNAs (miRs) can also affect TET-mediated 5hmC formation by direct downregulation of TET expression. Finally, TETs are connected to and regulated by chromatin-related proteins. For example, TETs bind the OGT GlcNAc transferase, which can glycosylate and possibly stabilize TETs. (From Delatte et al. (2014), with permission from Wiley). (b) L-Ascorbic acid-mediated modification of the epigenome. 1. CpG islands of promoter X-gene is hypermethylated (5mC) hence transcriptionally repressed. Vitamin C induce TET1/2/3, which demethylates 5mC to unmethylated cytosine (5C) leading to transcriptionally active gene promoter. 2. Vitamin C demethylates methylated histone in TET independent way to unmethylated histone in nucleosome structure. (c) Vitamin C-mediated miRNA upregulation, which further maintains the pluripotent state of mouse embryonic stem cells. (From Nur et al. (2021), with permission from Taylor & Francis). **TET** Ten-eleven translocation

The first demethylation step was identified, when the involvement of TET1 in the oxidation of 5mc to 5hmc was demonstrated (Box Fig. 1b, c). Later studies show oxidation of 5mc to 5hmc by TET2 and TET3 (Nur et al. 2021). Variation in VC bioavailability thus can influence the demethylation of both DNA and histone, further leading to different phenotypic presentations. How VC deficiency can be involved through epigenetic dysregulation in embryonic and postnatal development as well as in various diseases such as neurodegeneration or cancer is reviewed by Wang et al. (2016). Likely, a comparable mechanism takes place also in lower vertebrates and invertebrates, because the signature motif for this histone demethylases is conserved from *Saccharomyces cerevisiae* to humans (Tsukada et al. 2006). In fact, it is also found in fishes, for instance, in three-spined sticklebacks (Leveelahti et al. 2011); a systematic survey in fishes and aquatic invertebrates, however, is not available.

Recently, Kolhe et al. (2018) and Ramezankhani et al. (2019) detected interplays between VC and miRNAs in regulatory pathways in human bone marrow stromal cells and breast cancer—this finding may serve as an incentive for studies in aquatic animals.

Many animals are able to produce VC, since it can be derived from glucose (Ching et al. 2015). For those who lack the ability of VC synthesis, an adequate dietary supply is mandatory. The latter group includes many teleost fishes. It was originally assumed that no fish species can synthesize VC and that VC synthesis first appeared in amphibians (Chatterjee 1973). There were simultaneous claims that some teleosts can synthesize VC (Yamamoto et al. 1978; Soliman et al. 1985). However, the proofs were based on a non-robust enzyme assay with L-gulonono- γ -lactone oxidase (GLO). Alternatively, it is accepted to clone and sequence *glo* and then to determine its tissue expression (Ching et al. 2015). So far, *glo* has been found in cartilaginous fishes (*Chondrichthyes*) and primitive *Osteichthyes*

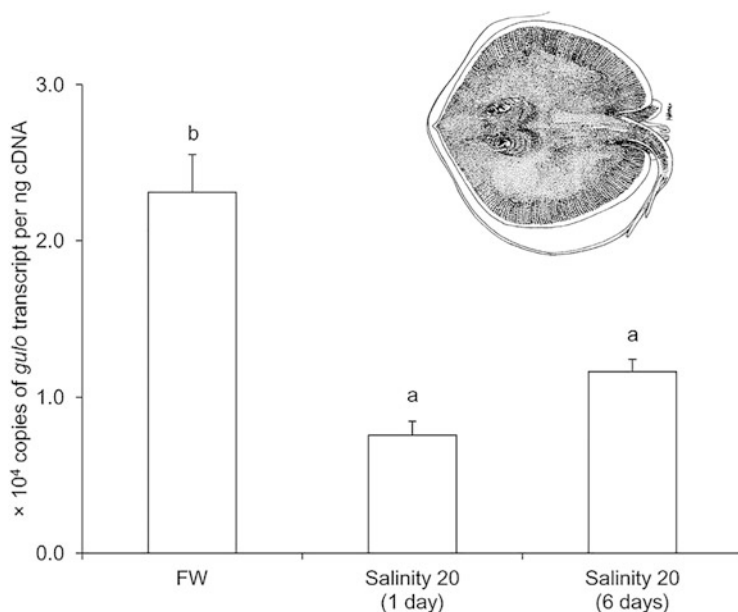


Fig. 34.3 Quantitative mRNA expression of *gulonolactone oxidase* (*glo*, here abbreviated as *gulo*) from the kidney of *Fluvitrygon* (*Himantura*) *signifer* kept in freshwater (FW) or exposed to brackish water (salinity 20) for 1 or 6 days after a 4-day progressive increase in salinity. The results are mean + SEM. Different letters indicate significant differences ($P < 0.05$). (From Wong et al. (2013), credit Public Library of Science; image credit FAO)

(lobe-finned fish, *Sarcopterygii*, and primitive *Actinopterygii*, such as *Acipenseriformes*) (Cho et al. 2007).

VC biosynthesis takes place in liver and kidney. Moreover, very high concentrations of ascorbate and dehydroascorbate are found also in the brain of the African lungfish (*Protopterus annectens*), indicating its importance to protect this organ against oxidative stress (Ching et al. 2014). Moreau and Dabrowski (2003) provided evidence that the antioxidant status of tissues controls the tuning of GLO activity in white sturgeon (*Acipenser transmontanus*) with α -tocopherol downregulating this activity.

Later, Wong et al. (2013) cloned and sequenced *glo* from kidneys of the euryhaline freshwater white-rimmed stingray (*Fluvitrygon* (*Himantura*) *signifer*, Fig. 34.3) that has only a short history of freshwater invasion. To this stingray, living in freshwater is a greater oxidative stress than living in brackish water; however, due to its ability to increase the capacity of VC synthesis in response to hyposalinity (Fig. 34.3), the successful invasion of the freshwater environment appears feasible.

Due to the lack of a functioning *glo* system, most teleost fishes cannot synthesize VC. Remains of the nonfunctional gene with many mutations are, however, still present in the genomes of several species, e.g., common carp (Ocalewicz et al. 2010). The importance of VC during ontogeny can easily be seen by high mortalities in offspring from VC-deficient broodstock (Waagbø 2010). In *Acipenser persicus*, *glo* transcription

and GLO activity are highest at the embryonic stage and start to decline from the hatching of larvae to the rest of the development (Akbarzadeh et al. 2011, 2013).

34.1 Effects on Life History Traits

The VC requirement varies with fish or shrimp species and strain, size, developmental stage, diet, and experimental conditions. Molina-Poveda (2016) lists VC (as ascorbic acid) requirements for fishes from 0.005 g kg⁻¹ (*Oncorhynchus mykiss*) to 0.75 g kg⁻¹ (*Labeo rohita*) and for shrimps from 0.002 g kg⁻¹ (*Penaeus monodon*) to 10 g kg⁻¹ (*Marsupenaeus japonicus*). Low requirement (between 0.01 and 0.025 g VC equivalent kg⁻¹) is found in channel catfish, hybrid striped bass (*Morone chrysops* × *M. saxatilis*) or hybrid tilapia (Dawood and Koshio (2018), with references therein).

In VC-fed crustaceans, the focus of the studies has been put on stress tolerance, female fecundity, and metamorphosis success. Details can be found in Table 34.1. More individual data as well as the derivatives applied as supplement are reviewed by Dawood and Koshio (2018).

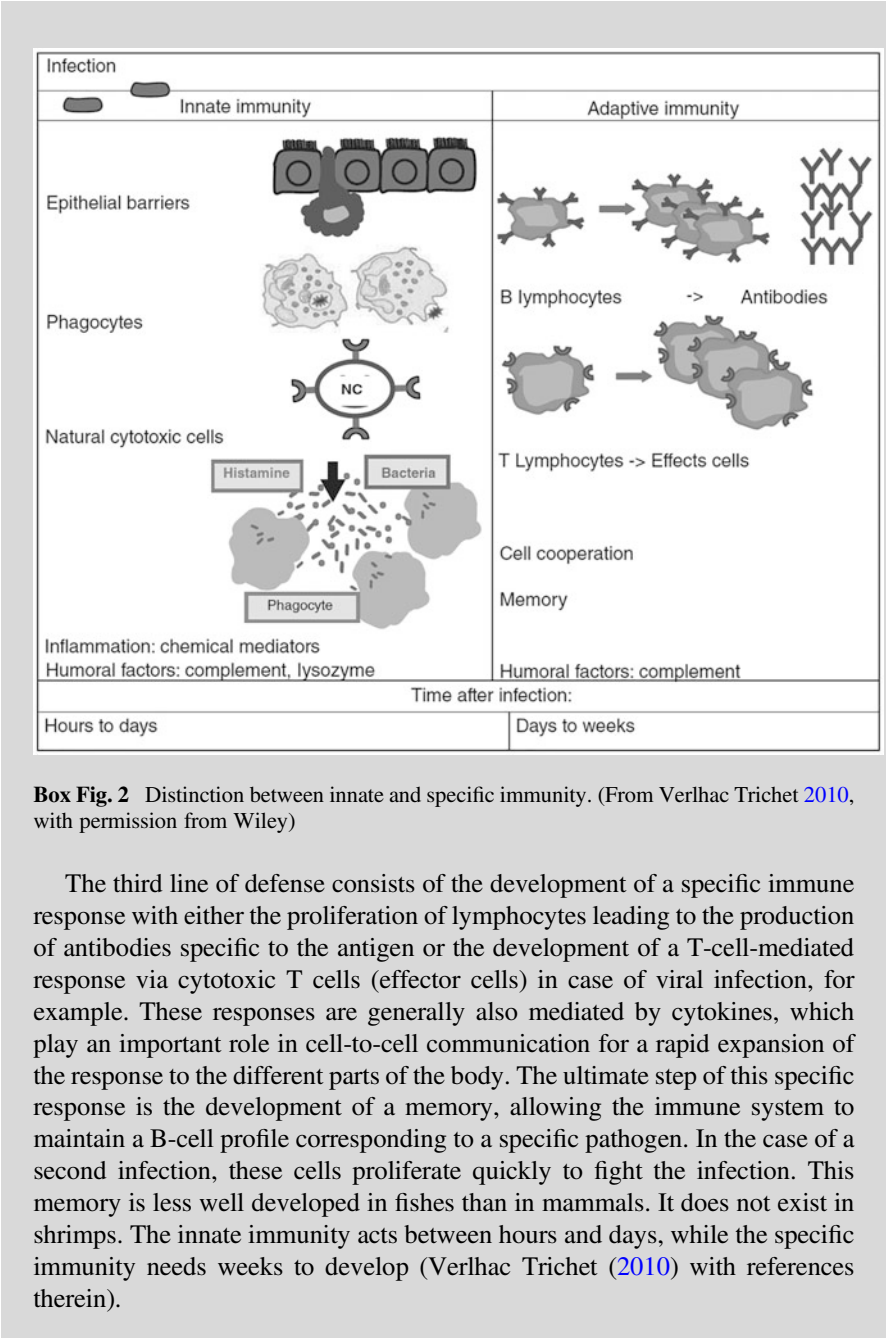
Verlhac Trichet et al. (2015) presented evidence of which compartment of the immune system is triggered in selected fish species. VC is paramount in improving the innate and specific (or adaptive) immunity. To understand the various potential modes of action, the distinction between these two immunity systems is briefly sketched in the Box below.

Box: The Fish Immune System and Inflammation in Brief

Fishes have an important first line of defense consisting of epithelial barriers such as skin, scales, mucus membranes (gastrointestinal tract, secretions of mucus) (Box Fig. 2), and physiological barriers like stomach pH, gut microflora, and chemical mediators secreted by the mucus (among others: defensins, lysozyme, transferrin, complement system (Chap. 2)). Involvement of cells like phagocytes, natural cytotoxic cells (NCC), and inflammatory response through the release of chemical mediators represent a second line of defense that is initiated if the pathogen has been able to pass the epithelial and physiological barriers. The actors of the inflammatory response are interferon (IFN), interleukins (ILs), chemokines,¹ and factors like tumor-necrosis factor alpha (TNF-α). Pathogen-associated molecular patterns (PAMP) of recognition are important elements of the innate immunity, involving different receptor types (Box Fig. 2).

(continued)

¹Small cytokines or signaling proteins secreted by cells able to induce directed chemotaxis in responsive cells.



(continued)

Inflammation is a normal reaction towards harmful stimuli and plays a critical role during infections or wounds. Inflammation follows a biphasic stage starting with the initiation phase, where cells are attracted towards the affected side and release pro-inflammatory signal molecules such as interleukin (IL)-1 β , IL-8, TNF- α , and ROS (Kany et al. 2019). These signal molecules recruit more immune cells towards the side of inflammation. In the resolving phase, anti-inflammatory molecules, e.g., IL-10, TGF β (Opal and DePalo 2000; Zhang et al. 2019a) are released to end the inflammation. In chronic inflammation, the resolving phase is delayed or blocked, which can be caused by diseases or exposure to hazardous environmental pollutants (Prata et al. 2020).

The use of dietary VC in fishes and invertebrates improves their resistance to infections (Table 34.1); the underlying mechanisms, however, are not yet fully understood. During challenge of unvaccinated fishes, the nonspecific immune response is more important for disease resistance than the specific immune response. In vertebrate phagocytic cells, the oxygen-dependent defense mechanism consists in the generation of reactive oxygen and nitrogen species (ROS, NO \cdot) with powerful microbicidal activity (Steinberg 2012). The phenomenon known as respiratory (or oxidative) burst can be elicited by soluble components, such as lectins² and lipopolysaccharides, or by particulate phagocytic stimuli, such as β -glucan (Dawood et al. 2015). The stimulation of the phagocytic cell membrane leads to increased consumption of oxygen with O $_2$ reduction giving rise to \cdot O $_2^-$. Verlhac and Gabaudan (1994) showed that the respiratory burst of salmonid head kidney leucocytes, stimulated by β -glucan, is enhanced by VC. VC is rapidly expended during bacterial infections and phagocytosis. Correspondingly, VC utilization increases during the stress responses (Dawood and Koshio 2018).

Another result of the VC-mediated oxygen and nitrogen activation is the increase of lymphokine production (Misra et al. 2007), the second line of immune defense. Lymphokines are cytokines that are produced by a type of immune cells known as a lymphocyte. They are protein mediators typically produced by T cells to direct the immune system response by signaling between its cells. Important lymphokines secreted by the T helper cell include interleukin 2, interleukin 3, interleukin 4, interleukin 5, and interleukin 6 (IL2...IL6). In the absence of lymphokines from the helper T cells, the remainder of the immune system is almost paralyzed (Guyton and Hall 2011).

Reinventing hormesis with contrasting effects of low and high dietary doses (Chap. 22), Han et al. (2019) identified that VC regulates liver and muscle unsaturated FAs content in a feedback way through FA synthesis- and metabolism-related

²Lectins are carbohydrate-binding proteins, highly specific for sugar moieties and perform recognition on the cellular and molecular level. Lectins also mediate attachment and binding of bacteria and viruses to their intended targets (Takeuchi and Akira 2010).

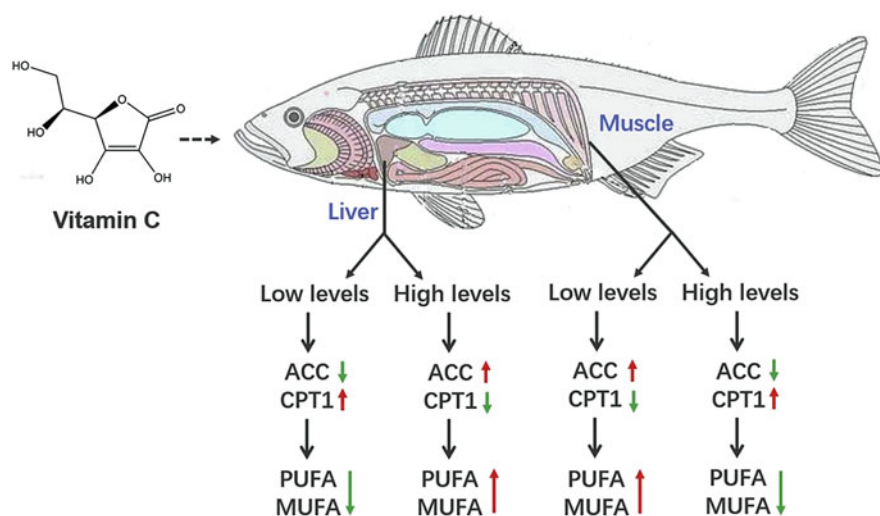


Fig. 34.4 Hormetic function of vitamin C on fatty acids in liver and muscle of juvenile grass carps via related genes. (From Han et al. (2019), with permission from Elsevier). ACC, acetyl CoA carboxylase; CPT1, carnitine palmitoyltransferase I; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid

genes. Additionally, VC elicits inverse results of FAs composition of liver and muscle, owing to the contrasting effects of VC at high and low levels and tissue specificity (Fig. 34.4). This paper may serve as an incentive for the integration of hormetic considerations into aquatic animal nutrition; that is, applying well-graded dietary doses combined with highly sophisticated analyses of underlying mechanisms of the response. The eventual aim is to shed light on the often-observed contrasting results of low and high dietary doses and to solve this obvious conflict.

34.2 Interactions

Interactions between VC and several micronutrients in diets and fish tissues modulate the demand for VC. The strongest interactions are found for antioxidant vitamins and astaxanthin, iron, and copper. Even the opposite, namely, synergisms, is feasible. By combining different vitamins or by combining vitamins with prebiotics as well as minerals (e.g., Se) in the diets, a potentiation of beneficial effects can be expected compared to the effects of single compounds.

34.2.1 Vitamins

More than five decades ago, Tappel (1968) hypothesized that there is an interaction between VC and VE in that these vitamins act synergistically to prevent LPO. As a reducing agent, VC reacts with intermediates of α -tocopherol to reduce VE to its initial state and, thereby, to repair oxidized membranes (Gey 1998). Thus, VC can have a sparing effect on tissue VE levels and VE demands of an animal. Therefore, dietary VC is most often combined with VE in order to spare VE or to fortify the beneficial effects of the single vitamins.

34.2.1.1 Vitamin E

The most prominent interaction between VC and VE is their concerted action as hydro- and lipophilic antioxidants, particularly to prevent from or to repair oxidized membranes. The major step is the reduction of the VE radical by VC. The active and polar phenol group of α -tocopherol resides at the interface between lipid and aqueous compartments, for example, at the membrane surface. In this position, it can react with the water-soluble VC (Buettner 1993).

In nutritional studies with aquatic animals, one focus is put on the sparing effect of VC—with inconsistent results. Gatlin et al. (1986) showed in channel catfish that the VC status does not modulate the severity of VE deficiencies. In contrast, juvenile hybrid striped basses exhibit interactions between VC and VE in terms of growth, feed efficiency, survival, tissue vitamin levels, and oxidation status (Sealey and Gatlin 2002b) indicating the presence of an *in vivo* VC sparing of VE as well as VE sparing of VC. The mechanism behind these interactions appears to be the quenching of ROS by one vitamin that would otherwise consume the other antioxidant vitamin. This mechanism appears to prevail also in studies in gilthead seabream (Montero et al. 1999), channel catfish (Yildirim-Aksoy et al. 2008), angel fish (*Pterophyllum scalare*) (Norouzitallab et al. 2009), Nile tilapia (Lim et al. 2010), European seabass (Betancor et al. 2012), hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) (Pitaksong et al. 2013), Japanese flounder (*P. olivaceus*) (Gao et al. 2014), Caspian brown trout (*Salmo caspius*) (Khara et al. 2016), discus fish (*Symphysodon (haraldi) aequifasciatus*) (Liu et al. 2019), and also in kuruma shrimp (*M. japonicus*) (Nguyen et al. 2012).

One study of sparing effects deserves particular attention. Chen et al. (2004) identified sparing of VE on VC-diet in golden shiner (*Notemigonus crysoleucas*, Fig. 34.5a). In this fish, darkening of the skin indicates VE deficiency. The authors fed graded levels of dietary VC to VE-deficient fishes and found that elevated dietary VC reduces the incidence and severity of VE deficiency symptoms in a dose-dependent manner (Fig. 34.5a). Improved hematological parameters, muscle histology, alternative complement activity, and cumulative mortality following exposure to heat stress also indicate this sparing effect.

In red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegelii*), Ji et al. (2003) showed that VC, but not VE, influences lipid metabolism.

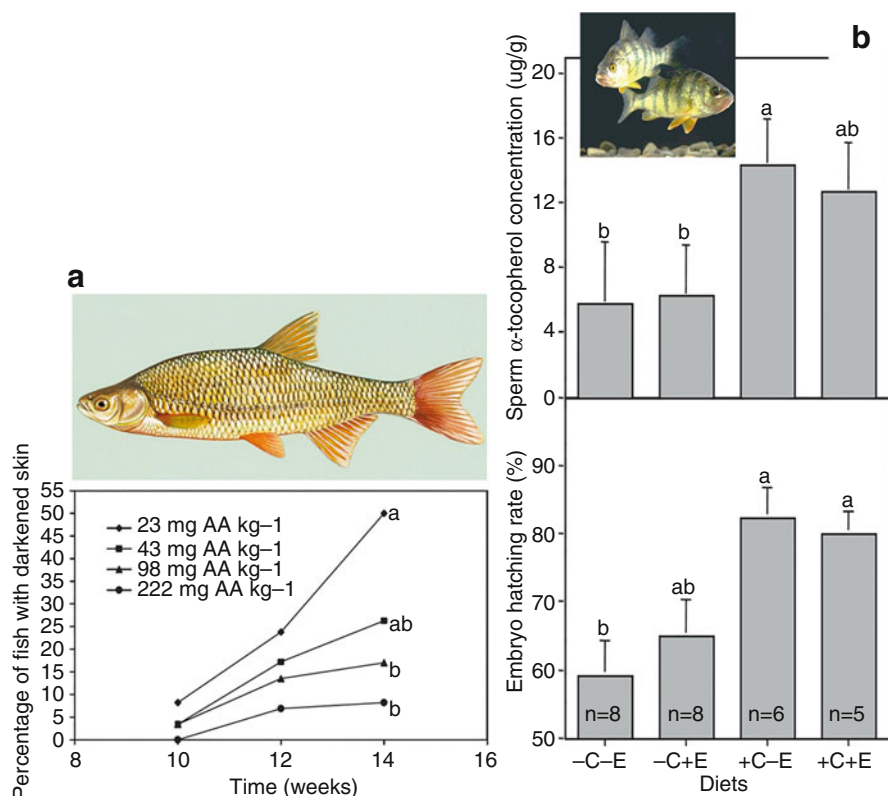


Fig. 34.5 (a) Percentage of golden shiners with darkened skin after consuming α -tocopherol-unsupplemented diets with four levels of ascorbic acid for 10–14 weeks. Different superscripts indicate significant differences ($P < 0.05$). (From Chen et al. (2004), with permission from Elsevier; image credit US Fish and Wildlife Service & Duane Raver). **(b)** Sperm α -tocopherol concentrations and effect of diets on embryo hatching rate in yellow perch fed four semi-purified experimental diets for 32 weeks. The diets were formulated to contain low or high vitamin E levels of 5 and 160 mg kg⁻¹ without and with vitamin C supplementation (250 mg kg⁻¹) designated as diets -C - E, -C + E, +C - E, or +C + E, respectively. Different superscripts indicate significant differences ($P < 0.05$). (From Lee and Dabrowski (2004), with permission from Elsevier; image credit Hadal, United States Department of Agriculture)

Furthermore, VC accelerates VE absorption and prevents VE oxidation. The ability to behave as a lipid antioxidant appears to be stronger in VC than in VE. In terms of vitality of red seabream, the combination of VC and VE fortifies the beneficial effect.

Supplementation of dietary VC and VE does not only accelerate growth in yellow perch (*Perca flavescens*) but also improves the semen quality within one maturation cycle (Lee and Dabrowski 2004). VC can spare sperm VE. Sperm VE content, in turn, controls the hatching of the embryos (Fig. 34.5b). Increased egg hatching rate and larval viability occurs also in penaeid shrimps on this dietary vitamin combination (Boonyaratpalin 1998).

Stress resistance and immunity can increase if VC and VE are combined in the diets (Table 34.2). These responses comprise increasing phagocytosis and pathogen

Table 34.2 Dietary combination of vitamin C and vitamin E modulates survival and immune responses in selected invertebrates and fishes

Species	Preparation, mg kg ⁻¹	Response	References
Invertebrates			
<i>Apostichopus japonicus</i>	Binary mixtures: C500 – 1000 E100 – 200	Growth↔ ^a	Gao et al. (2013)
<i>Litopenaeus vannamei</i>	Binary mixtures: C up to 2500 E up to 600	Survival after low-salinity stress↑	Li et al. (2017)
	Complex vitamin & PUFA mix	Maturation↑, spawning↑, Larval survival↑	Wouters et al. (1999)
	Binary mixtures: C up to 700 E up to 900 & PUFA mix	Larval survival↔, LPO↔	Ruff et al. (2001)
<i>Marsupenaeus japonicus</i>	Binary mixtures: C: 0, 500, 1000 E: 0, 300, 600	Egg size↑	Nguyen et al. (2012)
	C1000 + E150 C2500 + E5000	Brain lipofuscin↓	Castro et al. (2002)
<i>Penaeus merguensis</i>	Complex vitamin mix with C8750 + E350	Survival + growth of juveniles↑	Sedgwick (1980)
Fishes			
<i>Arapaima gigas</i>	Binary mixture: C800 + E500	Red blood cell indices↔	de Menezes et al. (2006)
<i>Argyrosomus regius</i>	Binary mixtures: C up to 3600 E up to 3000 + 0.4%, 3.0% dw HUFA	Growth↑, PUFA oxidation↓	El Kertaoui et al. (2017)
<i>Clarias macrocephalus</i> × <i>C. gariepinus</i>	Binary mixtures: C up to 1000 E up to 125	Growth↑, stress recovery↑, immunosuppression↓	Pitaksong et al. (2013)
<i>Ictalurus punctatus</i>	Binary mixtures: C up to 2000 E up to 500	Hematological indices↑	Yildirim-Aksoy et al. (2008)
<i>Morone chrysops</i> × <i>M. saxatilis</i>	2 binary mixtures: C250 + E30 C2500 + E300	Resistance against <i>Streptococcus iniae</i> infection↑	Sealey and Gatlin (2002b)
<i>Notemigonus crysoleucas</i>	Binary mixtures: C up to 222 E up to 38 (VE sparing, Fig. 34.5)	Alternative complement activity↑, survival↑	Chen et al. (2004)
<i>Oncorhynchus mykiss</i>	Binary mixtures C up to 2000 E up to 800	Macrophage activity↑ Mortality after infections↓	Wahli et al. (1998)
<i>Oreochromis niloticus</i>	5 tertiary mixtures with Se C up to 2000 E up to 240	Growth↑, mortality after infection↔	Kim et al. (2003)

(continued)

Table 34.2 (continued)

Species	Preparation, mg kg ⁻¹	Response	References
<i>Piaractus mesopotamicus</i>	Binary mixtures: C 0, 250, 500 E 0, 250, 500	Hematological indices↑ Mortality after infection↔	Garcia et al. (2009, 2011)
<i>Salmo caspius</i>	C300 + E30 C300 + E40	Growth↑, survival↑	Khara et al. (2016)
<i>S. salar</i>	Binary mixtures: C 0, 7.5, 15, 30, 45, 60 (phosphate) E 0, 150, 300 (acetate)	VE regeneration by VC↑	Hamre et al. (1997)
<i>Sparus aurata</i>	C 3000 + E 1200	Macrophage activity↑	Ortuño et al. (2001)
	C 250 (monophosphate) + E 250 (acetate)	Immunodepression↓	Montero et al. (1999)
<i>Terapon jarbua</i>	C 100 + E 100	Cu stress resistance↑	Vijayavel et al. (2006)

↑, support; ↔, indifferent or apparently ineffective; ↓, retardation or reduction; LPO, lipid peroxidation

^aProbably sufficient vitamin supply in the basic feed

killing capacity, as well as increasing antibody production and immunological memory (Pohlenz and Gatlin 2014) and reversal of the immunosuppression after pathogen challenges.

The study of Wahli et al. (1998) deserves a closer look. The authors tested various combinations of VC and VE on immune response and disease resistance in rainbow trout. Complement levels show no modulation; however, the combination of high dietary doses of VC and VE stimulate lymphoproliferation and macrophage oxidative burst activity. In the disease resistance experiments, the best survival in trouts infected with the viral hemorrhagic septicemia virus is achieved with diets containing both vitamins at high levels. Similar, but less impressive, results are obtained also with *Yersinia ruckeri* and *Ichthyophthirius multifiliis* challenges.

34.2.2 Further Interactions

Cross talk of VC is not restricted to vitamins but occurs also with prebiotics and minerals. Darias et al. (2011) showed that dietary VC levels lower and higher than 0.05 g kg⁻¹ reduce larval growth, skeletogenesis, and bone mineralization, indicating a cross talk between VC and vitamin D. Such disturbances are associated with skeletal abnormalities.

34.2.2.1 Prebiotics: β -Glucan

β -Glucans, insoluble polysaccharides derived from yeast cell walls, can potentiate the nonspecific immunity and are effective in antibacterial defense (\rightarrow AAN III Chapters on 'Functional Food'). Therefore, VC is often combined with β -glucans. Verlhac et al. (1996) applied such a combination to rainbow trout. As expected, it transiently stimulates phagocytosis (Fig. 34.6a). Similarly, Madsen and Dalsgaard (1999) found that the dietary combination of glucan and high VC doses protects trouts against *Flavobacterium psychrophilum* infections.

Increased immunity translates into elevated survival of fishes after pathogen challenge. Diets supplemented with 0.6 g VC kg⁻¹ and 0.1% of β -glucan fed for at least 15 days increase survival of Nile tilapia (Fig. 34.6b) (Barros et al. 2014). Moreover, often the transport-induced stress is even more severe than a bacterial challenge; a dietary supplementation may be a good prophylactic measure against transport stress. In fact, the combination of VC and β -glucan results in an additive effect on growth, immunity, and oxidative status in *Pagrus major*. The authors recommend a treatment of 0.4 g kg⁻¹ VC and 0.5 g kg⁻¹ β -glucan to induce appropriate immune response (Dawood et al. 2017).

Beneficial effects of the combination of VC and β -glucan as dietary immunostimulants apply also to invertebrates as recently shown in Pacific white shrimps with improved nonspecific immunity (Wu et al. 2016). A dose of 0.2 g kg⁻¹ VC plus 1 g kg⁻¹ β -glucan is appropriate.

34.2.2.2 Minerals

As a reducing and chelating agent, VC interacts with minerals in diets and tissues (Waagbø et al. 2001). VC enhances the intestinal absorption of Fe and Se, reduces the absorption of Cu, Ni, and Mn, but has only minor effects on Ca, Zn, and Co absorption, and has no effect on Cd and Hg. In invertebrates, different uptake mechanisms may prevail for the individual metals; Wang et al. (2016) found different absorption for Cu and Fe in *Apostichopus japonicus*. As likely mechanism, the authors point out the reducing capacity of VC under physiological conditions, keeping Fe in a favorable state, while Cu is kept in an unfavorable state for absorption.

Four decades ago, Mahajan and Agrawal (1980) studied Ca uptake in experimentally induced scorbutic spotted snakeheads (*C. punctata*) fed VC-deficient diet for 210 days. When compared with a parallel control on synthetic diet, the results show decreased absorption and utilization of Ca by gill, skin, muscle, and bone of the scorbutic fishes from surrounding water. This deficiency in Ca uptake and collagen synthesis explains skeletal deformities (scoliosis) found often also in other fishes.

One of the best-studied interactions of VC with minerals is its involvement in Fe metabolism. Deficiency of VC leads to reduced serum Fe and redistribution of tissue

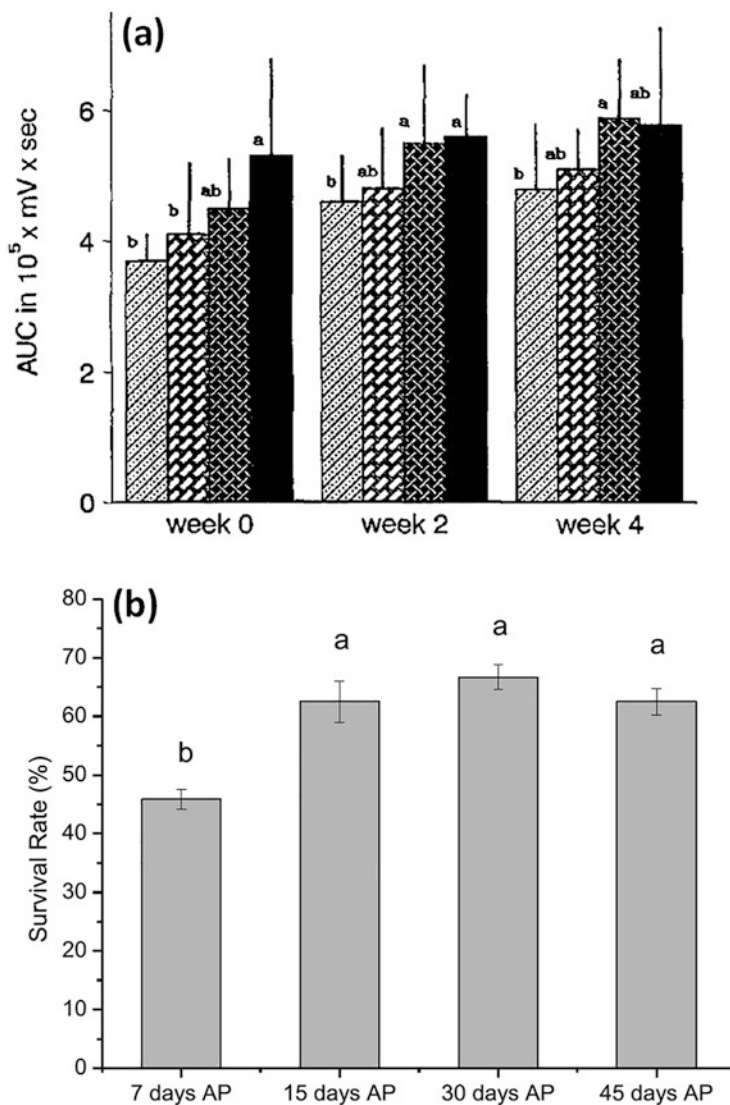


Fig. 34.6 (a) Effect of glucan (zymosan[®]) and vitamin C (150; 1000; 4000 VC equivalents kg^{-1}) on the phagocytosis measured by chemiluminescence in rainbow trout at weeks 0, 2, and 4 after the end of the experimental feeding. Different letters indicate significant differences at $P < 0.05$. (From Verlhac et al. (1996), with permission from Elsevier). (b) Survival rates of Nile tilapia fed a diet supplemented with β -glucan and vitamin C and challenged with *Aeromonas hydrophila*. Different superscripts indicate significant differences. 7 days AP, fishes were fed the control diet for 38 days and the test diet for 7 days; 15 days AP, fishes were fed the control diet for 30 days and the test diet for 15 days; 30 days AP, fishes were fed the control diet for 15 days and the test diet for 30 days; 45 days AP, fishes were fed the test diet for 45 days. (From Barros et al. (2014), with permission from Elsevier)

Fe stores as described in rainbow trout (Hilton et al. 1978). This translates into reduction in both hemoglobin and hematocrit levels (Hilton 1989). The underlying mechanisms is likely the lack of suitable reducing power needed in Fe mobilization from storages (Waagbø et al. 2001).

The interaction between VC and Fe has been revisited—with mixed results. Andersen et al. (1998) found that VC and Fe supplementation has no effect on growth, hematological parameters, antioxidant status, or health in Atlantic salmon smolts. In *Colossoma macropomum*, no modulation in hematological parameters occurs if fed diets with graded levels VC and Fe (Aride et al. 2010). In contrast, Lim et al. (2000) detected that Fe deficiency in channel catfish can be accelerated by supplementation of VC as can be seen by the decrease in weight gain and survival. Also Adel and Khara (2016) reported on an interaction between VC and Fe in rainbow trout fingerlings. Diets supplemented with both micronutrients lead to improved growth and immunity.

For invertebrates, obviously no information on interaction between VC and Fe is available (Waagbø et al. 2001), a deficit that has not changed since then.

34.3 Hypo- and Hypervitaminosis

34.3.1 Hypovitaminosis

In marine and freshwater fishes, requiring VC, hypovitaminosis C is well understood. Hemorrhages, scoliosis, or broken backbone syndromes are very common (Verlhac Trichet et al. 2015). A few more deficiency symptoms are listed in Table 34.3.

Table 34.3 Symptoms of vitamin C deficiency in selected fishes

Species	Affected trait	References
<i>Anguilla</i> spp.	Hemorrhage in fins, head, skin, lower jaw erosion	Satoh (2002)
<i>Cyprinus carpio</i>	Caudal fin erosion and deformed gill arches in larval stage, poor growth	Takeuchi et al. (2002)
<i>Ictalurus punctatus</i>	Scoliosis, lordosis, internal and external hemorrhage, fin erosion, reduced bone collagen formation	Robinson and Li (2002)
<i>Lates calcarifer</i>	Gill hemorrhages, distortion of gill filaments and hyperplasia, short operculum, short snout, exophthalmia, loss of equilibrium, scoliosis, lordosis, broken back, fatty liver, muscle degeneration and hemorrhage, low blood parameters, low tissue hydroxyproline	Boonyaratpalin and Williams (2002)
<i>Lutjanus guttatus</i>	Fin erosion, dark skin, desquamation, erratic swimming	Chávez-Sánchez et al. (2014)
<i>Seriola quinqueradiata</i>	Scoliosis, dark coloration, ataxia, hemorrhage in body surface, hypochromic anemia	Masumoto (2002)

In addition to phenotypic studies, Xu et al. (2016) elucidated signaling pathways of dietary VC deficiency in juvenile grass carps infected by *Flavobacterium columnare*. The authors discovered a complex network of VC targets that depress gill physical as well as immune barriers (Fig. 34.7).

Under infection of *F. columnare*, dietary VC deficiency:

1. Reduces antioxidant capacity by decreasing the antioxidant enzyme activities and mRNA levels, related to the Nrf2 signaling pathway, a master regulator of the antioxidant response.
2. Induces apoptosis by upregulating *caspase-3*, *caspase-7*, *caspase-8*, and *caspase-9* mRNA levels.
3. Interacts with intercellular tight junctional complexes (TJs) by upregulating mRNA levels of *claudin-12*, *claudin-15a*, and *claudin-15b*, and downregulating mRNA levels of *occludin*, *zo-1*, *zo-2*, *claudin-c*, *claudin-3c*, *claudin-7a*, and *claudin-7b*³.
4. Reduces immunity through decreasing lysozyme and acid phosphatase activities and C3, C4, and IgM contents.
5. Aggravates inflammation in the gills.

In addition to these pathways, Kirkwood et al. (2012) reported that, in zebrafishes, VC deficiency activates the purine nucleotide cycle, since levels of inosine monophosphate (IMP) and AMP deaminase (AMPD) activity are enhanced in VC-deficient fish (more information about nucleotides, Chap. 38). AMPD is the enzyme that irreversibly deaminates AMP to form IMP. The authors found a 47% increase in AMP deaminase activity in the VC-deficient zebrafishes, complementary to the 44-fold increase in IMP concentration. This paper indicates that VC is crucial for maintaining cellular energy homeostasis.

34.3.2 Hypervitaminosis

Surplus of micronutrients such as VC, but also vitamin E, pyridoxine, riboflavin, pantothenic acid, and folic acid, have shown beneficial influences on immunity and disease resistance in some, but no effects in other studies (Table 34.1) (Sealey and Gatlin 1999). With water-soluble vitamins, the current belief is that only a certain amount can be absorbed, and if an excess is ingested, it will be excreted (WHO 1999). This means that a hypervitaminosis C is thought to be very unlikely to occur. However, evidence from mammalian studies is accumulating that dietary uptake of excess VC can cause adverse effects. Therefore, also VC excess should be taken seriously and reconsidered.

³Transmembrane proteins that are important components of the tight junctions; plasma-membrane protein located at the tight junctions; *zonula occludens*, tight junction protein; together with the Claudin group of proteins, the main component of the tight junctions.

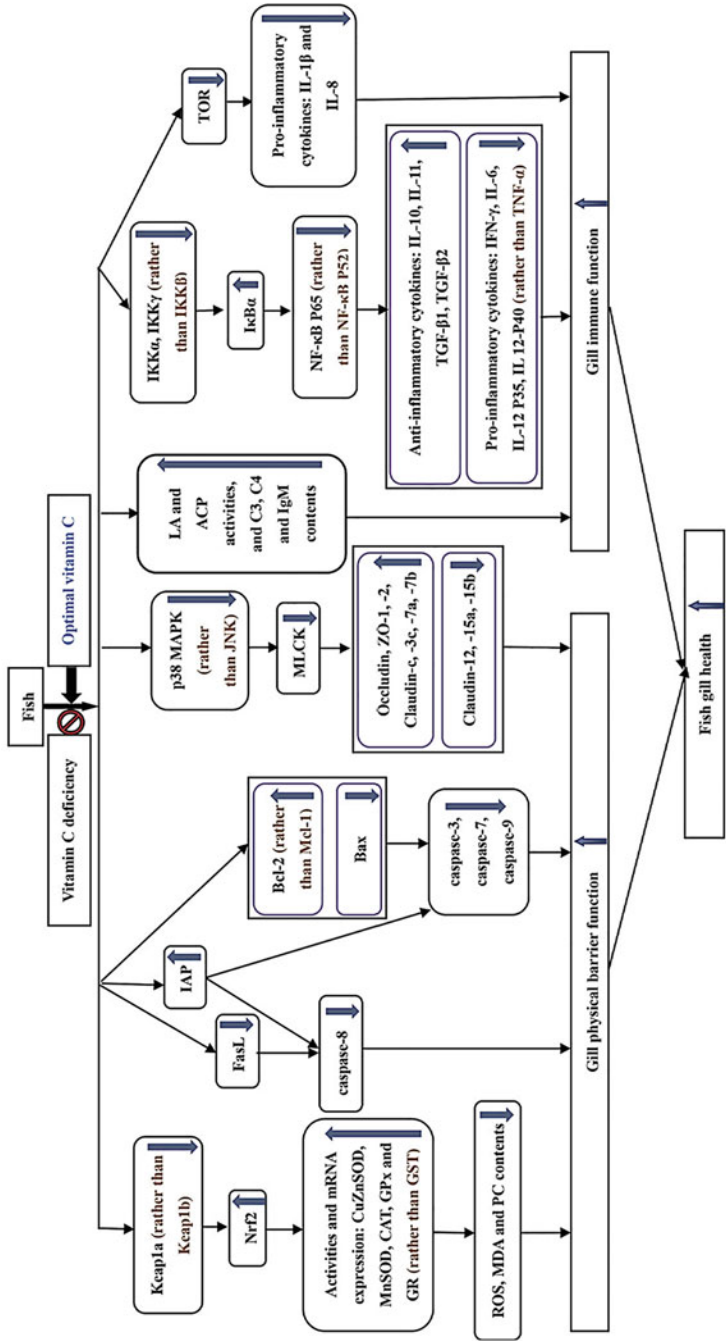


Fig. 34.7 The potential action pathways of appropriate vitamin C supply in the gill physical barriers and immune barriers of fish, exemplified in juvenile grass carp. (From Xu et al. (2016), with permission from Elsevier)

Already several decades ago, Liu et al. (1989) reported that channel catfish needs only moderate dietary VC levels to achieve optimal defense response against *Edw. ictaluri* infection. However, adverse symptoms of excess supply of water-soluble vitamins were beyond considerations at that time; consequently, extreme dietary doses were not tested, and the likely optimum curve was not completed. Nevertheless, skeletal abnormalities, jaw malformations, albinism, and melanism caused by high concentrations of VC have later been reported in olive flounder (*Paralichthys olivaceus*) and in striped trumpeter (*Latris lineata*) (Negm et al. 2014; Takeuchi 2014), provided experimental flaws can be excluded.

Based on his “mitohormesis” hypothesis (\rightarrow AAN III ‘Modes of Action of Selected Plant Compounds’), Ristow et al. (2009) proved adverse effects of excess dietary VC in mammals. It is well understood that exercise promotes longevity and ameliorates insulin resistance. However, exercise also increases the mitochondrial formation of ROS. The authors show that antioxidant supplements prevent the induction of molecular regulators of endogenous antioxidant defense by physical exercise. Therefore, they propose that transiently increased levels of ROS reflect a potentially health-promoting process, rather than an adverse oxidative stress.

Since ROS are comprehensively involved in cell-internal and cell-to-cell communication as well as lipogenic and adipogenic processes (Chap. 24), it can easily be deduced that the reported lacking induction of molecular regulators by dietary excess of antioxidants adversely affects a plethora of pathways (Lykkesfeldt and Poulsen 2010; Saul et al. 2011; Ristow and Schmeisser 2014). This fundamental mechanism applies also to aquatic animals; however, this concept finds its way only very slowly into studies of their nutrition. In general, the nutrition has to overcome “the industry-driven public obsession with antioxidants,” as Gutteridge and Halliwell (2010) accurately phrase the general overconsumption of VC.

Only a few papers raised concerns or presented evidence that aquatic animals are no exception to this rule. Moreau et al. (1999) suspected that in *Acipenser fulvescens*, a fish able to synthesize VC, large doses of dietary VC can be pro-oxidant in vivo. A clear proof of this assumption, however, continues to be missing.

Subsequently, Wu et al. (2014) found that, in the Pacific abalone *H. discus hannai*, excessive dietary VC (0.8 and 5.0 g kg⁻¹) induces oxidative stress by downregulating certain antioxidant enzymes, such as CAT and mu-GST. In *Macrobrachium malcolmsonii*, fed dietary 0.025–0.1 g kg⁻¹ VC, Asaikkutti et al. (2016) discovered significant improvements in growth, digestive enzyme activities, and muscle biochemical compositions. However, if fed dietary VC levels >0.2 g kg⁻¹, prawns show poor performance (Fig. 34.8a). Although somewhat weaker, Asaikkutti et al. (2018) describe an optimum of dietary VC also for the sister species, *M. rosenbergii* (Fig. 34.8b).

In juvenile golden pompano (*Trachinotus ovatus*), Zhang et al. (2019b) detected that excess VC causes a decline in *hsp70* transcription. HSP70 assists the folding of nascent polypeptide chains, acts as a molecular chaperone, and mediates repair and degradation of altered or denatured proteins after a stress (Bukau and Horwich 1998). The result in golden pompano agrees well with those in juvenile loach

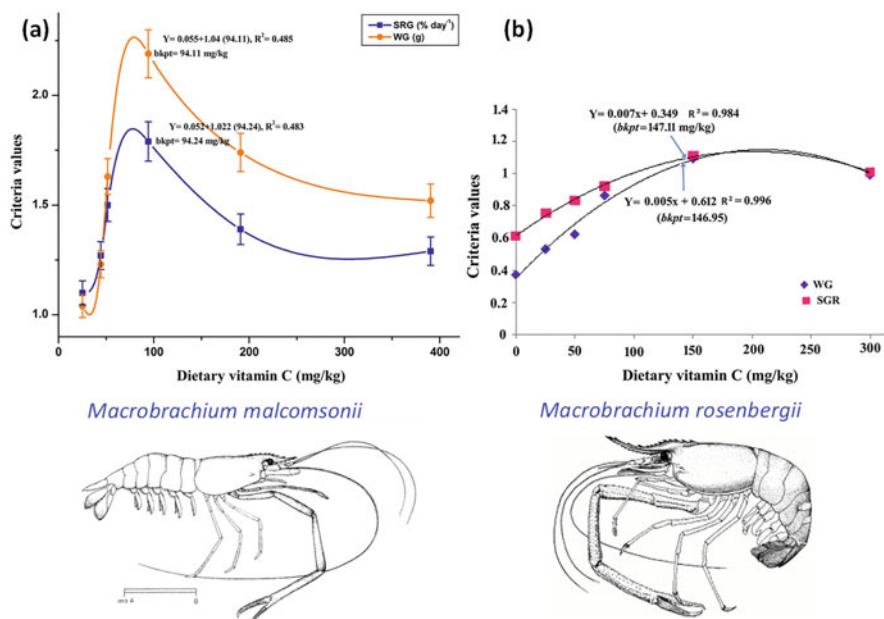


Fig. 34.8 Relationship between weight gain (WG) specific growth rate (SGR) on dietary vitamin C levels based on a broken-line regression analysis, where X represents dietary vitamin C requirement of (a) *Macrobrachium malcolmsonii* and (b) *M. rosenbergii* and containing graded levels of vitamin C for 90 days. The term “criteria values” represents the values of a selected parameter, such as WG and SGR. (From Asaikkutti et al. (2016), credit Elsevier, and from Asaikkutti et al. (2018), with permission from Springer Nature; images credit FAO)

(*Misgurnus anguillicaudatus*): Excess VC supplementation downregulates *hsp70* and the antioxidant master genes *nrf2* (Zhao et al. 2017).

Effect studies of dietary excess VC are still in their infancy and future studies have to figure out how fishes and invertebrates in general respond to dietary VC excess.

34.4 Concluding Remarks

Doubtless, VC is a vitally important nutrient, required in limited amounts by any organism. The modes of action start to be well understood on the transcriptomics level. These studies indicate that multiple genes and metabolic pathways are involved in the observed effects, which, on the phenotypic level, comprise the classical life history traits: growth, fertility, or stress and pathogen resistance. The immunological study by Xu et al. (2016) on molecular effects of VC-deficiency in juvenile grass carps can serve as role model of how to study the common mode of vitamin action. It certainly will initiate future studies of further vitamins in required,

deficient, or excess doses also in other aquatic organisms. In addition, interactions of VC with further nutrients and minerals should be considered. The topic of interactions is only scarcely documented mostly on the phenotypic level.

The question arises whether excess of VC is without adverse effects in fishes and invertebrates. In this chapter, we have focused on this phenomenon and presented the few available studies, mainly on the phenotypic levels. Especially the excessively fed invertebrates, *Haliotis discus hannai* and *Macrobrachium malcolmsonii*, respond by increased oxidative stress or reduced growth, respectively. These reports raise the question, whether or not previous studies applied sufficiently high dietary excess VC doses to identify potentially adverse effects. Vice versa, if there are different susceptibilities of aquatic animals towards dietary VC excess, it is mandatory to identify the underlying regulating pathways and to apply sufficiently graded dietary doses. Verification via biomolecular studies are needed to understand the underlying modes of action and, thereby, proving the generalizability of this extraordinary, as compared to other vertebrates.

It appears that VC modulates the intestinal microflora in favor of beneficial microorganisms, such as lactic acid bacteria and *Bacillus* spp. and in disfavor of potentially pathogenic microorganisms, such as *E. coli*. This aspect lacks systematic studies; the few available studies are like an interesting hand waving, which draws the attention to this effect. Furthermore, the feedback of intestinal microflora on the vitamin supply of wild and farmed aquatic animals is not yet addressed. This applies to the original gut microflora as well as that microflora modulated by pre- or probiotics. It can be expected that it differs between wild and farmed animals and that the aquaculture industries can learn from knowing the wild conspecifics better.

Since the miRNAs lost their blemish as junk RNA, this epigenetic pathway is slowly entering the aquatic phase. Available information shows that this translational modification of mRNA significantly contributes to the malnutrition effects as shown with deficient and excess (that equals toxic) dietary supply of α -tocopherol in tilapia. This study will surely serve as incentive for further similar and proceeding studies. Moreover, one has to keep in mind that the translational action of miRNAs is only one mechanism from the epigenetic machinery. Diet-induced methylations of DNA and histones as well as acetylation of histones deserve intensive investigations, particularly because several of the water-soluble vitamins are major methylation substrates.

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Chapter 35

Vitamin D—‘Keep the Orthopedist Away!’



Abstract Vitamin D (VD) comprises five fat-soluble secosteroids responsible for enhancing intestinal absorption of ions. In concert with two peptide hormones, its well-established function comprises calcium metabolism, bone formation, and bone mineralization. Moreover, VD is related to innate and adaptive immunity with production of anti-inflammatory cytokines. It is also important as regulator of lipid metabolism. Interactions of VD and lipophilic compounds are documented, but interactions occur also between VD and hydrophilic vitamin C affecting ossification; the latter interaction deserves future studies. Moreover, mammalian studies point out the epigenetic role of VD via DNA methylation and histone modification. In zebra fish, also miRNAs have been identified as mediators of VD signaling. As compared to mammals, much less is understood about the biomolecular role of VD in aquatic organisms.

Vitamin D (VD) comprises five fat-soluble secosteroids responsible for enhancing intestinal absorption of ions. Its well-established function comprises calcium metabolism, bone formation, and bone mineralization in concert with two peptide hormones, calcitonin, and parathyroid hormone. VD plays an important role also in maintaining the immune homeostasis, decreasing inflammation, and inducing cell differentiation. Vice versa, VD deficiency increases pathogen susceptibility (Soto-Dávila et al. 2020). Furthermore, although commonly considered antiproliferative, Han et al. (2019) identified VD as mitogenic for cardiomyocytes and other cell types in zebrafish. These authors indicate also a VD-mediated mechanism regulating organ size and regeneration.

A comprehensive overview of VD and VD receptor (VDR) functions is provided by Sundar and Rahman (2011) (Fig. 35.1). This overview draws the attention to environmental agents in deregulating the functions of VD and its receptor. Airborne agents, such as fine particulate matter (cigarette smoke) in the original paper, can easily be replaced by food and environmental agents such as so-called antinutritional factors, metals, or natural and synthetic waterborne xenobiotics, which activate the VDR to fit the situation of the exposed aquatic animals. The major cellular and molecular functions affected by VD/VDR include calcemic effects, antimicrobial peptide gene transcription, tissue remodeling, immune modulation, autoantibody

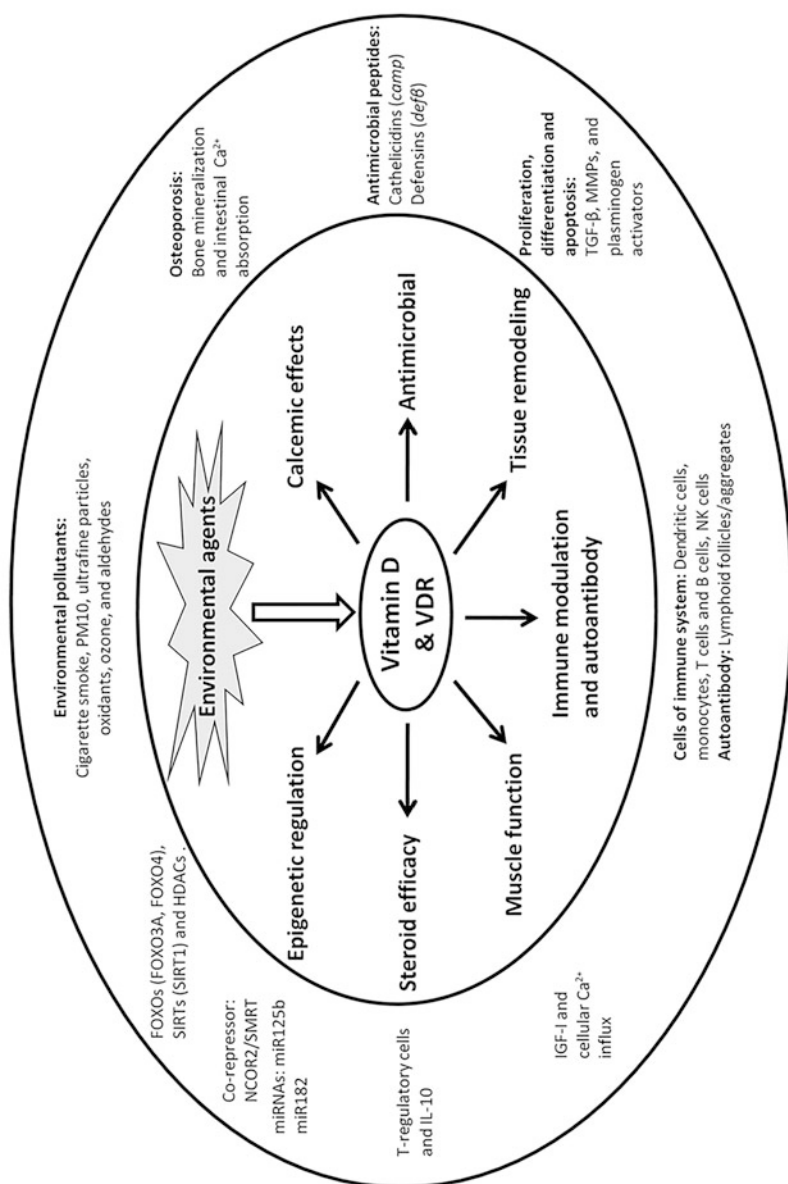


Fig. 35.1 Role of vitamin D/VDR (vitamin D receptor) in dietary and environmental trigger-mediated deregulation of cellular and molecular functions. (From Sundar and Rahman (2011), credit Frontiers Media SA). Environmental agents can easily be supplemented by food items

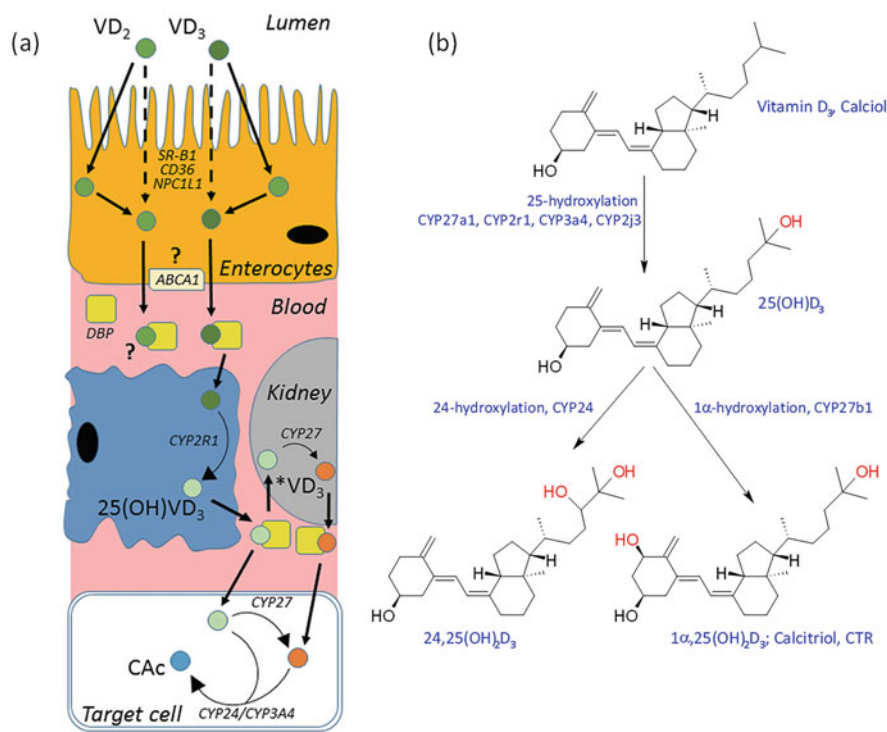


Fig. 35.2 (a) Overview of the metabolism of vitamin D (VD) in various cells/tissues and the cholecalciferol signaling pathway (CSP) from absorption to target cells. *ABCA1* ATP binding cassette A1; *CAC*, calcitric acid; *CD36*, cluster determinant 36; *CYP2R1*, cytochrome P450 family 2 subfamily R member 1; *CYP24*, cytochrome P450 family 24; *CYP27*, cytochrome P450 family 27; *CYP3A4*, cytochrome P450 family 3 subfamily A member 4; *DBP*, vitamin D binding protein; *NPC1L1*, Niemann-Pick C1-like 1; *SRB1*, scavenger receptor class B member 1; VD_2 , ergocalciferol; VD_3 , calcitriol; $*VD_3$, $1,25(OH)_2VD_3$ (1,25-dihydroxyvitamin D_3 , calcitriol); $25(OH)VD_3$, 25-dihydroxyvitamin D_3 or cholecalciferol. (From Fernández et al. (2018), with permission from Springer Nature). (b) Structures of vitamin D_3 (=cholecalciferol or calcitriol) and its activation; $1\alpha,25(OH)_2D_3$ (simplified: $1,25(OH)_2D_3$, CTR) and $24,25(OH)_2D_3$ are the most polar forms of VD; the most active form is CTR in most animals, whereas $25(OH)D_3$ is the active form in several mollusks. (Redrawn and modified after Prosser and Jones (2004), with permission from Elsevier)

production, muscle function, steroid efficacy, and epigenetic regulation. Detailed studies in fishes are not yet available but appear to be promising issues.

The most important compound in the VD group is VD_3 , also known as cholecalciferol or calcitriol (Fig. 35.2b). Contrary to mammals, which are able to photosynthesize VD in their skin, aquatic animals obtain the required VD_3 almost exclusively from dietary sources (Lock et al. 2010). Only one study indicates that rainbow trouts can produce VD in their skin when exposed to blue visible light (440–480 nm) (Pierens and Fraser 2015); however, this production does not reach actual demands.

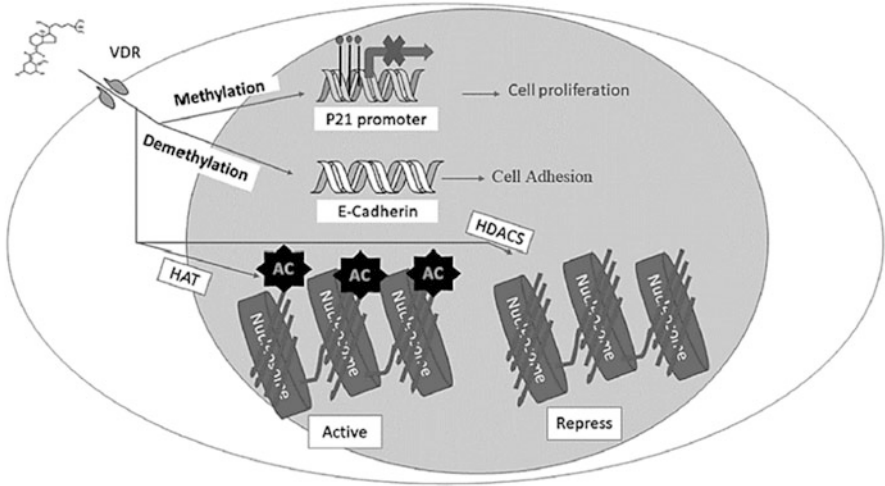


Fig. 35.3 Vitamin D uses VDR to enter the cell and access the nuclear material of epigenome. It can repress p21 promoter transcription; the transcripts accelerate cell proliferation and can demethylate of E-cadherin promoter. It can also activate both HAT (leads active transcription) and HDACs (leads repressed transcription). (From Nur et al. (2021), with permission from Taylor & Francis). HAT, histone acetyltransferase; HDACs, histone deacetylases

Planktonic VD_3 accumulates in the aquatic food chain. Fishes store large quantities of VD in their liver and fatty tissues without metabolizing it (Takeuchi et al. 1984). To activate VD_3 , it has to be hydroxylated in two steps by microsomal cytochrome P450-dependent enzymes to form $1,25 (\text{OH})_2\text{D}_3$ (also calcitriol, CTR) and, to a lesser degree, $24,25 (\text{OH})_2\text{D}_3$ (Fig. 35.2b). Both metabolites are essential for bone formation (Ornøy et al. 1978). In an educational cartoon, Fig. 35.2a sketches the location of this metabolism (Fernández et al. 2018), and Bouillon and Bikle (2019) point out that the VD endocrine system is important also for operational functions in most cells of the body. It is much more complex than initially thought and still incompletely understood.

Meanwhile, the role of VD in epigenetics gains clarity—at least in mammals (Fig. 35.3): VD is involved in DNA methylation as well as histone modification (Nur et al. 2021). VD_3 activates histone acetyltransferases (HATs) involved in active transcription and histone deacetylases (HDACs), which are involved in transcription repression (Fig. 35.3). Also histone methylation is regulated by VD_3 , so far exemplified only in cancer progression (Nur et al. (2021) and references therein).

Compared to mammals, much less is understood about the role of VD in aquatic organisms. VD is crucial to preserve Ca and P homeostasis and to maintain skeletal integrity. The VD_3 receptor adsorbs CTR. Upon activation by CTR, it forms a heterodimer with the retinoid-X-receptor (RXR) and regulates gene expression by interacting with specific DNA sequences upstream of VD responsive genes, namely, the TATA box, the core promoter sequence (Lock et al. 2010).

VD acts directly also on osteoblasts, the resident bone-forming cells of the skeleton, to inhibit proliferation, modulate differentiation, and regulate mineralization of the extracellular matrix (Darias et al. 2011a). In European seabass larvae, Darias et al. (2010) found an effect of VD₃ in intestinal maturation that influences the transcription of a gene of the transient receptor potential (*trp*) family, namely *trpv6*, which is one the most predominant Ca²⁺ transporter in the intestine (Nijenhuis et al. 2005), thereby indirectly influencing the intestinal absorption of Ca²⁺.

In zebra fish, Craig et al. (2012) provided a detailed study of VD action in developing individuals. In response to CTR, the numbers of RNAs increase from 4 (dpf 2) to 2407 (dpf 7). At dpf 7, in CTR-treated larvae, pharyngeal cartilage and mineralization are enlarged. In developing embryos/larvae, CTR alters RNAs encoding proteins that central in diverse pathways during development: transcription factors, leptin, peptide hormones, and RNAs encoding proteins of fatty acid, amino acid, xenobiotic metabolism.

Moreover, miRNAs are involved in the action of VD₃, as studied in zebra fish in vivo (Craig et al. 2014). The authors verified the role of *miR-125b* targeting *cyp24a1*. CYP24 enzymes hydroxylate 25 (OH)D₃ (Fig. 35.2). Several more, often cancer- and disease-related, miRNAs are identified as mediators of VD signaling (Nunez Lopez et al. 2017). In zebra fish, Merrigan and Kennedy (2017) showed the ability of VD₃ as VDR agonist to selectively inhibit developmental angiogenesis in the larval eye: VD₃ elicits increased ocular *miR-21* expression.

The role of CTR is even more complex (Peng et al. 2017): It promotes fatty acid oxidation in adipose tissues of zebrafish (Fig. 35.4), indicating its important role as regulator of lipid metabolism. Depletion of the hydroxylation enzymes CYP2R1 and CYP27B1 (Fig. 35.2b, Fig. 35.4) results in CTR deficiency, retarded growth, and excessive visceral adipose tissue (VAT). On the other hand, sufficient CTR regulates lipid metabolism, controlling mitochondrial biogenesis and oxidative metabolism in zebrafish VAT. The retinoid X receptor is also central in the action of vitamin A and a cross talk emerges (Chap. 32).

Deficient as well as excess VD₃ leads to varying pathological symptoms. In their classical study, Taveekijakarn et al. (1996) showed in Japanese Amago salmon (*Oncorhynchus rhodurus*) that deficient individuals have a thinned epidermis consisting of atrophied and necrotic epidermal cells over the basal cells. In lesions of the caudal peduncle, the skin and the underlying musculature are extensively necrotized. The liver shows degenerated and necrotic hepatocytes. Respiratory epithelium and cardiac muscle fibers are swollen. Hypercalcemia is evident. No pathological changes are detected in the eyes, brain, alimentary tract, and, contrary to other fishes, bones. The pathological changes can be reversed after several weeks of feeding of VD₃-sufficient diets.

In lampreys and other vertebrates lacking calcified structures, the existence of VDR points out primordial functions. In the sea lamprey (*Petromyzon marinus*), Whitfield et al. (2003) found that VDR transactivates a reporter gene linked to a VD-responsive element from *cyp3A4*, which encodes a P450 enzyme likely involved in general xenobiotic detoxification (Kutuzova and DeLuca 2007). Due to the wide

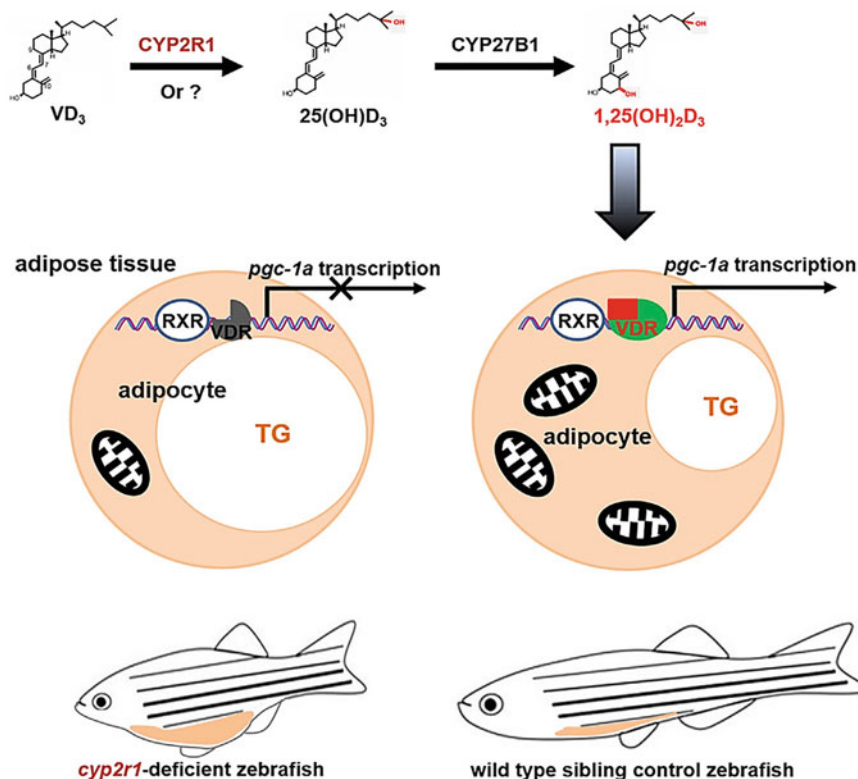


Fig. 35.4 Involvement of 1,25 (OH)₂D₃ (CTR) in lipid metabolism in zebra fish. (From Peng et al. (2017), credit Elsevier). RXR, retinoid X receptor; VDR, vitamin D receptor

distribution of VDR in animal tissues and with a focus on human issues, Whitfield et al. (2005) update the VDR function as follows:

1. Stimulation of intestinal Ca and P absorption to prevent rickets/osteomalacia.
2. Enhancement of bone remodeling via osteoblast-induced osteoclast maturation.
3. Differentiation of skin cells and maintenance of the hair cycle.
4. Repression/induction of CYP enzymes for CTR hormone synthesis/degradation as well as for the promotion of secondary bile acid detoxification as found also in aquatic mollusks (Cruzeiro et al. 2016).
5. Modulation of the immune system.
6. Potential anticancer actions via the control of epithelial cell growth, differentiation, and apoptosis.
7. Finally, VD interactions are involved in healthful aging with CYP24A1 catalyzing 1 α -hydroxylation.

35.1 Skeletal Development

The most severe phenotypic symptoms of VD_3 malnutrition are visible in skeletal developments, since a disruption of the mineralization process induces skeletal deformities. A few, but impressive, studies do exist on the role of dietary VD in the development of skeletal deformities in fishes. Haga et al. (2004) found 41.6% deformed juveniles of Japanese flounder fed a control diet containing $1.8 \text{ IU}^1 \text{ VD}_3 \text{ g}^{-1}$ diet and 52% of vertebral deformities when fed 21 IU CTR g^{-1} diet, mostly winding of the vertebrae caused by abnormal calcification and impairment of its rigidity and hypermelanosis on the blind side of the flounder. Darias et al. (2010) showed that European seabass larvae exhibit lower deformities when diets contained $28 \text{ IU VD}_3 \text{ g}^{-1}$ diet (20% deformities) while lower levels ($11 \text{ IU VD}_3 \text{ g}^{-1}$) and levels higher than $42 \text{ IU VD}_3 \text{ g}^{-1}$ induce vertebral and branchiostegal rays deformities, whereas pugheadness and deformities of the jaws and caudal fin are maximized at the low VD_3 dose (Fig. 35.5).

In contrast to the obviously rather sensitive European seabass, salmonids and cyprinids respond to dietary excess supply by VD_3 storage in various organs (Darias et al. 2011a). Horvli et al. (1998) identified deposition of VD_3 not only in liver, but also in intestine, kidney, spleen, gills, muscles, skin, and plasma of Atlantic salmon (Table 35.1). This dose-dependent accumulation of VD_3 occurs also in livers of bastard halibut and common carp (Takeuchi et al. 1991).

No evidences of skeletal deformities by dietary VD_3 excess have also been found in rainbow trout and brook trout. Inconsistent results can be due to differences in the stage of development when the experiments were performed (Darias et al. 2011a).

35.2 Requirements

35.2.1 Invertebrates

Pioneering studies on VD functions in invertebrates are available for terrestrial species, such as the amphipod *Cryptorchestia (Orchestia) cavimana* (Meyran et al. 1991), whereas in *Cryptorchestia*, CTR is involved in the Ca metabolism, its precursor $25 (\text{OH})\text{D}_3$ (Fig. 35.2) appears to be active in marine mollusks (Kriajev et al. 1994). $25 (\text{OH})\text{D}_3$ is regarded only as storage form of VD and devoid of biological activity. Li et al. (2019) confirm this assumption for the freshwater mussel (*Hyriopsis cumingii*).

For juvenile giant tiger prawns (*Penaeus monodon*), the requirement is $100 \mu\text{g kg}^{-1}$ (Shiau and Hwang 1994). Wen et al. (2015) showed that the VD_3 demand of juvenile *L. vannamei* at low salinity should be 6366 IU kg^{-1} ($\sim 160 \text{ mg kg}^{-1}$), similar to the recommended dietary supply for broodstock

¹Vitamin D: 1 IU (international unit) = 0.025 microgram (μg) cholecalciferol or ergocalciferol.

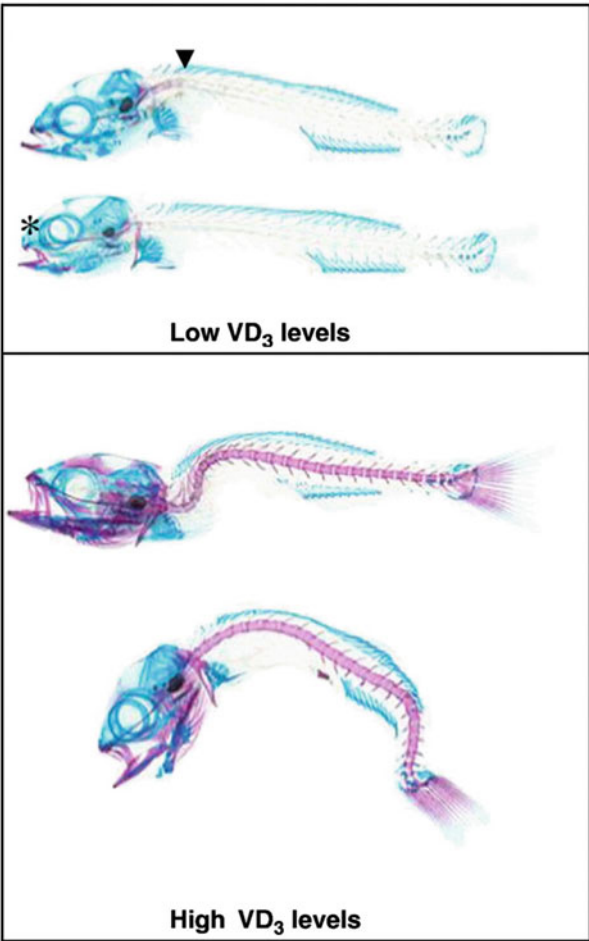


Fig. 35.5 Skeletal disorders observed at 45 days past hatch in European seabass (*Dicentrarchus labrax*) larvae induced by inadequate levels of vitamin D₃ (VD₃). Low VD₃ content, especially provoked poor mineralization, pugheadness (*), and kyphosis (arrowhead). Kyphosis and scoliosis were maximized at high VD₃ levels. (From Darias et al. (2011a), with permission from Elsevier)

Table 35.1 Vitamin D₃ concentrations (μg g^{−1}) in tissues of Atlantic salmon fed three dietary levels I–III of VD₃ for 11 weeks. (From Horvli et al. (1998), with permission from Wiley)

	Initial	I	II	III
Diet		0.04 ± 0.01	2.21 ± 0.35	28.68 ± 5.62
Plasma	0.03 ± 0.00	Nd	0.44 ± 0.03	4.04 ± 0.53
Intestines	0.09 ± 0.01	0.04 ± 0.01	0.64 ± 0.11	6.04 ± 1.09
Liver	0.08 ± 0.01	Not detectable	0.57 ± 0.20	0.35 ± 0.83
Posterior kidney	0.05 ± 0.02	0.07 ± 0.02	0.38 ± 0.21	2.68 ± 0.83
Anterior kidney	0.04 ± 0.01	Not detectable	0.28 ± 0.03	2.73 ± 0.35
Spleen	0.06 ± 0.02	0.13 ± 0.10	0.26 ± 0.05	2.21 ± 0.22
Gills	0.04 ± 0.01	0.01 ± 0.00	0.15 ± 0.03	1.62 ± 0.30
Skin	0.03 ± 0.03	0.01 ± 0.01	0.12 ± 0.05	1.58 ± 0.14

Table 35.2 Vitamin D₃ concentrations in experimental artificial penaeid shrimp broodstock diets. (From Wouters et al. (2001))

Species	Vitamin D ₃ concentrations, mg kg ⁻¹ diet
<i>Marsupenaeus japonicus</i>	19
<i>Litopenaeus stylirostris</i>	20
<i>L. vannamei</i>	125
<i>Fenneropenaeus indicus</i>	125–150
<i>F. chinensis</i>	240
<i>Penaeus monodon</i>	948

(Table 35.2) ranging from 19 to 948 mg kg⁻¹ diet. The latter figure is an extreme value. Chinese mitten crab larvae develop best with VD₃ supplementation between 4825 and 5918 IU kg⁻¹ (~120–150 mg kg⁻¹) (Liu et al. 2021).

35.2.2 Fishes

The requirement of VD spans from apparently zero, as detected in Pacific salmon (*Oncorhynchus* spp.) or yellowtail (*Seriola lalandi*), to 60 µg kg⁻¹ in rainbow trout (Molina-Poveda 2016). Wang et al. (2017) determined the dietary VD₃ requirement of juvenile Siberian sturgeon (*Acipenser baerii*) as 1400 to ~1700 IU kg⁻¹ (35 and 42 µg kg⁻¹) per diet based on weight gain (WG) and osteocalcin, respectively. Higher VD₃ supplies result in increased crude lipid and ash levels. A significantly higher dietary requirement (5430 IU kg⁻¹ = 126 µg kg⁻¹) applies to Wuchang bream (*Megalobrama amblycephala*) (Miao et al. 2015a).

The question of whether some freshwater fishes seem to not depend on dietary VD₃ supply appears to be answered: The previous studies leading to this “paradigm” lack methodological robustness and describe artifacts instead (Dabrowski and Portella 2005). The same seems to apply to studies of Pacific salmon, yellowtail, and other species (Lock et al. 2010). Small amounts of VD metabolites in the diet can be sufficient to maintain normal growth in these fishes.

To demonstrate the VD₃-mediated immune stimulation, the study by Dioguardi et al. (2017) is representative: In a clear dosis-effect relationship, VD₃ stimulates the innate immune status of European seabass, shown with its phagocytosis ability in leucocytes of the peritoneal cavity (Fig. 35.6).

Recently, Cheng et al. (2020a, 2020b) presented evidence of immune modulating effects of dietary VD₃ in yellow catfish (*Tachysurus (Pelteobagrus) fulvidraco*). VD₃ reduces the mortality of this catfish after *Edwardsiella ictaluri* challenge. Dietary VD₃ mainly downregulates the type I interferon, i.e., *ifn-β* and pro-inflammatory cytokines *tnf-α*, *il1-β*, *il-6*, and *il-8*, and upregulates the anti-inflammatory cytokine *il-10* (Fig. 35.7a) (Cheng et al. 2020b).

In the companion paper, Cheng et al. (2020a) extended this issue to the spleen by reporting that dietary VD₃ regulates innate and, most likely, also adapted immune

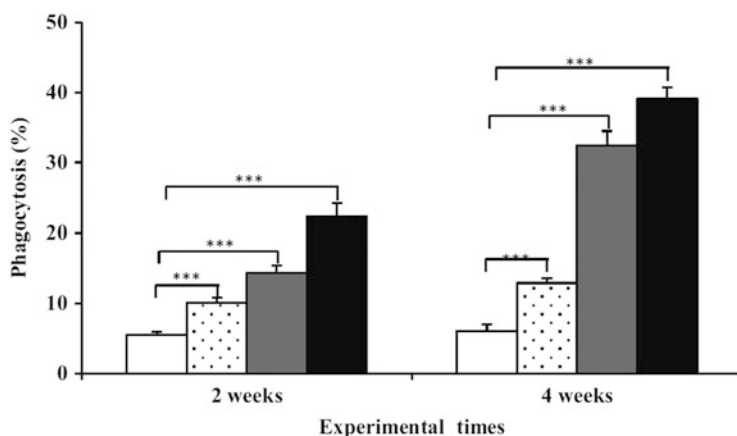


Fig. 35.6 Phagocytic ability (%) of peritoneal cavity leucocytes from European seabass specimens fed with different vitamin D₃-supplemented diets: control (white columns); 3750 (dotted columns); 18,750 (gray columns); or 37,500 U kg⁻¹ (black columns), after 2 and 4 weeks of experiment. Values are means + SEM ($n = 6$), asterisks denote significant differences ($P \leq 0.001$). (From Dioguardi et al. (2017), with permission from Springer Nature)

response after infection with *E. ictaluri* by inhibiting the classical and alternative NF- κ B activation signaling pathways (Fig. 35.7b). NF- κ B (nuclear transcription factor kappa-B) responds to stimuli such as stress, cytokines, reactive oxygen species, heavy metals, UV irradiation, oxidized low-density lipoprotein, and bacterial or viral antigens. Until recently, only a single NF- κ B signaling pathway was known, whereby NF- κ B activity is stimulated by pro-inflammatory cytokines, such as TNF- α and IL-1, as well as by pathogen-associated molecular patterns (PAMPs). However recently, a second pathway is discovered. This alternative pathway is activated by certain members of the TNF cytokine family but not by TNF- α . The recent results indicate that classical and alternative pathways to NF- κ B activation have distinct regulatory functions; one is mostly involved in innate immunity and the other in adaptive immunity (Bonizzi and Karin 2004).

The yellow catfish study get support by a recent one in juvenile black carp (*Mylopharyngodon piceus*) (Wu et al. 2020). Adequate dietary VD₃ (from 410 to 1480 IU kg⁻¹) improves innate immunity through increasing the expression levels of nonspecific defense genes (*interferon- α* , *lysozyme*, *hepcidin*, *natural resistance-associated macrophage protein*, *complement components 3* and *9*) in black carp juveniles (Fig. 35.8).

35.2.2.1 Hypervitaminosis D

Fishes respond differently to dietary VD₃ excess. Symptoms of VD₃ hypervitaminosis and intoxication are listed in Table 35.3; however, threshold values of intoxication are missing, and the applied graded dietary VD₃ steps appear to be too coarse.

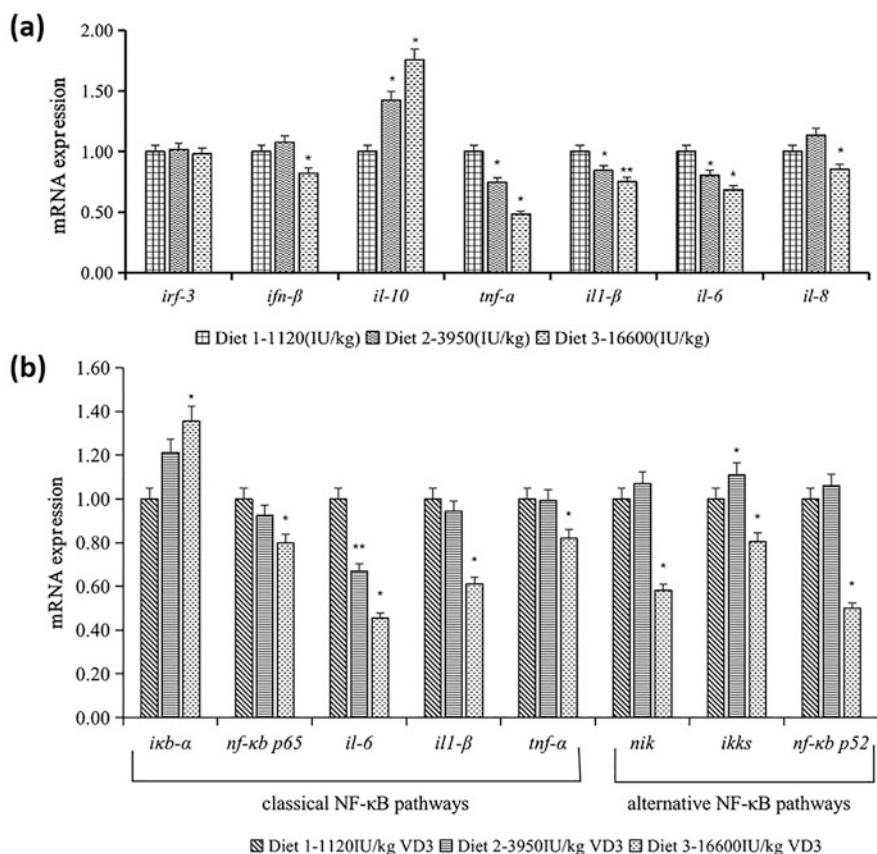


Fig. 35.7 (a) Differentially expressed key genes in the immune-related pathway in the head kidney of the yellow catfish after challenge with *Edwardsiella ictaluri*. (From Cheng et al. (2020b), with permission from Elsevier). (b) Expression of key genes in classical and alternative NF-κB activation pathways in the spleen of yellow catfish after *E. ictaluri* infection. Yellow catfish were fed for 12 weeks with diets containing 1120 IU kg⁻¹, 3950 IU kg⁻¹, and 16,600 IU kg⁻¹ of VD₃. The spleen tissues were collected for qPCR validation after 72 h of intraperitoneal injection of 3.14×10^8 CFU mL⁻¹ *E. ictaluri*. Values are means ($n = 6$), and the error bars represent 95% confidence intervals, asterisks represent significant difference (* $P < 0.05$, ** $P < 0.01$). (From Cheng et al. (2020a), with permission from Elsevier)

Sturgeons and salmonids appear to be VD tolerant. In Siberian sturgeon juveniles, no adverse effects of excess VD₃ are detected in feed conversion, specific growth rate, and survival rate (Wang et al. 2017). Also, Atlantic salmon fry reared on a diet with three different levels of VD₃ (0.2, 5, and 57 mg VD₃ kg⁻¹) show no significant differences in WG, length, specific growth rate, mortality, or kidney Ca concentration between the different dietary groups (Graff et al. 2002). No skeletal malformations or histopathological changes are recorded. No effect of excess VD₃ is detected in WG, feed efficiency, or total mortalities in rainbow trout (Hilton and

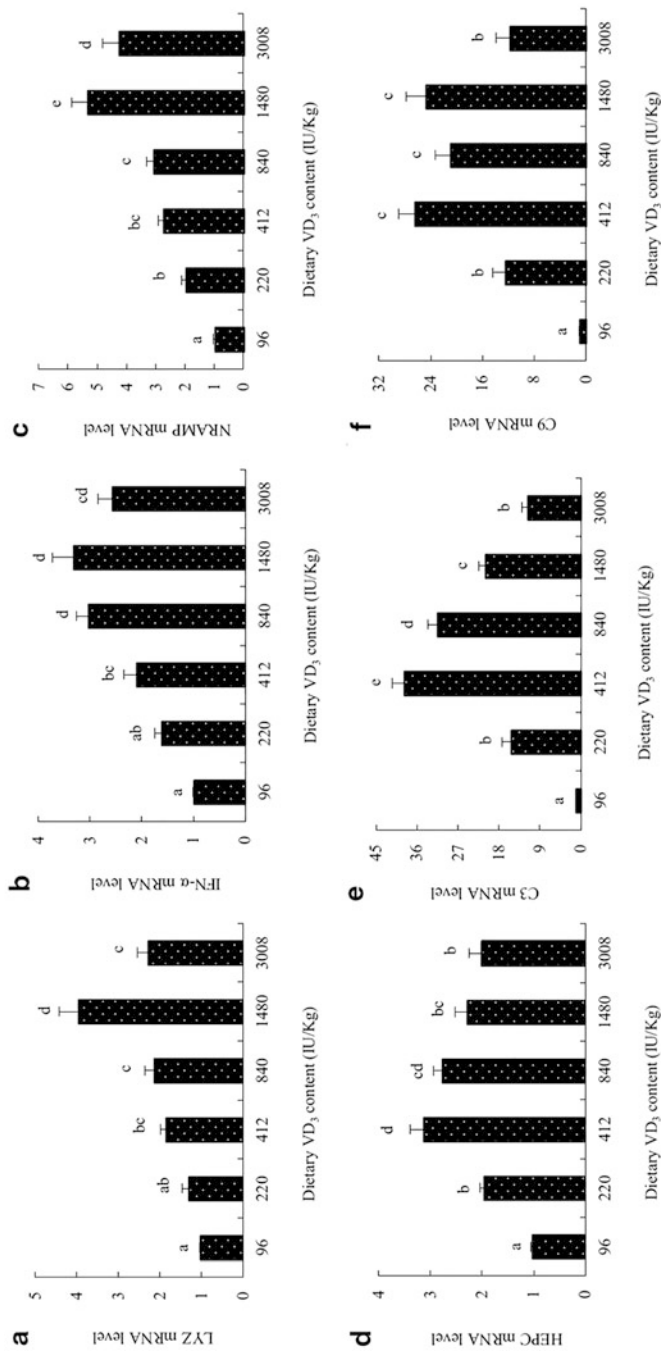


Fig. 35.8 Effects of dietary VD_3 on the mRNA expression levels of immune-related genes in the liver of juvenile black carps. Values are means \pm SEM ($n = 3$). Different superscripts indicate significant differences ($P < 0.05$). *Lysozyme*, *LYZ*; *interferon- α* , *IFN- α* ; *natural resistance-associated macrophage protein*, *NRAMP*; *hepcidin*, *HEPC*; *complement component 3*, *C3*; *complement component 9*, *C9*. For functions of the genes, refer to the glossary (From Wu et al. (2020), with permission from Springer Nature)

Table 35.3 Symptoms of hypervitaminosis D in selected fish species (from Andrews et al. (1980); Darias et al. (2011a))

Fish species	Observed effects
Trout, salmon	Elevated alkaline phosphatase activity
Brook trout (<i>Salvelinus fontinalis</i>)	Impaired growth, lethargy and dark coloration
Juvenile Japanese flounder (<i>Paralichthys olivaceus</i>)	Hypermelanosis on the blind side; vertebral deformities
Juvenile European seabass (<i>Dicentrarchus labrax</i>)	Skeletal malformations
Juvenile channel catfish (<i>Ictalurus punctatus</i>)	Depressed growth rate

Ferguson 1982). None of the fishes shows any sign of hypercalcemia; and no overt signs of renal calcinosis are detected in any of the trouts. In contrast, Vielma et al. (1998) fed young rainbow trouts diets supplemented with 50,000 or 250,000 IU kg⁻¹ VD₃, respectively, and found depressed WG.

Although the studies above were carried over a relatively short period, chronically megadoses are studied systematically only lately. In Wuchang bream, Miao et al. (2015b) detected that, although this species can tolerate high doses of dietary VD₃ (up to 200,000 IU = 5000 mg kg⁻¹), its glycolipid metabolism, immune function, antistress function, and resistance to pathogenic infections are adversely affected. In contrast to the two salmonids and Wuchang bream, European seabass (*D. labrax*) larvae show already signs of VD₃ intoxication at dietary levels slightly above 1 mg kg⁻¹ (Darias et al. 2011a).

35.3 Interaction with Nutrients

Vitamins A, D, E, and K are lipophilic; and studies of the demand of vitamins have mostly been performed based on single vitamin administration. However, interactions between vitamins do occur and deserve intensive attention (Fernández et al. 2018). For example, CTR and retinoic acid (RA), the active metabolite of vitamin A, have several tissues, such as bones, as common target, and interactions between these vitamins or their metabolites are most likely. To function in target tissues, CTR binds to VDR, and the ligand-bound receptor, either as a heterodimer with the retinoic acid X-receptor, or as a homodimer, activates the expression of various genes. Many of these genes encode proteins that alter transepithelial Ca transport (Craig et al. 2012). Therefore, an integrated approach has been carried out by Ørnsrud et al. (2009) in Atlantic salmon showing that RA injections reduce plasma CTR values, in line with a direct involvement of RA in CTR metabolism. While gene expression of a bone matrix protein and a bone-specific collagen are downregulated by RA, alkaline phosphatase mRNA expression is upregulated. Ørnsrud et al. (2009) assume that this issue influences bone strength and development.

Unexpectedly, an interference of fat-soluble VD₃ and water-soluble VC was found leading to skeletal malformations in the European seabass (Darias et al.

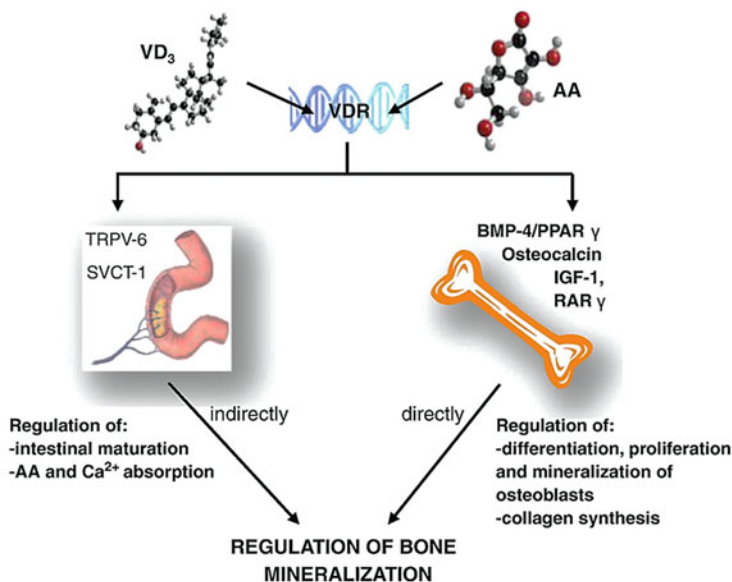


Fig. 35.9 Diagrammatic representation of the role of VD₃ and VC (ascorbic acid, AA) in the regulation of several genes involved in skeletogenesis and, particularly, in bone mineralization. (From Darias et al. (2011a) with permission from Elsevier)

(2010, 2011b)). Not only VD₃ but also VC (AA) interacts with the VDR (Fig. 35.9). VD and VC influence the larval ossification through the regulation of osteoblast determination/differentiation and mineralization processes. Both delay and acceleration of the ossification process induce 50–60% malformations. The underlying mechanism is a disruption of the mineralization process that induces the skeletal deformities by downregulating *vdr* (Darias et al. 2011b). This downregulation reduces Ca²⁺ absorption in the gut and reduces/delays mineralization and maturation of bones (Fig. 35.9). Since increasing VC contents in the diets also downregulates the transcription of one retinoic acid receptor (RAR _{γ}), even the cartilage formation is affected (Darias et al. 2011a). With respect to VD requirement, the European seabass may present an extreme example; however, it clearly points out significant interferences between vitamins even of contrasting solubility. This interaction has not been predicted so far; it can function as a lighthouse study for detailed research in other fishes and aquatic invertebrates.

35.4 Concluding Remarks

Doubtless, vitamins are vitally important nutrients, required in limited amounts by an organism. Recommendations for vitamin supplementation in diets for aquatic animals are based upon the demands in carefully performed feeding experiments

(NRC 1998, 2011; Waagbø et al. 2001). The biomolecular modes of action of the major vitamins start to be well understood. The corresponding studies indicate that multiple genes and metabolic pathways are involved in the observed effects, which, on the phenotypic level, comprise the classical life history traits: growth, fertility, or pathogen and stress resistance.

Since long, it is well documented that the requirement of lipophilic vitamins follows optimum curves: Not only deficient but also excess supply is malnutrition and hinders the development of fishes and aquatic invertebrates, whereby the underlying mechanism is more than only oxidative stress. Since the controlling function of microRNAs is acknowledged, this epigenetic pathway is slowly entering the aquatic phase. The available information shows that this translational modification of mRNA significantly contributes to malnutrition effects as shown with deficient and excess (equals toxic) dietary supply of α -tocopherol in tilapia (Chap. 36). Equivalent studies with VD are lacking. Overall, the modes of action of dietary VD excess deserve attention.

The same applies to the role of the intestinal microbiota, a déjà vu claim because it has to be repeated with each individual vitamin. In their review, Forgie et al. (2019) point out an intriguing recent finding in mammals. More mechanistic research is required to understand the impact of vitamins on immune responsiveness, especially with respect to the microbe-host gut axis under deficient and in excess conditions. Experiments in germ-free mice models confirm that the microbiota influences VD metabolism by lowering fibroblast growth factor 23 through increased activation of TNF- α in the colon. The fact that the presence of the microbial community or mono-colonization with *Citrobacter rodentium* increase serum VD levels highlights their role on host Ca homeostasis and bone formation. Research indicates that proper regulation of vitamins and minerals is central for establishing a proper immune response and intestinal barrier function. Similar to vitamin and mineral deficiencies, excessive supplementation can impair the host's ability to resist enteric infections by altering intestinal integrity or enhancing pathogen fitness.

Mice are avant-garde to aquatic animals; but the latter will follow.

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Chapter 36

Vitamin E—‘Keep Stress Away!’



Abstract Vitamin E (VE) exists in eight different forms, four tocopherols, and four tocotrienols. Tocopherols have saturated and tocotrienols unsaturated isoprenoid side chains with three double bonds. The forms of tocopherol as well as tocotrienol differ in number and position of the methyl groups on the aromatic ring. VE is a strong lipophilic antioxidant and essential for normal somatic and neurodevelopment. Consequently, embryonic VE deficiency causes lasting perturbations in lipid, antioxidant, and energy metabolism. Dietary VE controls a complex response network of stress defense, particularly oxidative stress, immune response, and molecular repair via miRNAs, pointing out that epigenetics starts to be understood in aquatic animals. VE stimulates cell proliferation in immunopoietic organs, strengthens immunity, and enhances the expression of immune-related genes. Consequently, pathogen and parasite resistance improves upon optimal VE diets. VE deficiency leads to downregulation of antimicrobial peptides and anti-inflammatory cytokines and to upregulation of pro-inflammatory cytokines. Furthermore, it adversely affects the one carbon metabolism. VE excess is intoxication with adverse effects on reproduction, growth, innate immunity, hatching, and larval survival with well-documented biomolecular modes of action. The role of gut microbiota in VE nutrition and metabolism in aquatic animals needs future attention.

Vitamin E (VE) exists in eight different forms, four tocopherols (α ... δ) and four tocotrienols (α ... δ) (Fig. 36.1a), which differ in number and position of the methyl groups on the aromatic ring.

All feature a substituted aromatic ring, with a hydroxyl group that can donate a hydrogen atom to reduce free radicals and a hydrophobic side chain, which allows for penetration into biomembranes. Tocopherols have saturated side chains, whereas tocotrienols have unsaturated isoprenoid side chains with three double bonds (Fig. 36.1a). VE is essential for vertebrate embryogenesis (Head et al. 2021).

The compound with the highest biopotency is α -tocopherol. As dietary demand, the NRC (2011) lists a span from 25 (rainbow trout) to 200 mg kg⁻¹ (grass carp) for fishes and values oscillating around 90 mg kg⁻¹ for penaeid shrimps, such as

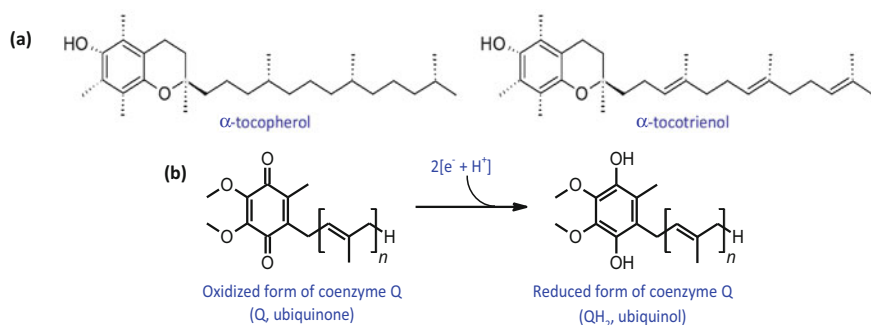


Fig. 36.1 (a) Structures of α -tocopherol and α -tocotrienol; (b) reduction of ubiquinone (Q, coenzyme Q) to ubiquinol (QH₂)

Penaeus monodon, *Fenneropenaeus chinensis*, or *Litopenaeus vannamei*¹ (He et al. 1992). The relatively low dietary requirement of these penaeids clearly contrasts that value reported for two Argentine penaeid shrimps, *Pleoticus muelleri* and *Artemesia loginaris* (Table 36.1), two inhabitants of cold waters of the South West Atlantic. This comparison points out an interesting ecophysiological aspect: Aquatic animals need high levels of polyunsaturated fatty acids (PUFAs) to maintain cell membrane fluidity, termed homeoviscous adaptation. To keep membranes intact, α -tocopherol and ascorbic acids (VC) jointly prevent or reduce lipid peroxidation (LPO) (Chap. 34). The colder the environmental temperature, the higher is the PUFA requirement of a poikilothermic organism and its subsequent dietary requirement of the lipophilic antioxidant α -tocopherol (Gimenez and Fenucci 2002).

VE deficiency causes increased oxidative stress and a secondary depletion of VC, resulting in severe damage to muscle tissue by LPO; thus clearly demonstrating the antioxidant activity of VE (Lebold et al. 2013; McDougall et al. 2017). Increased levels of oxidized ω 3 and ω 6 lipid derivatives and impaired muscle function in terms of reduced swimming behavior are the symptoms. Moreover, Motorykin et al. (2014) reported that in VE-deficient fishes, additional VC deficiency is associated with induction of stress response, astrogliosis², and shift from glycolysis to glutaminolysis³ as an alternative mechanism to satisfy cellular energy requirements.

The well-known antioxidant property allows VE to serve as lipophilic antidote in cases that the major toxic action is oxidative stress. In fact, VE supplementation reduces the toxicity induced by cylindrospermopsin in Nile tilapia and recovers

¹Classical conversion from mg into IU: 1 IU = 0.67 mg for *d*- α -tocopherol (natural); = 0.9 mg for *dl*- α -tocopherol (synthetic). New guidelines, which are not applied here, require that vitamin E content be determined based on the source of vitamin E and that it be reported as mg α -tocopherol rather than in IU. (<https://dsid.od.nih.gov/Conversions.php>, accessed September, 2020).

²Abnormal increase in the number of astrocytes due to the destruction of nearby CNS neurons.

³A series of biochemical reactions by which glutamine is lysed to glutamate, aspartate, CO₂, pyruvate, lactate, alanine, and citrate (Krebs and Bellamy 1960).

Table 36.1 Typical vitamin E effects on life history traits in selected aquatic animals

Species	Trait	Estimated VE requirement, mg kg ⁻¹	References
Invertebrates			
<i>Apostichopus japonicus</i>	Growth↑	41	Ko et al. (2009)
	WG↑	165→187	Li et al. (2020)
<i>Artemisia loginaris</i>	Growth↑	1500	Gimenez and Fenucci (2002)
<i>D. pulex</i>	Defense morphs↑	Not mentioned	Khlebovich and Degtyarev (2005)
<i>Eriocheir sinensis</i>	Immunity↑, antioxidant capacity↑	100	Wang et al. (2015)
<i>Fenneropenaeus indicus</i>	Growth↔	100–300	Ouraji et al. (2011)
<i>Litopenaeus vannamei</i>	Survival↔ Growth↑	200→890 200	Ruff et al. (2001)
<i>Macrobrachium nipponense</i>	Fecundity↑, antioxidant capacity↑, vitellogenins↑	80→160	Li et al. (2018b)
	Damage of hepatopancreas & oocytes↑	640	
<i>Marsupenaeus japonicus</i>	Fecundity↔, hatching rates↑, metamorphosis nauplii→zoea I↑	300	Nguyen et al. (2012)
<i>Moina macrocopa</i>	Reproduction↑	2.0 mg L ⁻¹	Conklin and Provasoli (1977)
<i>Pleoticus muelleri</i>	Growth↑, survival↑	1750	Fernández-Gimenez et al. (2004)
Fishes			
<i>Acipenser transmontanus</i>	Ascorbic acid synthesis↓	>20 nmol g ⁻¹	Moreau and Dabrowski (2003)
<i>Anguilla japonica</i>	AST↓, ALT↓, LYZ↑	0→400; optim. 212	Shahkar et al. (2018)
<i>Arapaima gigas</i>	Physiological status↔		de Menezes et al. (2006)
<i>Argyrosomus regius</i>	WG↑, antioxidant enzymes↑	300 + 70 VC	Ruiz et al. (2019)
<i>Channa argus</i> × <i>C. maculata</i>	Growth↑, AOC↑, innate immunity↑	10→320	Zhao et al. (2018)
<i>Ctenopharyngodon idella</i>	Growth↑	100	Li et al. (2014)
	Growth↑, disease resistance↑, immunity↑, structural integrity of immune organs↑	0→225	Pan et al. (2017)

(continued)

Table 36.1 (continued)

Species	Trait	Estimated VE requirement, mg kg ⁻¹	References
<i>Cyprinus carpio</i>	Muscle dystrophy↓	500	Watanabe et al. (1970)
	Growth↑, larvae production↑	200	Salih et al. (2020)
<i>Danio rerio</i>	ω3 PUFA content↓	1.1	Lebold et al. (2011)
	<i>ppara</i> ↑, <i>wnt10b</i> ↑, <i>β-catenin</i> ↑	50; 100	Liu et al. (2020)
<i>Dicentrarchus labrax</i>	Compensatory growth↑, protein carbonyls↑	600	Costantini et al. (2018)
<i>Huso huso</i>	WG↑, SGR↑, FER↓	0→400; optim. ~28	Amlashi et al. (2011)
<i>Labeo rohita</i>	Nitrite resistance↑	100→300	Ciji et al. (2013)
<i>Larimichthys crocea</i>	Growth↑	0–800	Yi et al. (2018)
<i>Latris lineata</i>	Growth↑, survival↔	437→1040	Brown et al. (2005)
<i>Micropterus salmoides</i>	Innate immunity↑	73→108	Li et al. (2018a)
<i>Misgurnus anguillicaudatus</i>	Growth↑, LPO↓	136	Zhang et al. (2016)
<i>Oncorhynchus mykiss</i>	WG↑, stress resistance↑	0, 80, 500	Naderi et al. (2017)
<i>Oreochromis niloticus</i>	<i>Streptococcus iniae</i> challenge↔	100→200	Lim et al. (2009)
	<i>il-1β</i> ↑, <i>tnf-α</i> ↔, PA ↔, SOD↑, LPO↓		Dawood et al. (2020)
<i>O. niloticus</i> GIFT	Growth↑, survival↑, AOC↑	0→1000	Wu et al. (2017)
<i>O. niloticus</i> x <i>O. aureus</i>	WG↑ on oxidized oil	Optim. 42	Huang and Huang (2004)
<i>Nibea albiflora</i>	<i>V. alginolyticus</i> resistance↑	15→70	Wang et al. (2019)
	<i>V. alginolyticus</i> resistance↓	290	
<i>Paralichthys olivaceus</i>	<i>Edwardsiella tarda</i> resistance↑	213	Wang et al. (2006)
<i>Perca flavescens</i>	Survival↑	160	Lee and Dabrowski (2003)
<i>Piaractus mesopotamicus</i>	Stress-related immunosuppression↓ (Fig. 36.2)	450	Belo et al. (2005)
	WG↑, SGR↑	0–600 Optim at 150	Sado et al. (2013)

(continued)

Table 36.1 (continued)

Species	Trait	Estimated VE requirement, mg kg ⁻¹	References
<i>Pseudoplatystoma reticulatum</i>	Immunity↑	3→300 Optim at 167	Zanon et al. (2018)
<i>Pterophyllum scalare</i>	Growth↑, survival↑, stress resistance↑	Enriched <i>Artemia</i> larvae	Norouzitallab et al. (2009)
<i>Rachycentron canadum</i>	Growth↑, <i>aco</i> ↑, <i>ppara</i> ↑, PUFAs↑	0→67	Xu et al. (2020)
<i>Salmo salar</i>	Pathogen (<i>Piscirickettsia salmonis</i>) resistance↑	0.015, 0.65 + ARA	Dantagnan et al. (2017)
<i>Salmo caspius</i>	Alternative complement pathway↑	1000	Sotoudeh et al. (2016)
<i>Scophthalmus maximus</i>	Sperm quality↑	721.6	Xu et al. (2015)
	Follicle-stimulating hormone <i>fsh</i> -β↑, luteinizing hormone <i>lh</i> -β↑	In vitro pituitary cells	Huang et al. (2019)
<i>Sillago sihama</i>	WG↑, immunity↑	100	Huang et al. (2020)
<i>Sparus aurata</i>	Non-specific immunity↑ (Fig. 36.5)	600→1800 acetate Optim. @ 1200	Ortuño et al. (2000)
	Hemolytic complement activity↑	26	Ortuño et al. (2001)
<i>Stizostedion vitreum</i>	Survival↔	17	Kolkovski et al. (2000)
<i>Tachysurus (Pelteobagrus) fulvidraco</i>	Growth↑, pathogen resistance↑	33, 46	Lu et al. (2016)
<i>Takifugu obscurus</i>	Growth↑, immunity↑, ammonia stress resistance↑	0→300	Cheng et al. (2018)

↑, support/upregulation; ↔, indifferent or ineffective; ↓, retardation/reduction; AOC, antioxidant capacity; LPO, lipid peroxidation; SOD, superoxide dismutase activity; PA, phagocytosis activity; FER, feed efficient ratio; SGR, specific growth rate; WG, weight gain; ARA,arachidonic acid; PUFA, polyunsaturated fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LYZ, lysozyme activity; *aco aconitase*, catalyzes interconversion of citrate and isocitrate in the tricarboxylic acid (TCA) cycle; *ppara peroxisome proliferator-activated receptor α*, ligand-activated transcription factor; key regulator of lipid metabolism; *wnt10b* encodes secreted signaling proteins, which have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis; *β-catenin*, a dual function protein, involved in regulation and coordination of cell-cell adhesion and gene transcription; VC, vitamin C

biotransformation and antioxidant enzymes as well as the glutathione content to basal levels (Guzmán-Guillén et al. 2015). The antidote function applies even to strong natural xenobiotics, such as aflatoxin B₁ (Abdel-Hamid and Firgany 2015). Further, mainly beneficial, effects of dietary VE on life history traits are collected in Table 36.1.

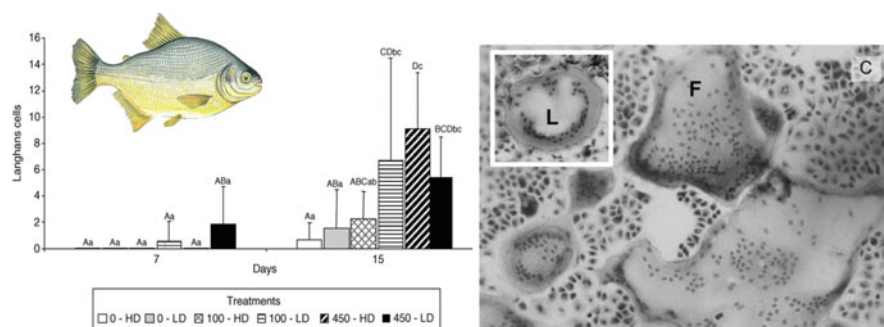


Fig. 36.2 Number of Langhans giant cells counted on the coverslips in pacu (*Piaractus mesopotamicus*) fed with 0, 100, or 450 mg of vitamin E and subjected to high or low stocking densities (HD and LD, respectively). Data are presented as means + SD. Statistical differences ($P < 0.05$) between treatments are indicated by capital letters for comparison of data from different experimental days and lower case letters for comparison within each day of analysis. *Micrograph* 15 days after implantation, foreign body (F), and Langhans (L) giant cells (inset) have developed ($\times 200$). (From Belo et al. (2005) with permission from Elsevier; image credit ambientebrasil). Langhans giant cells are formed by the fusion of epithelioid cells (macrophages) and contain nuclei, arranged in a horseshoe-shaped pattern in the cell periphery

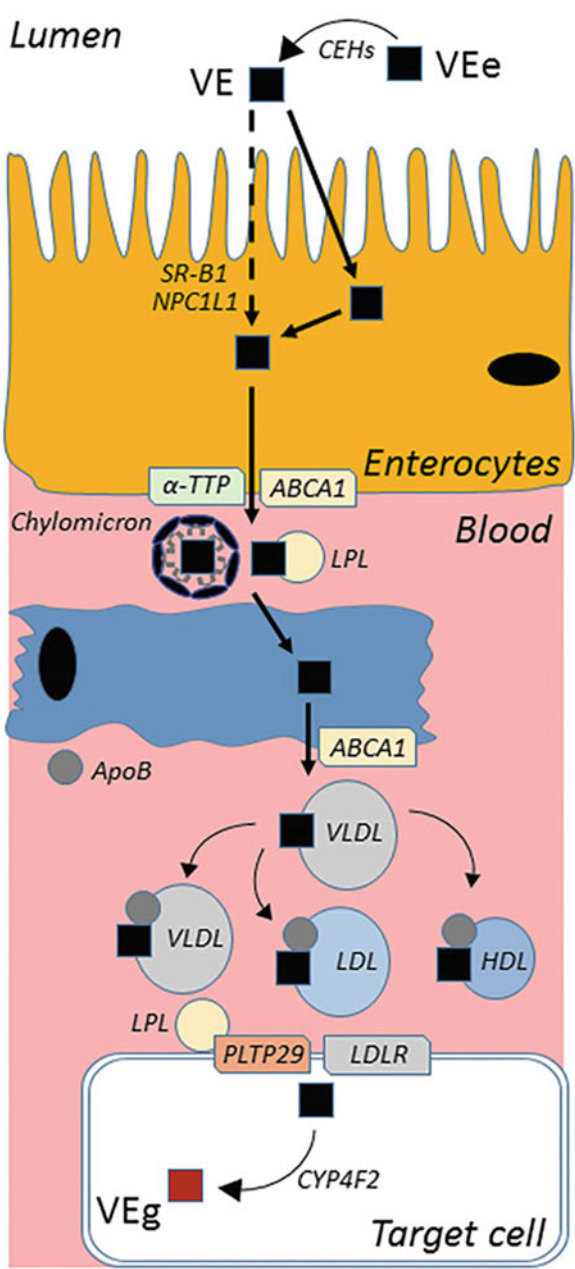
To understand the molecular mechanism of VE on gonad development in *Macrobrachium nipponense*, Li et al. (2018b) measured the transcription of two vitellogenin genes, *vg* and *vgr*, in ovaries. Both genes have the highest transcription in females on 160 mg VE kg⁻¹ diet. Deficient, as well as excess dietary VE contents reduce the transcription of the vitellogenin genes. Noteworthy, excess VE has adverse effects: individuals on 640 mg VE kg⁻¹ possess visible impairment of hepatopancreas tubules confirming the toxic character of dietary excess VE (Li et al. 2018b).

Histologically, Belo et al. (2005) demonstrated that dietary VE improves the immune response also of pacu (*Piaractus mesopotamicus*) by increasing macrophage recruitment and giant cell formation in the foreign body granulomatous reaction. This can be seen with Langhans giant cells in the skin (Fig. 36.2).

There is only limited knowledge about the specific absorption and transport of VE in fishes (Hamre 2011); these processes, however, should be comparable to those in mammals (Fig. 36.3). The primary site of VE absorption is the small intestine, where esterified forms of the VE are hydrolyzed and absorbed as free alcohols. Regardless of the VE form, higher VE intake generally leads to higher absorption but with lower efficiencies. Like other hydrophobic substances, VE appears to be absorbed by (i) passive diffusion dependent on the formation of micelles in the intestinal lumen and (ii) receptor-mediated transport (Fernández et al. 2018).

The proper absorption of VE requires the presence of fat in the lumen of the gut and the secretion of pancreatic esterases for the release of free fatty acids (FAs) from dietary triglycerides, bile acids for the formation of mixed micelles, and esterases for the hydrolytic cleavage of tocopheryl esters (Fernández et al. 2018).

Fig. 36.3 Overview of the metabolism of vitamin E (VE) and the tocopherol signaling pathway (TSP) from absorption to target cells. *ABCA1*, ATP-binding cassette A1; *ApoB*, apolipoprotein B (proteins that bind lipids (oil-soluble substances such as fat and cholesterol) to form lipoproteins. They transport lipids (and fat soluble vitamins) in blood, cerebrospinal fluid, and lymph (Nelson and Cox 2005)); α -*TTP*, α -tocopherol transfer protein; *CEHs*, carboxyl ester hydrolases; *CYP4F2*, cytochrome P450 family 4 subfamily F member 2; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein; *LAP*, low-density lipoprotein receptor; *LPL*, lipoprotein lipase; *NPC1/11*, Niemann-Pick C1-like 1 (*NPC1/11* is a protein found on the gastrointestinal tract epithelial cells as well as in hepatocytes (Temel et al. 2007)); *PLTP29*, phospholipid transfer protein 29; *SR-B1*, scavenger receptor class B member 1; *VE*, vitamin E; *VEe*, vitamin E esters; *VEg*, vitamin E glucuronides; *VLDL*, very low-density lipoprotein. (From Fernández et al. (2018), with permission from Springer Nature)



36.1 Epigenetics

First studies prove epigenetic pathways in VE action. The involvement of miRNAs in the action of VE has been documented in fish (Tang et al. 2013). The authors studied eight hepatic miRNAs in Nile tilapia on diets differing in VE content. All miRNAs are upregulated after excessive supplementation of VE (Fig. 36.4). Furthermore, all miRNAs have more than one target gene, ranging from 10 (*miR-146a*) to 302 (*miR-21*). One of the responding miRNAs, *miR-122*, is involved in various metabolic processes, such as cholesterol and FA biosynthesis, oxidative stress, in general, and inhibition of FA oxidation, in particular. Excessive dietary VE upregulates hepatic *miR-122*, whereas VE deficiency downregulates it. GO annotation of the target genes shows that high percentages of targets attend macromolecular complex and membrane-enclosed lumen (Fig. 36.4, Cellular component), with functions as regulators, such as enzyme regulator, transcription regulator, translation regulator, and other functions, including electron carrier and molecular transducer (Fig. 36.4, molecular function). The upregulated biological processes include “metabolic processes,” “response to stimulus,” and “immune system processes” (Fig. 36.4, Biological process). It becomes obvious that dietary VE controls a complex response network of stress defense, particularly oxidative stress, immune response, and molecular repair via miRNAs.

In a subsequent study in common carp and channel catfish, Tang et al. (2020) tested the hypothesis that *miR-155* (Fig. 36.4) is a regulator of immunity, antibacterial, and antiviral effects as found in mammals. In fact, *miR-155* regulates the expressions of cytokines, including *tnf-α*, *il-1β*, *ifn-γ*, *il-6*, and *il-10*⁴. Therefore, the effect of VE on immunity deserves a closer look.

36.2 Immunity

Vitamin E supplementation stimulates cell proliferation in immunopoietic organs, increases the number of antibody-producing plasma cells, and stimulates T helper lymphocytes (Blazer 1992). Consequently, the resistance against various kinds of infections improves upon optimal VE diets. Hardie et al. (1990) tested the immune response in Atlantic salmon fed different VE diets and challenged with *A. salmonicida*. Fishes on VE-depleted diets show increased mortality due to compromised complement activity. In the same line of evidence, decreased mortality occurs in rainbow trouts infected by the protozoan parasite *Ichthyophthirius multifiliis* and fed sufficient VE (Wahli et al. 1998). The same laboratory shows that also leucocyte phagocytosis is modulated by dietary VE (Fig. 36.5) (Clerton

⁴*tnf-α* and *il-1β*, pro-inflammatory cytokines; *ifn-γ*, critical for innate and adaptive immunity against viral, some bacterial, and protozoal infections; *il-6*, acts as both (mostly) pro-inflammatory cytokine and anti-inflammatory myokine; *il-10*, anti-inflammatory cytokine.

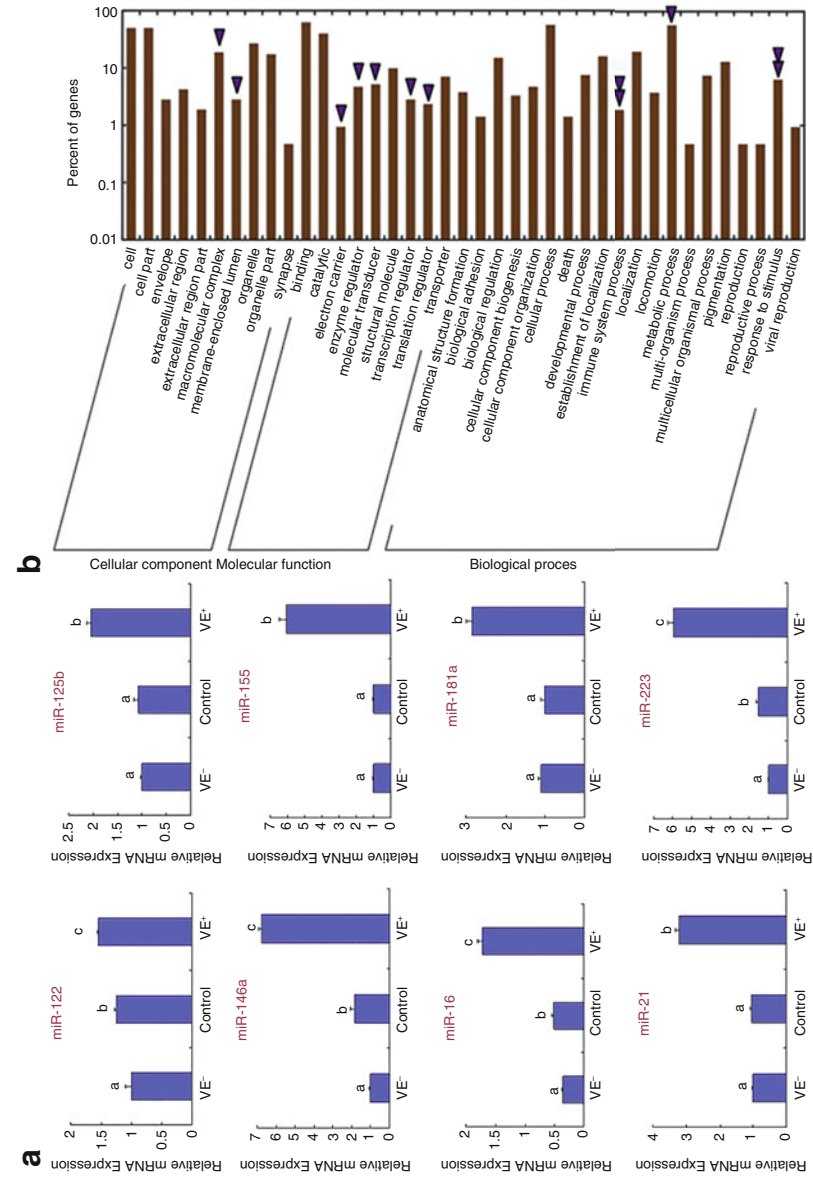


Fig. 36.4 (a) Effects of VE-deficient or VE-excessive diets on expressions of miRNAs in the liver of Nile tilapia. Different superscripts indicate significant differences ($P < 0.05$). (b) Analysis of the corresponding targets of the eight VE-responsive miRNAs. The cellular locations, molecular functions, and biological processes are grouped together to provide a global review of the targets. Significant items of the targets related to antioxidative defense via VE and miRNAs bear triangular symbols. (From Tang et al. (2013), with permission from Elsevier)

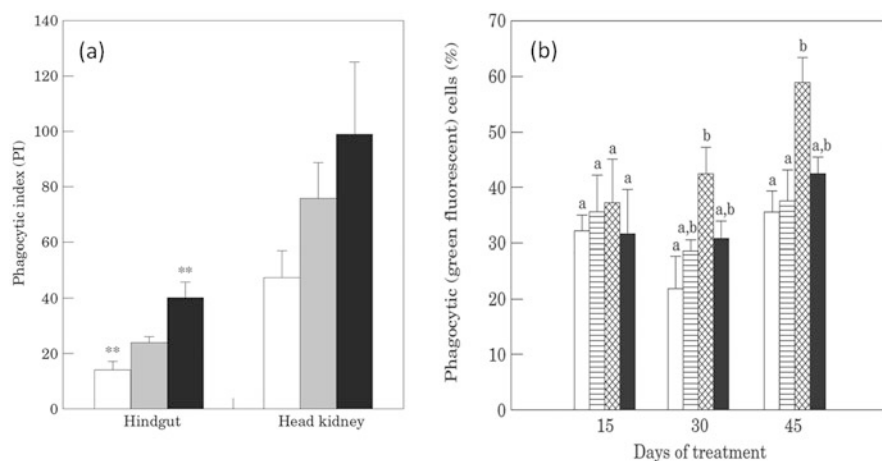


Fig. 36.5 (a) Effects of vitamin E on intestinal and head kidney cell phagocytosis activity in rainbow trout. Three groups of 25 fishes were fed three diets with graded vitamin E: white 0, gray 28, black 295 mg kg⁻¹, from day 0 to 80. After this period, the phagocytic activity was assessed from intestinal cells (hindgut) and from macrophage-enriched head kidney cells. Each histogram represents the average of four experiments (\pm SEM). **Significant difference as compared to the E-28 group at $P < 0.05$. (From Clerton et al. (2001), with permission from Elsevier). (b) Phagocytosis of FITC-labeled *Vibrio anguillarum* by leucocytes of gilthead seabream head kidney. Vitamin E (mg kg⁻¹) supplemental doses: □ 0, control; ▨ 600; ▩ 1200; ■ 1800. Data are means \pm SE. Different letters indicate statistical differences. (From Ortuño et al. (2000), with permission from Elsevier). FITC, fluorescein isothiocyanate

et al. 2001). Conversely, phagocytosis is reduced in individuals on zero VE diet, clearly proving the immunostimulatory effect of VE.

Much higher dietary doses were applied in a gilthead seabream study (Ortuño et al. 2000): Fishes on 600 mg VE kg⁻¹ show no enhancement in any of their immune parameters. On 1200 mg VE kg⁻¹ diet, however, serum hemolytic and phagocytic activity of head kidney leucocytes are enhanced (Fig. 36.5b). The highest dietary VE dose does not elicit any immunostimulation—obviously a hypervitaminosis effect.

An increasing number of studies identify potential biomolecular pathways of VE action. Niu et al. (2014) reported that moderate doses of dietary VE (480 mg kg⁻¹) enhance nonspecific immunity and expression of immune-related genes in sub-adult turbot (*c3*, *il-1 β* , and *tnf- α* , Fig. 36.6). This finding appears to be representative of fishes in general.

Consistent with the immune comprising effects of diets deficient in other vitamins (Chaps. 32, 33, 34), Pan et al. (2017) reported that VE deficiency depresses fish growth, disease resistance, and immunity as well as structural integrity of immune organs in juvenile grass carps (details below).

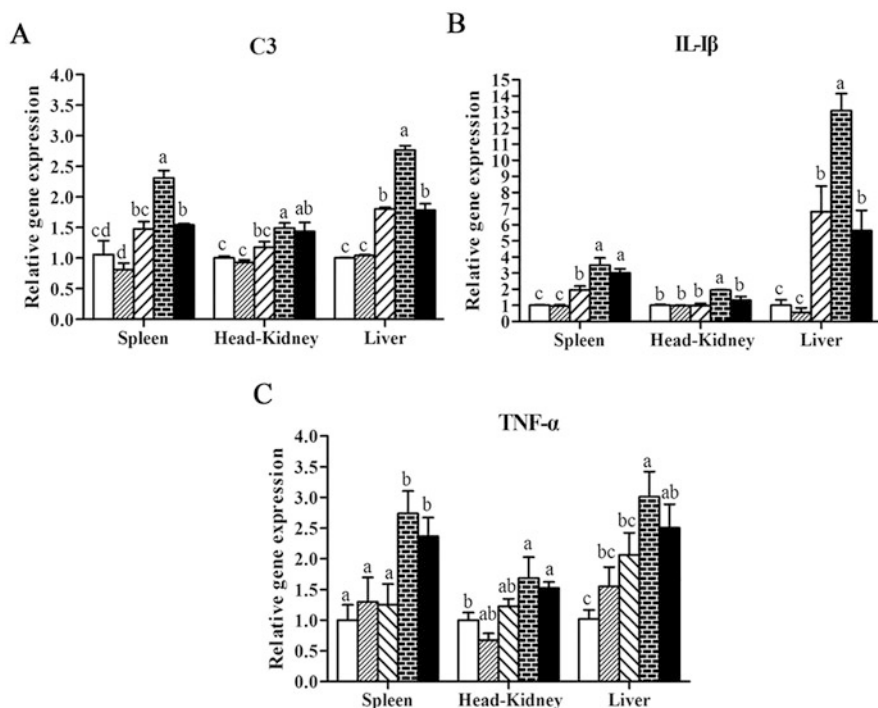


Fig. 36.6 Relative mRNA expression of *complement component 3 (c3)* (a), pro-inflammatory *il1β* (b), and pro-inflammatory *tnf-α* (c) in the spleen, head kidney, and liver of subadult turbot fed with graded levels of vitamin E. Data are presented as means \pm SEM. Different superscripts indicate statistically differences ($P < 0.05$). (From Niu et al. (2014), with permission from Elsevier). Columns from left to right: control, 120, 240, 480, 960 mg VE kg⁻¹

36.3 Interactions

The interactions of the two major antioxidative vitamins, VC and VE, have already been set out in Chap. 34. Therefore, the following section focuses on the interaction of VE with further lipophilic nutrients. As mentioned above, the VE demands depend on concomitant dietary lipids with an increase with rising lipid content. For instance, the optimum dietary VE requirements of juvenile grouper (*Epinephelus malabaricus*) are 61–68 and 104–115 mg kg⁻¹ diet in 4% and 9% lipid diets, respectively (Lin and Shiau 2005).

Several studies are dedicated to the remediation of oxidative stress caused by high-fat diets. One illustrative example: dietary VE in turbot broodstock acts via attenuating LPO, renewing antioxidant enzyme activities and nonspecific immune responses, and modulating the expression of stress proteins (*hsp70*, *hsp90*) and immune-related genes (*c3*, *tnf-α*, *il-1β*, *tlr22*) (Jia et al. 2017). The gene modulation

clearly indicates that the ameliorating VE effect is based not only on its antioxidative property but also on interaction with immune-related pathways.

36.4 Hypo- and Hypervitaminosis

36.4.1 Hypovitaminosis

In marine and freshwater fishes, hypovitaminosis E is well documented on the phenotypic as well as the biochemical and biomolecular levels. As deficiency signs are listed, among others, for:

- Barramundi: muscular atrophy, susceptibility to disease (Boonyaratpalin and Williams 2002)
- Channel catfish: muscular dystrophy, exudative diathesis, skin depigmentation, erythrocyte hemolysis, splenic and pancreatic hemosiderosis, fatty liver, ceroid deposition (Robinson and Li 2002)
- Yellowtail: dark coloration, ataxia, hemorrhage in fins and opercula (Masumoto 2002)
- Common carp: muscular dystrophy, exophthalmia, lordosis, kidney degeneration, pancreatic degeneration (Takeuchi et al. 2002)
- Eels: hemorrhage in fins and skin and dermatitis (Satoh 2002)

Traber and coworkers extensively describe hypovitaminosis E in zebra fish as a general vertebrate model. The consequences of deficient VE are serious, lasting, and more complex than anticipated. VE deficiency decreases long-chain $\omega 6$ and $\omega 3$ PUFAs by 20% (Lebold et al. 2011). In VE-deficient (E[−]) individuals, the long-chain-PUFA status is impaired, as assessed by a ~60% lower docosahexaenoic acid (DHA): α -linolenic acid (ALA) ratio and a ~50% lower arachidonic acid (ARA)/linoleic acid (LIN) ratio. *Fads2* and *elovl2* transcription is doubled in E[−] compared to E⁺ fishes. Thus, inadequate VE status leads to depletion of PUFA that may be a result of either or both increased LPO (Choi et al. 2015) and an impaired ability to synthesize sufficient PUFAs, such as LIN, ARA, and especially the $\omega 3$ LC-PUFAs, eicosapentaenoic acid and DHA.

E[−] zebra fishes produce embryos with increased morphologic abnormalities and elevated mortality (Miller et al. 2012) (\rightarrow AAN I 'Transgenerational Effects' (Steinberg 2018)). One major abnormality is an uninflated swim bladder, combined with changes to the phospholipid (PL) and lysophospholipid (lyso-PL) profile of developing embryos (McDougall et al. 2016). Lyso-PLs are significant substrates for the synthesis, remodeling, and repair of membrane PLs. VE deficiency depletes the lyso-PL status by approximately 60% (Choi et al. 2015). Furthermore, a selective decrease in DHA-containing PLs and lyso-PLs during the development occurs (McDougall et al. 2016). This paper proves that critical lipids are protected by α -tocopherol and points out that their depletion are involved in the mechanism (s) leading to observed swimming defects and increased mortality.

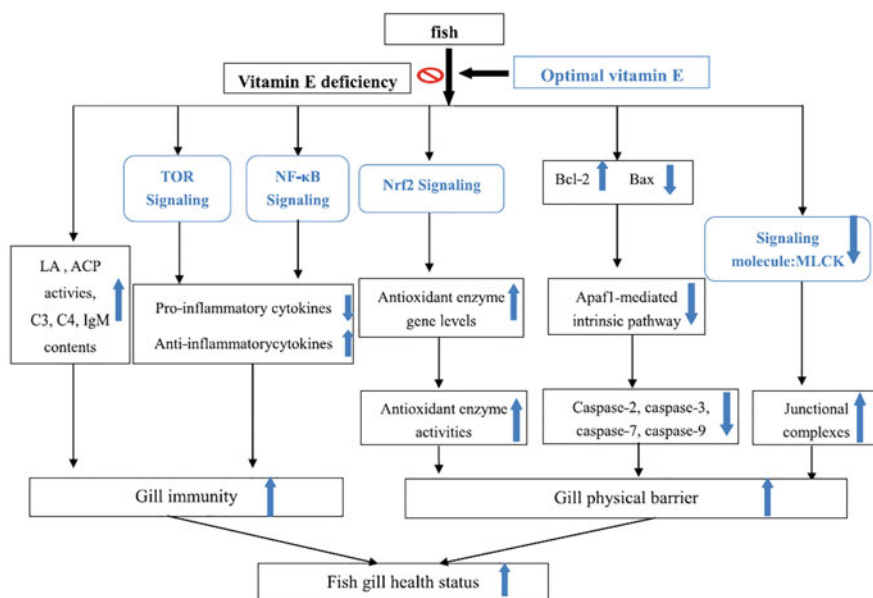


Fig. 36.7 The potential action pathways of vitamin E-regulated gills health of young grass carps. (From Pan et al. (2018), with permission from Elsevier)

VE deficiency affects even neuronal development and immune response. It causes severe developmental impairment at early embryonic stages (Head et al. 2020), since VE is required for development of the brain and peripheral nervous system in zebrafish embryos. A companion paper points out major metabolic dysfunctions occurring as early as 12 hpf. Simultaneously, E- embryos experience LPO disruption to membrane structure and signaling pathways (Head et al. 2021). Furthermore, due to increased phospholipid and choline utilization, which causes methyl donor depletion and energy metabolism derangements, E- embryos experience deficiencies in methyl donors, such as choline, betaine, and SAM (Zhang et al. 2021).

For young grass carps, Pan et al. (2018) show that VE deficiency (below 130–140 mg kg⁻¹) depresses gill immune response and physical barrier referring to NF-κB, TOR, Nrf2, and MLCK signaling in fishes under infection of *Flavobacterium columnare* (Fig. 36.7). Dietary VE deficiency:

1. Increases gill rot morbidity and aggravates gill pathological response in young grass carp after pathogen exposure
2. Impairs gill immune barrier function by decreasing antibacterial compounds, inducing gill inflammation *via* upregulating pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-15, IL-17D, IFN-γ2, TNF-α) and downregulating anti-inflammatory cytokines (IL-4/13A, IL-4/13B, IL-10, TGF-β1)
3. Disturbs the integrity of fish gill epithelial cells by oxidative stress: reduction of GSH content and CuZnSOD, MnSOD, GPx, GST, and GR activities. The decline in antioxidant enzyme activities is related to reduced transcriptions

4. Induces caspase-dependent apoptosis
5. Disrupts gill tight junction barriers by downregulating *claudin-3*, *-b*, *-c*, *-11a*, *occludin*, *zo-1*, and *zo-2* mRNA, related to the increased pro-inflammatory cytokines

36.4.2 Hypervitaminosis

Even more alarming than hypovitaminosis E is hypervitaminosis E, since it is a kind of intoxication. On the phenotypic level, Erdogan and Arslan (2019) reported that optimal dietary VE supplementation (220 mg kg^{-1}) does not only promote optimal reproduction (duplication of egg numbers, improved fertilization and hatching rates) but also supports best possible growth in actively spawning adult pindani (*Chindongo (Pseudotropheus) socolofi*). However, increasing the VE dose only up to 270 mg kg^{-1} reduces female fecundity. The numbers of eggs drop from 40 to 28 per female.

These findings are supported by reported adverse effects of dietary VE excess on reproductive, growth, innate immunity, hatching, and larval survival in rainbow trout (Puangkaew et al. 2004), goldfish (James et al. 2008), Pabda catfish (*Ompok pabda*, Fig. 36.8) (Mollah and Sarowar 2009), grass carp (Li et al. 2014), or Nile tilapia (Nascimento et al. 2014).

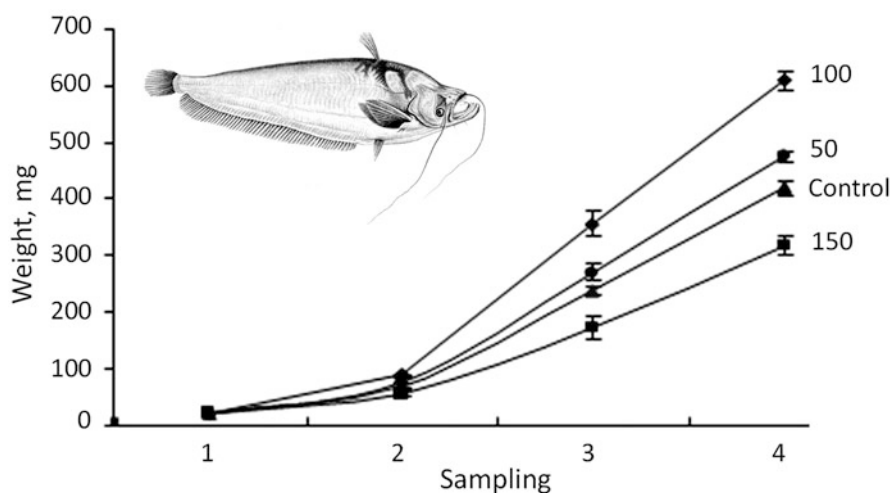


Fig. 36.8 Weights of *Ompok pabda* larvae produced from brood fish reared on different dietary vitamin E levels (in mg kg^{-1} diet). Sampling was done at 7 days interval (Vertical bars \pm SD) (from Mollah and Sarowar (2009), credit Bangladesh Academy of Sciences; image credit Cuvier and Valenciennes (1769–1865))

Certain VE metabolites, or VE itself, possess prooxidant properties (Pearson et al. 2006). In fact, excess VE proves prooxidative in vivo in *Acipenser fulvescens* (Moreau et al. 1999). Similarly, Ji et al. (2003) discovered a tendency of prooxidation in black seabream (*Acanthopagrus schlegelii*) on high VE diets. Indications of prooxidation are also found in rainbow trout (Puangkaew et al. 2004), olive flounder (Gao et al. 2014), and pond loach (*Misgurnus anguillicaudatus*) (Zhang et al. 2016). In subadult turbot, the reduced immunity after dietary uptake of excess VE is well reflected on the biomolecular level: Individuals show reduced expression of at least one of three major immunity genes (Fig. 36.6) (Niu et al. 2014). Likewise, very high dietary VE doses (1.0 g VE kg⁻¹ diet) induce apoptosis, oxidative stress, and inflammation in olive flounder, as *tnf-α*, *il-6*, *lysozyme*, and *cytochrome oxidase subunit III* transcription increases (Kim et al. 2017).

36.5 Coenzyme Q

Coenzyme Q (Fig. 36.1b), also known as ubiquinone (because it is a *ubiquitous quinone* in biological systems), is a coenzyme family that comprises fat-soluble, vitamin-like substances. It is a quinone derivative with a long tail consisting of five-carbon isoprene units that account for its hydrophobic nature. The number of isoprene units in the tail depends on the species. The most common form contains 10 isoprene units (coenzyme Q₁₀). As components of the mitochondrial respiratory chain, ubiquinones act as antioxidants, suppressing the formation of ROS by passing electrons to the ultimate acceptor, molecular oxygen (Berg et al. 2015). Coenzyme Q₁₀-concentration in the red microalga *Porphyridium purpureum* can amount up to ~2.0 mg L⁻¹ (Klein et al. 2012). In red macroalgae, even higher Q₁₀ concentrations can be found: *Porphyra* sp. contain 3.9 mg kg⁻¹ (Paiva et al. 2014) or *Pterocladia capillacea* 8.3 mg kg⁻¹ (Paiva et al. 2016), respectively.

Q₁₀ has been tested in aquafeeds. El Basuini et al. (2020) fed graded levels of Q₁₀ to Nile tilapia and found that dietary inclusion of Q₁₀ at levels of ≥20 mg kg⁻¹ diet improves growth and digestion, health (increased antibacterial peptide content and lysozyme activity), and compensates for harmful stress (reduced cortisol level). Also resistance against *Streptococcus agalactiae* increases (El Basuini et al. 2021).

The alteration in growth, feed efficiency, and digestive enzymes may be due to the indirect effect of Q₁₀ on resynthesis of VE. Moreover, the role of Q₁₀ in the electron transport chain makes it a primary factor in carbohydrates, protein, and lipid metabolism. Also, the impacts of Q₁₀ dietary incorporation on hormone activities (insulin, glucagon, and cortisone) support the decisive role in enhancing the performance of fishes (El Basuini et al. (2021) and references therein).

36.6 Concluding Remarks

The epigenetics in VE nutrition of aquatic animals shows that dietary VE controls a complex response network of stress defense via miRNAs and IC metabolism. Furthermore, a single miRNA targets more than one single gene. The stimulus of VE-deficient or VE-excessive diets results in changes of related miRNAs and their subsequent translational regulation of genes in different metabolic pathways. Many biomolecular details remain unclear so far. Currently, only one laboratory shows how complex the biomolecular fine-tuning of gene transcription and translation is (Tang et al. 2013, 2020). In VE action, more genes and complex miRNA networks are involved than previously anticipated.

Three more aspects deserve mention: VE excess/toxicity, VE deficiencies, and the role of gut microbiota.

On the phenotypic levels, adverse effects of dietary VE excess are often and well documented; or, vice versa, beneficial effects of dietary VE administration follow optimum curves (Sect. 36.4.2, Table 36.1, Figs. 36.2, 36.6, and 36.8). Studies on underlying biomolecular pathways focus on immune compromising actions and oxidative stress. These findings can serve as useful platform to start comprehensive mechanistic studies. From a brief look into medical literature (Hale et al. 1995; Miller III et al. 2005; Swift et al. 2014), it can easily be hypothesized that dietary VE excess causes a variety of severe side effects that wait to be discovered also in aquatic animals. First comprehensive studies in zebrafish elucidate that VE deficiency disrupts gene expression networks during fish development (Head et al. 2021).

In a recent book chapter, Traber (2019) summarizes existing knowledge gaps of VE deficiencies: The discoveries from VE-deficient zebrafishes show that oxidative and metabolic damage, along with behavioral and morphological abnormalities, is caused by inadequate VE status. Importantly, VE inadequacy drives secondary deficiencies that cause developmental defects, especially neural tube defects. Specifically, the relationship of VE, oxidative damage, and metabolic control systems involved in neurogenesis deserve attention.

As claimed already in previous chapters, the role of gut microbiota in vitamin production and metabolism in farmed aquatic animals has not yet been addressed adequately. Nonetheless, mammalian studies exemplify that α -tocopherol consumption influences gut microbiota composition by increasing the share of *Bacteroidetes* in disfavor of *Firmicutes* (Choi et al. 2020). Although the meaning of the *Firmicutes*/*Bacteroidetes* ratio is controversially discussed (Mariat et al. 2009) (although an increase in *Bacteroidetes* is often interpreted as beneficial), this fact cannot serve as excuse to ignore the role of gut microbiota in VE nutrition of aquatic animals. Furthermore, the gut microbiota influences synthesis, metabolism, and transport of fat-soluble vitamins including their bioactive metabolites that are either introduced with the diet or released into the gut via circulation. A better understanding of these interactions and their impact on intestinal and metabolic homeostasis (Stacchiotti et al. 2020) will be pivotal to design new and more efficient aquafeeds.

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Chapter 37

Vitamin K—‘Keep the Hematologist Away!’



Abstract Vitamin K (VK) comprises a group of fat-soluble vitamins known for their role in assisting proper blood coagulation. The major sources of VK are diets and probably intestinal microbiota; the latter contribution remains controversial. Crustaceans appear to have a higher VK requirement than fishes; VK is required for larval development. VK improves fish gonad maturation and sperm quality via circulating small noncoding RNAs. The VK target genes, central in embryonic development, are beginning to be understood. Adverse VK deficiency-mediated effects can be passed to succeeding generations. Effect studies of dietary VK excess are lacking, as do comprehensive studies in invertebrates as well as on the contribution of the intestinal microflora in VK provision and metabolism.

Vitamin K (VK)¹ comprises a group of structurally similar, fat-soluble vitamins best known for their role in assisting proper blood coagulation. Furthermore, VK is associated with Ca deposition, and it is important in the health of bone in vertebrates and calcified structures in invertebrates (Fernández et al. 2018). VK deficiency can lead to excessive bleeding. Moreover, VK is a ligand for the pregnane X receptor (PXR)²; and vice versa, the PXR takes part in VK metabolism, since the transcription of its gene (*pxr*) responds to dietary VK supply (Marques et al. 2017).

All K vitamins share a quinone ring but differ in the length and degree of saturation of the carbon tail and the number of side chains. **Phylloquinone**, vitamin K₁ (VK₁, Fig. 37.1), is a polycyclic aromatic ketone. It is found naturally in a wide variety of green plants, particularly leaves, since it functions as an electron acceptor in the photosynthetic chain.

In **menaquinones** (VK₂), the numbers of side chains are indicated in the name, e.g. MK-4 means that four isoprene units comprise the side chain, and MK-7

¹The “K” derives from its German name, “Koagulationsvitamin” (coagulation vitamin).

²Pregnane X receptor is a nuclear receptor sensing the presence of xenobiotic substances and in response upregulating the expression of proteins involved in the detoxification and clearance of these substances (Kliewer et al. 2002).

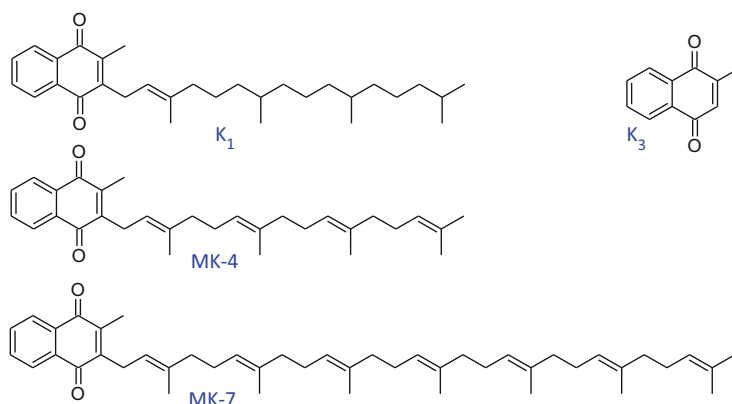


Fig. 37.1 Structure of phyloquinone (vitamin K₁); menaquinone-4 (MK-4, vitamin K₂); menaquinone-7 (MK-7); menadione (vitamin K₃)

possesses seven isoprene units (Fig. 37.1). Menaquinones include products of bacterial biosynthesis.

Menadione (Fig. 37.1, VK₃). Water-soluble salts of the synthetic menadione are used in animal diets, including aquafeed (Fernández-Gimenez et al. 2016).

The dietary VK requirements strongly depend on the chemical form and animal species considered, ranging from 0.1 mg in channel catfish to <10–20 mg kg⁻¹ diet in Atlantic salmon (Krossøy et al. 2009; Beato et al. 2020) and in shrimps from 30–40 mg kg⁻¹ in *Penaeus monodon* (Shiau and Liu 1994b) to 185 mg kg⁻¹ in *Fenneropenaeus chinensis* (Shiau and Liu 1994a).

A detailed recommendation has been developed for juvenile large yellow croaker (*Larimichthys (Pseudosciaena) crocea*): Dietary supplementation of menadione sodium bisulfate is not necessary for short-term survival and growth (56 days). However, with regard to blood coagulation, a minimum of dietary menadione sodium bisulfate (<3.5 mg kg⁻¹) is recommended. Furthermore, for the maximum accumulation of MK-4 in muscle and liver, the minimum dietary VK contents are ~10.5 mg kg⁻¹ (Cheng et al. 2015). With ~3.0 mg kg⁻¹ diet, the requirement of Jian carps complies well with that of juvenile large yellow croakers (Yuan et al. 2016).

37.1 Functional Studies

Until recently, the focus of most functional studies of dietary VK has been laid on the health of calcified structures; most recent studies, however, expand to genetic and epigenetic regulatory pathways. Fernández et al. (2019) show in Senegalese sole that VK supplementation improves fish gonad maturation and sperm quality, indicating a complex regulation of nutritional status and reproductive performance through circulating small noncoding RNAs. Dietary VK supplementation in soles with a reproductive disorder regulates plasma levels of different steroid hormones and reduces DNA fragmentation in sperms. Moreover, dietary phyloquinone

supplementation can lead to DNA methylation of the Niemann-Pick C1-like 1 gene in humans (Westerman et al. 2020).

37.1.1 Invertebrates

In crustaceans, VK is also required for larval development, as shown in a variety of penaeid shrimps (Fernández-Gimenez et al. 2016). Presumably, VK plays the same role in invertebrates as in vertebrates: support of Ca and P transport. Shiau and Liu (1994b) found in the midgut gland of *P. monodon* evidence of a carboxylase and a protein precursor dependent on the VK₃ levels. An increased weight gain (WG) in *P. monodon* occurs on a 35 mg VK₃ kg⁻¹ diet.

Fenneropenaeus chinensis responds beneficially to graded dietary VK₃, however, on a higher VK₃ level than fishes. Optimal WG for juveniles is found at 185 mg VK₃ kg⁻¹ (Shiau and Liu 1994a). In contrast, no effect occurs in *Litopenaeus vannamei* (He et al. 1992), *P. muelleri*, or *A. loginaris* (Fernández-Gimenez et al. 2016) on VK₃-containing diet. This indicates that closely related species have different specific requirements. The underlying mechanism remains obscure.

In Japanese abalone (*Haliotis discus hannai*), Tan and Mai (2001) showed that Ca deposition in soft body or shell is relatively constant regardless of dietary VK contents with the role of VK in the mineralization of shells being still obscure.

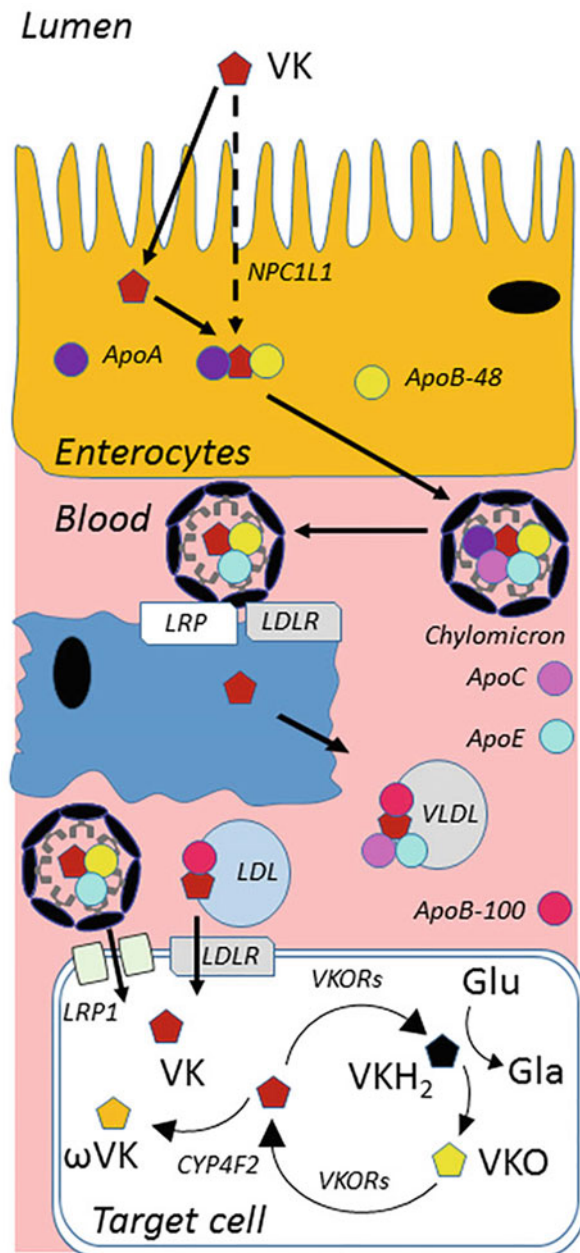
Summer mortality affects the productivity of the greenlip abalone (*Haliotis laevis*) in land-based Australian farms. It is associated with suppression of the immune system and likely related to VK deficiency. Therefore, Thomson et al. (2018) tried to alleviate mortality of this abalone by dietary supplementation of VK₁ (0.0–5.0 mg kg⁻¹), however, without any success.

37.1.2 Fishes

In fishes, VK is mainly concentrated in liver and gonads, with only small amounts in muscles (Udagawa 2000). VK deficiency results in anemia and prolonged coagulation time. In addition, skeletal deformities are one of the major bottlenecks in aquaculture, and several studies show the potential of VK in skeletal development (see reviews by Lall and Lewis-McCrea (2007) and Krossøy et al. (2011)). Dietary VK supplementation improves larval skeletal development as shown in haddock (*Melanogrammus aeglefinus*) (Roy and Lall 2007) or Senegalese sole (*Solea senegalensis*) (Richard et al. 2014). In the latter study, the authors confirm that VK deficiency is correlated with altered expression of molecular players of the VK cycle: *ggcx*³ and *pxr* are expressed, indicating that PXR is the nuclear receptor binding VK.

³ γ -glutamyl carboxylase catalyzes the posttranslational modification of vitamin K-dependent proteins involved in coagulation and in regulating Ca²⁺ homeostasis (Fusaro et al. 2017).

Fig. 37.2 Overview of the metabolism of vitamin K (VK) and the (phyto-/mena-) quinone signaling pathway (QSP) from absorption to target cells. *ApoA*, apolipoprotein A (Apolipoproteins are proteins that bind lipidic compounds to form lipoproteins. They transport lipids (and fat-soluble vitamins) in the blood, cerebrospinal fluid, and lymph (Nelson and Cox 2005)); *ApoB-48*, apolipoprotein B 48; *ApoB-100*, apolipoprotein B 100; *ApoC*, apolipoprotein C; *ApoE*, apolipoprotein E; *CYP4F2*, cytochrome P450 family 4 subfamily F member 2; *Gla*, c-carboxyl glutamate; *Glu*, glutamic acid; *LDL*, low-density lipoprotein; *LDLR*, low-density lipoprotein receptor; *LRP*, low-density lipoprotein receptor-related protein; *LRP1*, low-density lipoprotein receptor-related protein 1; *NPC1L1*, Niemann-Pick C1-like 1 (Niemann-Pick C1-like 1 (NPC1L1) is a protein found on the gastrointestinal tract epithelial cells as well as in hepatocytes); *VK*, vitamin K; *VKH2*, reduced vitamin K; *VKO*, vitamin K epoxide; *VKORs*, vitamin K epoxide reductases; *VLDL*, very low-density lipoproteins; *xVK*, hydroxyvitamin K1. (From Fernández et al. (2018), with permission from Springer Nature)



A comprehensive picture is provided by Fernández et al. (2018) (Fig. 37.2): The major sources of VK are diets and intestinal microbiota, although the contribution of colonic flora to VK provision remains controversial. Absorption of VK mainly takes

place in the jejunum and ileum in the mixed micelle complexes with bile salts and less likely in the colon. The absorption of VK from the diet is low, indicating a high efficiency of hepatic VK recycling during the synthesis of blood clotting factors and raising the question of whether a similar efficiency is obtained in other tissues, such as bone and vessel wall. Nevertheless, while high intakes of phyloquinone suppress the colonic production of all higher menaquinones, high menaquinone-4 intake specifically induces very high menaquinone-8 concentrations. These results point out a tight control of VK metabolism as depicted in Fig. 37.2.

Alarming, adverse VK deficiency-mediated effects can even be passed to succeeding generations. Udagawa (2004) investigated the effect of parental VK deficiency on bone development in mummichog (*Fundulus heteroclitus*) larvae. Since VK is acting as a cofactor for GGCX, therefore central in Ca^{2+} homeostasis, VK-deficiency affects larval bone formation: Larvae from parents on VK-deficient diet have an abnormal vertebral formation, whereas larvae from parents on appropriate VK diet show significantly fewer abnormalities (Fig. 37.3 left). This VK deficiency can be identified histologically: Fig. 37.3a, b shows the bone structure 5 days after hatching. The bone surface of the VK-enriched larvae is smooth and has regular circles, whereas that of the VK-deficient larvae is neither uniformly thick nor smooth and shows distorted circles. Overall, VK-deficient diets decrease bone quality, and this lowered bone quality is passed to the progeny.

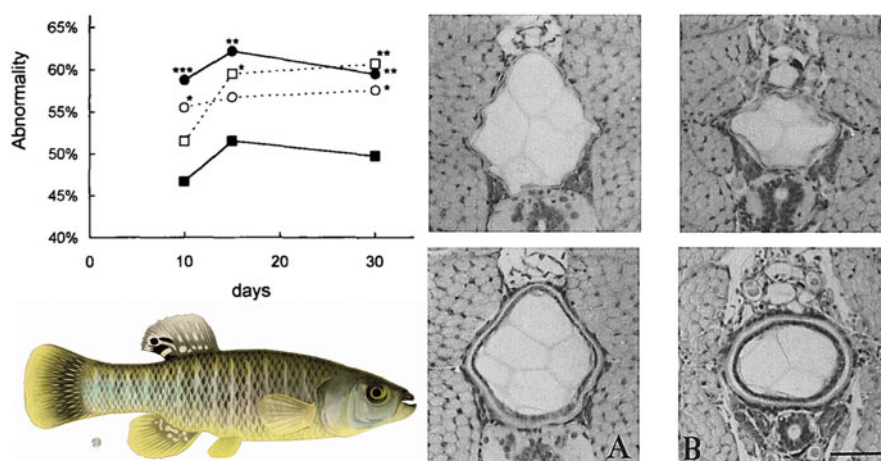


Fig. 37.3 Left Larval vertebral abnormalities in different feeding groups of mummichog (*Fundulus heteroclitus*). ● Vitamin K-deficient egg fed vitamin K-deficient diet; ■ vitamin K-containing egg fed vitamin K-supplemented diet = control group; □ vitamin K-containing egg fed vitamin K-deficient diet; ○ vitamin K-deficient egg fed vitamin K-supplemented diet. ** denote significant differences (* $P < 0.005$; ** $P < 0.01$; *** $P < 0.001$). Right Micrographs of cross section of vertebral bone, showing the effect of vitamin K in egg on the bone structure, 5 days after hatching. (a) Vitamin K-deficient individuals; (b) vitamin K-supplemented individuals. Bar equals 1 μm . (From Udagawa (2004) with permission from Wiley; image credit Ellen Edmondson, Hugh H. Chrisp, and New York State Department of Environmental Conservation)

VK exerts its biological functions through two pathways mentioned above. One acts as cofactor of the GGCX enzyme, which promotes the conversion of glutamate into γ -carboxyglutamate residues in VK-dependent proteins. Carboxylation requires the abstraction of a proton from the four-carbon of the glutamate residue by reduced VK and results in the conversion of VK into VK epoxide. The VK epoxide must be recycled into VK before it can be reused, a reaction that is catalyzed by two VK 2,3-epoxide reductases (Vkors), the Vkor complex subunit 1 (Vkorc1) and Vkorc1-like 1 (Vkorc1l1) (Beato et al. 2020).

In this context, VK recycling through Vkors is essential. Beato et al. (2020) determined the expression patterns of VK epoxide reductase complex subunit 1 (*vkorc1*) and vkorc1 like 1 (*vkorc1l1*) during the ontogeny of Senegalese sole larvae and in early juveniles under different physiological conditions (Fig. 37.4). During larval development, expression of *ssvkorc1* shows a slight increase during absence or low feed intake. Expression of *ssvkorc1l1* continuously decreases until 24 h post-fertilization and remains constant afterwards. Both *ssvkors* are ubiquitously expressed in adult tissues, and the highest expression is found in liver for *ssvkorc1* and ovary and brain for *ssvkorc1l1*. Expression of *ssvkorc1* and *ssvkorc1l1* is differentially regulated under physiological conditions related to fasting and refeeding but also under VK dietary supplementation and induced deficiency (Fig. 37.4). Based on the expression profiles of *vkors* gene during larval development and at adult stage, Senegalese sole has higher dietary VK requirements during embryogenesis and pre- and pro-metamorphosis and for proper gametogenesis than during juvenile stages.

37.2 Concluding Remarks

The forms of VK are fat-soluble and may interact with other lipophilic vitamins. Most known interactions of lipophilic vitamins were identified in studies using mammalian species, raising the need for specific research efforts on this issue in fish species, preferentially farmed ones, since nutritional requirements identified in model fish species do not simply translate to farmed fishes (Fernández et al. 2018). VK nutrition of aquatic invertebrates has to move into focus; up to date, it has been neglected almost completely.

In total, comprehensive information about VK requirement is scarce, although symptoms of VK-deficient nutrition are drastic and well documented on the phenotypic level. The underlying pathways and potential side effects, however, remain obscure. Effects of excess dietary VK have not yet been described in aquatic animals. Nevertheless, menadione, as the most common dietary VK supplement, is a well-known prooxidant and acts as apoptosis-inducing agent (Monks et al. 1992). Therefore, adverse effects of excess dietary VK have to be anticipated; this lack of information, however, may be due to the fact that, in chickens, effective and toxic dosage are separated by three orders of magnitude (Oduho et al. 1993). In the same line of negligence lies the lack of knowledge about the involvement of intestinal

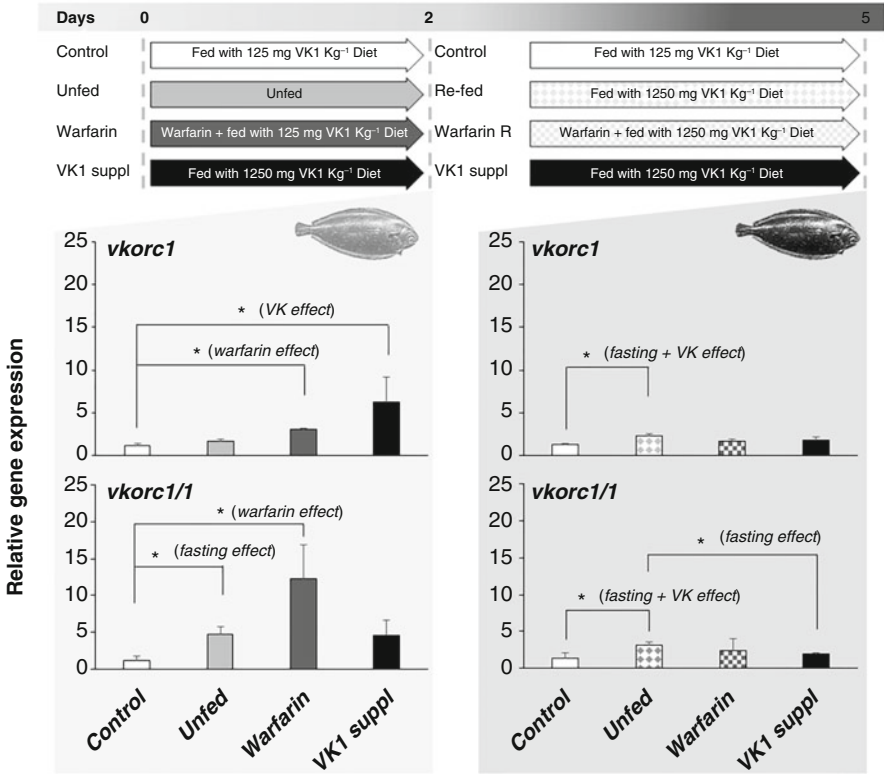


Fig. 37.4 Experimental design and relative gene expression of Senegalese sole *vitamin K epoxide reductase complex subunit 1 (ssvkorc1)* and *vkorc1 like 1 (ssvkorc1l1)* in juveniles cultured under different physiological conditions. Top image: experimental design indicating the physiological conditions to which Senegalese sole juveniles were subjected, from 0 to 2 days and from 2 to 5 days. Gray shadow graphs: mean and standard deviations values of *ssvkorc1* (upper image) and *ssvkorc1l1* (bottom image) transcript levels at 2 and 5 sampling days. Transcript levels were determined by qPCR and normalized using *ubiquitin (ubq)* gene expression levels. Levels in the control group were used as reference and set to 1. Left side of the graphs: juveniles fed with 125 mg VK1 kg⁻¹ diet (control), kept unfed (unfed), exposed to 25 mg L⁻¹ of warfarin while fed with 125 mg VK1 kg⁻¹ diet (Warfarin), (Warfarin is a VK antagonist, thus, functioning as anticoagulant drug (Bevans et al. 2013)) or fed with 1250 mg VK1 kg⁻¹ diet (VK1 suppl) for 2 days. Right side of the graphs: juveniles fed with 125 mg VK1 kg⁻¹ diet for 5 days (control), fed with 1250 mg VK1 kg⁻¹ diet for 3 days after being kept unfed for two days (re-fed), fed with 1250 mg VK1 kg⁻¹ diet for 3 days while being exposed to 25 mg L⁻¹ of warfarin (warfarin R), or fed with 1250 mg VK1 kg⁻¹ diet (VK1 suppl) for 5 days of experiment. At 2 days, all experimental groups were compared to the control group, while at 5 days all experimental groups were compared to the control and VK1 suppl group. Asterisks on the top denote significant differences between the two experimental groups compared ($n = 3$; student's t -test; $P < 0.05$). Text in brackets indicates the effect studied within each comparison. (From Beato et al. (2020), credit MDPI AG, Basel)

microbiota and potential controlling epigenetic mechanisms in VK provisioning and functioning. Although the general information about intestinal microbes as one major source of VK is available (Fernández et al. 2018) and recent studies in mammals show that dietary VK influences gut microbiota composition and metabolic activity (Ellis et al. 2021), little progress has been made to empirically address this issue. In addition, the combination of VK and epigenetics appears to be overlooked with aquatic animals.

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Chapter 38

Nucleotides—‘*Only for Fitness Fans?*’



Abstract As monomers, nucleotides (NT) play a central role in metabolism at the cellular level by providing energy; as polymers, they store and transmit genetic information. They are considered “semi-essential” nutrients, since under stress conditions, de novo synthesis of NTs may become limiting and dietary supplementation necessary. Dietary NTs can improve growth in early developmental stages, enhance larval quality via broodstock fortification, alter intestinal structures, increase stress tolerance, and modulate innate responses and strengthen adaptive immune leading to enhanced resistance to infections. In low fishmeal aquafeeds, NTs have been used as functional additives. NTs are found to reduce adverse effects of alternative proteins. The contribution of the intestinal microbiome in providing or metabolizing nucleotides is being understood, but it needs much more attention. Furthermore, the missing scientific treatment of epigenetics in NT nutrition of aquatic animals has to be pointed out.

Nucleotides (NTs) are ubiquitous, low molecular weight intracellular organic compounds with considerable structural diversity that serve as the monomers, or sub-units, of nucleic acids. NTs are composed of a nitrogenous base, a five-carbon sugar (ribose or deoxyribose)—called nucleosides (Fig. 38.1)—and at least one phosphate group. Traditionally, NTs have not been considered essential nutrients. As dietary supplements, only natural NTs find application; synthetic NTs are usually not administered via diets, but via intraperitoneal injection as a kind of vaccination; therefore, studies of these compounds are not included here.

Nucleotides are base units for DNA and RNA synthesis during cell construction and provide energy for normal cellular processes. Therefore, they are essential for growth, development, and immune strengthening. Under stressful conditions, additional NTs can be needed for further signal transduction or immune cell proliferation (Carver and Walker 1995). Although much less details are disclosed than in mammals, beneficial effects of dietary NTs do also occur in fishes (Li and Gatlin 2006). Particularly, adenosine is increasingly well understood for its immunoprotective and anti-inflammatory activities (Haskó and Cronstein 2013).

Three pathways of how an animal can obtain the required NTs do exist. These include recycling from dead cells known as the salvage pathway, direct de novo

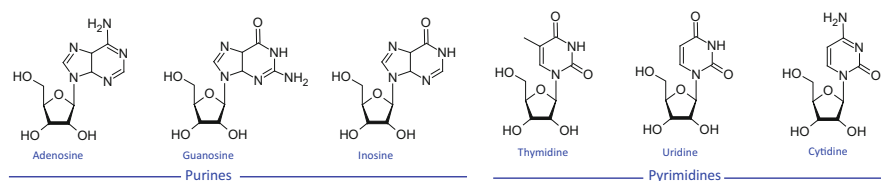


Fig. 38.1 Structures of the six nitrogenous bases attached to ribose (RNA nucleosides)

synthesis from amino acids (AAs), or through diet (Quan et al. 1990). As NTs can be synthesized endogenously, requirements may be met under normal conditions, and no signs of deficiency are observed when aquatic animals feed NT-free diets (Dawood et al. 2018), NTs have been considered non-nutritional. However, both the salvage and de novo pathways are complex multistage processes with energy requirement. Therefore, under stressful conditions, such as infection or during fast growth and development, de novo synthesis of NTs may become limiting and dietary supplementation necessary (Do Huu et al. 2012). Based on epidemiological and clinical studies in which dietary supplementation with NTs revealed beneficial effects, Grimble (1996) was one of the first to coin the term “essential nutrients” for dietary NTs; other authors prefer the term “semi-essential” or “conditional nutrients” (Do Huu et al. 2012). The essentiality of NTs is apparent during the following conditions: rapid growth, malnutrition, infection, or injury (Fontana et al. 2018).

The role of NTs in aquafeed has gained interest only recently. Exogenous NTs show promise as dietary supplements to enhance immunity and disease resistance of farmed animals. They can improve growth in early development stages, enhance larval quality via broodstock fortification, alter intestinal structure, increase stress tolerance, and modulate innate and adaptive immunity with enhanced resistance to viral, bacterial, and parasitic infection (Fig. 38.2) (Hossain et al. 2020a).

Some examples are going to depict actual highlights of these nutrients in diets for aquatic invertebrates and fishes.

38.1 Invertebrates

Invertebrates are tested with NT-enriched diets only quite late (Table 38.1). Li et al. (2007b) hypothesized a beneficial role of dietary NTs in *Litopenaeus vannamei*. However, their findings are vague and may have been masked, because the shrimps consume not only NTs, but also microbial biomass, which likely contains high concentrations of nucleic acids. Moreover, fishmeal (FM) in the test diets is also high in NTs. Clear evidence of physiological benefits of dietary NTs are reported later by Do Huu et al. (2012), Shankar et al. (2012), and Guo et al. (2016), who fed graded NT diets to *L. vannamei*, *Penaeus monodon*, or *Macrobrachium rosenbergii*. Guo et al. (2016) showed that 90 mg kg^{-1} NTs is optimal for gut health, immune

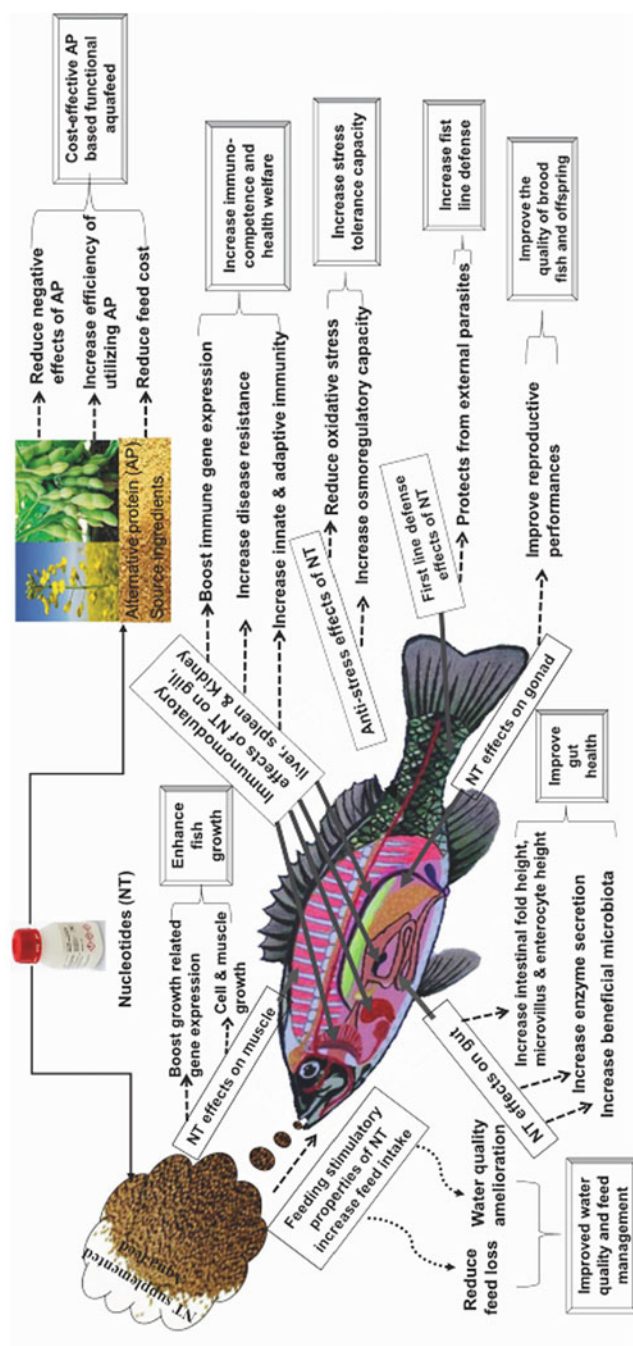


Fig. 38.2 Nucleotide application as potential functional feed additives in aquaculture and its effects on fish. (From Hossain et al. (2020a), with permission from Wiley)

Table 38.1 Effects of dietary nucleotides on life history traits in selected aquatic animals

Species	Effect on life history trait	Nucleotide	Optimal/ tested dose %	References
Invertebrates				
<i>Apostichopus japonicus</i>	Growth ↑, phagocytosis ↑, disease resistance ↑	Rovimax NX [®]	0.04	Wei et al. (2015)
	Growth ↑, non-specific immunity ↑	Guanosine	0.06	Chen et al. (2018)
<i>Astacus leptodactylus</i>	Growth ↑	Vannagen [®]	0.01 → 0.03	Safari et al. (2015)
<i>Babylonia areolata</i>	Growth ↑, pathogen resistance ↑	NuPro [®]	0.1, 0.2	Chaitanawisutti et al. (2011)
<i>Litopenaeus vannamei</i>	Growth ↔, reproduction (↑)	NM	0.04	Arshadi et al. (2018)
	Growth ↑	NM	0.04	Li et al. (2007b)
	Gut health ↑, immune response ↑, disease resistance ↑	NM	0.09	Guo et al. (2016)
	Hyposensitivity tolerance ↑	Optimūn [®]	0.2 → 0.5	Murthy et al. (2009)
	Growth ↑, innate immunity ↑, intestinal morphology ↑	Yeast, enriched with NM (Guangdong Hinabiotech Co.)	1.0 → 5.0	Xiong et al. (2018)
	Growth ↑ ↑, survival ↑	GMP	0.1	Yong et al. (2020)
	Growth ↑, survival ↑	IMP		
	Growth ↑, survival ↑	GMP + IMP		
	On SBM diet: best WG @ GMP	IMP	0.1	Mok et al. (2021)
		IMP+GMP	0.1	
		Blend of 3 NTs	0.1	
		Blend of 4 NTs	0.1	
<i>Macrobrachium rosenbergii</i>	Growth ↑	NM	0.15 → 0.3	Shankar et al. (2012)
<i>Marsupenaeus japonicus</i>	Innate immunity ↑	Vertex IG20 [®]	5.0	Biswas et al. (2012)

<i>Penaeus monodon</i>	Growth↑ Day 21 Day 42 Day 63		Optimûn®	0.56 0.46 0.45	Do Huu et al. (2012)
Fishes					
<i>Carassius gibelio</i> var. CAS III	Resistance against <i>Aeromonas hydrophila</i> ↑		5'-IMP	0.2 in FM-based feed 0.1 in SB-based feed	Zhang et al. (2019)
<i>Ctenopharyngodon idella</i>	Growth↑, transcription & activity of antioxidant enzymes↑, anti-apoptotic ability↑; intestinal immunity↑, intestinal inflammation↓, pathogen resistance↑		AMP, GMP, UMP, IMP, CMP 1:1:1:1:1	0.02 → 0.1	Tie et al. (2019, 2021a, b)
<i>Cyprinus carpio</i>	Resistance against <i>Aer. Hydrophila</i> ↑		Ribonuclease cleaved yeast RNA	0.015	Sakai et al. (2001)
<i>Danio rerio</i>	Growth↑, FA oxidation↓		NM	0.1	Guo et al. (2017)
<i>Dicentrarchus labrax</i>	Feed intake, growth↔		Yeast-RNA extract	0.01, 0.02	Peres and Oliva-Teles (2003)
	Growth↔, gut morphology↑		Mixture (Lalide® yeast)	0 → 0.3	Bowyer et al. (2019)
<i>Epalzeorhynchus bicolor</i>	Resistance against <i>streptococcus iniae</i> ↑		Aquagen®	0.02	Russo et al. (2006)
<i>Epinephelus malabaricus</i>	Growth↑, respiratory burst↑		NM, individual NTs	0.15	Lin et al. (2009)
<i>Gadus morhua</i>	Growth↑		NT-enriched <i>Artemia</i>		Lanes et al. (2012)
<i>Gibelion (Catla) catla</i>	Immunity↑		Yeast NTs	0.8	Jha et al. (2007)
<i>Huso huso</i>	Cortisol↓		Optimûn®	0.25 → 0.35	Yousefi et al. (2012)
	Growth↑			0.15 → 0.50	Abtahi et al. (2013)

(continued)

Table 38.1 (continued)

Species	Effect on life history trait	Nucleotide	Optimal/ tested dose %	References
<i>Ictalurus punctatus</i>	Effect on life history trait			
	Phagocytic activity↑, pathogen resistance↔	<i>Saccharomyces cerevisiae</i>	2.7	Duncan and Klesius (1996)
<i>Labeo rohita</i>	Pathogen resistance↓, stress resistance↑	NM	0.1	Welker et al. (2011)
	Resistance against <i>A. hydrophila</i> ↑	RNA supplementation	0.4	Choudhury et al. (2005)
<i>Lates calcarifer</i>	Growth↑, innate immunity↑, resistance against <i>A. hydrophila</i> ↑	RNA-loaded chitosan nanoparticles	0.4	Ferozekhan et al. (2014)
	Growth↑ @ 30 °C Growth↔ @ 37 °C	Optimûn®	0.2	Glencross and Rutherford (2010)
<i>Morone chrysops</i> × <i>M. saxatilis</i>	<i>S. Iniae</i> resistance↑	Ascogen P®	0.5	Li et al. (2004)
	Innate immunity↑, stress resistance↑	AMP C. Impex	0.5	de Cruz et al. (2020a)
	B-lymphocytes↑	IMP C. Impex	0.5	
	No synergism No synergism	AMP + IMP AMP + IMP	0.5 + 0.5 0.25 + 0.25	
<i>Odontesthes argentinensis</i>	Growth↑, innate immunity↑	AMP	0.5	de Cruz et al. (2020b)
	Survival↑, growth↑, stress resistance↑	NT-enriched <i>Artemia</i>		Führ et al. (2016)
<i>Oncorhynchus mykiss</i>	Growth↔, survival↔, Ig↓	Optimûn®	0.2	Yousefi et al. (2016)
	Antioxidant capacity↑, immune responses↑	Yeast extract (NuPro)® as FM replacement 20 → 60%		Özlier-Hunt et al. (2016)
	Lymphocytes, virus resistance↑, plasma cortisol↓	Optimûn®	0.03	Burrells et al. (2001a)
	Cortisol↓, immunity↑, pathogen resistance↑	Optimûn®	0.5	Leonardi et al. (2003)
	Growth↑, IgM↑, survival↑, stress resistance↑	Optimûn®	0.2	Tahmasebi-Kohyani et al. (2011, 2012)
Metabolic & structural muscle proteins↑				

						Keyvanshokoh and Tahmasebi-Kohyani (2012)
	LPO↓, low-density lipoprotein↓, high-density lipoprotein↑	Optimūn®		0.15, 0.2		Mohebbi et al. (2013)
	Growth↓, feed intake↓	Adenine		1.54		Rumsey et al. (1992)
	On plant diet: Growth↔	IMP, AMP		0.15 each		Ridwanudin et al. (2019)
	On plant diet: Growth↔; 18:3ω3 → 18:4ω3 + 20:4ω3	GMP, UMP, or CMP		0.15 each		
	Pathogen & parasite resistance↑	Optimūn®		0.2		Burrells et al. (2001a)
	Growth↔; muscular ARA & DHA↑	UMP or CMP		0.15 each		Ridwanudin et al. (2021)
<i>O. kistutch</i>	Pathogen & parasite resistance↑	Optimūn®		0.2		Burrells et al. (2001a)
<i>Oreochromis niloticus</i>	Survival↑, growth↑, pathogen resistance↔	Yeast extract (NuPro)®		0.4 → 0.6		Berto et al. (2016)
	Growth↔, feed utilization↔, disease resistance↑	AccelerAid*		0.1		Barros et al. (2015)
	Survival↑, growth↑, antioxidative response↑, disease resistance↑	Semipurified IMP		0.2		Kader et al. (2018)
<i>O. niloticus</i> ♀♀	<i>Ghrelin</i> ↑, <i>Igf</i> ↑ (Fig. 38.3)	Nucleoforce fish®		0 → 0.25		Selim et al. (2020)
<i>Oreochromis niloticus</i> × <i>O. aureus</i>	@0.5–0.75: Growth↑, reproduction↑	Ascogen®		0 → 1.0		de Lima et al. (2020)
	Growth↑, pathogen resistance↑	Ascogen S®		0.2 → 0.5		Ramadan et al. (1994)
	Immune responses↑, survival↑	Rovimax NX®		0.01, 0.02		Shiau et al. (2015)
	Growth↑, antioxidant status↑, immunity↑, intestine growth↑	Yeast-derived NT mixture		0 → 1.2		Xu et al. (2015)
<i>Pagrus major</i>	Growth↑, hematological profile↑, immune functions↑, stress tolerance↑	UMP		0.48 → 0.59		Hossain et al. (2016a, b, c, 2017a, b; 2018a)
	Growth↑, digestibility↑, innate immunity↑, stress resistance↑	AMP		0.2 → 0.4		

(continued)

Table 38.1 (continued)

Species	Effect on life history trait	Nucleotide	Optimal/ tested dose %	References
	Effect on life history trait			
	Transcription of <i>igs</i> ↔, stress resistance↑	Nucleoside by-products inosine	0.3 0.1	
	Growth↑, innate & adaptive immunity↑, stress resistance↑	CMP	0.48, 0.50	
	Growth↑, immune response↑, stress resistance↑, gut morphology↑	Inosine, IMP	0.4	
	Growth↑, survival↑, innate immunity↑, stress resistance↑	GMP	0.45, 0.48	
	Growth on SPC↑	GMP	0.4	Hossain and Koshio (2017)
	WG↑, innate immunity↑, oxstress resistance↑, best @IMP	AMP GMP or IMP for 56 days	0.2 0.4 0.4	Hossain et al. (2021)
	Growth↑, immunity↑, oxstress resistance↑	CMP for 150 days	0.2; 0.4	Hossain et al. (2020b)
<i>Pangasianodon hypophthalmus</i>	Growth↔, stress resistance↔, intestinal microbiota↑, immunity↑	Optimūn®	0.25 → 1.0	Yaghobi et al. (2015)
<i>Paralichthys olivaceus</i>	Innate immunity↑, disease resistance↑	IMP	0.4	Song et al. (2012)
<i>Salmo salar</i>	Growth↑, survival↑, intestinal folds↑, plasma chloride↓, sea lice infection↓	Optimūn®	0.2	Burrells et al. (2001a)
	Stress resistance↑, growth↑, gut histology↔	Maxi-Gen® Plus	0.6	Fu et al. (2017)
<i>S. caspius</i>	Cortisol↓, glucose↓, stress resistance↑	Optimūn®	0.25	Kenari et al. (2013)
<i>Sander lucioperca</i>	Innate cellular & humoral immunity↑, <i>Aer. Salmonicida</i> resistance↑, nutrient accumulation↔	Extracts of <i>Saccharomyces cerevisiae</i> (NuPro®, Alltech Inc., Nicholasville, USA)	2.0 → 6.0 4.0 2.0	Jarmolowicz et al. (2013, 2018)
	Growth↑			
	Innate immunity↑		4.0	Kowalska et al. (2015)

<i>Sciaenops ocellatus</i>	Transient growth↑, pathogen survival↑	NM, Optimûn®	0.03 → 0.3 Optim. 0.2	Li et al. (2007a)
<i>Scophthalmus maximus</i>	Gut morphology↑	Ascogen P®	1.0	Cheng et al. (2011)
	Igm↑, rag-I↑	Optimûn®	0.03	Low et al. (2003)
	Growth↔, immunity↑, intestinal structure↑	NM	0.03 → 0.10	Peng et al. (2013)
	Growth↔, feed utilization↔	Vannagen®	0.02	Fuchs et al. (2015)
	Growth↑, antioxidative capacity↑, intestinal morphology	Rovimax NX®	0.02 → 0.04	Meng et al. (2017)
<i>Seriola dumerili</i>	Immunity ↔, SGR↓ best @ 0.1 IMP or IMP +GMP	IMP GMP IMP+GMP	0.05 → 0.2 0.05 → 0.2 0.05 + 0.05	Li et al. (2018)
	Digestive enzymes: best @ 0.05 + 0.05	GMP IMP+GMP	0.05 → 0.2 0.05 + 0.05	Xia et al. (2020)
	Growth↑, survival↑, feed utilization↑, immunity↑, oxidative stress response↑, intestinal morphology↑	Inosine	0.54 → 0.67	Hossain et al. (2017d)
	Blood chemistry↑, non-specific immunity↑, oxidative stress↓, intestinal morphology↑	Nucleoside by- products (mainly inosine)	0 → 9.0	Hossain et al. (2017c)
	Growth on SBM↑	Inosine	0.6	Hossain et al. (2018b)
<i>Siniperca chuatsi</i>	<i>Pomc</i> ↓ (uptake of artificial diet↑) ^a	IMP	0.4	Li et al. (2019) ^b
<i>Solea solea</i>	Growth↔	Vannagen®	0.04	Palermo et al. (2013)
<i>Sparus aurata</i>	Feeding↑, growth↑, survival ↑	IMP	3.0	Oliva-Teles et al. (2006)

(continued)

Table 38.1 (continued)

Species	Effect on life history trait	Nucleotide	Optimal/ tested dose %	References
<i>Tachysurus</i> (<i>Pelteobagrus</i>) <i>fulvidraco</i>	Growth↑, antioxidant Responses↑	NM	0.15	Zhao et al. (2017)
<i>Thunnus orientalis</i>	Feeding↑, growth↑, survival↑	IMP	3.0	Haga et al. (2011)

↑ beneficial effect/improvement; ↔ no obvious effect; ↓ adverse effect/reduction; ® commercial preparation; **FM** fishmeal; **LPO** lipid peroxidation; **NM** NT mixture; **AMP** adenosine mono-phosphate; **GMP** guanosine mono-phosphate; **UMP** uridine mono-phosphate; **IMP** inosine mono-phosphate; **CMP** cytidine mono-phosphate; **Ig** total immunoglobulin; **SGR** specific growth rate; **oxstress** oxidative stress; **SB** soybean; **SBM** soybean meal; **SPC** Soy protein concentrate; **ARA** arachidonic acid; **DHA** docosahexaenoic acid

^aPro-opiomelanocortin a precursor polypeptide which cleavage gives rise to several peptide hormones. POMC neuron stimulation results in satiety. Therefore, POMC is an anorexigenic, rather than an orectic neuropeptide in mammals and fishes (Volkoff et al. 2005; Mineur et al. 2011)

^bBased on a wrong classification of pro-opiomelanocortin as orectic neuropeptide, Li et al. (2019) drew the wrong conclusion that IMP does not stimulate feed uptake in *Siniperca chuatsi* as recently communicated to Aquaculture Research (Steinberg 2021)

response, and disease resistance in *L. vannamei*. In the sister species *P. monodon*, dietary NT supports growth with the optimal NT content of 0.51% (Do Huu et al. 2012). The requirement decreases with increasing juvenile age (Table 38.1). In their subsequent paper, the authors report that all purine NTs (guanine, adenine, and inosine (a rare, but often nutritionally applied RNA nucleoside, Fig. 38.1)) support growth of this shrimp, but guanine is most effective (Do Huu et al. 2013).

To continue this line of evidence, in juveniles of the narrow clawed crayfish (*Astacus leptodactylus*), dietary NTs exert beneficial effects on growth, feed utilization, and immune response against air exposure (Safari et al. 2015). Wei et al. (2015) confirm that a diet supplemented with 375 mg kg⁻¹ NTs is able to enhance survival after pathogen challenge and growth of the Japanese sea cucumber (*Apostichopus japonicus*).

38.2 Fishes

Since long, NTs have successfully been applied as dietary chemo-attractants (Mackie and Adron 1978) and feeding stimulants (Kubitza et al. 1997; Lim et al. 2016), whereby inosine-5-monophosphate (IMP) is the most effective NT. Comparable approaches with invertebrates appeared promising (Harpaz et al. 1987; Lee and Meyers 1996); however, NTs often prove to be less effective than AAs.

Research into potential growth and health benefits of dietary NTs in farmed fishes began in the early 1990s. After the pioneering study in rainbow trout (Rumsey et al. 1992), NTs in aquaculture are found to improve growth, immunity, and pathogen defense, particularly under stress conditions (Table 38.1). The numbers of scientific papers dealing with dietary NTs in finfishes have been relatively limited since the comprehensive review of Li and Gatlin (2006) but increase since then. We focus on potential mechanisms behind the described beneficial effects.

Dietary NTs promote growth. Nile tilapia shows increased transcription of two major growth-promoting genes, *ghrelin* and *insulin-like growth factor (igf)*, on diets supplemented with NTs (Fig. 38.3) (Selim et al. 2020). Ghrelin acts as orexigenic hormone, stimulating feed acquisition and growth.

Applying proteomics, Keyvanshokoh and Tahmasebi-Kohyani (2012) shed further light on the mechanisms of enhanced growth. Dietary NTs cause differential expression of muscle metabolic proteins including glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, adenylate kinase, nucleoside diphosphate kinase, and triosephosphate isomerase. In addition to the metabolic enzymes, the structural protein troponin-T-1 increases in treated fishes.

Since aquaculture suffers from heavy losses of larvae due to diseases, any parameter that strengthens defense mechanisms has major impacts on future recruitment. Nutrition is such a crucial parameter, and Low et al. (2003) tested NT feeding in turbot larvae in order to increase immunity. The authors analyzed the expression of a number of immune genes, such as *immunoglobulin M (igm)* and *recombinase*

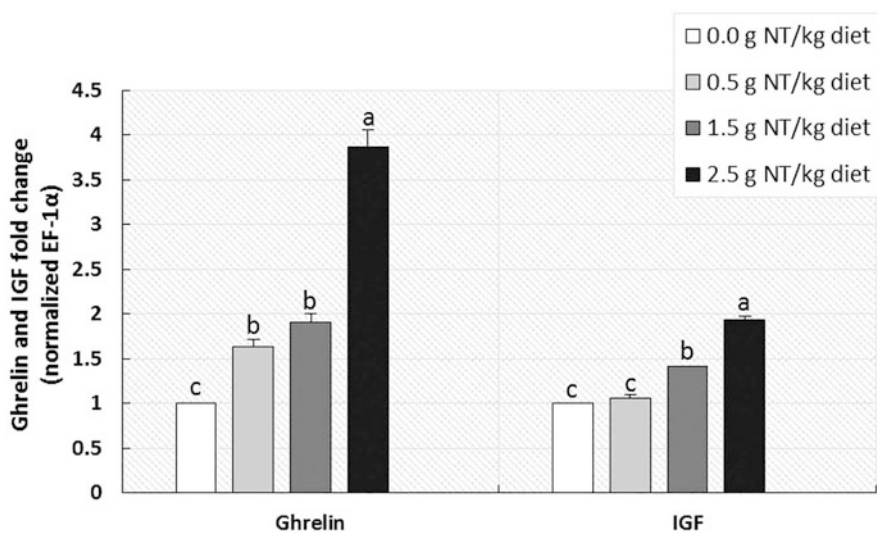


Fig. 38.3 The quantitative expressions of *ghrelin* and *insulin-like growth factor (igf)* extracted from the stomach and intestine, respectively, of Nile tilapia fed diets enriched with different levels of dietary yeast NTs (Nucleoforce Fish®) for 60 days. (From Selim et al. (2020), with permission from Taylor & Francis)

activating gene 1 (rag-1), crucial for lymphocyte maturation (Hansen and Kaattari 1995), and genes important for non-specific defenses (*transferrin*, *lysozyme*, *cytokines*). *Igm* and *rag-1* expression increases in the gill and spleen of fishes on NT-supplemented diet, but decreases in kidney (Fig. 38.4). *Lysozyme* expression decreases in the spleen and kidney of individuals on NT-supplemented diet (Fig. 38.4). In contrast, a second cytokine gene examined, *interleukin-1β*, shows an increased expression in the kidney of the NT-supplemented group (not shown). Overall, a tissue-specific transcription becomes obvious.

Similarly, feeding striped catfish (*Pangasianodon hypophthalmus*) a NT-containing diet improves alternative complement (ACH_{50}) activity and increases IgM levels, whereas growth parameters are not improved. Furthermore, dietary NT supplementation affects the intestinal morphology, whereby the folds and enterocyte heights in the mid-intestine increase, while the *microvilli* length is not affected. The best NT levels are 0.25 and 0.5% (Table 38.1).

As an additional mechanism, Guo et al. (2017) identified bioenergetics underlying the growth-promotion effect of NTs in zebra fish and the associated roles played by the intestinal microbiota. NT supplementation at 0.1% increases weight (WG) and energy gains of zebra fish by 10% and 25%, respectively. The standard metabolic rate is 28% lower in NT-fed fishes than in controls. NT supplementation downregulates the inflammatory tone in head kidneys. Moreover, NT-fed fishes have a 51% lower intestinal expression of *fasting-induced adipose factor (fiaf)* than control ones, which is consistent with decreased expression of key genes involved

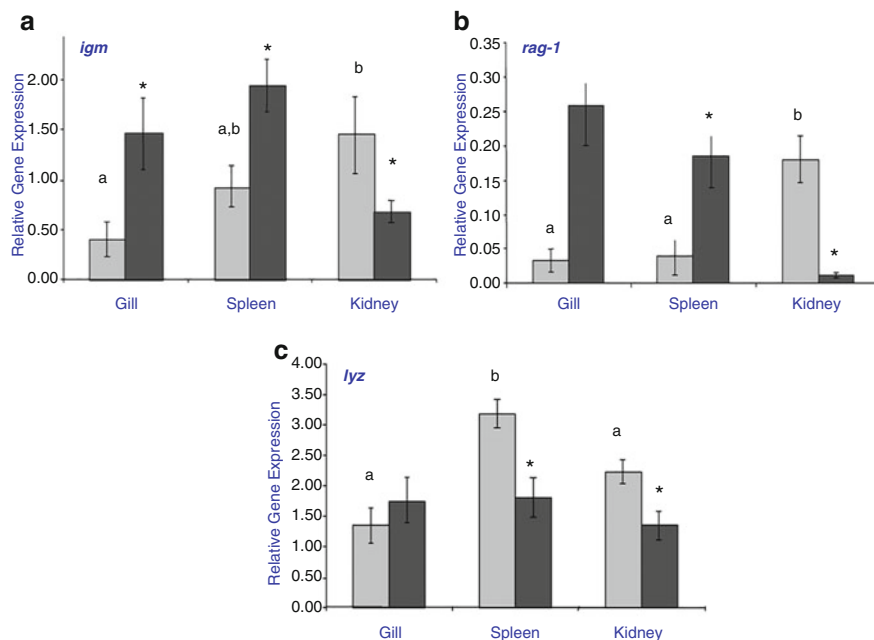


Fig. 38.4 NT-supplemented diet modulates the expression level of turbot *immunoglobulin (igm, a)*, *recombinae activating gene-1 (rag-1, b)*, and *lysozyme (lyz, c)* in different turbot immune tissues. Data are mean \pm S.E. * $P < 0.05$ relative to the respective control. Different superscripts indicate significant differences ($P < 0.05$) between tissues. (From Low et al. (2003) with permission from Elsevier). Light gray, control diet; dark gray, NT diet

in fatty acid (FA) oxidation [*carnitine:palmitoyl transferase 1a (cpt1a)* and *medium-chain acyl coenzyme A dehydrogenase (mcad)*] in liver and muscle. Germ-free zebra fishes colonized with microbiota from NT-fed fish have a 25% lower standard metabolic rate than those colonized by control microbiota, whereas direct NT feeding of germ-free zebra fish does not affect the standard metabolic rate at all. Furthermore, germ-free zebra fishes colonized with NT microbiota exhibit downregulated inflammatory tone and 33% lower *fiaf* expression than their control counterparts. Overall, the growth-promoting effect of dietary NTs involves two intestinal microbiota-mediated mechanisms that result in reduced standard metabolic rate: (i) lower inflammatory tone and (ii) reduced FA oxidation associated with increased microbial suppression of intestinal *fiaf*.

In addition to improved growth and strengthened immunity, dietary NT addition reduces the susceptibility to a variety of stressors (Burrells et al. 2001b). To figure out these effects in rainbow trouts, Tahmasebi-Kohyani et al. (2012) fed graded dietary NTs and subjected the individuals to crowding stress. Stressed control fish show typical primary (high cortisol) and secondary (high glucose and ionic disturbance) stress symptoms. This finding complies well with studies by Leonardi et al. (2003) and Barton et al. (1980) in rainbow trouts stressed by pathogens or handling,

respectively. Likewise, Yousefi et al. (2012) reported that stress-induced cortisol concentrations decrease in beluga sturgeons (*Huso huso*) on dietary NTs.

As mentioned, dietary NTs may spare the cost of de novo NT synthesis and optimize the function of rapidly dividing cells, such as lymphocytes. In fact, external NTs increase the expression of *rag-1* and *igm* in B lymphocytes residing in the spleen or kidney (Low et al. 2003), which are essential for antibody secretion and robust development of adaptive immunity (Pohlenz and Gatlin 2014).

Except for channel catfish, even pathogen resistance increases with dietary NT supplementation (Table 38.1). The reason for the divergent results in *Ictalurus* remains undisclosed. Likewise, the lack of growth improvement in Nile tilapia (Barros et al. 2015), turbot (Fuchs et al. 2015), or European seabass (Peres and Oliva-Teles 2003) on NT-enriched diet merits further evaluation. Overall, dietary NTs enhance immune response and disease resistance in many aquatic animals (Table 38.1): *A. japonicus*, *B. areolata*, *L. vannamei*, *M. japonicus*, *C. catla*, *E. bicolor*, *L. rohita*, *M. chrysops* × *M. saxatilis*, *O. mykiss*, *O. kisutch*, *O. niloticus*, *O. niloticus* × *O. aureus*, *P. major*, *P. hypophthalmus*, *S. salar*, *S. ocellatus*, *S. maximus*, and *S. dumerili*.

The beneficial effect may be of only transient nature. Li et al. (2007a) documented that juvenile red drum fed diets supplemented with various levels of purified NTs show enhanced WG and feed efficiency during the first week. However, these effects level off during the following three weeks of feeding. Corresponding features occur in Atlantic salmon smolts (Fu et al. 2017) and Pacific white shrimp (Guo et al. 2016). This occasionally observed transient beneficial effect of dietary NTs contributes to the conventional controversy about the general dietary applicability of NTs.

Most of the effective doses lie below 1% feed dry weight. In several cases, the effective doses range even one order of magnitude below this figure: in *A. leptodactylus*, *D. labrax*, or *S. maximus* (Table 38.1). Even the opposite trend can be found: *M. japonicus* and *I. punctatus* need NT doses between 2.7 and 5.0% with only moderate beneficial effect (*I. punctatus*)—most likely, the applied doses are above the dietary optimum.

Like many other macro- and micronutrients, the relationship between dietary NT contents and traits of farmed animals is characterized by optimum curves indicating that high doses exert detrimental effects in fed animals. Besides rainbow trout or striped catfish, this can clearly be seen also in turbot (Meng et al. 2017): 0.30 g kg⁻¹ of dietary NTs improves growth, antioxidative capacity, feed utilization, and intestinal morphology of individuals fed with low FM diet. Dietary excess (3.0 g kg⁻¹), however, causes oxidative stress and morphological damages in the intestine combined with growth reduction.

Another example: the transcriptions of *mnsod*, *cat*, *gpx1a*, *gpx4b*, *gstp1*, *gstp2*, *nrf2*, and *tor* in the muscle of grass carp show clear optima (Fig. 38.5) (Tie et al. 2019). Which mechanisms do apply? So far, mechanistic explanations for the adverse effects of dietary NT excess are lacking and deserve evaluation; toxicity cannot be ruled out (Gatlin and Li 2007); however, proves for this assumption are

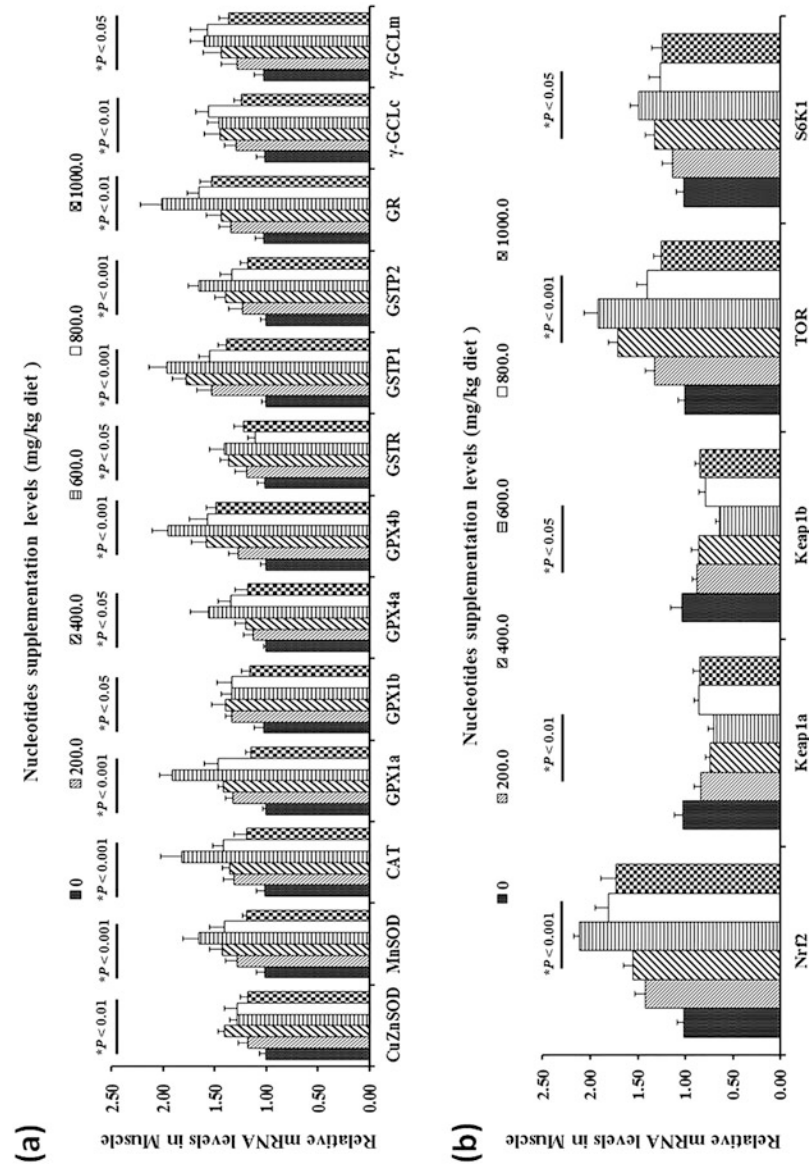
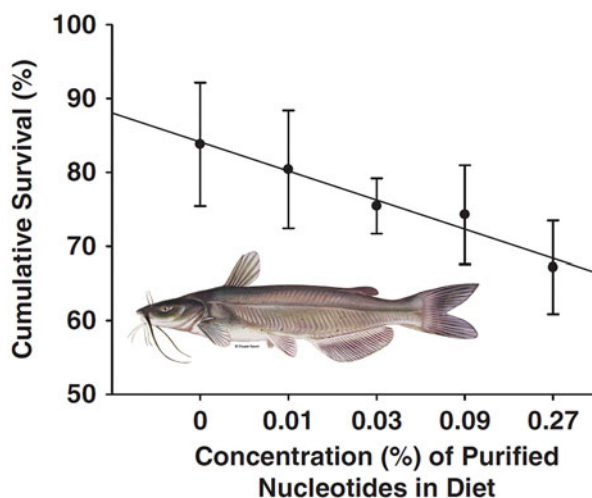


Fig. 38.5 Effect of NTs on the relative gene expression of antioxidant enzymes (a) and antioxidant signaling-related factors (b) in the muscle of grass carp. Values as mean \pm SE, $n = 6$. * P -values indicate a significant linear or quadratic dose response relationship ($P < 0.05$). CuZnSOD, copper, zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; cat, catalase; gpx, glutathione peroxidase; Nr12, NF-E2-related factor 2; Keap1, Kelch-like-ECH-associated protein 1; tor, target of rapamycin; s6k1, ribosomal protein s6 kinase polypeptide 1. (From Tie et al. (2019), with permission from Elsevier)

Fig. 38.6 Mean cumulative survival ($\% \pm$ SE) 21 days of channel catfish after challenge with *Edwardsiella ictaluri*. The linear response was significant. Catfish fed the 0% control diet had significantly higher survival than catfish fed the 0.9% or 2.7% NT diets (from Welker et al. (2011); with permission from Wiley; image credit US Fish and Wildlife Service and Duane Raver)



sparse. It can easily be predicted that excess NT also interferes with the transcription of other regulatory pathways, likely immunity (Fig. 38.6).

Li and Gatlin (2006) reported that salmonids and seabass may be able to tolerate high dietary levels of nucleic acids and/or yeast by virtue of their active hepatic uricase.¹ Rumsey et al. (1992) found that yeast RNA extract up to 4.1% of diet does not depress the growth of rainbow trout. Other studies report lower values as adverse doses for rainbow trout. It appears very difficult to explain the inconsistency concerning growth-depressing effects of dietary NTs in these trout studies, because sources, forms, levels, and bioavailability of NTs applied in the studies are too diverse (Li and Gatlin 2006).

Some three decades ago, Rumsey et al. (1992) showed that dietary NTs can be detrimental. The authors reported that free base adenine is a potent inhibitor of feed intake and growth in trouts; a high mortality demonstrates that it probably is toxic as well. In the same line of evidence and in contrast to beneficial effects of dietary NT amendments on immunity and pathogen resistance, Welker et al. (2011) report that the addition of a dietary mixture of five NTs produces a dose-dependent reduction in survival of channel catfish exposed to *Edwardsiella ictaluri* (Fig. 38.6). The authors hypothesize that adverse effects of NT supplementation on immune gene expression contribute to this dose-dependent decrease in disease resistance.

¹Uricase or urate oxidase is a copper-binding enzyme that catalyzes the oxidation of uric acid to 5-hydroxyisourate and H_2O_2 . Subsequent hydrolysis and decarboxylation lead to the formation of allantoin as the end product of purine metabolism (Nuki 2012).

38.3 Nucleotides and Immunity²

As documented, various, but not all, reports indicate enhancement of immune response by promoting the expression of specific and nonspecific immune genes. Hypothesized reasons include dietary provision of physiologically required levels of NTs due to limited synthetic capacity of certain tissues (e.g. lymphoid tissue), inadequate energetic expenditure for de novo synthesis, or immunoendocrine interactions (Li and Gatlin 2006).

The NTs contribute to the circulating pool of NT available to stimulate leukocytes production (Carver and Walker 1995), which are the important cells of the immune system that protect the body against infectious diseases (Fig. 38.7). Moreover, it is thought that dietary NTs are important for optimal function of the cellular immune response such as increased interferon- γ production (INF- γ), decreased production of interleukin-2 (IL-2) and natural killer cells, and increased resistance to bacterial or fungal infection. NTs enhance the expression of genes which are important for nonspecific defenses (*transferrin*, *lysozyme*, and cytokines) (Low et al. 2003). The authors identified also increased transcription of *immunoglobulin M (igm)* and *recombinase activating gene 1 (rag-1)* in gills and spleens of fishes on NT-supplemented diets, whereas *lysozyme* and *transferrin* remain unaffected. In seemingly contrast, a second cytokine gene examined, *interleukin-1 β* , shows increased expression in kidneys of the NT-supplemented groups. *IL- β* is a pro-inflammatory cytokine and central in the initiation phase, where cells are attracted towards the affected side and release pro-inflammatory signal to recruit more immune cells towards the side of inflammation (Kany et al. 2019). In the subsequent resolving phase, anti-inflammatory molecules are released to end the inflammation (Opal and DePalo 2000).

Research on fishes has triggered the hypothesis that the intestinal microbiota plays a crucial role in improving immunity (Guo et al. 2017); however, even microbiota-independent pathways of immunostimulation seem to exist (Guo et al. 2019). To identify the role of the gut microbiota, this laboratory tested the effects of dietary NTs on disease resistance and innate immunity of zebra fish. The authors confirm that dietary NTs can enhance both mucosal and systematic innate immune responses. The results show also that, while NTs induce alteration in the microbiota, the NT-altered microbiota does not necessarily contribute much to the immunostimulation; rather, dietary NTs can directly interact with host tissues and stimulate innate immune responses (Guo et al. 2019).

Detailed reports on dietary NTs affecting immunity in invertebrates are rare. Xiong et al. (2018) identified changes in immune enzyme gene expression in shrimps induced by NT-rich yeast diets; the expression levels of *lysozyme* and *alkaline phosphatase* are upregulated in Pacific white shrimp fed 30 g kg⁻¹ diets with NT-rich yeast. Also Biswas et al. (2012) reported that *lysozyme* transcription increases after feeding NT-rich baker's yeast extracts to kuruma shrimp (*Marsupenaeus japonicus*). To get more information about the modes of action of dietary NT on the innate immunity of shrimps, further detailed research is needed.

²Extract taken from Hossain et al. (2020a) with permission from Wiley; appropriate citations added.

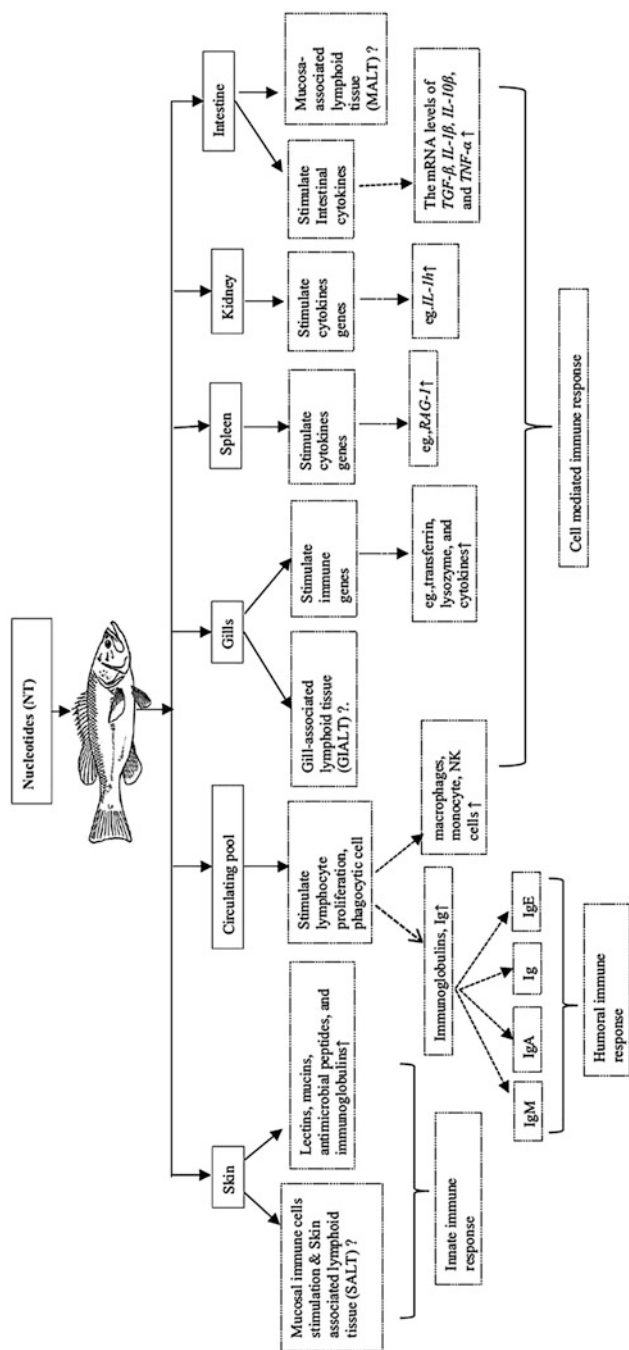


Fig. 38.7 Hypothesized mechanism of immunomodulation by NTs. NT interacts with mononuclear phagocytic cell and stimulates lymphocyte proliferation, which elevates the immunoglobulin level resulting enhanced innate immune responses. Skin, gill, and intestinal mucosa-associated lymphoid tissues (SALT, GALT, MALT) also probably affected by NT (?) NT enhanced the expression of certain genes from different immune organs like form gills (*transferrin*, *lysozyme*, and *cytokines*), spleen (*recombinase-activating gene 1*, *rag-1*), and kidney (*interleukin-1 h*, *il-1 h*) which are important for nonspecific defenses of fish (Low et al. 2003). NT also affects the mRNA levels of intestinal cytokines *transforming growth factor β* (*tgf-β*), interleukins *il-1β*, *il-10β*, and *tumor necrosis factor α* (*tnf-α*) in comparison with the control *ef-1a* levels (Reda et al. 2018). (From Hossain et al. 2020a), with permission from Wiley)

38.3.1 RNA Yeast Extract

While probiotic bacterial properties have been largely studied and consolidated, the beneficial properties of yeasts and their RNA cell compounds in aquaculture deserve more attention. Falcinelli et al. (2018) reported on a suitable potential use of RNA extracts from the yeast *Kluyveromyces (fragilis) marxianus* for improving growth and survival as well as reducing the inflammation and oxidative stress in zebra fishes. In fact, the yeast extract modulates the expression of genes involved in the immune system: pro-inflammatory cytokine genes (*il-1*, *il-8*) are down- and anti-inflammatory genes (*il-10*, *tnf-α*) are transiently upregulated. Moreover, the RNA yeast extract results in the amelioration of gut architecture. A similar cytokines communication network acts in Nile tilapia (Reda et al. 2018). Pro- as well as anti-inflammatory genes are upregulated in a dosis-dependent manner (Fig. 38.8).

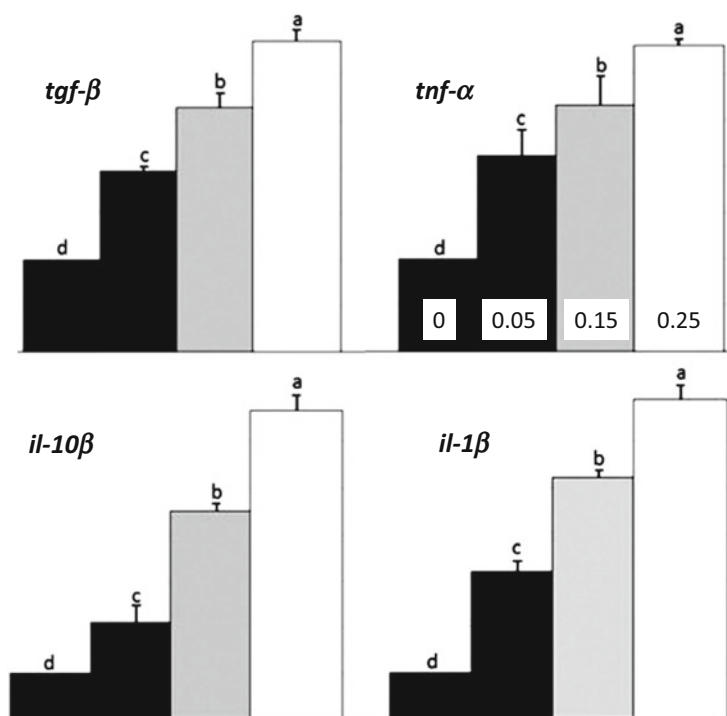


Fig. 38.8 Relative gene expression of intestinal cytokines (pro-inflammatory *il-1β*, *tnf-α*, and anti-inflammatory *il-10β*, *tgf-β*) of *Oreochromis niloticus* fed graded dietary yeast NTs (Nucleoforce Fish®) after a 30 days. Groups with different letters are significantly different ($P < 0.05$, one-way ANOVA). (Reconstructed from Reda et al. (2018), with permission from Elsevier). Figures in the column indicate % NT

38.4 Nucleotides and Fishmeal Replacement

Several cost-effective alternative protein sources have been investigated as substitutes for FM (Gatlin et al. 2007; Tacon and Metian 2008). Utilization of conventional plant protein sources in carnivorous fish feeds face a number of challenges due to imbalanced AA profiles and anti-nutritional factors (ANFs; see our concerns in AAN I ‘Chrononutrition’ (Steinberg 2018)) that can affect growth performance and health status of fishes (Francis et al. 2001; Gatlin et al. 2007).

Among the applied plant proteins, soy proteins are widely used as the most cost-effective alternative protein in diets for many farmed animals due to their relatively high content of available protein, relatively well-balanced AA profile, reasonable price, and steady supply of SBs (Hossain and Koshio 2017). Several approaches try to overcome some of the major limitations. Soy protein concentrate (SPC) is one product obtained during the processing of mature SBs with reduced ANFs and improved functional and nutritional properties.

The substitution of FM with SPC in diets for marine carnivores can reduce feed intake, growth, feed utilization efficiency, immunity, and intestinal health (Hossain and Koshio 2017). To overcome these drawbacks, the authors tested dietary supplementations with various NTs supporting optimum feed intake, optimum health, and growth. In fact, supplementation of GMP increases the efficiency of utilizing SPC ($\leq 66.7\%$) as a sole protein source for juvenile red seabreams (Hossain and Koshio 2017). In comparison with complete FM-based diet, fish growth, feed utilization, and blood chemical parameters do not differ on feed with up to 66.7% FM replaced by SPC plus GMP-supplementation. Noteworthy, this supplementation reveals even improved immune responses. In a subsequent paper, Hossain et al. (2018b) extended the repertory of tested species and NTs by showing that inosine supplementation is similarly successful in juvenile amberjack (*Seriola dumerili*) on soybean meal (SBM) and SBM protein. At least half of the FM can be replaced with SBM without compromising growth and health performance. Successful extension to further farmed fish species and the other NTs can be expected.

38.5 Concluding Remarks

Besides pointing out missing scientific treatment of epigenetics in the NT nutrition of aquatic animals, methodological aspects and the issue of dietary NT excess deserve mention—as mentioned several times in previous chapters.

Intriguing studies from one laboratory point out the important role of the gut microbiota in effect enhancement of NTs as nutrients exemplified in zebra fish (Guo et al. 2017, 2019); and one may argue that many, if not all, of the described beneficial effects of dietary NTs are influenced or modulated by gut microbiota although this micro-ecosystem was not considered in the corresponding studies. One may

continue that the nutritional effects of NTs can still be aggravated, if the role of the gut microbiota is better understood and controlled.

Most effective doses of dietary NTs lie below 1% dry feed. In several cases, the effective doses range even one order of magnitude below this figure. However, a comparison of the different studies is hampered, because many, particularly the older ones, applied various commercial NT products without declaring byproducts. These studies did not recalculate the effective doses of the NTs. A common protocol, e.g. retroactive accounting of the effective NT content or, even better, the application of individual pure NTs or defined mixtures would significantly improve the generalizability of the reported findings and applicability of NTs in aquafeed. This step seems overdue to solve apparent contradictions in NT requirement, even from the same laboratory (e.g., *S. maximus*). Up-to-date methods on the biomolecular and biochemical level are to be applied.

Since roughly one decade ago, leading laboratories abandoned commercial NT products in their studies and use mixtures of pure NTs, or pure individual compounds (reviewed by Li et al. (2015)). Nevertheless, many recent papers continue to apply, obviously for pragmatical and financial reasons, commercial products, and thereby reducing their scientific value.

Furthermore, many studies used only one dietary NT dose rather than graded levels in order to evaluate clear dose-response relationships and to identify potential hormetic relationships. Furthermore, if graded levels are fed, many studies report that at least one of the studied life traits follows an optimum dose-response curve indicating that an excess of dietary NTs has the potential of adversity and, thereby, counteracts the implication of feeding NTs. This becomes obvious in studies of *P. major*, *O. mykiss*, *S. maximus*, *P. hypophthalmus*, and invertebrates, such as *A. japonicus*, or *P. monodon*. Future progress in identifying molecular mechanisms of NT signaling may provide useful information to address these issues including optimal dosage. Particularly, the biomolecular regulation of dietary excess that leads to adverse effects deserves future attention.

To evaluate diet-mediated immune responses of aquatic animals and classify supplementations as pro- or anti-inflammatory, it is necessary to monitor the response kinetics. It does not suffice to check only one time point since, in the initiation phase, the response starts with the expression of pro-inflammatory cytokines followed by the resolving phase, when anti-inflammatory molecules are released to end the inflammation. Consequently, if only the initiation phase is monitored, an anti-inflammatory dietary supplement risks to be classified pro-inflammatory. This risk points out the necessity of reassessing several classifications of dietary supplements—not only of nucleotides.

Aiming to develop low FM-based functional aquafeeds, NTs have been used as additives in alternative protein-based diets. NTs are found to reduce the adverse effects of alternative proteins and lead to improving the efficiency of utilizing alternative protein through increasing growth and health performances of farmed organisms. However, extensive research information is still required regarding the use of NTs in alternative carbohydrate- and lipid-based diets to develop cost-effective and ecofriendly functional aquafeed (Hossain et al. 2020a).

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Chapter 39

Enzymes—‘Digestive Assistance from Aliens’



Abstract To digest substrates, which are not easily digested by nonruminant animals exogenous enzymes are well accepted as feed additives in aquafeed formulations. Currently, mainly enzymes targeting viscous cereals and microbial phytases are available. Recent studies show that exogenous enzymes, presumably not directly connected to immunity, not only extend the availability of food resources but also improve the innate immunity of fishes. Furthermore, dietary enzyme supplements may have a beneficial effect on digestive microbial ecology by reducing undigested substrates and antinutritive factors, and possibly by producing oligosaccharides from dietary NSPs with prebiotic potential. A few studies point out the role of chitinase in the immune response. This aspect deserves future attention. Research on exogenous enzyme supplementation to aquafeed is neither extensive nor comprehensive. Particularly, the application of commercial enzyme products with varying composition and quality standards increases the risk of missing repeatability in different laboratories or at different times. At present, the perspective of increasing productivity of farmed aquatic animals and of exploiting new feed sources is being supplemented by considering feed, intestinal microbes, and host animals as one entity.

Plant feedstuffs contain many nutrients or substrates, which are not easily digested by nonruminant animals because specific digestive enzymes are lacking. Major substrates are complex carbohydrates, such as hemicellulose and cellulose. In addition to these non-starch polysaccharides (NSP), these feedstuffs include phytic acid (PA, Fig. 39.1a) and protease inhibitors. In a classical trial, larval gilthead seabream benefited from the dietary supplement of commercial pancreatic enzymes in terms of growth and short-term survival (Kolkovski et al. 1993); however, the same approach failed later in European seabass (Kolkovski et al. 1997).

Meanwhile, exogenous enzymes are well accepted as feed additives in aquafeeds. They are supplemented in order to:

- Initiate or improve the degradation of substrates with specific bonds that are not cleaved by endogenous digestive enzymes or gut microbes.
- Degrade so-called anti-nutritional factors (refer to concerns → AANI “Chrononutrition,” Steinberg (2018)), which usually lower nutrient digestion.

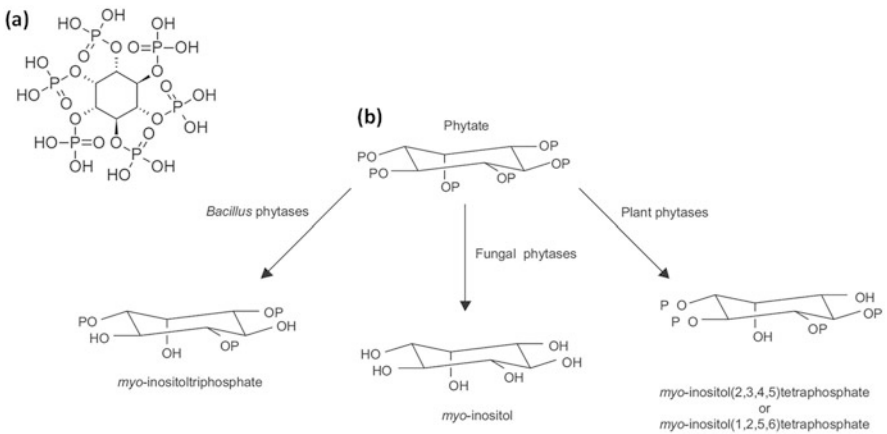


Fig. 39.1 (a) Phytic acid; (b) schematic diagram showing end products resulting from phytate hydrolysis mediated by different phytases (from Rao et al. (2009) with permission from Taylor & Francis). PO, OP phosphorus esters

Table 39.1 Enzymes, applied as aquafeed supplements

Enzyme	Target substrate	Target feedstuffs
Phytases	Phytates	Grains, oilseeds, plants
Proteases	Proteins	Plant proteins
Lipases	Lipids, oils	Seed oils and lipids
Carbohydrases	Combined carbohydrates in general	Cereals, grains, legumes, plant materials, fibers
Glucanases	Glucose polysaccharides	
Amylases	Amylose (starch)	
Cellulases	Cellulose	
Chitinase	Chitin	

- Change the composition of the gut microflora and reduce the share of pathogenic microbes.
- Augment endogenous digestive enzymes resulting in improved digestion. This is especially true for young animals with developing digestive systems (Ravindran and Son 2011).
- Reduce excretory and feed wastes in aquaculture, for instance, phosphorus (P) from phytates (Cho and Bureau 2001).

Major enzymes, potentially applied as feed supplements, are listed in Table 39.1; currently, only two distinct major categories of enzymes are commercially available for use in the aquafeed industry: Enzymes targeting viscous cereals and microbial phytases (PAses).

39.1 Phytases

Phytases (PAses) are most often supplemented, since many cereal and legume seeds and their products contain 1–2% PA. PA represents approximately 60% of the total P content. PA-bound P (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, Fig. 39.1a) is poorly available to monogastrics. Even worse, PA weakens the antimicrobial activity and aggravates the inflammatory status of head kidney, spleen, and skin as found in on-growing grass carps (Zhong et al. 2020). Furthermore, due to high negative charge, phytates have a strong affinity to interact with metals and reduce their bioavailability. This results in chelation of positively charged divalent metal ions (e.g. Fe^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , Mg^{2+} , Mn^{2+}). This challenge justifies research on PAses from different microbial sources for minimizing anti-nutritional effects of phytates and enhancing growth by improving P assimilation (Joshi and Satyanarayana 2017).

Bacteria, fungi, and plants possess PAses with different substrate specificity and products (Fig. 39.1b). Numerous studies show that dietary incorporation of microbial PAses improves the digestibility of P in most diets containing PA. Although PAses-producing bacteria have been isolated from various parts of the gastrointestinal tract of fishes (Roy et al. 2009; Dan and Ray 2014), the utilization of plant phytate can still be improved by adding PAses-producing bacterial symbionts from fish guts to aquafeeds (Roy et al. 2014). The current state of knowledge, however, appears to be too premature to allow general and applicable conclusions.

To substantiate this concern, we refer to a study in *Macrobrachium rosenbergii*. High decrease in protein, lipid, and energy utilization occurs in those individuals receiving the highest inclusion of PA (Rasid et al. 2017). Inclusions of up to 11.3 g PA kg^{-1} are “safe,” but levels exceeding this value exert a series of adverse symptoms except weight gain (WG) pointing out the necessity of studying the pathways in detail.

Microbial and fungal PAses [from bacteria, such as *Escherichia coli*, *Pseudomonas* spp., *Klebsiella* spp.; various *Bacillus* spp. (*B. amyloliquefaciens*, *B. amyloliquefaciens*, *B. licheniformis*, *B. mycoides*, *B. pseudomycoides*, *B. subtilis*, *B. thuringiensis*, *B. velezensis*), and yeasts (*Schwanniomyces occidentalis*, *Pichia anomala*, *Arxula adenivorans*)] belong to the best-studied dietary PAses supplements and have often been reviewed: Cho and Bureau (2001); Cao et al. (2007); Kumar et al. (2012); Dersjant-Li et al. (2015); Joshi and Satyanarayana (2017); Lemos and Tacon (2017); Romano and Kumar (2018). The use of PAses is appropriate only for diets with digestible P contents below the requirement of the farmed fishes and containing elevated levels of plant ingredients, i.e., in which a high proportion of the P is phytate-P (Cho and Bureau 2001). The *in vivo* efficacy of PAses is determined by their intrinsic properties, such as activity at different pHs, resistance against proteolytic degradation, and thermal stability (Sinha et al. 2011).

Some recent examples may illustrate the dietary supplementation of PAses in plant-based aquafeeds for carnivorous or omnivorous fishes. In *Pangasius*

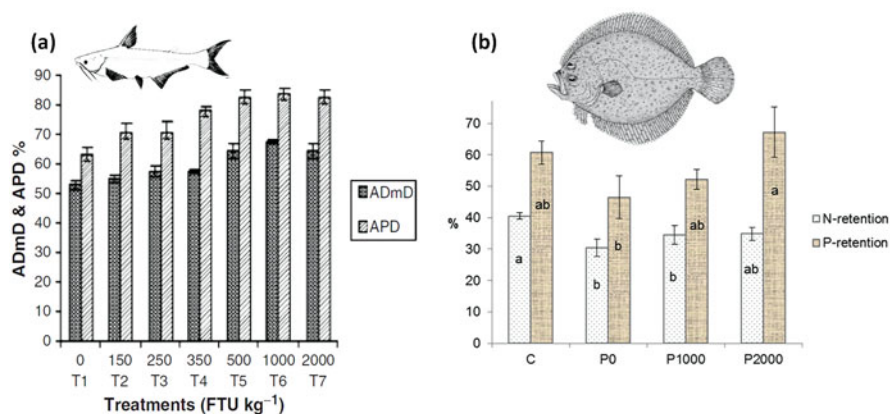


Fig. 39.2 (a) Effect of phytase supplementation on apparent dry matter (ADmD) and apparent protein (APD) digestibility of *Pangasius pangasius* fingerlings (from Debnath et al. (2005a) with permission from Wiley). (b) Retention of nitrogen (N) and phosphorus (P) in juvenile turbot fed experimental diets supplemented with phytase; different letters within one parameter (N- or P-retention) show significant differences ($P < 0.05$) (from von Danwitz et al. (2016) with permission from Elsevier). P0, P1000, P2000 phytase supplementation in FTU kg⁻¹. Images credit FAO

pangasius fingerlings, Debnath et al. (2005a) found that increasing PAsE supplementation improves both apparent dry matter and apparent protein digestibility: Dietary microbial PAsE supplementation at 500 FTU¹ kg⁻¹ diet with 35% crude protein increases growth, dry matter, and crude protein digestibilities (Fig. 39.2a).

The amount of supplemented PAsE is species-specific: Microbial PAsE supplementation with 2000 FTU kg⁻¹ improves growth, nutrient utilization, and phosphorus mineralization of juvenile red seabream (*Pagrus major*) on soybean (SB) diets (Laining et al. 2012); also the P requirement can be met for optimal growth. Similarly, in juvenile turbot (*Scophthalmus maximus*) on rapeseed protein concentrate (RPC) feed intake and growth improve on diets with 1000 and 2000 FTU kg⁻¹ PAsE (von Danwitz et al. 2016). Individuals fed 2000 FTU kg⁻¹ show feed conversion ratio (FCR), protein efficiency ratio (PER), and apparent digestibility coefficient (ADC) of protein comparable to controls. Moreover, slightly increased retention of N and significantly increased P retention are observed in fishes fed 2000 FTU kg⁻¹ (Fig. 39.2b). Therefore, PAsE supplementation is a solution to increase the incorporation of plant proteins in diets and reduce concomitant nutrient emissions into the waterways.

Further studies, with mixed success, are listed in Table 39.2; some reasons for lacking success will be discussed below.

Interestingly, the carnivorous European seabass is able to identify different food qualities (Fortes-Silva et al. 2011a). It prefers FM diet to herbal feeds (soybean meal,

¹One unit of phytase activity (FTU) is defined as the amount which liberates one μM of inorganic P per minute from an excess of sodium phytate at 37 °C and pH 5.5 (Kim and Lei 2005).

Table 39.2 Effects of pretreatment or dietary supplementation with microbial or fungal phytase on life history traits of selected aquatic animals

Species, weight	Plant Feedstuff	Phytase level, FTU kg ⁻¹ (source)	Effects	Reference
Invertebrates				
<i>Litopenaeus vannamei</i> , ~0.27 g	SB	Pretreatment Recombined from <i>Bacillus subtilis</i>	WG↑, survival↔	Cheng et al. (2013)
<i>L. vannamei</i> , ~0.22 g		Diet 500 – 2000 (Quantum® Blue 5 G, Vista feed ingredients)	Growth↔, FCR↔, ADC _F ↑, P retention↔, protein retention↔	Qiu and Davis (2017b)
<i>Macrobrachium rosenbergii</i> , 2.28 g	Peanut, rice, tapioca	Diet 250, 500, 750, (Natuphos®)	WG↑, PER↑ max @ 250	Biradar et al. (2017)
<i>Marsupenaeus japonicus</i> , 1.75 g	SB	Diet 200, <i>Aspergillus oryzae</i>	WG↓, SGR↓, FCR↑	Bulbul et al. (2015)
<i>Penaeus monodon</i> , 0.14 g		Diet 500 (Natuphos®)	Growth↔, ω3 PUFA↓, ω6 PUFA↑	Biswas et al. (2007)
<i>P. monodon</i> , ~1.2 g	SB, corn, rice, wheat	Diet 500; 1000; 1500 (Natuphos®)	Optim. growth↑ @ 1000, SR↔	Rachmawati and Samidjan (2016)
<i>Procambarus clarkii</i> , ~5.4 g	SB	Diet 1000 (Natuphos®)	WG↑, ACD _N ↑, P excretion↓	Wan et al. (2017)
Fishes				
<i>Carassius carassius</i> , ~1.5 g	SB	Diet 200, 300, 500, <i>Pedobacter</i> sp. MJ11 (PhyMJ11®, SunHY Biology, Wuhan)	WG↑, FCR↑, PER↑, ADC _F ↑	Nie et al. (2017)
<i>C. gibelio</i> , ~30 g	SB, RS, wheat	Diet 500; <i>Pedobacter</i> sp. MJ1	ADC _P ↑, ADC _F ↑ @ 50% replacement	Liu et al. (2012)
<i>Cirrhinus mrigala</i> , ~5 g	Sfl	Diet 0; 500; 1000; 1500; 2000 (Phyzyme® XP, Danisco animal nutrition, Vaasa, Finland)	Max. WG, max. ADC _N @ 1000	Hussain et al. (2017b)
<i>Clarias gariepinus</i> , 44 – 60 g; 76 – 82 g	SB	Pretreatment 1000 or Diet 15; 380; 750; 1000 (Natuphos® 5000)	ADC _F ↑, P retention↑	Van Weerd et al. (1999)
<i>C. gariepinus</i> , ~11.6 g	Peanut	Diet 0 – 1000 (Natuphos® 5000 L)	WG↑, bone phosphorus↑ optimum @ 335 – 485	Akpolih et al. (2017)

(continued)

Table 39.2 (continued)

Species, weight	Plant Feedstuff	Phytase level, FTU kg ⁻¹ (source)	Effects	Reference
<i>Ctenopharyngodon idella</i> , ~55 g	SB, RS, CS, wheat	Diet 500; <i>Pedobacter</i> sp. MJ11 (PhyMJ11®)	WG↔, SGR↔, PER↔, FCR↔	Liu et al. (2013b)
<i>Cyprinus carpio</i> , ~45 g	RS, SB, macrophytes, algae	Diet 0.3%; Phytase-A from <i>Peniophora lycii</i> , <i>Aspergillus oryzae</i> (Ronozyme® P5000 CT) Phytase-B from <i>A. niger</i> (Phytase Aska-25, 2500)	Digestibility of RS meal↑, digestibility of SBM↑	Watanabe et al. (2016)
<i>C. carpio</i> , 40 g	SB, wheat	Diet 500; 1000 (Natuphos®)	ADC _F ↑, P excretion↓	Schäfer et al. (1995)
<i>C. carpio</i> , 4.9 g	SB	Diet 750 (Ronozyme® P5000, DSM)	WG↑, ADC _P ↑	Phromkunthong et al. (2010)
<i>C. carpio</i> , ~115 g	Corn, SB, wheat, linseed, safflower	Diet 1000; 2000; 4000 (Ronozyme® P)	WG↔, SGR↔, FCR↔, P excretion↓, bone P↑	Nwanna and Schwarz (2007)
<i>C. carpio</i> , ~40 g		Diet 0; 500; 750; 1000 (Ronozyme® P)		Nwanna and Schwarz (2008)
<i>C. carpio</i> , ~6.5 g	SB	Diet 500 (Ronozyme® P)	WG↑	Sardar et al. (2007)
<i>C. carpio</i> , ~31 g, ~131 g	Corn, SB, wheat, linseed, safflower	Pretreatment 4000 (Natuphos®, BASF, or Ronozyme®, DSM)	WG↑, SGR↑, FCR↓	Nwanna et al. (2007, 2008)
<i>Danio rerio</i> , ~0.3 g	SB	Neutral PAs from <i>Pedobacter</i> : 0; 500; 1000; 1500	WG↑, SGR↑, FCR↓, SR↔, off-spring(!)	Liu et al. (2013a)
<i>Dicentrarchus labrax</i> , 13.5 g	SB	Pretreatment 1000; 2000, <i>A. niger</i> (Natuphos®)	ADC _P ↑	Oliva-Teles et al. (1998)
<i>D. labrax</i> , ~265 g		Diet 1500 (Natuphos®)	WG↔	Fortes-Silva et al. (2011b)
~7.5 g	High protein distillers dried grain, 0 → 50%	Diet 0.05% (Axta PHY®), <i>Buttiauxella</i> sp. (Enterobacteriaceae)	WG↑, SGR↑, FCR↓, F/L, AST↓, ALT↓, ALP↔, CHOL↑, LYZ↓, TAC↑	Goda et al. (2020)

<i>Gibelion (Catla) catla</i> , 8 g	<i>Moringa oleifera</i> , horseradish tree	Diet 0; 300; 600; 900; 1200; 1500 (Phyzyme® XP 10000, Danisco Animal Nutrition, Finland)	ADC _N ↑, optim. WG @ 900	Hussain et al. (2017c)
<i>Ictalurus punctatus</i> , ~6.5 g	SB, corn, wheat	Diet 0; 500; 1000; 2000; 4000 (Natuphos®)	FCR↔, WG↑, bone P↑, FCR↓, P excretion↓	Li and Robinson (1997)
765 g	SB, CS, corn	Diet 1000, 3000 (Natuphos®)	P retention↑	Eya and Lovell (1997)
23 g	SB, CS	Diet 250, 500 (Natuphos®)	Replacement of Ca phosphate↓	Robinson et al. (2002)
12 g	SB, corn, wheat	Diet 0; 500; 1000; 2000; 4000; 8000 (Natuphos®)	WG↔, FCR↔, PER↔, ADC _P ↔	Yan et al. (2002)
~1.7 g	SB, CS, RS, wheat	Diet 0; 300; 500; 1000; 1500; 2000 (Natuphos®)	WG↑, SGR↑, FCR↓, PER↑	Chen et al. (2019)
<i>I. punctatus</i> ♀ × <i>I. furcatus</i> ♂, 59 g	Commercial 28% protein diet (south fresh feeds, Demopolis, AL, USA)	Diet 2500; 5000 (AB Vista, Plantation, FL, USA)	Optim. WG@500. Surplus PAse: WG↔, P excretion↑	Li et al. (2019a)
<i>Labeo rohita</i> , ~13.5 g	SB	Diet 0; 750; 1000 (Phyzyme®xP, Damisco animal nutrition)	WG↑, mineralization↑	Shah et al. (2016)
~1.85 g	Sesame	Diet 10, 20, 30, 40, 50 (Biophos-TS)	P retention↑, P excretion↓	Roy et al. (2016)
Juvenile	Sfl	Diet 1000 (Ronozyne®)	ADC _N ↑	Rabia et al. (2017)
Fingerling	Corn	Diet 0; 250; 500; 750; 1000; 1250; 1500; (Phyzyme® XP 10000)	ADC _M ↑, ADC _{En} ↑, ADC _L ↑; optim @ 750	Hussain et al. (2011)
7.0 g	Canola	Diet 0; 250; 500; 750; 1000; 1250; 1500 (Phyzyme® XP 10000)	ADC _{mn} ↑, optim WG @ 750	Hussain et al. (2017a)
7.6 g		Diet 0, 1000, 2000 (Phyzyme® XP 10000)	ADC _M ↑	Habib et al. (2018)
~13 g	SB, wheat	Diet 0, 500 (Natuphos®)	Bone mineralization↑, ADC _M ↑, ADC _{mn} ↑, blood parameters↑	Baruah et al. (2005, 2007, 2009)

(continued)

Table 39.2 (continued)

Species, weight	Plant Feedstuff	Phytase level, FTU kg ⁻¹ (source)	Effects	Reference
7 g	CS	Diet 0; 250; 500; 750; 1000; 1250; 1500 (Phyzyme® XP 10000)	ADC _F ↑, ADC _M ↑, optim. WG@750	Hussain et al. (2015)
<i>Morone saxatilis</i> , 25 g, ~160 g	SB, corn, wheat	Diet 2400 (Natuphos®)	Bone mineralization↑, serum P↑	Powers Hughes and Soares Jr (1998)
400 g	SB, corn	Diet 0; 1000; 2000 (Natuphos®)	WG↑, ADC _M ↑, ADC _P ↔, SR↔, P excretion↓	Papatryphon et al. (1999)
3 years old	SB, corn, wheat	Diet 1000 (Natuphos®)	ADC _P ↑, ADC _F ↑	Papatryphon and Soares Jr (2001)
<i>Oncorhynchus mykiss</i> , 1.9 g	SB	Pretreatment 2‰ (Alko Biotechnologies, Rajamaki, Finland)	WG↑, ADC _F ↑, P excretion↓	Cain and Garling (1995)
120 g	Sfl, SB	Diet 4000; <i>E. coli</i> (Quantum®, AB Vista, Germany)	ADC _P ↑, ADC _N ↑, P excretion↓	Morales et al. (2016)
0.74 g	SB, linseed	Diet 0.08% (Ronozyme®)	WG↔, ADC _L ↓, P excretion↓	Reyes et al. (2015)
~200 g	SB, wheat, <i>Faba</i> beans, corn, Sfl, RS	Diet 2800 <i>Aspergillus oryzae</i> (Ronozyme®P, DSM Nutritional Products, Basel, Switzerland)	ADC _P ↑, P excretion↓	Greiling et al. (2019)
42 g	Diverse plant products	Diet 500; 1000; 2000 <i>Aspergillus oryzae</i> (Ronozyme HiPhos®)	ADC _F ↑, P excretion↓	Verlhaac-Trichet et al. (2014)
200 – 250 g	SB products	Diet 1000 (Natuphos®)	ADC _F ↑, growth↔	Rodelutschord and Pfeffer (1995)
4 – 7 g	Peanut, CS, SB, corn, canola	Diet 3750 (Finase® S 2X, Alko)	ADC _F ↑	Riche and Brown (1996)
~12 g	SB, corn	Pretreatment 0.7‰ (Finase®)	WG↔, protein content↔	Ranseyer et al. (1999)

74 g	Canola	Pretreatment? (Finase®)	ADC _P ↔, ADC _{DM} ↓	Mwachireya et al. (1999)
17.9 g	Canola	Diet 0; 500; 1500; 4500; (Natuphos®)	P excretion ↓	Forster et al. (1999)
~225 g	Barley, canola, wheat	Diet 500 (Natuphos®)	ADC _M ↑	Cheng and Hardy (2002)
~170 g	SB	Diet 200; 400; 600; 800; 1000 (Natuphos®)	ADC _P ↑, ADC _F ↑, optim. @ 400	Cheng and Hardy (2003)
~1.7 g	SB	Pretreatment 0 – 3000 (Natuphos®)	ADC _N ↑ optim. @ 1000 – 2000	Wang et al. (2009)
~45 g	Corn, SB, wheat, canola, pea	Diet encapsulated 3000 (Natuphos®)	WG↑, ADC _P ↑, ADC _F ↑, ADC _N ↑	Vandenberg et al. (2011)
~51 g	SB, corn gluten, canola, wheat	Diet 2000 (Natuphos®)	ADC _{DM} ↑, ACD _P ↑, ADC _M ↑	Vandenberg et al. (2012)
35–42 g	SB, wheat	Diet 0; 500; 1500; <i>Pichia pastoris</i> (Syngenta) or <i>E. coli</i> in corn	WG↓, SGR↓; vertebral mineral content↑ with corn PASE	Fox and Davies (2011)
~50 g	SB, Sfl	Diet 0; 1400 (Ronozyme®)	P excretion ↓	Dalsgaard et al. (2009)
~100 g	SB	Diet 0; 500; 1000; 2000; 4000 (Ronozyme®P)	ADC _M ↑, ADC _P ↑, ADC _F ↑	Cheng et al. (2004)
~130 g	DDGS	Diet 0; 300; 600; 900; 1200 (Natuphos®)	ADC _P ↑, ADC _F ↑	Cheng and Hardy (2004)
4.1 g	SB	Diet 0.08% (Ronozyme®P5000)	WG↑, P excretion↓ optim. @ 75% FM substitution	Castro et al. (2011)
1.2 g		Diet 0.04% (Ronozyme®P)	SGR↔, FCR↔, PER↔, P excretion ↓	Ávila et al. (2015)
~52 g		Diet 0; 1500 (Natuphos®)	P retention↑, P excretion↓	Vielma et al. (1998)

(continued)

Table 39.2 (continued)

Species, weight	Plant Feedstuff	Phytase level, FTU kg ⁻¹ (source)	Effects	Reference
~250 g		Diet 0; 1200 (Natuphos®)	WG↔, P excretion↓	Vielma et al. (2000)
~20 g		Diet 0; 500; 1000; 2000; 4000 (Ronozyme®P)	ACD _P ↑, ACD _F ↑	Vielma et al. (2004)
~1 g	Canola, SB	Diet 0.1% <i>Pichia pastoris</i>	WG↔, SGR↔, ACDs↔	Yigit and Keser (2016)
<i>Oreochromis niloticus</i> , <1 g	SB, wheat, corn, cassava	Pretreatment 1000 (Habio®, Sichuan Habio Bioengineering)	WG↓, FCR↓, PER↓, ACD _F ↑, ADC _P ↔	Cao et al. (2008)
13.5 g	SB, wheat, corn	Diet 500; 750; 1000; 1250 (Ronozyme®P)	WG↑, SGR↑, FCR↓, PER↑, ADC _F ↑, ADC _P ↑; optimum @ 750	Liebert and Portz (2005)
42 g	Low P plant-based diets @ 11 °C or 15 °C	Diet <i>E. coli</i> 0; 500; 2500 (Quantum® Blue)	P utilization↑, protein utilization↑, environmental pollution↓ even @ 11 °C	Lee et al. (2020b)
133 g	Diverse plant products	Diet 500; 1000; 2000 <i>Aspergillus oryzae</i> (Ronozyme®HiPhos)	ADC _F ↑, P excretion↓	Verhaac-Trichet et al. (2014)
~42 g	SB	<i>Bacillus paralicheniformis</i>	Growth↑, immunity↑: <i>Il-1</i> ↑, <i>il-4</i> ↑, <i>il-12</i> ↑	Makled et al. (2019)
~2 g	SB, corn, wheat	Diet 0; 500; 1000 (Natuphos®)	WG↑, ADC↑ max @ Ca: P = 0.6:1 & 500 or 1000	Hassaan et al. (2013)
GIFT males, 42 g	SB, wheat, RS, Sfl, corn, rice	Diet 500–1300; <i>Buttiauxella</i> sp., (Danisco animal nutrition, Marlborough, UK), Diet Phytase + xylanase 500–1300	Growth↑, SGR↑, FCR↔	Maas et al. (2018)
Males, ~23 g	Corn, SB, wheat	Diet 500–1300 (Quantum® Blue)	Growth↑↑, SGR↑↑, FCR↔	Abo Norag et al. (2018)
~68 g	SB	Pretreatment 5000 (Natuphos®)	ADC _P ↔, availability of essential AAs↓	Riche et al. (2001)
~12 g		Diet 500; 1000 (Phytasonovo® CT, Novo Nordisk, Denmark)	SGR↑, FCR↓	Goda et al. (2002)

~41 g	Wheat bran Sfl meal Citrus pulp	Diet 1000 (<i>Battiauxella</i> sp.; Danisco Animal Nutrition)	WG↑ WG↑ WG↔	Maas et al. (2019)
<i>Pagrus major</i> , 1.3	SB	Diet 2000 (Ronozyme®)	P requirement met in P-deficient diets	Laining et al. (2012)
<i>Pangasianodon hypophthalmus</i> , ~1.8 g	SB, corn, rice, wheat	Diet 0 ; 150; 300; 450; 600 (Natuphos®)	SGR↑, EFU↑, PER↑, ADC _P ↑, ADC _F ↑; optim @ ~300; SR↔	Rachnawati and Samidjan (2018)
<i>Pelteobagrus fulvidraco</i> , 3.5 g	SB, RS, CS, wheat	Diet 1000 (<i>E. coli</i> , Sunhy Biology Company, Wuhan, China)	Sparing of <50% dietary P	Cheng et al. (2016)
<i>P. fulvidraco</i> , 1.3 g		Diet 1000 (Cibenza® Phos, Novus International, Shanghai, China)	SGR↑, WG↑, ADC _F ↑, ADC _P ↑, ADC _{DM} ↑, ACD _M ↑, FCR↓, P excretion↓	Zhu et al. (2014)
<i>Piaractus mesopotamicus</i> , ~14 g	FM-free, mixed plant-based diet	Diet 500 ; 1000; 2000 microbial PASE (Ronozyme®)	Growth↔, blood parameters↔	Bacchetta et al. (2020)
<i>Rhamdia quelen</i> , 237 ± 54 g	Corn, sorghum, wheat	Diet 0 ; 1500 (Ronozyme®)	ADC _F ↑ corn, wheat bran ADC _{En} ↑ corn, sorghum	Signor et al. (2016)
~16 g	Sfl, partial substitution	Diet 0 ; 1500; <i>Aspergillus niger</i> (Natuphos®)	WG↔, hematological parameters↔	Weiler et al. (2019)
<i>Salmo salar</i> , ~100 g	SB	Pretreatment (Natuphos®)	ADC _F ↑, ADC _M ↑	Storebakken et al. (1998)
~140 g		Pretreatment 2900 or coating , <i>E. coli</i> (Quantum 5000®)	ADC _F ↑, ADC _P ↔, mineral body content↓; coating inefficient	Denstadli et al. (2007)
~100 g	Canola	Diet 0 ; 2000 (Natuphos®)	ADC _F ↑	Sajjadi and Carter (2004)
~45 g	SB	Diet 250 ; 500; 1000; 4000; pretreatment 250; 500; 1000 (Natuphos®)	WG↓ < 1000, SR↔, ADC _F ↑ independent of PASE application	Carter and Sajjadi (2011)
~235 g	SB, wheat, <i>Faba</i> beans, corn, RS Sfl	Diet 2800 <i>Aspergillus oryzae</i> (Ronozyme®)	ADC _P ↑, P excretion↓	Greiling et al. (2019)

(continued)

Table 39.2 (continued)

Species, weight	Plant Feedstuff	Phytase level, FTU kg ⁻¹ (source)	Effects	Reference
<i>Sebastes schlegeli</i> , ~7.2 g	SB	Diet 1000; 2000; pretreatment 1000 (Natuphos®)	ADC _F ↑, ADC _P ↔, ADC _{DM} ↔	Yoo et al. (2005)
<i>Sparus aurata</i> , ~52 g	SB, corn	Diet 0.02%, <i>Aspergillus ficuum</i> (Sigma P-9792®)	ADC _N ↔	Robaina et al. (1998)
~58 g	SB, pea, broad bean, Sfl	Diet 2500, <i>Pichia pastoris</i> (Quantum®, AB Enzymes, Darmstadt, Germany)	ADC _F ↑, stomach protease↑, intestinal protease↔	Morales et al. (2014)
<i>Tandanus tandanus</i> 6.0 g 12.7 g	SB Replac 30% FM Replac 45% FM	Diet (Ronozyme®) 1000 – 3000 3000	WG↑, P excretion↓ WG↔, P excretion↓	Huynh and Nuegoda (2011)

↑ support/improvement/increase; ↔ no apparent effect; ↓ decrease/reduction; FCR, food conversion ratio; FI, feed intake; WG, weight gain; SGR, specific growth rate; PER, protein efficiency ratio; ADC, apparent digestibility coefficient; ADC_{DM}, ADC of dry matter; ADC_{En}, ACD of energy; ADC_F, ADC of phosphorus; ADC_L, ACD of lipids; ADC_{mN}, ADC of macronutrients; ADC_N, ADC of minerals; ADC_P, ADC of protein; EFU, efficiency of feed utilization; SR, survival rate; DDGS, dried distillers grains with solubles; RBC, red blood cell count; WBC, white blood cell count; PUFA, polyunsaturated fatty acid; AST, serum aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CHOL, cholesterol; LYZ, lysozyme activity; TAC, total antioxidant capacity; CS, cottonseed; RS, rapeseed; SB, soybean; SBM, soybean meal; Sfl, sunflower

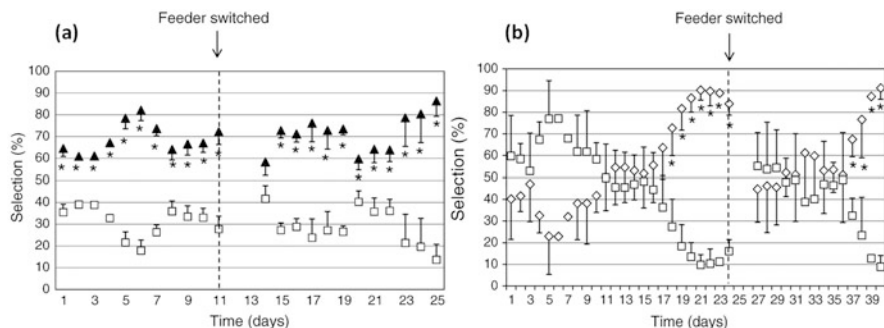


Fig. 39.3 Dietary selection experiments in European seabass juveniles. **(a)** Selection of fishmeal, FM (▲), and soybean meal, SBM + 1500 IU kg⁻¹ (◇), and **(b)** selection of soybean meal, SBM (□), and SBM + 1500 IU kg⁻¹ (◇), considering the total of diets as 100% and the corresponding standard errors (SEM) of three groups (11 seabass per group), in the 22 days and two phases of the experimental period. After feed demand had stabilized (day 10 and day 23, respectively), feeds were switched between feeders. The preference for the complete fishmeal diet, represented by the asterisk, was significant ($P < 0.05$). (From Fortes-Silva et al. (2011b), with permission from Elsevier)

SBM) supplemented with PAs, SBM+) (Fig. 39.3a). It can differentiate even between isoproteic diets containing SBM or SBM + PAs (SBM+) (Fig. 39.3b). After switching the diets between feeders for the treatments, fishes quickly respond to the preferred diet, and the pattern of intake is reestablished. Supplemented PAs improve the nutritional proprieties of a plant-based diet with increased P and Ca retention in bones.

This study agrees well with an earlier one of the same laboratory, which reported that omnivorous tilapia prefers a diet supplemented with exogenous PAs (Fortes-Silva et al. 2010). Nile tilapia clearly prefers a diet with 1500 IU kg⁻¹ PAs, compared to a control diet or a diet with more PAs.

39.1.1 Limitations and Concerns

Beneficial effects of PAs addition on protein and mineral digestibility are observed in many fish species. Despite the growing body of information on P sparing by dietary PAs in plant-based feeds, there remain discrepancies in the results, because of lacking success, or even adverse outcomes (Table 39.2): refer to *L. vannamei*, *Marsupenaeus japonicus*, *C. carpio*, *I. punctatus*, *O. mykiss*, *O. niloticus*. Generally, such discrepancies can be attributed to differences in the PAs treatment, diet composition, selected species, or strain of the selected species. However, this statement is too coarse and does not really fill the gap of lacking mechanistic studies. Many studies worked with the famous developmental stage “juveniles,” disregarding that, in a given species, the tested weight of juveniles can span more

than two orders of magnitude as seen in *O. mykiss*, *I. punctatus*, and *O. niloticus*. However, the requirement of a nutrient is not constant and changes not only with ontogenetic development, but also with growth/age (Koven et al. 1993; D'Abramo 1998; Cahu and Zambonino Infante 2001; Pfeiffer 2017).

Table 39.2 spells out significant differences in suitability of dietary PAs supplementation. Romano and Kumar (2018) discuss huge differences in the efficacy of PAse in the same species: when using plant-based diets, PAse at 3000 or 2000 FTU kg⁻¹ (microbial 6-PAse; *Aspergillus oryzae*) improves growth, feeding efficiency, and digestibility of various ingredients in *O. mykiss* (Vandenberg et al. 2012; Verlhac-Trichet et al. 2014). In contrast, lower doses of 1000 or 1400 FTU kg⁻¹ in the same species have no effect on growth or feeding efficiencies when using a plant-based diet, but do improve P digestibility (Dalsgaard et al. 2009; Yigit et al. 2018). These discrepancies may be related to differences in the PAse levels; however, this assumption does not explain inconsistent findings in tilapia, in which PAse (1000 FTU kg⁻¹; *A. niger*) improves P utilization and modulates the gut microbial community but causes intestinal inflammation and upregulation of stress-related genes (Hu et al. 2016). This combination of simultaneous beneficial and harmful effects in tilapia is considered the cause for lacking growth improvement. This issue will be revisited below.

Furthermore, two separate studies in tilapia show that PAse additions in SBM-based diets reduce lysine and alanine digestibility as well as protein utilization (Riche et al. 2001). In contrast, Liebert and Portz (2005) found that a similar phytate type and level in a SBM-based diet improves tilapia growth and nutrient utilization. Possibly, this discrepancy is due to differences in the pretreatment: Liebert and Portz (2005) mixed PAse directly with the dietary ingredients, whereas Riche et al. pretreated SBM with PAse by incubation and activated by citrate addition. Most likely, the degradation of PA has made the amino acids (AAs) susceptible to destruction by high heat conditions during pellet manufacture.

Some more mechanistic reasons may apply. Morales et al. (2011) presented evidence that the PAse sources matter. Under the acidic-pepsin environment of stomachs of fishes, such as rainbow trout, *E. coli* PAse is more active and stable than PAse from *Peniophora lycii* (plant pathogen fungus) (Fig. 39.4). The presence of digestive proteases determines the efficacy of PAses. Actually, the stability of most plant and fungal PAses decreases dramatically at pHs below 4 and above 7.5, while the majority of the corresponding microbial enzymes are stable even at pHs above 8.0 and below 3.0 (Greiner and Konietzny 2006).

Dalsgaard et al. (2009) revisited another critical issue: Phytase supplementation is advantageous to fishes and the environment only if supplemented to low-P diets containing a large share of plant-derived protein. Conversely, the authors demonstrate that fungal PAse should not be supplemented to diets in which the available P level already meets the demand of the fishes, because this will lead to a significant increase of P waste output.

Furthermore, information is lacking about the site at which the enzyme acts on the substrate within the gastrointestinal tract. As mentioned, PAse has optimal activity in alkaline as well as acidic pH ranges. The pH of the gastrointestinal tract of fish

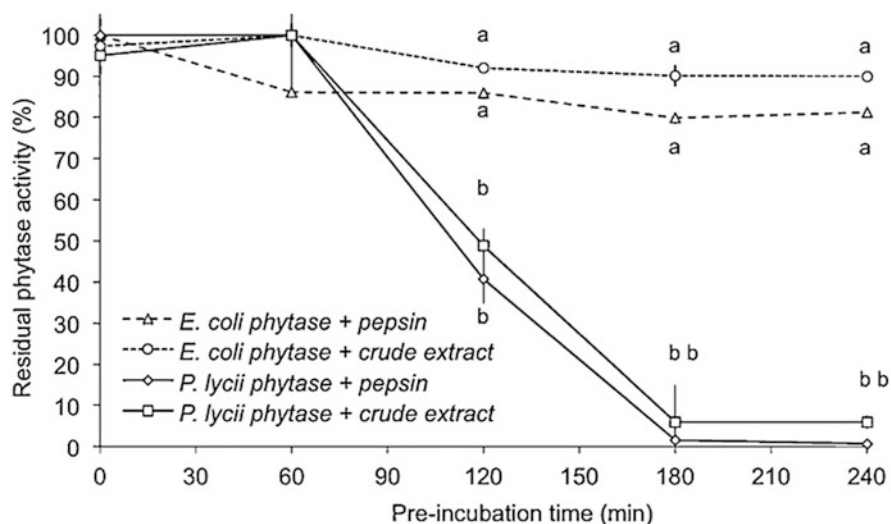


Fig. 39.4 Residual activity of *E. coli* and *Peniophora lycii* phytase in the presence of pepsin or gastric crude extract from rainbow trout stomach hydrolysis throughout incubation time. The incubation was performed by adding 1 FTU phytase to a protease solution with 5000 U from porcine pepsin or gastric crude extract from fish, performed at pH 2.0 (HCl); 16 °C. The results are plotted as means \pm SE (triplicates). Different letters, for each time, indicate significant differences ($P < 0.05$) between phytases. (From Morales et al. (2011), with permission from Elsevier)

depends on the presence or absence of a stomach. Hence, PAs activity is species-specific. Moreover, the use of PAs as a feed additive is limited due to several factors like inactivation at the high temperatures required for pelleting (>80 °C), loss of activity during storage, and narrow optimum pH range. The inherent PAs content of various plant ingredients needs to be studied in detail. Hence, lots of empirical data are required to define optimal doses of dietary PAs for different species (Debnath et al. 2005b).

Do benefits to aquatic animals exist from PAs super-dosing? From terrestrial livestock, it is well understood that super-dosing increases productivity via increased P and Fe availability (Ushasree et al. 2017; Laird et al. 2018). In contrast, Li et al. (2019a) reported that PAs super-dosing does not have additional benefits in terms of growth promotion and anemia prevention in hybrid catfish. Even more alarmingly, Hussain et al. (2016) showed that the apparent digestibility of all studied minerals in *Labeo rohita* fingerlings fed canola meal-based diets, supplemented with PAs, follow optimum curves. The optimal PAs level (740 FTU kg^{-1}) results in a P digestibility of 71%, whereas higher PAs levels reduce it to some 50%. Supportingly, Biradar et al. (2017) reported that dietary excess PAs reduces growth and protein efficiency in *M. rosenbergii* (Table 39.2). The same applies to growth in *P. monodon* (Rachmawati and Samidjan 2016), or *C. mrigala* (Hussain et al. 2017b) (Table 39.2).

Mechanisms for adverse effects of dietary PAs surplus are going to be increasingly well understood. One potential mechanism can be gut stress and inflammation (Hu et al. 2016): in hybrid tilapia, the supplementation of microbial PA (1000 FTU kg^{-1}) causes upregulation of pro-inflammatory *tnf- α* and *tgf- β* and stress-related *hsp70*. This obviously enhanced intestinal inflammation and stress contributes to the failure of PA as a beneficial feed additive. Moreover, it points out that this aspect may have been overlooked in many past studies. Vice versa, if the hybrid tilapia findings should remain the unlikely single case of adversity, it would be crucial to figure out the underlying mechanism of high PA tolerance in the other species.

39.2 Depolymerizing Enzymes

Plant-based ingredients used as a replacement of FM in diets for farmed fishes typically contain remnants of dietary fibers (NSP and lignin). Dietary fibers are poorly utilized by carnivorous fish because they lack the corresponding endogenous enzymes (Krogdahl et al. 2005), and because their intestinal microflora has limited capacity to degrade this stuff. Rather than being a dietary nutrient, dietary fibers appear to have various antinutritive effects by reducing the uptake of other nutrients (Krogdahl et al. 2005). Therefore, various exogenous, depolymerizing enzymes have been applied to improve the digestibility of plant-based diets by assisting in the breakdown of NSP (Dalsgaard et al. 2016).

39.2.1 Carbohydrases

Carbohydrases (CHOases) are enzymes that catalyze the breakdown of polymeric carbohydrates into simple sugars. In aquaculture, the use of carbohydrases has not been as common as in poultry and swine nutrition; therefore, research on exogenous CHOases supplementation in aquaculture nutrition is not extensive (Castillo and Gatlin 2015).

In fishes, the presence of digestive enzymes that specifically hydrolyze the β -glycosidic bonds of NSP seems to be very low or nonexistent (NRC 2011). Some of these NSP (Table 39.3) are part of cell walls, thus shielding substrates from contact with the digestive enzymes, or part of cell contents where their presence may interfere with digestion and absorption due to their chemical nature. Furthermore, NSP are known to increase the hydrolysis of bile salts and hence reduce fat utilization. Their cleavage, in turn, improves fat digestibility (Castillo and Gatlin 2015).

In addition to enhanced fat metabolism, CHOases also improve energy utilization by shifting absorption of energy-yielding nutrients to the proximal intestine. The shift in nutrient utilization to the more proximal intestine decreases host-microbe competition for nutrients (\rightarrow AAN I "The Intestinal Microbiota" (Steinberg 2018))

Table 39.3 Classification of non-starch polysaccharides found in commonly used plant feedstuffs for aquaculture feeds (from Sinha et al. (2011) and Castillo and Gatlin (2015) with permissions from Elsevier)

Category	Monomeric residue	Linkage	Plant feedstuffs
Cellulose	Glucose	β —(1 \rightarrow 4)	Cereals (barley, corn, wheat), legumes (SB, lupin), cottonseed, and rapeseed/canola
Noncellulosic polymers			
Arabinoxylans	Arabinose, xylose	β —(1 \rightarrow 4)-linked xylose units	Cereals (barley, corn, wheat, oat, sorghum)
Mixed-linked β -glucans	Glucose	β —(1 \rightarrow 3) and β —(1 \rightarrow 4)	Barley, oat
Pectic polysaccharides			
Arabinans	Arabinose	α —(1 \rightarrow 5)	Cereal coproducts
Galactans	Galactose	β —(1 \rightarrow 4)	Lupin, SB
Arabinogalactans (type I)	Arabinose, galactose	β —(1 \rightarrow 4)	Grain legumes (SB, lupin)
Arabinogalactans (type II)	Arabinose, galactose	β —(1 \rightarrow 3,6)	Canola/rapeseed cotyledon

and ensures availability of nutrients where absorption efficiency is greatest. Furthermore, CHOase supplementation increases gut health in animals on high-NSP diets. NSP-induced increases in digesta viscosity encourage slower diffusion rates, accumulation of particulate matter for microbial adhesion, and elevated flow of solids. These factors encourage slow shedding of microorganisms and increased proliferation of harmful bacteria. Therefore, by reducing digesta viscosity, CHOase supplementation reverses these adverse effects. Moreover, by increasing the proportion of lactic and organic acids, reducing ammonia production, and increasing volatile fatty acid (short-chain FA) concentration, indicative of hydrolytic fragmentation of NSP (Chap. 26), CHOases promote and support growth of beneficial bacteria, thereby improving gut and overall health of the animal, even in enzyme mixtures (Table 39.6) (Castillo and Gatlin (2015) and references therein). Studies on the effects of dietary CHOases are collected in Table 39.4.

Noteworthy, Shi et al. (2017) showed that *Chlorella* sp. meal can replace 100% of FM protein in diets for crucian carp (*Carassius carassius*) indicated by acceptable growth and feed utilization provided that cellulase is added in appropriate quantities ($<1.5 \text{ g kg}^{-1}$). This fact indicates that additional nutrients can be mobilized by this and most likely other omnivorous freshwater fishes.

39.2.1.1 Glucanases

Glucanases break down glucans, polysaccharides made of D-glucose subunits. Glucans comprise α - and β -forms of the glycosidic bond. Major α -glucans are

Table 39.4 Effects of exogenous carbohydrases in several farmed species

Species	Enzymes	Feedstuff(s)	Selected effects	Reference
<i>Litopenaeus vannamei</i>	NSPase*	SBs	Growth↔, feed digestibility↔	Qiu and Davis (2017a)
	Xylanase + β -mannanase		Growth↑, nutrient utilization↑	Yao et al. (2019)
<i>Bidyanus bidyanus</i>	α -amylase (Natustarch®)	Wheat starch	Digestibility of raw wheat starch↑	Stone et al. (2003)
	β -glucanase+- β -xylanase (Natugrain-blend®)	Wheat + dehulled lupin	No effect	
<i>Carassius carassius</i>	Cellulase, max. 1.0–1.5 g kg ⁻¹	<i>Chlorella</i>	Growth↑, feed utilization↑	Shi et al. (2017)
<i>Cyprinus carpio</i>	Multienzyme (Hostazyme X® NOREL-Misr co., Giza, Egypt)	FM-reduced, SB, sun-flower, wheat	WG↑, intestinal health↑, AOC↑ Optim. @ 0.1%	Monier (2020)
<i>Ctenopharyngodon idella</i>	Cellulase	Duckweed & wheat flour	Growth↑, digestive enzymes↑, intestinal microbiota↑	Zhou et al. (2013)
	Xylanase	Plant-derived	Growth↑, intestinal microbiota↑, enteritis↓	Jin et al. (2020)
<i>Huso huso</i>	Mix: PASE, lipase, xylanase, cellulase, endo-1,3 (4)- β -glucanase, α -amylase, protease (Kemzyme-Kemin industries, Belgium)	SBM	SBM-induced histopathological impairments↓	Sohrabnezhad et al. (2017)
<i>Diplodus sargus</i>	NSPase (Natugrain® TS)	SBs	Nutrients utilization↑, amylase↑, lipase↑, protease↔	Magalhães et al. (2016)
<i>Labeo rohita</i>	α -amylase	Non-gelatinized corn flour	Growth↑, muscle growth↑, ADC _{DM} ↑, immunity↑	Kumar et al. (2005)
		Gelatinized corn flour ^a	No effect	
	Xylanase + PASE 1:1	De-oiled rice bran	WG↓, FCR↑	Ranjan et al. (2018b)
<i>Lateolabrax japonicus</i>	NSPases (WX + VP, DSM, Shanghai)	White FM, SBM, RS meal, peanut meal, meat & bone meal	Growth↑, N retention↑, NH ₄ ⁺ excretion↓	Ai et al. (2007)

(continued)

Table 39.4 (continued)

Species	Eenzymes	Feedstuff(s)	Selected effects	Reference
<i>Lutjanus stellatus</i>	NSPase	<i>Gracilaria lemaneiformis</i>	WG↑, SGR↑, FER↑	Zhu et al. (2016)
<i>Oncorhynchus mykiss</i>	NSPase	SBs, lupins	WG↔, nutrient Digestibility↑	Dalsgaard et al. (2016)
<i>Oreochromis niloticus</i> × <i>O. aureus</i>	NSPase	SB, RS, CS meal	SGR↑, ADC _{DM} ↑	Li et al. (2009)
<i>O. niloticus</i>	Cellulase	Canola	WG↔, nutrient digestibility↔	Yigit and Olmez (2011)
	Xylanase	Wheat bran Sfl meal Citrus pulp	WG↑ WG↑ WG↔	Maas et al. (2019)
<i>Mugil cephalus</i>	Pretreatment with glucanase + Pase	Multi-plant preparation	AA, sugar availability↑, P availability↓	Martínez et al. (2019)
<i>Salmo caspius</i>	2 NSPases (Natuzyne®), β-mannase (Hemicell®)	Commercial trout diet	WG↑, FER↑	ali Zamini et al. (2014)
<i>Siganus canaliculatus</i>	NSPase	Macroalgae	WG↑, innate immunity↑, pathogen resistance↑	Xie et al. (2018)
<i>Sparus aurata</i>	NSPase (Natuzyne®TS, BASF)	100% replacement of SBM by DDGS	FER↑, nitrogen retention↑, energy retention↑, hepatic LPO↓	Diógenes et al. (2019)
<i>Trachinotus carolinus</i>	NSPase (Rovabio Excel LC®)	Several SB products	Growth↔	Roe et al. (2019)
<i>T. marginatus</i>	Rovabio® MAX AP ^b	SB, wheat	WG↑, SGR↔, FCR↔, PER↔, survival↔	Simião et al. (2018)

↑ support/improvement/increase; ↔ no apparent effect; ↓ decrease/reduction; ADC_{DM}, apparent digestibility coefficient of dry matter; NSPase, non-starch carbohydrases enzyme complex; WG, weight gain; SGR, specific growth rate; FER, feed efficiency ratio; FCR, food conversion ratio; PER, protein efficiency rate; DDGS, distillers dried grains with solubles; LPO, lipid peroxidation; WX, commercial product mainly xylanase; VP, commercial product including mainly glucanase, pentosanase, and cellulase; RS, rapeseed; SBM, soybean meal; Sfl, sunflower; AA, amino acid
^aDuring the gelatinization process, starch granules are modified making the digestion more complete (Chap. 21 “Box: Raw vs. Gelatinized Starch”)

^bCocktail of endo-1,4-xylanase, α-arabinofuranosidase, β-xylosidase, feruloyl esterase, endo-1,5-arabinase, endo-1,4-glucanase [cellulase], cellobiohydrolase, β-glucosidase, polygalacturonase, pectinesterase, rhamnogalacturonase, endo-1,4β-mannanase, 6-phytase, aspartate protease, and metalloprotease

glycogen (α -1,4- and α -1,6-glucan) and starches, such as amylose (α -1,4-glucan, unbranched), or amylopectin (α -1,6-glucan, branched) (Kossmann and Lloyd 2000; Ball and Morell 2003); and major β -glucans comprise cellulose (β -1,4-glucan), chitin (β -1,4-glucan), or laminarin (β -1,3- + β -1,6-glucan) (Read et al. 1996; Klemm et al. 2005; Rinaudo 2006). Dalsgaard et al. (2016) showed that supplementing β -glucanase to a diet for juvenile rainbow trouts containing 344 g kg⁻¹ SBM causes a breakdown of NSP structures, followed by an increase in apparent digestibilities of mannose, galactose, and uronic acids, but not of xylose.

Amylases

Amylases are amylose (starch) hydrolyzing enzymes. Most studies used multi-enzyme complexes as a dietary supplement (Castillo and Gatlin 2015); the scarce single enzyme studies with dietary amylases reveal mixed results. Feeding success may be lacking as found in Atlantic salmon parr fed a diet containing a commercial α -amylase (Carter et al. 1992), or starch becomes fully available only after pretreatment—even to omnivorous fishes, such as silver perch (*Badyanys badyanus*) (Stone et al. 2003) (Chap. 21).

The addition of α -amylase to diets of rohu carp improves WG, muscle growth, apparent digestibility coefficient of dry matter, and immunity (Table 39.4) (Kumar et al. 2005). In contrast to previous findings in silver perch (Stone et al. 2003) or rainbow trout (Bergot and Breque 1983), only non-gelatinized corn flour displays beneficial effects (Kumar and Chakravarty 2018).

The few available studies on dietary amylase in shrimp feeds report consistently beneficial effects. Maugle et al. (1983) found in *M. japonicus* that any α -amylase supplementation increases growth and modulates hepatopancreatic glycogen contents. Up to 30% of dietary protein (casein) can be substituted by starch without substantial reduction in WG or feed efficiency, if α -amylase is added. In the same line of evidence, postlarvae of *M. rosenbergii* fed a diet amended with 0.2% commercial amylase enhances food conversion, assimilation efficiency, metabolism, and growth efficiency (Patil et al. 2006).

Cellulases and Hemicellulases

Cellulases comprise several enzymes produced mainly by fungi, bacteria, protozoans, and a number of invertebrates that catalyze the decomposition of cellulose and of some related polysaccharides (Cragg et al. (2015), →AAN III "Plant Materials"). They are widely distributed in invertebrates that feed on algae and macrophytes (Linton 2020) (Chap. 22).

Many fish species are unable to secrete cellulases and high levels of dietary fibers result in low energy yield and sub-optimal protein utilization of plant feed even with high protein contents, such as canola meal. Therefore, a supplementation with cellulases appears to be the method of choice to overcome this obstacle. However,

corresponding studies are rare and show only mixed success. For instance, the supplementation of canola meal-containing feed with cellulases for rainbow trout or Nile tilapia fry turns out unsuccessful (Yigit and Olmez 2011; Yigit and Keser 2016). In contrast to carnivorous trout and omnivorous tilapia, herbivorous grass carp responds positively to dietary cellulase addition by increased digestive enzyme activities, improved intestinal flora, and promoted growth (Zhou et al. 2013). Taken together, the application of cellulases is far from being fully appropriate to digest ingested fibers; therefore, the knowledge of cellulase supplementation needs to be further developed.

Chitinases

Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin, a long-chain polymer of *N*-acetylglucosamine (a derivative of glucose). Chitinases are found not only in chitin-containing organisms but also in widely diverse organisms that are not composed of chitin (Hamid et al. 2013). In these animals, they are used not only for digestion purposes. This may be the major reason, why chitinases as feed supplement were scarcely tested in the past (Mali et al. 2004). Studying a cnidarian, these authors found that its chitinase takes action in host defense against chitinous pathogens, but not in digestion. After this pioneering paper, evidence supporting this chitinase-based defense hypothesis has been presented for *M. japonicus* (Pan et al. 2005), *M. rosenbergii* (Ramalingam and Ramarani 2006), *Crassostrea gigas* (Badariotti et al. 2007), *Gorgonia ventalina* (Douglas et al. 2007), *L. vannamei* (Huang et al. 2010), *Mytilus galloprovincialis* (Venier et al. 2011), *Exopalaemon carinicauda* (Duan et al. 2014), *Macrophthalmus japonicus* (Nikapitiya et al. 2015), *P. monodon* (Zhou et al. 2018), or *Portunus trituberculatus* (Song et al. 2020). Furthermore, it is found even in Arctic lamprey (*Lethenteron camtschaticum* (*Lampetra japonica*)) (Liu et al. 2009).

The few available results of dietary chitinase are intriguing. Zhang et al. (2012) produced a recombinant protein of grouper (*Epinephelus coioides*) chitinase1 and added it to a diet containing chitin from shrimp shells. The recombinant protein displays beneficial effects on growth, immune defense, and oxidative burst (Fig. 39.5); the mRNA expression and protein secretion of grouper *chitinase1* and *chitinase2* are stimulated in the spleen in response to bacterial lipopolysaccharide (LPS) challenge. This indicates the existence of an innate pathway for local defense against chitin-containing organisms. To a certain extent, even pathogens (*E. coli* and *Staphylococcus aureus*) are inhibited by the recombinant protein of grouper chitinase1. This is another confirmation of the above hypothesis established by Mali et al. (2004) and should be taken as an incentive to study this pathway of immune response more thoroughly in finfishes.

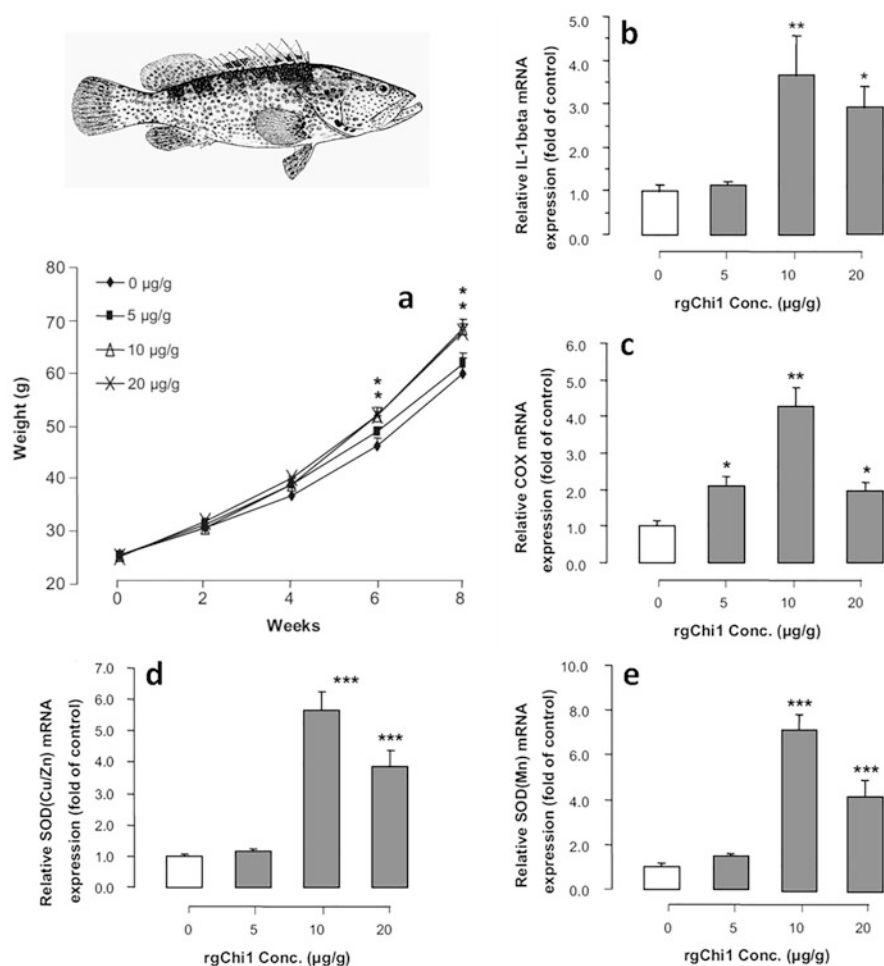


Fig. 39.5 Weight gain (a), expressions of pro-inflammatory cytokine *il-1β* (b) and pro-inflammatory *cyclooxygenase-2* (*cox-2*) (*Cyclooxygenase-2* or *prostaglandin-endoperoxide synthase 2* is an enzyme involved in the conversion of ARA to prostaglandin H_2 (Chap. 27) an important precursor of prostacyclin, which is expressed in inflammation) (c) in the spleen and expression of two *sods* (d, e) in the liver of orange-spotted grouper fed diet containing grouper chitinase1 (rgChi1) at three different dosages for 8 weeks. Each value is a mean of three replicates ($n = 20$). ** indicate significant differences (one-way ANOVA, followed by a Duncan test, $*P < 0.05$, $**P < 0.01$). (From Zhang et al. (2012), with permission from Elsevier; image credit FAO)

39.2.1.2 Xylanases

Hemicelluloses are one major component of plant cell walls. Their linear polysaccharide β -1,4-xylan are broken down by xylanases into xylose (Beg et al. 2001).

Only a few studies address the supplementation with xylanases, again with mixed results. In *B. bidyanus*, the dietary addition of a mixture of β -glucanase and β -xylanase does not have any effect on dry matter, energy, or protein digestibilities of diets or ingredients (Stone et al. 2003). In contrast, Jiang et al. (2014) found improved WG, food conversion, protein efficiency, and production of proteins and lipids as well as Ca and P retention in juvenile Jian carp with increasing levels of xylanase. Furthermore, dietary xylanase enhances also intestinal enzyme activities and influences the balance of intestinal microflora. Comparable beneficial results are also found in juvenile large yellow croaker with improved growth, optimized intestinal structure and microbiota composition, and enhanced carbohydrate utilization (Luo et al. 2020).

Hassaan et al. (2019b) reported that the addition of xylanase to the diet of Nile tilapia containing a high levels of sunflower meal improves growth, digestive enzymes, nutrient digestibility, nutrient retention, and structure of liver and intestine. In contrast, Maas et al. (2018) did not find accelerated growth in Nile tilapia on a diet based on mixed plant sources, although enhanced digestibilities of dry matter, crude protein, carbohydrates, and energy are recorded.

39.2.2 Pectinases

Pectinases are enzymes that break down pectin, a polysaccharide in plant cell walls. The supplementation of canola meal-containing feed with pectinases for rainbow trout fry is not successful in terms of growth promotion (Yigit and Keser 2016). On the contrary, pectinases in an enzyme mixture (containing protease, amylase, xylanase, β -glucanase, pectinase, cellulase, and PAse) supplemented to a non-fishmeal diet enhances feed intake and growth in adult red seabream (Matsukura et al. 2017). However, the specific effect of the supplemented pectinase remains obscure.

39.3 Proteases

Proteases are enzymes that catalyze the hydrolysis of proteins; usually several proteolytic enzymes are necessary for the complete breakdown of polypeptides to their AAs (IUPAC 2005–2017). Dietary proteolytic enzymes in aquaculture have gained increased attention in recent years, because of the need for better utilization of proteins from non-marine sources (Philipps-Wiemann 2018). Again, the results are mixed and recent studies are listed in Table 39.5.

Table 39.5 Selected recent studies using exogenous proteases in aquatic animals mainly fed plant-based diets

Species	Exogenous protease, level	Dietary plant basis	Effects	Reference
<i>Eriocheir sinensis</i>	125 → 175 mg kg ⁻¹¹ (AG175® JEFO Nutrition, Saint-Hyacinthe, QC, Canada)	Low FM diet with mixed plant protein sources	Nutrient retention↑	Kabir Chowdhury et al. (2018)
<i>Litopenaeus vannamei</i>	125 → 175 mg kg ⁻¹ (AG175®)	Low FM diet with SBM and peanut meal	Resistance against <i>Vibrio parahaemolyticus</i> ↑	Song et al. (2017)
	175 mg kg ⁻¹ (AG175®)	Commercial diet (40% crude protein, Tongwei Feed Co. LTD, Suzhou, China)	WG↑, nutrient utilization↑	Yao et al. (2019)
<i>Acipenser ruthenus</i>	Bromelain ^a , 0, 10, 20 g kg ⁻¹	Commercial feed 54% protein	Immunity↑	Wiszniewski et al. (2019)
<i>Clarias gariepinus</i>	1250 IU ^b	Commercial dry feed (Skretting “Gemma micro 150”)	Survival↑, growth↑	Kemigabo et al. (2019)
<i>Oncorhynchus mykiss</i>	250 mg kg ⁻¹ (Poultrygrow-250®)	Canola–pea Flax–pea	Apparent digestibility↑ Apparent digestibility↔	Drew et al. (2005)
	228 mg kg ⁻¹	Dehulled, solvent extracted SBM, Sfl meal, RS meal	Apparent digestibility of all nutrients in SB-based diets↑	Dalsgaard et al. (2012)
	500, 1000 mg kg ⁻¹	44% dehulled hexane extracted SB	Apparent digestibilities↔	Yigit et al. (2018)
	175 mg kg ⁻¹ (Jefo Nutrition Inc., Quebec, Canada)	Mixed protein sources	Apparent digestibilities↑, except crude protein	Lee et al. (2020a)
	Seafood processing wastes	Regular diet	Protease↑	Rodriguez et al. (2021)
<i>Oreochromis niloticus</i>	500 mg kg ⁻¹	FM-free diet, mixed plant sources	Growth↑, gut microbes↑, immunity↑	Hassaan et al. (2021)

(continued)

Table 39.5 (continued)

Species	Exogenous protease, level	Dietary plant basis	Effects	Reference
	Serine protease, 0, 200, 400 mg kg ⁻¹ (Ronozyme ProAct)	FM-free diet, 26, 28% protein	WG↑, protein digestibility↑	Ragaa et al. (2017)
	Seafood processing wastes	Regular diet	Protease↑	Rodriguez et al. (2017)
	175 mg kg ⁻¹ (AG175®)	FM-free diet	WG(↑)	Li et al. (2019b)
	0 → 2500 IU (Huvepharma, Antwerp, Belgium)	Cottonseed meal	WG↑, growth-related genes↑	Hassaan et al. (2019a)
	0 → 5000 IU	FM-free diet, mixed plant sources + 5 g kg ⁻¹ malic acid	WG↑ Chymotrypsin↑, trypsin↑, lipase↑	Hassaan et al. (2020)
<i>O. niloticus</i> GIFT	5700 IU ^c	FM-free diet, mixed plant sources	Intestinal health↑, innate immunity↑, pathogen resistance↑	Wu et al. (2020)
<i>O. niloticus</i> × <i>O. aureus</i>	1000 IU	FM-free diet, mixed plant sources	Growth↑	Huan et al. (2018)
<i>Pangasianodon hypophthalmus</i>	Pepsin, 0 → 1.0 g kg ⁻¹	Mixed plant sources	Growth↑, feed utilization↑, red blood & white cells↑	Islam et al. (2021)

↑ support/improvement/increase; ↔ no apparent effect; ↓ decrease/reduction; FM fishmeal; SBM soybean meal, WG weight gain; RS rapeseed

^aBromelain is a proteolytic enzyme extract derived from pineapples

^bOne definition is that unit (U) of protease is defined as the amount of enzyme that hydrolyzes 10 g L⁻¹ casein solution to yield 1 μg of tyrosine per minute at 40 °C at a pH of 7.5

^cExcessive protease exerts adverse effects on growth and health due to the damage to intestinal villi

39.4 Lipases

Lipases are esterases catalyzing the hydrolysis of lipids. Only a few studies consider exogenous lipases as a feed additive. An interesting approach is published by Ran et al. (2015): they cloned *lipG1*, a novel *Acinetobacter* lipase gene, from the intestinal of common carp, expressed it in *E. coli*, and evaluated the enzyme as an aquafeed additive for juvenile carps. It turns out that the supplementation of LIPG1 improves gut and hepatopancreas lipase activity of individuals on a palm oil diet. Consistently, improved FCR and growth are found in the LIPG1 group.

Adequate dietary supplementation with exogenous lipase improves even the immunity of fishes. This is shown in grass carp (Liu et al. 2016), Japanese eel

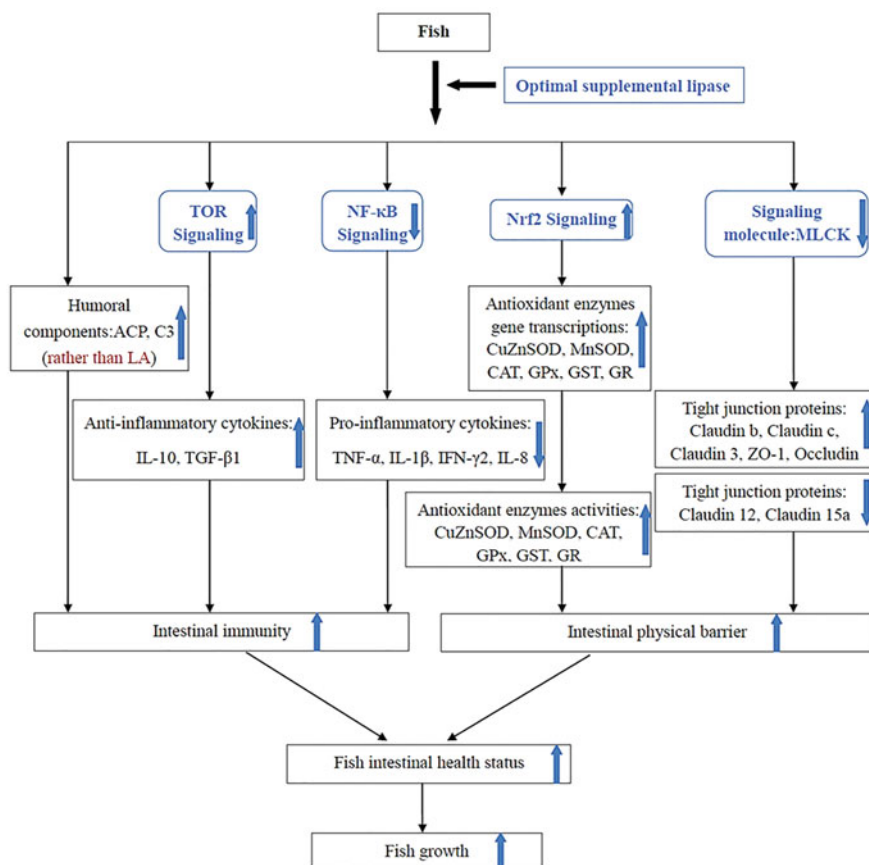


Fig. 39.6 The potential action pathways of supplemental lipase regulated intestinal health of young grass carps. (From Liu et al. (2016), with permission from Elsevier)

(Zheng et al. 2019), or hybrid sturgeon (Fei et al. 2018). The latter group reported that lipase of bacterial (*B. amyloliquefaciens*) or fungal origin (*Yarrowia lipolytica*) increases, besides docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) contents and WG, particularly skin mucus lysozyme activity, leukocytes phagocytosis activity, ROS level, serum alternative complement pathway activity in hybrid sturgeon. A similar success of the same dietary lipases takes place in Japanese eel in which improved immunity results in pathogen resistance against both *Vibrio anguillarum* and *A. hydrophila* (Zheng et al. 2019).

Liu et al. (2016) are able to sketch a lipase-mediated pathway of increased immunity (Fig. 39.6). Sufficient lipase supplementation (~ 1200 IU kg^{-1} diet) improves growth, intestinal function, and health status of young grass carps.

In low-protein and high-lipid diets, lipase supplementation:

- Improves fish intestinal immune response through increasing acid phosphatase (ACP) activities and complement 3 (C3) contents, downregulating pro-inflammatory cytokines (*tnf- α* , *il-1 β* , *ifn- γ 2*, *il-8*), and upregulating antimicrobial peptides (*leap-2*, *hepcidin*) and anti-inflammatory cytokines (*il-10*, *tgf- β 1*).
- Enhances the intestinal tight junction barrier function by upregulating *claudin b*, *claudin c*, and *claudin 3*, ZO-1 and *occludin* and downregulating *claudin 12*, and *claudin 15a*.
- Enhances intestinal antioxidant status by elevating the GSH content and activities and transcription of intestinal antioxidant enzymes (CuZnSOD, MnSOD, CAT, GPx, GST, and GR).

39.5 Enzyme Cocktails

In 1994, Carter et al. pioneered the supplementation of exogenous enzymes in plant-based fish diets. By adding an enzyme mix consisting of trypsin, alkaline protease, acid protease, amyloglucosidase, amylase, and cellulase, to a SBM-based diet (33% by weight) for Atlantic salmon, the authors reported the first success of improved growth and feed efficiency due to an exogenous enzyme mix. However, a few years later, the same authors did not find any effect on salmon growth performance by supplementing a high SBM-containing diet with PASE plus carbohydrase and proteolytic enzymes. These apparently contradictory results point out that the effects of exogenous carbohydrases may depend on the level of inclusion of plant feedstuffs in the diet and/or the mixture of different enzymes used (Castillo and Gatlin 2015). Different strains, as well as different ontogenetic and physiological stages of the tested animals cannot be excluded as further underlying reasons for the discrepancy (Chap. 40). Some more studies with enzyme cocktails, showing mixed success, are collected in Table 39.6.

Again, optimum curves apply to enzyme mixture, if complete dose-response relationships are taken instead of only one or two dietary doses. This can be seen with the commercial Natuzyne50® fed to Mozambique tilapia: If this enzyme cocktail is supplemented at more than 500 mg kg⁻¹ in the diet, growth and digestive enzyme activities decrease (Hlophe-Ginindza et al. 2016). Based on experiences with PASE, adverse effects by excess exogenous enzyme cocktails are likely due to oxidative stress and intestinal inflammation. However, this issue needs empirical substantiation.

Table 39.6 Selected studies using exogenous enzymes cocktails and zymogens^a in aquatic animals fed plant-based diets

Species	Exogenous enzyme mix	Dietary plant basis	Effects	Reference
<i>Macrobrachium rosenbergii</i>	Digestin®: Protease, amylase, lipase, cellulase	<i>Azolla pinnata</i> , mosquito fern	Growth↑, ACD↑, body lipid content↑	Goda et al. (2018)
<i>Penaues monodon</i>	Porzyme®: Xylanase, amylase, β-glucanase	Canola 20%	WG↔, FCR↔, PER↑	Buchanan et al. (1997)
		Canola 64%	WG↔, FCR↔, PER↔	
<i>Acipenser baerii</i>	Ronozyme®: Xylanase, protease, PAs	Comm. basal diet (12% protein)	WG↑, gut microbiota↔	Ghodrati et al. (2021)
<i>Chelon ramada</i>	Comm. zymogen: Pepsin, amylase, protease, lipase, hemicellulose (Nile Co., Egypt)	Comm. diet (40% protein) low wheat, SB, corn	Growth↑, survival↑	El-Dahhar (1999)
<i>Cyprinus carpio</i>	Endofeed®W: Xylanase, β-glucanases, cellulase, hemicellulase	Wheat, SB, CS	WG↓	Kazerani and Shahsavani (2011)
<i>Dicentrarchus labrax</i>	Solid-state fermentation of <i>A. niger</i> (Synergen®, Alltech): Protease, amylase, xylanase, β-glucanase, pectinase, cellulase, PAs; Natugrain®TS: β-xylanase, β-glucanase (BASF)	SB, corn, wheat, pea	ADC _{DM} ↑, ADC _P ↑, ADC _L ↑, ADC _E ↑, ADC _F ↑, digestive enzymes↔; Natugrain® more effective than Synergen®	Magalhães et al. (2018)
<i>Labeo rohita</i>	Mix: Xylanase, PAs	Rice	WG↑	Ranjan et al. (2018a)
<i>Lateolabrax japonicus</i>	Mix: PAs, glucanases, pentosanase, cellulase, xylanase	SB, RS, peanut	WG↑, FER↑, P retention↑, N retention↑	Ai et al. (2007)
	Mix: Protease, xylanase, glucanase, mannase, cellulase	FM, SBM	WG↑, FCR↓, LYZ↑	Huang et al. (2020)
<i>Mugil liza</i>	Phytase, xylanase	SB, wheat, rice	WG↔, ADC _L ↑, SB-induced enteritis↓	Ramos et al. (2017)
<i>Oncorhynchus mykiss</i>	Superzyme® CS: Xylanase, amylase, cellulase, protease, β-glucanase	SB	WG↔, ADC(↑)	Ogunkoya et al. (2006)

(continued)

Table 39.6 (continued)

Species	Exogenous enzyme mix	Dietary plant basis	Effects	Reference
	Individually applied: Pectinase, xylanase, cellulase Farmazyme®: Xylanase, β -glucanase, cellulase, pectinase	SB	WG \leftrightarrow , FCR \leftrightarrow , ADCs \leftrightarrow	Yigit et al. (2014)
	Mix: Hemicellulases, protease, α -galactosidase	Dehulled lupin	WG \leftrightarrow , ADC _{DM} \uparrow , ADC _P \uparrow , ADC _L \downarrow , ADC _E \downarrow , PER \uparrow	Farhangi and Carter (2007)
	Mix: Protease, β -glucanase, xylanase	SB, Sfl, RS	ADC \uparrow , ADC _L \uparrow , WG \leftrightarrow , N retention \leftrightarrow	Dalsgaard et al. (2012)
<i>Oreochromis mossambicus</i>	Natuzyme50®: α -amylase, β -glucanase, PAse, cellulase, xylanase, protease	Kikuyu grass (<i>Pennisetum clandestinum</i>)	WG \uparrow , SGR \uparrow , FCR \downarrow	Hlophe-Ginindza et al. (2016)
<i>O. niloticus</i> \times <i>O. aureus</i>	Mix: Protease, β -glucanase, xylanase	SB, RS, CS	GR \uparrow , FER \uparrow , gut enzyme production \uparrow	Lin et al. (2007)
<i>O. niloticus</i>	Roxazyme G2®: β -glucanase, xylanase; Avizyme® 1500: Xylanase, protease, amylase	SB, Sfl, yellow corn, rice	WG \leftrightarrow , SGR \uparrow , FCR \downarrow , PER \uparrow	Eleraky et al. (2016)
	Natuzyme®: α -amylase, β -glucanase, PAse, cellulase, xylanase, protease, pectinase	<i>Ulva lactuca</i> + L-carnitine	WG \leftrightarrow , FCR \leftrightarrow	Amer et al. (2020)
	Phytase @ 1000 FTU kg ⁻¹ + xylanase @ 6000 U kg ⁻¹	FM-free, mixed plant protein sources	WG \uparrow , nutrient digestibilities \uparrow , N & P retention \uparrow	Maas et al. (2021)
<i>Pagrus major</i>	Solid-state fermentation of <i>A. niger</i> (Alltech, Kentucky, USA): Protease, amylase, xylanase, β -glucanase, pectinase, cellulase, PAse	Corn, SB	WG \uparrow , SGR \leftrightarrow , FCR \downarrow , PER \leftrightarrow	Matsukura et al. (2017)

(continued)

Table 39.6 (continued)

Species	Exogenous enzyme mix	Dietary plant basis	Effects	Reference
<i>Salmo salar</i>	Mix: Trypsin, alkaline protease, acid protease, amyloglucosidase, amylase, cellulase	SB	WG↑, FER↑	Carter et al. (1994)
<i>Siganus canaliculatus</i>	Mix: Cellulase, xylanase, β-glucanase	<i>Ulva pertusa</i>	Gut microbiota: <i>Ruminococcus</i> ↑, <i>clostridium</i> ↑, <i>Lachnospiraceae</i> -related <i>Firmicutes</i> ↑	Zhang et al. (2018)

↑ support/improvement/increase; ↔ no apparent effect; ↓ decrease/reduction; WG, weight gain; GR, growth rate; FER, feed efficiency ratio; ADC, apparent digestibility coefficient; ADC_{DM}, ACD of dry matter; ADC_P, ADC of protein; ADC_L, ADC of lipid; ADC_E, ACD of energy; ADC_F, ACD of phosphorus; FCR, feed conversion ratio; PER, protein efficiency ratio; FM, fishmeal; CS, cottonseed; RS, rapeseed; SB, soybean; SBM, soybean meal; Sfl, sunflower; LYZ, lysozyme activity; AOC, antioxidant capacity

^aZymogens are inactive enzyme precursors that, following secretion, are chemically altered to the active form of the enzymes

39.6 Concluding Remarks

Based on the information gathered in this chapter and recent books and reviews (Nunes and Kumar 2018; Zheng et al. 2020), it is obvious that research on exogenous enzyme supplementation to aquafeeds is neither extensive nor comprehensive, probably except phytase studies. Particularly the application of commercial enzyme products with varying composition and quality increases the risk of failing repeatability in different laboratories or at different points of times.

With respect to NSP enzymes, Habte-Tsion and Kumar (2018) reviewed that dietary enzymes can have a beneficial effect on digestive the microbial community by reducing undigested substrates and antinutritive factors and possibly by producing oligosaccharides from dietary NSPs with prebiotic potential. Therefore, the possible effect of carbohydrases on gut health and gut microbiota of monogastrics should be pursued.

In aquaculture industries, the use of PAs to improve P utilization from phytates has emerged quite recently and is rather well documented. Conversely, the application of CHOases or pectinases deserves future studies; there exist only a few promising effects in improving nutrient digestibility by hydrolyzing non-starch polysaccharides present in plant feedstuffs. Therefore, the emerging picture remains fragmentary. Nonetheless, this topic is still relatively new and research to date is insufficient, since most papers do not transgress the stocktaking level based on a dichotomous question: Is the supplementation of exogenous enzyme beneficial or adverse? Most indicators of success are food production traits; often, the biomolecular basis (including epigenetics) of such assessment remains obscure; however, this

basis is indispensable to the understanding of beneficial as well as adverse effects and to optimization aquafeed for sustainable farming.

This approach is essential, since the few available comprehensive studies indicate that an appropriate dietary supply of exogenous enzymes influences not only growth and production traits but also health, immunity, as well as intestinal microbiota, and, most likely, also reproduction of farmed animals and their offspring. This is a crucial requirement for the sustainable farming of aquatic animals (Lieke et al. 2020; Dawood 2021; Reverter et al. 2021).

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Chapter 40

Intraspecific Variability—‘*The Apple May Be a PineApple*’



Abstract Referring to field and domestication studies and in addition to the discourse about individual specialization (→AAN I “Trophic Diversification and Speciation” (Steinberg, Aquatic animal nutrition: a mechanistic perspective from individuals to generations, Springer Nature Switzerland AG, Cham, 2018)), this chapter points out intraspecific variability of fishes and aquatic invertebrates. In terms of life history traits or reaction norms to environmental stimuli such as food, the extent of the variability between strains, clones, or breeding lines can significantly exceed those between related species: The intraspecific variability can significantly exceed the interspecific one. Several recent examples are presented. Due to epimutations, domestication can take place even within the first generation isolated from the wild and leads often to less robust and less resistant individuals. These issues serve as an incentive to request the standardization of biological minimal requirements of robust research and publications. Therefore, the genetic characterization/identification of the animals tested should be mandatory for future studies and publications to reduce inconsistencies and scientific less robust and less valuable papers.

A fish is a fish, and a salmon is a salmon; that simple, stupid!

After creating the preceding chapters, it is difficult to avoid the conclusion that this quote may have served as one major biological base or guideline of many papers studied—even in highly ranked journals. Laboratories working with, e.g., Atlantic salmon in North America, South America, Australia, Europe, or China, always identify their study object as *Salmo salar*—and advanced authors add the naming authority in its Latin form “Linnaeus” and, very brave ones, even the publication year “1758.” That simple! The same applies to further farmed salmonids or giant shrimp species.

Nature, however, is more complex and provides much more diversity and information even within a single taxon than anticipated by many researches, particularly those aiming at human food (mass) production. Genetic diversity may satisfy biologists but puzzles aquaculturists who are interested in the safe production of proteinaceous food. It becomes obvious that aquatic biologists and ecologists on the one hand and aquaculture scientists on the other hand live in different, incompatible worlds, except a few disciplinary border crossings.

Provokingly, the latter behave and design their research often like the Indian blind monks described in Chap. 1 examining an elephant that they never saw before. Like the monks, many researchers claim truth based on their limited, subjective experience as they ignore other people’s (here, other disciplines’) partial and subjective experiences, which may be equally true. Moreover, if, in empirical studies, already the starting basis tilts, can one expect robust, innovative, and enduring results? Robust results only will emerge by chance; rather, diverging, inconsistent, and even contrasting results are very likely to be obtained; they are common.

Each of the preceding chapters has depicted several examples of inconsistent or even contrasting results in individual species carrying the same name. In most instances, these divergences are not discussed in depth—if mentioned at all. Most of such studies are banned into tables. One major reason of the divergence in results is the huge intraspecific variability combined with epimutations due to domestication. These effects are mostly ignored or overlooked—at best. However, a fish is not simply a fish; and a species is more diverse than the uniform species description suggests.

In AAN I, we presented several striking examples of phenotypic plasticity and specialization on the individual level as the basis for radiation within populations. Specialization within a population shows that individuals clearly differ from each other—they are not all the same. This phenotypic differentiation can be traced back to its genomic or epigenomic basis. Furthermore, clear evidence emerges that the intraspecific variability of a given species can exceed even the variability between species of the same genus (interspecific variability). In fact, all populations in nature are composed of relatively specialized individuals that, with regard to nutrition, selectively consume a subset of their population’s diet—a phenomenon known as “individual specialization.” These specialized individuals serve as the basis for greater morphological, physiological, and ecological variations and are one basis of evolution (→AAN I “Trophic Diversification and Speciation” (Steinberg 2018)).

Ecologists have documented remarkable intraspecific variations inherent to natural systems: variability in juvenile growth rates, mortality rates, fecundities, time to reproductive maturity, outcomes of competitive interactions, and response to natural and synthetic xenobiotic chemicals. It has become increasingly apparent that at least some of these variations are due to differences in embryonic or larval experiences or epimutations. Such experiences include delayed metamorphosis, resistance to short-term starvation, short-term salinity stress, or exposure to sublethal levels of xenobiotics or ultraviolet irradiation (Pechenik 2006).

Consequently, this chapter will recall the phenomenon of intraspecific variability and present some more intriguing examples hoping that eventually “little strokes fell big oaks” will also apply to studies of aquatic animal nutrition. Therefore, we refer again to the striking example of intraspecific variability from AAN I. Gribble et al. (2014) published life span patterns in twelve isolates from the rotifer *Brachionus plicatilis* itself and sister species in response to dietary restriction. The authors tested the effects of chronic dietary restriction at multiple food levels or intermittent fasting. While chronic dietary restriction generally increases or does not change life span and total fecundity, intermittent fasting causes increased, unchanged, or decreased life span, depending upon the isolate, and decreases total fecundity in all but one isolate. Life

span under ad libitum feeding varies among isolates and predicts the life span response to dietary restriction: Longer-lived isolates under ad libitum are less likely to have an increase in life span under chronic dietary restriction and are more likely to have a shortened life span under intermittent fasting. Most intriguingly, the extent of intraspecific variability among the *B. plicatilis* strains clearly exceeds that between *B. plicatilis* and its sister species: *B. plicatilis* vs. *B. manjavacas* or *B. plicatilis* vs. *B. sp.*

Disregarding intraspecific variability may result in misleading or even wrong conclusions of species responses to environmental stimuli and challenges or to erroneous definitions of ecological requirements, in general, and nutritional demands, in particular. Such papers do by no means provide robust information; instead, they possess rather a low scientific value. They should be treated with caution when drawing general conclusions on the environmental requirements of the “species” studied. Moreover, the application of such knowledge may even lead to failing practice in aquaculture.

With respect to fishes, Stepien et al. (2015) provided a striking recent example of population differences and genetic diversity in two percid species across North America, namely yellow perch (*Perca flavescens*) and walleye (*Sander vitreus*). The two species differ in levels of genetic diversity, with walleye populations possessing overall higher genetic variability than yellow perch (Fig. 40.1). Genetic divergence patterns follow the opposite trend, with more pronounced differences occurring among closely spaced spawning aggregations of yellow perch than walleye. Results reveal broad-scale correspondence to isolation by geographic distance; however, their fine-scale population structures show less relationship, often with pronounced genetic differences among some nearby reproductive groups (Stepien et al. 2015). The genetic differences surely translate into various life history traits such as growth and fecundity; however, they have not been in the focus of this study.

The examples can easily be multiplied or expanded to life history traits. Using a high throughput behavior assay, Drew et al. (2012) detected significant variations among zebra fish strains in behavior related to fearfulness and predator avoidance (Fig. 40.2a, b). This variation shows a strong association with domestication history. While there is substantial interstrain variation in patterns of gene expression in the brain, far fewer genes are associated with domestication history (Fig. 40.2c). In this study, the number of genes associated with domestication in zebra fish is comparable to that associated with within-population behavioral variation in other fish species.

The zebra fish example shows that one prominent indication of animal personality is whether it adopts an active or passive defense strategy when faced with danger (Alderman 2016). A meta-analysis revealed that the personality trait “boldness” correlates positively with reproductive success in many species (Briffa and Weiss 2010). Given that active and passive defense strategies require markedly different energetic costs, Rupia et al. (2016) tested whether different metabolic profiles underscore bold and shy behavioral phenotypes in Japanese flounders. Actually, behavioral and metabolic differences translate well into divergent physiological responses during acute stress: Shy individuals adopt a passive response by reducing their oxygen consumption rates, whereas bold individuals adopt an active response by increasing their respiration rates. Some more examples of energy-dependent individual responses are available (Table 40.1).

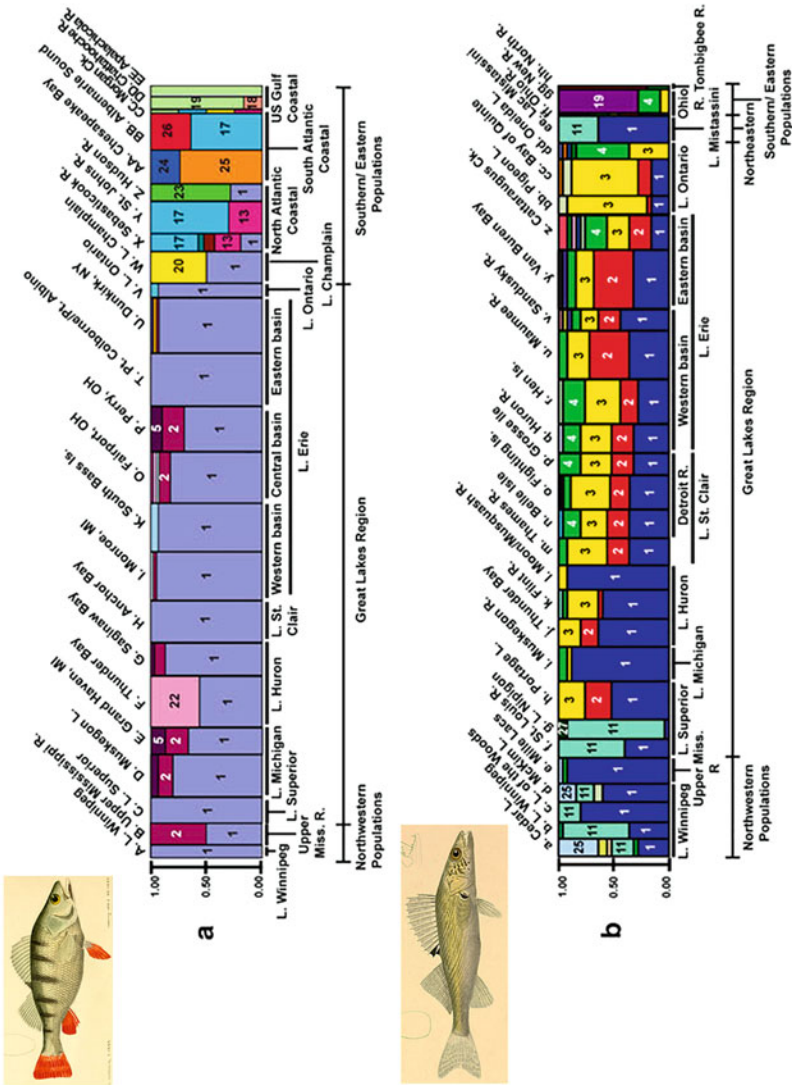


Fig. 40.1 Estimated population structure from mtDNA control region frequencies for (a) 26 yellow perch haplotypes and (b) 28 walleye haplotypes. Vertical black lines separate different spawning groups (lettered). Major geographic regions are indicated in the bottom rule for each chart. Note that there is no correspondence between the colors and haplotypes of yellow perch and walleye (a and b are entirely independent). (From Stepien et al. (2015), with permission from Springer Nature; images credit Cuvier and Valenciennes (1828–1849))

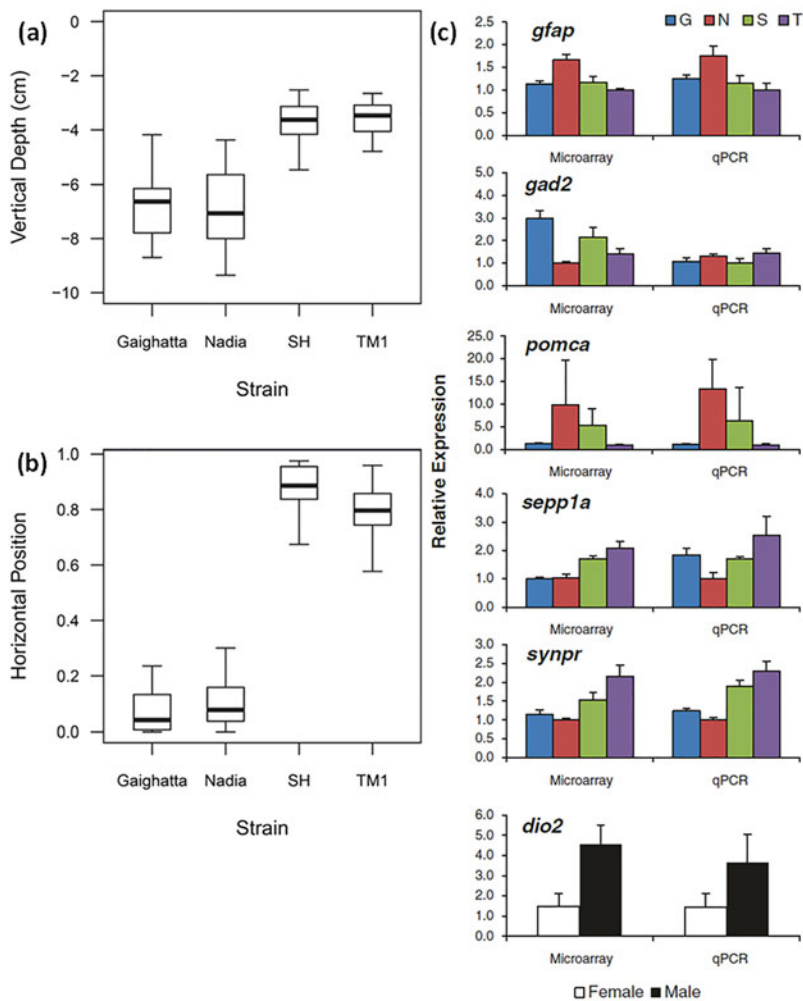


Fig. 40.2 Box plots of behavioral variations among two wild and two domesticated zebra fish strains. **(a)** Domesticated zebra fish [Scientific Hatcheries (SH) and TM1] had lower Vertical Depth, spending more time close to the water surface than wild zebra fish [Nadia and Gaighatta]. **(b)** Domesticated zebra fish also had higher levels of Horizontal Position, spending a larger portion of time within one body length of the front of the aquarium nearest the human observer. Boxes represent the 25th and 75th percentiles, while the median is indicated by the interior horizontal line. The 5th and 95th percentiles are represented by the whiskers. Significant differences among strains are represented by lower case letters within each figure. **(c)** qRT-PCR was used to validate microarray results for six genes. Microarray and qRT-PCR showed similar patterns for five of the six genes: *glial fibrillary acidic protein* (*gfap*), *proopiomelanocortin a* (*pomca*), *selenoprotein P1a* (*sepp1a*), *synaptoporin* (*synpr*), and *deiodinase, iodothyronine, type II* (*dio2*). Validation failed for *glutamate decarboxylase 2* (*gad2*), indicating that additional factors may be affecting hybridization to the microarray. G, Gaighatta; N, Nadia; S, Scientific Hatcheries; T, TM1. (From Drew et al. (2012), credit BMC Genomics)

Table 40.1 Diet–/energy-dependent personality trait in selected aquatic animals

Species, common name	Indicative trait	References
Invertebrates		
<i>Carcinus maenas</i> , Shore crab	Fight	Sneddon et al. (1998)
<i>Pacifastacus leniusculus</i> , Signal crayfish	Boldness	Pintor et al. (2008)
<i>Portunus trituberculatus</i> , Swimming crab	Boldness, activity, or hesitancy	Su et al. (2019)
Fishes		
<i>Cyprinus carpio</i> , Common carp	Personality traits unaffected	Liu and Fu (2017)
<i>Danio rerio</i> , Zebra fish	Proactivity (boldness)	Yuan et al. (2018)
<i>Dicentrarchus labrax</i> , European seabass	Shyness↑ at food↑ Boldness/exploration↑ at food supply unpredictable	Sébastien et al. (2016)
	Food↓ → contact to conspecifics↓	Aimon et al. (2019)
<i>Neogobius melanostomus</i> , Round goby	Boldness↑ → growth↓	Behrens et al. (2020)
<i>Paralichthys olivaceus</i> , Japanese (olive) flounder	Boldness	Rupia et al. (2016)
<i>Solea senegalensis</i> , Senegalese sole	Oxygen consumption↑ → latency to escape↑	Martins et al. (2011)

↑predictable supply; ↓deprivation/reduction

Subsequently, Holden and Brown (2018) identified baseline mRNA expression differences between zebra fish strains as a physiological interpretation of established genetic differences between strains. Differences occur between sexes and strains including lipid transport and circadian rhythms. The latter aspect is emphasized also by Tudorache et al. (2018), who report that large variation in circadian rhythmicity of zebra fish individuals may occur within populations. The observed correlation between coping style and circadian rhythmicity indicates that the level of rhythmicity forms an integral part of proactive or reactive coping styles.

Overall, in the absence of a practical understanding of intrapopulation baseline variation, the downstream interpretation of data becomes skewed; reproducibility becomes increasingly challenging, and the application of study results become more abstract (Holden and Brown 2018)—politely phrased.

40.1 Invertebrates

Growth of the freshwater keystone zooplankter *Daphnia* is seasonally limited by low availability of eicosapentaenoic acid (EPA). Brzeziński and von Elert (2007) identified intra- as well as interspecific genetic variability in *Daphnia* populations

indicated by the response to different EPA-based food qualities. Among 18 clones tested, ten respond to EPA limitation with significant reduction of juvenile growth rates, whereas eight are not affected.

Eventually, genetically based trade-offs in response to stoichiometric food quality influence competitive interactions and micro-evolutionary trajectories as demonstrated in *Daphnia pulicaria* (Jeyasingh et al. 2009). The authors show that stoichiometric mismatches between diet and consumer drive genotype-environment interactions: In multiple pairs of clones, the genetic variation at the *phosphoglucose isomerase* (*pgi*) locus indicates that *pgi*-heterozygotes outcompetes *pgi*-homozygotes under high phosphorus/carbon (P/C) conditions, whereas the opposite outcome occurs under low P/C conditions.

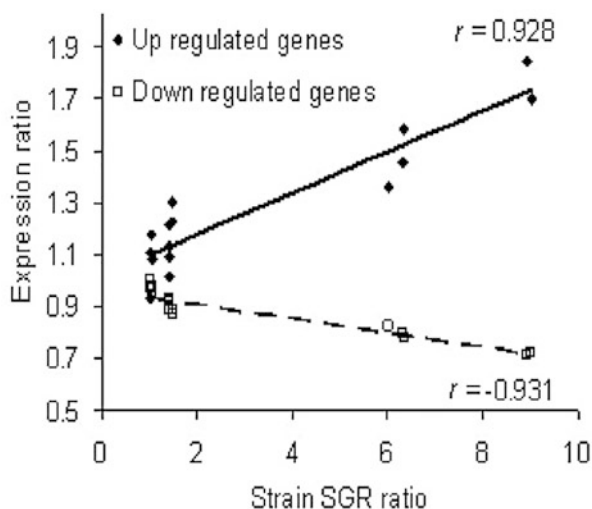
Studying *Brachionus* species different from those in the Gribble et al. (2014) paper, Schällicke et al. (2019) explored the potential of a single limiting biochemical nutrient to mediate variations in population growth in *B. calyciflorus* s.s. and *B. fernandoi*. Rotifers are fed a sterol-free algal supplemented with cholesterol-containing liposomes. Co-limitation by food quantity and biochemical quality results in different population growth rates among strains: The effect of cholesterol supplementation on population growth is strain-specific, but not species-specific. In a succeeding paper, Schällicke et al. (2020) continued their comparison by showing that rotifer strains of one species differ in their capacity to retain α -linolenic acid and EPA under PUFA starvation stronger than the two separate species.

Evidence is emerging that population epigenetic divergence can exceed genetic divergence and that this divergence can be inherited to the succeeding generation. Johnson and Kelly (2020) studied Eastern oyster (*Crassostrea virginica*) in the Northern Gulf of Mexico and identified differential methylation among genes known to exhibit high plasticity in response to environmental conditions (*sodium/hydrogen exchanger*, *glutathione synthetase*), to developmental timing (*protogenin B*), and DNA damage repair (*dnaJ* homologs). The variation in methylation reflects differences in the environments from which the individuals were collected. This paper reinforces the need to combine genomic and epigenomic data when seeking to understand divergent population responses to heterogeneous environments. One major constituent of the environment is food and one can predict that epigenetically divergent individuals respond differently to the same food quality; however, this study did not focus on this issue. Furthermore, a potential of intergenerational inheritance of the methylation patterns does exist as recently demonstrated in *C. gigas* following parental exposure to a herbicide (Rondon et al. 2017).

40.2 Farmed Fishes

From field population structure and genetics, a huge body of evidence of intraspecific variability is available as demonstrated in the two North American percids above; less, however, from aquaculture. Growth differences between individuals can be traced back to the biomolecular level. Growth rate can be genetically modified in

Fig. 40.3 Relationship between mRNA levels and growth among rainbow trout strains: strain growth ratios (SGR) vs. average intensity of mRNA levels for up- and downregulated genes among strain pairs. Correlation coefficients (r) are shown. (From Devlin et al. (2013), with permission from the Oxford University Press)



many vertebrates by domestication and selection and more recently by transgenesis overexpressing growth factor genes. Devlin et al. (2013) assessed levels of mRNA in the liver of wild-type and growth-modified strains of rainbow trout. The authors report a correlation of ratios of mRNA levels between strains vs. the ratio of growth rates seen for the same strains showing relationships for both upregulated and downregulated genes (Fig. 40.3).

In the same line of evidence, Opazo et al. (2017) compared gene expression patterns of growth genes in zebra fish larvae with different growth rates. The observed variability in growth rates is controlled by *igfbp-1* and *igf-1r* (Fig. 40.4).

Not only growth or behavior but also all life history traits reflect personality in a given species. So does the immune response. Overturf et al. (2003) compared five strains of rainbow trout challenged with infectious hematopoietic necrosis virus (IHNV). Measured by IHNV neutralization titers over a 12-week period, the results demonstrate wide differences (Fig. 40.5). Strain 1 gives the strongest response. Strains 4, 3, and 2 exhibit similar titers that are slightly decreasing in the order listed. Strain 5 has a homogenously weak virus neutralization response. Did this interesting paper really fertilize future research to characterize the studied aquatic animals more intensely in terms of molecular biology?

Total replacement of fishmeal-(FM)-based diet with plant-based diet affects the hepatic transcriptome. Geay et al. (2011) analyzed this issue in two European seabass half-sibfamilies. The half-sibfamilies exhibit similar growth rates when fed a FM diet but significantly different growth rates when fed an all-plant diet. Accordingly, several genes involved in macromolecule biosynthesis, and particularly in protein biosynthesis, are upregulated in the fast-growing half-sibfamily, whereas genes involved in the inflammatory response are upregulated in the slowly growing half-sibfamily.

In two isogenic lines of rainbow trout, Song et al. (2018a) identified striking differences in glucose metabolism. In addition to contrasting transcription of the

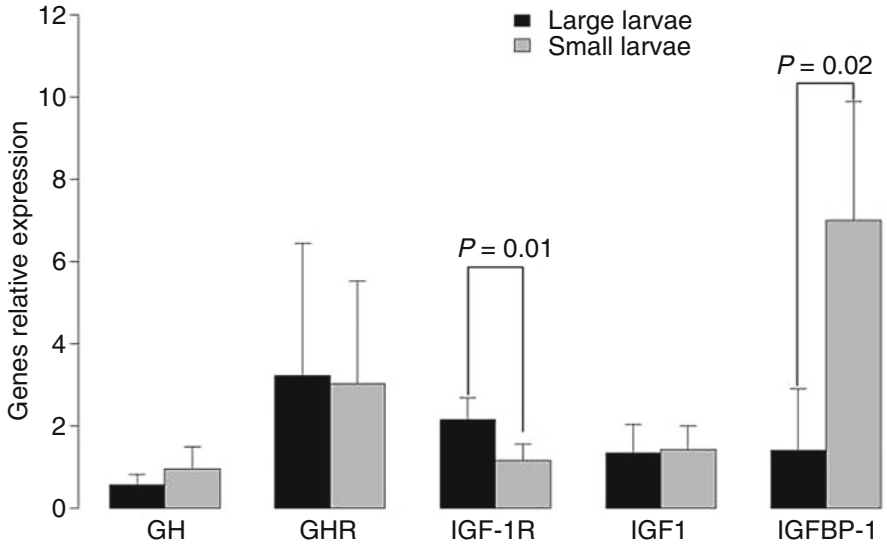


Fig. 40.4 Relative gene expression of growth hormone (*gh*), insulin-like growth factor 1 (*igf-1*), growth hormone receptor (*ghr*), insulin-like growth factor 1 receptor (*igf-1r*), insulin-like growth factor binding protein 1 (*igfbp-1*) in small- and large-body zebra fish larvae. Columns represent mean \pm SD, $n = 5$. The statistical significance was determined using the paired-sample Wilcoxon Mann-Whitney U signed-rank test ($P < 0.05$). (From Opazo et al. (2017), credit Escuela de Ciencias del Mar)

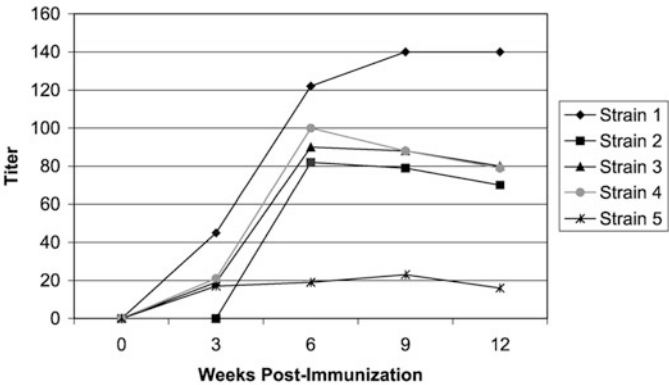


Fig. 40.5 Infectious hematopoietic necrosis virus (IHNV) neutralization titers for five rainbow trout strains. The measured level of antibody titer in fishes after challenging with IHNV lysate plotted against weeks post-challenge. (From Overturf et al. (2003), with permission from Elsevier)

transporter *glut2* (Chap. 16) in both strains, the lines differ in the expression of hepatic glycolytic enzymes, particularly of *gcka*, *gckb*, and *pkl* (Fig. 40.6). Moreover, also differences occur in the gene expression of hepatic gluconeogenesis

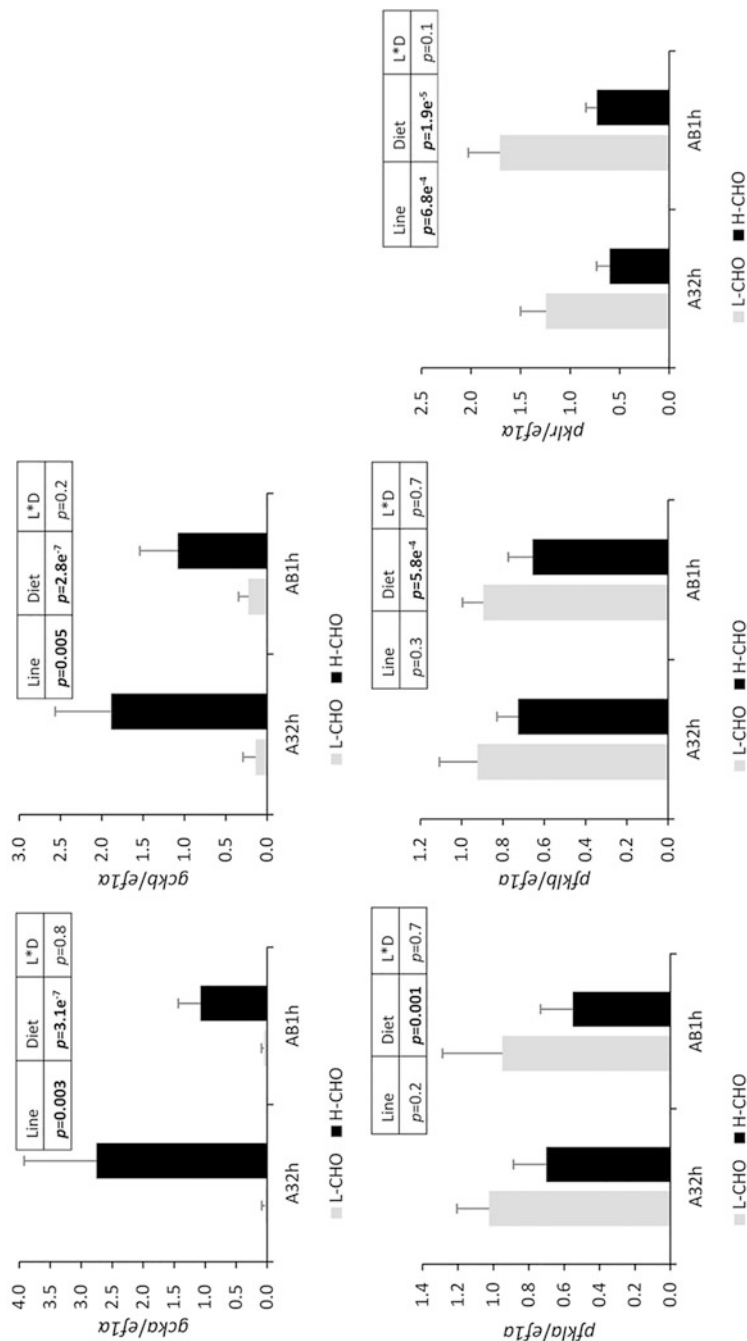


Fig. 40.6 mRNA levels of selected glycolytic enzymes in the liver of two lines of rainbow trout fed with low carbohydrate diet (L-CHO) and high carbohydrate diet (H-CHO). *Gcka*, *gckb* glucokinase paralogs; *pfkl* *6-phosphofructokinase*, liver type; *pfkl* *pyruvate kinase*, liver type. Data are presented as mean \pm SD (n = 6); statistical differences of *gcka*, *gckb*, *pfkl*, and *pkl* were evaluated by two-way ANOVA ($p < 0.05$, values in bold). (From Song et al. (2018a), credit Company of Biologists)

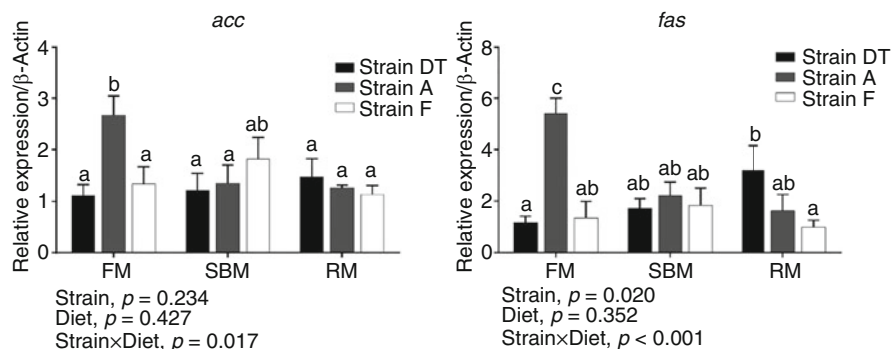


Fig. 40.7 Relative gene expressions of *acetyl-CoA carboxylase* (*acc*) and *fatty acid synthase* (*fas*) in the hepatopancreas of the three gibel carp (*Carassius gibelio*) strains fed with different protein sources (fishmeal **FM**; soybean meal **SBM**; rapeseed meal **RM**) at 8 hours after the last feeding. Columns represent means \pm SEM ($n = 3$). When an interaction was significant, lower case letters (a, b) represent the difference among all groups ($P < 0.05$). (From Xu et al. (2019), with permission from Wiley)

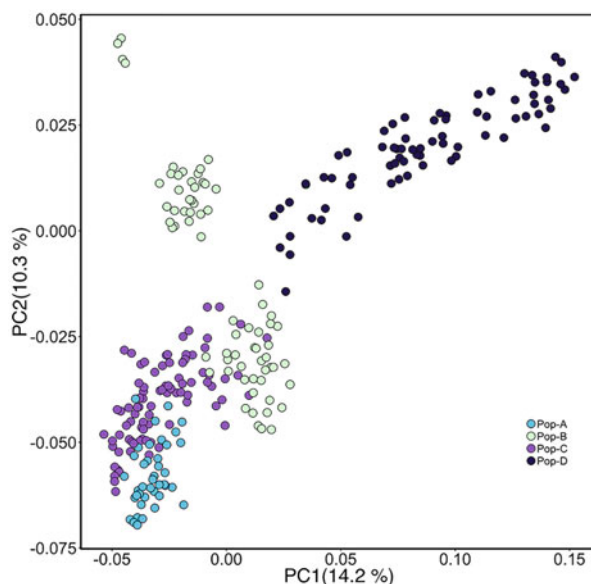
enzymes and one lipogenesis enzyme (*adenosine triphosphate citrate lyase*). In two other isogenic lines of rainbow trout, Song et al. (2018b) identified genetic variability of even more metabolic genes, namely of those involved in EPA and docosahexaenoic acid (DHA) biosynthesis and cholesterol metabolism.

Comparing three strains of gibel carp fed three different protein sources (FM, soybean meal (SBM), and rapeseed meal (RM)), Xu et al. (2019) identified strain-specific differences in gene transcription. Strain DT on the RM diet shows elevated *fas* mRNA level (Fig. 40.7), while high transcript levels of *acc* and *fas* are observed in strain A on the FM diet. All three strains show different transcriptional regulation of FA synthesis in response to FM and plant-based diets.

A not necessarily expected result is worked out in gilthead seabream (Piazzone et al. 2020). The influence of the selection for heritable growth depends on intestinal bacterial populations. Despite the genomic heterogeneity, gilthead seabream families selected for heritable growth are more robust. They adapt better to dietary changes, reshaping their intestines, with no significant effects on their growth and health parameters (Perera et al. 2019), and can cope more efficiently with pathogens. These animals also harbor a plastic microbiota, which effectively adapts to the metabolic challenges induced by dietary changes.

Some more illustrative examples: Kajungiro et al. (2019) demonstrated the variable population structure and high genetic diversity within and between local Nile tilapia lines cultured in Tanzania. The authors investigated seven populations from Karanga, Igunga, Ruhila, Fisheries Education and Training Agency (FETA), Tanzania Fisheries Research Institute (TAFIRI), Kunduchi, and Lake Victoria. Double-digest restriction site-associated DNA libraries were prepared from 140 individual fishes and sequenced resulting in the identification of 2180 informative single nucleotide polymorphisms (SNPs). Strong genetic differentiation between the closely related populations occurs: Individual fishes from FETA, Lake Victoria,

Fig. 40.8 Principal components analysis (PCA) of genetic differentiation among individual Atlantic salmon with a common genetic origin. Each point represents one individual, and different colors represent populations. (From López et al. (2019), credit Frontiers Media)



Igunga, and most of the individual fishes from Kunduchi form a group of genetically similar animals. All TAFIRI fishes form a different group and are distinct from the other populations.

A marine equivalent, López et al. (2019) performed a principal components analysis based on genotypes to look at the genetic relationship among four populations of Atlantic salmon with a common genetic origin (Fig. 40.8). Artificial selection through genetic improvement programs imposes distinctive signatures on the salmon genome. Population A (Pop-A) and Pop-C show a close genetic relationship to each other and are most distant to Pop-D along PC1. Pop-B lies between the Pop-A/Pop-C cluster and Pop-D along PC1, with some overlap with Pop-C, which has been introduced into the same region as Pop-B.

Furthermore, several regions are identified that harbor genes, such as *kind1*¹ and *chp2*,² which are associated with growth-related traits, or the *kcnb2*³ gene related to the immune system in Atlantic salmon, making them particularly relevant in the context of aquaculture (López et al. 2019). Overall, low genetic variation between populations occurs but is high within populations.

¹Kinase non-catalytic C-lobe domain containing 1 protein; knockdown of this gene in senescent umbilical vein endothelial cells partially reversed the senescence (Zhang et al. 2014).

²Calcineurin like EF-hand protein 2: Calcineurin is a calcium-dependent serine-threonine phosphatase and plays a pivotal role in the information flow from local or global calcium signals to effectors that control immediate cellular responses and alter gene transcription (Yáñez et al. 2012).

³Potassium voltage-gated channel subfamily B member 2: shown to be affected by parasite-driven selection (Zueva et al. 2014).

40.2.1 Domestication

Animal husbandry combined with biomolecular, physiological, and behavioral studies have identified high degrees and complexity of biological changes induced by domestication, including modulations of the epigenome (Rodríguez Barreto et al. 2019; Milla et al. 2021). Domestication mirrors very rapid evolution under controlled conditions (Vogt 2021). Farmed animals do no longer reflect the properties and capabilities of their wild conspecifics. Instead, they have acclimatized to the convenient–harsh conditions farmers are providing them. These conditions are convenient, because the animals do not have to forage for food; rather, food is usually supplied in sufficient quantities and acceptable qualities. Consequently, the corresponding parts of the brain and behavior become reduced. Furthermore, the food quality does not change seasonally as in nature; instead, it is designed to improve growth and protein production, and accelerate reproductive performances. This implies that several metabolic, epigenetic, and genetic functions may be reduced or even lost in farmed conspecifics (Frost et al. 2006). This can take place directly or indirectly via the intestinal microbiome. Moreover, domesticated fishes may develop diseases or malformations linked to genetic drift or inbreeding (Teletchea 2015).

Domestication triggers the development of distinguishable isolated populations with strongly modulated traits (some are listed in Table 40.2, more examples can be found in the recent review by Milla et al. (2021)). Due to required maximal short-term production, farmed individuals are kept at convenient water temperatures and do not have to cope with circadian or seasonal temperature fluctuations, and an escape from aquafarms into the wild may be a temperature shock. It can be expected that farmed individuals have reduced phenotypic plasticity; they have lost part of their fitness traits to survive and reproduce in the wild. On the other hand, farmed animals have to stand the crowding stress due to the lack of hiding opportunities, which are available in nature. This implies that they are subject not only to metabolic but also behavioral modifications.

Species farmed for human consumption will progressively be strongly modified from their wild congeners because the goal is to promote “commodity traits,” i.e., traits that aquaculturists find desirable (Teletchea 2016). For example, the study of Chen et al. (2017) in *Perca fluviatilis* revealed that domestication has altered the digestive system. By downregulating proteolytic enzyme and metabolic pathways for protein absorption, captive *P. fluviatilis* are able to reduce unnecessary energy expended for digestion because they are supplied with unlimited feed. In other words, the most streamlined and most opportunistic, but not the most versatile, flexible, and robust individuals continue to be farmed. Therefore, captive fishes significantly differ from their wild conspecifics in terms of phenotype, metabolism, epigenetics, genetics, behavior, etc. (Teletchea 2019).

Epigenetic changes can alter the phenotype during domestication with a breathtaking speed. A recent paper on Nile tilapia presents evidence that such changes take place even within a single generation. Konstantinidis et al. (2020) demonstrated for

Table 40.2 Comparison of traits, other than fatty acid patterns and population markers, in selected aquatic species modulated by farming

Species, common name	Modulated trait in farmed individuals	References
Invertebrates		
<i>Litopenaeus vannamei</i> , Pacific white shrimp	Energy transformation into biomass↓	Arena et al. (2003)
<i>Penaeus monodon</i> , Black tiger shrimp	Genetic diversity↓, effective population size↓	Dixon et al. (2008)
	Sensitivity against white spot syndrome virus (WSSV)↑	Alam and Pálsson (2016)
Fishes		
<i>Anguilla japonica</i> , Japanese eel	<i>Gh</i> ↓, <i>lh</i> ↓, <i>shbg</i> ↓	Higuchi et al. (2019)
<i>Danio rerio</i> , Zebra fish	Microbiome: Wild: <i>Dothideomycetes</i> ↑ Farmed: <i>Saccharomycetes</i> ↑	Siriyappagounder et al. (2018)
<i>Dicentrarchus labrax</i> , European seabass	Macrophage centers↑ ^a	Kurtovic et al. (2008)
	ω3PUFA↓, ω6PUFA↑, MUFA↑	Fuentes et al. (2010)
<i>Huso dauricus</i> , Kaluga	Diversity of intestinal microbiota↓	Lv et al. (2018)
<i>Labeo rohita</i> , Rohu carp	Genetic diversity↓	Gjerde et al. (2002)
<i>Oncorhynchus mykiss</i> , Rainbow trout	Growth↑	Martens et al. (2014)
<i>O. tshawytscha</i> , Chinook salmon	Modulation of genetic architecture and plasticity of DNA methylation	Venney et al. (2020)
<i>Perca fluviatilis</i> , European perch	Modulation of egg quality	De Almeida et al. (2019)
<i>Salmo salar</i> , Atlantic salmon	Growth↑, genetically based appetite↑	Harvey et al. (2016)
<i>Sander lucioperca</i> , Pikeperch	Immune-related proteins↓, heat shock proteins↓; modulation of offspring nervous system via egg proteins	Nynca et al. (2020)
<i>Seriola lalandi</i> , Yellowtail amberjack	Microbiome: Diversity↑, <i>Proteobacteria</i> ↓, <i>Bacteroidetes</i> ↓, <i>Firmicutes</i> ↑, <i>Actinobacteria</i> ↑	Ramírez and Romero (2017)
<i>Sparus aurata</i> , Gilthead seabream	Critical swimming speed↓	Basaran et al. (2007)

↑ increase, support; **PUFA** polyunsaturated fatty acid; **MUFA** monounsaturated fatty acid; ↓ reduction; *gh* growth hormone; *lh* luteinizing hormone; *shbg* sex hormone-binding globulin

^aMacrophage centers (MC) are physiological features in fish spleen and kidney and indicative of contamination and health (Meinelt et al. 1997). Later, Montero et al. (1999) found that stressful aquaculture situations resulted in increased numbers of splenic and kidney MC

the first time that muscle DNA hydroxymethylation differs significantly between wild fish and their progeny reared in captivity. Differentially hydroxymethylated cytosines (DhmCs) occur mostly within gene bodies, which indicate their functional

role in epigenetic regulation. These DhmcCs are associated with immune-, neuronal-, and growth-related processes. Concurrently, thousands of genes are differentially expressed, with upregulation of immune-related genes in the wild and upregulation of metabolic and muscle-specific genes in the first generation of fish undergoing domestication. Another remarkable finding is the hyperhydroxymethylation of the first intron of *myosin-Vb* and its connection with the upregulated *phosphatase and tensin homolog (pten)* gene in the wild group. Myosin Vb is associated with plasma membrane recycling systems (Lapierre et al. 2001) and *pten* antagonizes the PI3K-Akt signaling pathway and, therefore, muscle atrophy or cancer.

Evidence of fast domestication via epigenetics is accumulating further. For instance, farming influences the genome of European seabass in the initial stages of domestication by inducing epimutations (Anastasiadi and Piferrer 2019). The term “epimutation” has been coined by Calo et al. (2014) and underscores the ability of an organism to adapt to the environment through transient phenotypic variation (epimutation). This plasticity evokes a broader phenotypic repertoire including the ability to reverse epimutations when selective pressures are relaxed (Steinberg 2012).

Anastasiadi and Piferrer (2019) continue that the epimutations of the European seabass are established already during early life and involve genes related to developmental processes that are expressed in embryonic structures including the neural crest. The epimutations persist until adulthood in different tissues and some result in measurable changes in gene expression. Epimutated genes include those connected with the domestication syndrome, such as jaw deformities. Epimutations significantly associate with SNPs, indicating that they can be integrated into the genome as genetic polymorphisms due to the hypermutability of the CpGs and its neighboring sequences.

Information about changes in protein composition of eggs in response to domestication is very limited. Such data, however, are prerequisite for improvements in the reproduction of domesticated fishes. In pikeperch, Nynca et al. (2020) showed that domestication influences proteins involved in metabolism, immune response, and protein folding. The changes in metabolic proteins in eggs from domesticated females reflect the acclimation during the domestication process to commercial diets, which have profoundly distinct compositions from natural food. Reduced abundance of proteins related to the immune response in eggs from the domesticated population indicates that domestication leads to disturbances in defense mechanisms. In turn, the lower heat shock (stress) protein content in eggs of domesticated fishes points out their adaptation to stable farming conditions with reduced environmental stresses.

Furthermore, in eggs from domesticated individuals, proteins involved in purine metabolism are downregulated, such as a nucleoside diphosphate kinase (NME2). Purine metabolism is a known factor for the nuclear maturation of oocytes through both follicle cells and oocytes in mammals. The NME protein family is involved in multiple physiological and pathological processes such as cell differentiation, proliferation, development, and cilia functions. This finding shows that the purine metabolism in domesticated fish is disturbed. Therefore, the nucleoside diphosphate

kinase can reflect the domestication process in pikeperch and is likely a universal marker of domestication (Nynca et al. 2020)—an intriguing hypothesis to test in other farmed fishes.

Overall, changes by domestication are manifest on microbiome, genome, and epigenome levels even in short periods. If such clear epigenetic modifications take place already within the first generation—what will happen within more than one generation in the long term?

40.3 Perspective: Genotyping

As one basis of individuality/personality, genetic diversity can be reflected by biomolecular markers. Quantitative trait *loci* (QTLs, (Gutierrez and Houston 2017)) need to be identified, validated across strains, lines, and populations. Genetic and epigenetic regulations of important traits in farmed animals need to be determined, and techniques for marker-assisted selection, causal gene/mutation-assisted selection, genome selection, and genome editing using CRISPR⁴ and other techniques must be applied to aquaculture. Major progress has been made in genomics for dozens of fish and invertebrate (shellfish) species including the development of genetic linkage maps, physical maps, microarrays, SNP (Liu et al. 2017a, 2017b) arrays, transcriptome databases, and various stages of genome reference sequences (Abdelrahman et al. 2017).

Box: Requirement of Biological Studies

The discomfort about the lack of standards required for biological studies and publications with its eventual outcome of diverse and contrasting results of supposed identical taxa is widespread. For instance, In their excellent review on fish meal replacement, already Hua and Bureau (2012) meta-analyzed flaws in common (esoteric sensu Kuhn (1970)) research of aquatic animals nutrition. Their concerns, however, are not restricted to fishmeal replacement studies and deserve to be quoted verbatim. “It has been common practice to evaluate plant protein ingredients through experimentation and simple inferential statistical analysis. However, there are several weaknesses associated with this approach. Firstly, only a limited number of experimental diets can be investigated per experiment, thus only a limited number of factors can be evaluated per experiment. Secondly, significant variations exist across studies with regard

(continued)

⁴Clustered regularly interspaced short palindromic repeats (CRISPRs) were described in bacteria and archaea, before being extensively studied and used for gene-editing experiments. CRISPRs are 21 to 37 bp identical repeats that are separated from each other by a nonrepetitive DNA sequence called spacer DNA (Doudna and Charpentier 2014; Kirchner and Schneider 2015).

to dietary nutrient compositions (and/or anti-nutritional factors) and nutrient digestibility as well as differences in fish strains, life stage and body weight, body composition, feed intake, growth, feed efficiency, rearing conditions, etc. Thirdly, using fishmeal control diets as a comparison basis across studies may not be valid since the composition and nutritive value of fishmeal is known to vary according to many factors, such as fish species, raw material type (whole fish vs. processing by-product), seasons, and processing temperature. Therefore, the fishmeal replacement level achieved in one study cannot be extrapolated with confidence to other conditions and thus becomes meaningless. Fourthly, results have been presented and evaluated in different ways of expression. For example, the growth response of experimental fish is expressed as weight gain, specific growth rate, thermal growth coefficient, feed efficiency, and feed conversion ratio. Finally, interpretation and inference of experimental results overly rely on null hypothesis testing of the absence of effect of the experimental diets from the fishmeal control diet. A typical experimental design consists of a control diet with high fish meal levels and a series of experimental diets formulated with plant protein ingredients replacing either % fish meal or % protein of fish meal. Under these conditions, the evaluation of the nutritive value of the test ingredients is not sufficiently robust or specific. The same level of test ingredients may or may not support the optimal growth performance, depending on nutrient composition of the diets” (Hua and Bureau (2012), extract taken with permission).

A minimum general standard for publications in bioscience research has been published by the ARRIVE consortium: Animal Research: Reporting of In Vivo Experiments) (Kilkenny et al. 2010) and updated 10 years later by Percie du Sert et al. (2020). The guidelines have been published in several leading bioscience research journals simultaneously, and publishers have already endorsed the guidelines by including them in their journal Instructions to Authors subsequent to publication (Kilkenny et al. 2010). The reason for these guidelines is that, in recent years, concerns about the reproducibility of research findings have been raised by scientists, funders, research users, and policy makers.

Based on the experience, however, we made during writing this book, we have to go even one step further as the ARRIVE consortium. In addition to the unified rules of reporting results, a robust characterization of the studied biological material is mandatory.

Accelerated by the introduction of Next-Generation Sequencing (NGS, Liu (2017)), a set of genomic tools and resources is now available including reference genome sequences and their annotations (including coding and noncoding regulatory elements), genome-wide polymorphic markers, efficient genotyping platforms, high-density and high-resolution linkage maps, and transcriptome resources including non-coding transcripts (Li et al. 2017). Besides classical techniques with

allozymes, mitochondrial DNA, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, SNP, and expressed sequence tags (EST) markers are available (Liu and Cordes 2004).

While SNP chips or genotyping-by-sequencing data contain only a subset of the polymorphic variants available in a species, whole genome sequencing data provide access to complete information on all the variants of an individual. Although these techniques are successfully applied to molecular systematics, population genetics, evolutionary biology, molecular ecology, conservation genetics, and seafood safety monitoring, they translate only slowly into aquacultural science and practice. Moreover, although these techniques are applied for genetic improvements (Fig. 40.9) (Liu et al. 2014; Nguyen 2016; Yue and Wang 2017), most nutritional papers still refer only to species and strain names, naming authorities, and year of naming, thereby enlarging the catalog of diverging and even contrasting results of farmed animal reactions to aquafeeds and feeding practice. And the audience is faced with problems identifying the studied biological material. Obviously, it still applies: “A fish is a fish; and a salmon is a salmon.”

One step forward, linkage maps provide a framework of the order and spacing of genetic markers and serve as a starting point for the mapping of QTLs for traits of commercial interest and for the development of marker-assisted selection programs (Liu and Cordes 2004). In other words, new generation sequencing techniques facilitate the development of abundant genetic markers that have not only multifaceted applications for selected breeding of aquatic species but also for characterizing the subspecies or strain level and identifying the used farmed animals.

Gratacap et al. (2019) go even one step further by pleading for genome editing. Selective breeding programs are enabling genetic improvement of production traits, such as disease resistance, but progress is limited by the heritability of the trait and generation interval of the species. New breeding techniques, including the application of genome editing using CRISPR/Cas9, have the potential to expedite sustainable genetic improvement in aquaculture. Genome editing can rapidly introduce favorable changes to the genome, such as fixing alleles at existing trait loci, creating *de novo* alleles, or introducing alleles from other strains or species. The high fecundity and external fertilization of most aquaculture species can facilitate genome editing for research and application.

To banish the reluctance or even resistance toward new techniques, Barrera-Redondo et al. (2020) published a field guide for beginners of genomic, transcriptomic, and epigenomic tools to study the domestication of animals. The authors summarize the theoretical and technical background needed to carry out domestication genomics, starting from the acquisition of a reference genome and genome assembly, to the sampling design for population genomics, transcriptomics, epigenomics, and experimental validation of domestication-related genes (Fig. 40.10).

Finally, only one recommendation has to be made. In order to prevent or, at least, retard the increase of the pile of often repetitive and partly inconsistent and contrasting studies with “identical” species and to provide robust information

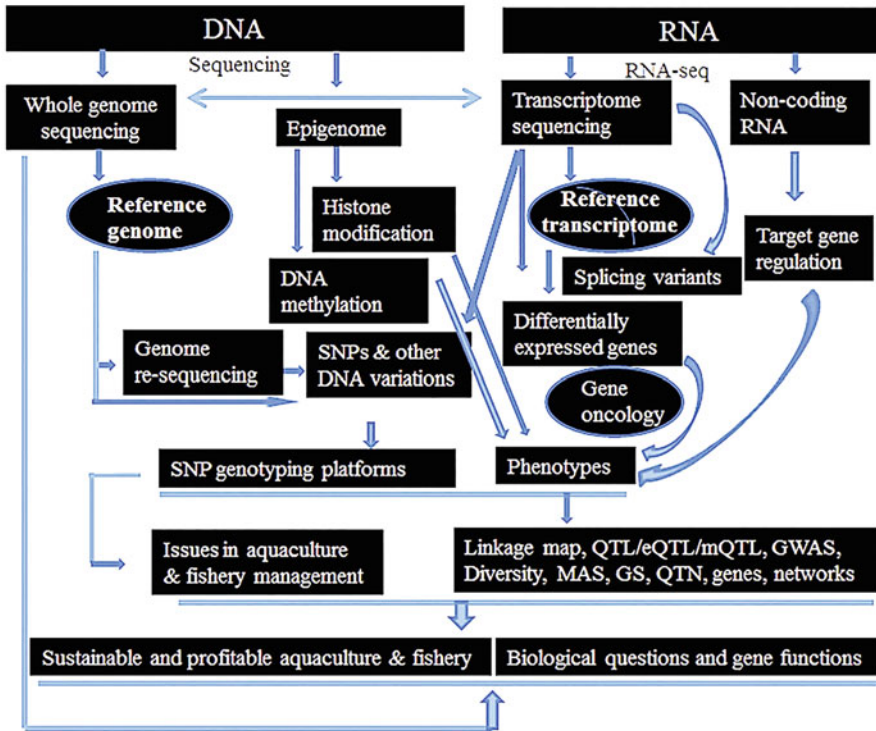


Fig. 40.9 An overview of applications of whole genome (DNA) and transcriptome (RNA) sequencing in aquaculture and fisheries. Next-generation sequencing (NGS) techniques have many potential applications in aquaculture. General applications include the generation of reference genomes, transcriptomes and genomic resources, as well as the developing of single nucleotide polymorphisms (SNPs) and other DNA markers. The reference genomes, transcriptomes, and DNA markers make many downstream specific applications possible. Specific applications include applications of DNA markers to construct linkage maps, to identify quantitative trait loci (QTL) for important traits, QTL for expressed genes (eQTL) and methylation QTL (mQTL), and to conduct genome-wide association studies (GWAS) to identify DNA markers associated with traits of interests. These DNA markers associated with a trait of interest can then be used in selecting progenies carrying favorable alleles via marker-assisted selection (MAS) or genomic selection (GS). To develop the functional or perfect gene-based markers (QTN), NGS of cDNAs of contrasting genotypes for the trait of interest can be used to identify polymorphisms (QTN) in candidate genes involved in or associated with the trait. The expression mapping of these candidate genes, together with phenotyping of the segregating populations developed from the contrasting genotypes, will provide expression QTLs (eQTLs), and markers associated with these eQTLs should thus serve as the perfect markers for MAS and GS in breeding. Another important application of DNA markers generated by NGS is to analyze genetic diversity and relationships in cultured and wild populations, which is critically important for the genetic improvement of aquaculture species. (From Yue and Wang (2017), with permission from Elsevier)

about nutritional effects on life history traits in well-characterized aquatic animals, highly ranked journals should request meaningful and robust biomolecular characterizations of the studied animals. Scientific societies of aquaculture and fish and

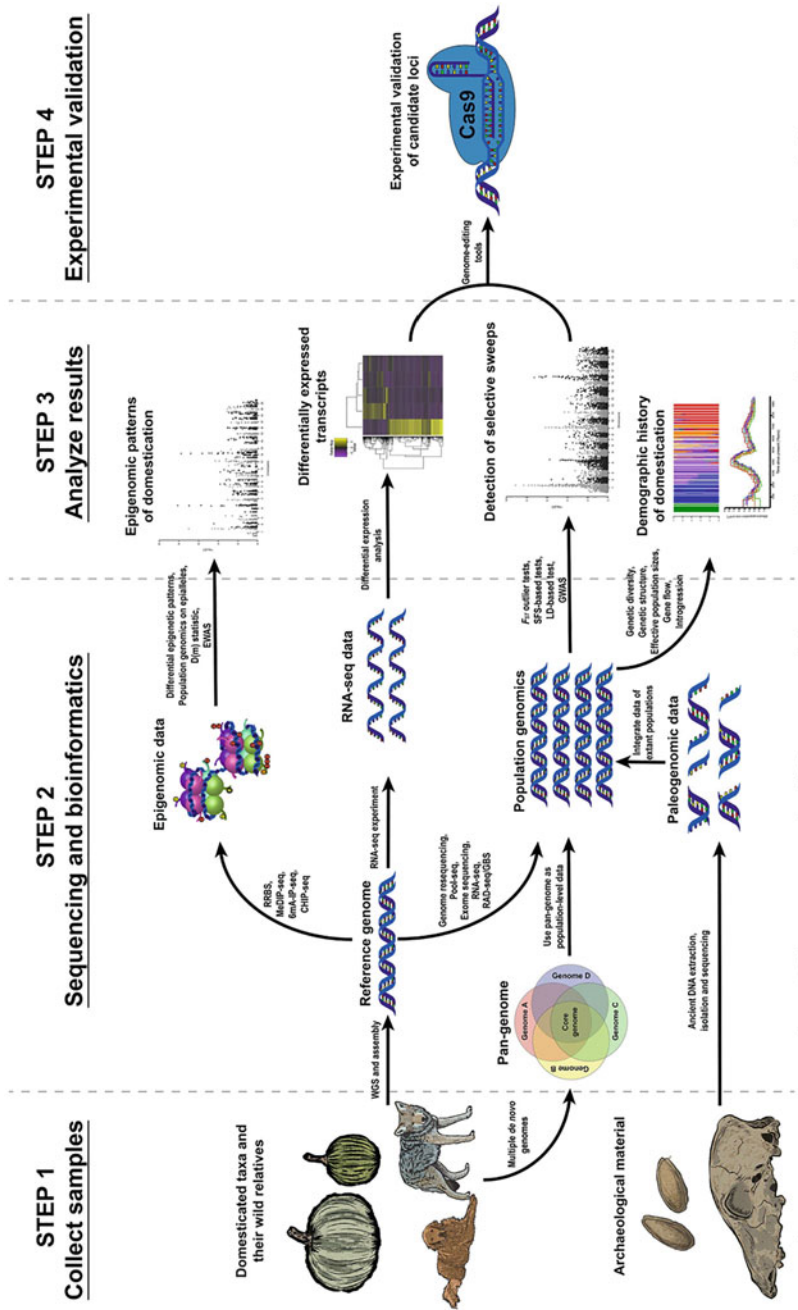


Fig. 40.10 Proposed workflows to study different problems related to the strain domestication of plants and animals through genomic, transcriptomic, and epigenomic tools. (From Barrera-Redondo et al. (2020), credit Frontiers Media)

invertebrate breeding as well as the responsive journal and book editors have to define which biomolecular traits should be mandatory for which type of study or paper—as the ARRIVE consortium did more generally. Eventually, it will be possible to relate nutritional effects to intrinsic genotypes with their specific transient epigenomes and microbiomes.

A variety of fast, robust, and affordable techniques as well as comprehensive bioinformatics databases and programs are available: They wait to be applied as a matter of future routine in studies of aquatic animals and their nutrition. This is one appropriate step towards the ultimate goal of characterizing and understanding the novel mini-ecosystem: the holo-omic interactions between the host and its gut microbiota (Limborg et al. 2018).

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Abbreviations

4E-BP1, 4E-BP2	eIF4E-binding protein-1 and -2 (eukaryotic translation initiation factor 4E-binding proteins), translation repressor proteins
4-HNE	4-hydroxynonenal: a natural byproduct of lipid peroxidation
5-HT	5-hydroxytryptamine = serotonin
5-HT ₁ receptor(s)	Subfamily of 5-HT receptors binding the endogenous neurotransmitter serotonin
5-HTP	5-hydroxytryptophan
AAs	Amino acids
<i>abcb4</i>	Encodes multidrug resistance protein 3, a membrane-bound transporter
<i>abcg2</i>	Encodes ATP-binding cassette sub-family G member 2, a membrane-bound transporter
<i>acca</i>	Encodes acetyl-CoA carboxylase α , a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA through its two catalytic activities, biotin carboxylase, and carboxyltransferase
ACh	Acetylcholine
<i>aco</i>	Encodes aconitase; catalyzes interconversion of citrate and isocitrate in the tricarboxylic acid (TCA) cycle (Yarian et al. 2005)
ADC	Apparent digestibility coefficient
adipocyte	Also lipocytes or fat cells: cells storing lipids
Adipokine	Or adipocytokine, cell signaling protein (cytokine) secreted by adipose tissue. The first adipokine to be discovered was leptin (Conde et al. 2011)
ADP	Adenosine diphosphate
AKP	Alkaline phosphatase

AKT/FOXO3a signaling	Muscle protein degradation is primarily through the activation of the ubiquitin-proteasome pathway (UPP), which can degrade most cell proteins and contribute to 75% protein degradation in muscle. The AKT-dependent regulation of the forkhead box O3a (FOXO3a) protein plays a critical role in UPP pathway. AKT phosphorylates FOXO3a, leading to the exclusion of phosphorylated FOXO3a proteins from the nucleus and the suppression of their transcriptional functions, which decreases muscle protein degradation (White et al. 2013)
ALA	α -linolenic acid, a fatty acid 18:3 ω 3
ALP	Alkaline phosphatase
ALT	Alanine transaminase catalyzes the transfer of an amino group from L-alanine to α -ketoglutarate, the products of this reversible transamination reaction being pyruvate and L-glutamate. The ratio AST/ALT is applied as a biomarker for hepatic health
Alternative complement	Alternative complement activity: one of three complement pathways that opsonize and kill pathogens
AMPK	Adenosine monophosphate-activated protein kinase. The AMPK and TOR (target-of-rapamycin) pathways are interlinked, opposing signaling pathways involved in sensing the availability of nutrients and energy and regulation of cell growth. AMPK (<i>Yin</i> , or the “dark side”) is switched on by lack of energy or nutrients and inhibits cell growth, while TOR (<i>Yang</i> , or the “bright side”) is switched on by nutrient availability and promotes cell growth (González et al. 2020)
ANF	Antinutritional factor
Anorexigen	Appetite suppressing drug or food constituent
AP	Alkaline phosphatase
Apo A-I	Apolipoprotein A-I is pivotal in the cholesterol homeostasis and has anti-inflammatory properties. In addition to its role in innate immunity and inflammation, it participates in the removal of damaged and apoptotic cells (Kravitz et al. 2005)
APU	Apparent protein utilization
ARE	Antioxidant response element: short sequence of DNA within a gene promoter region that is able to bind specific transcription factors and regulate transcription of genes

Arg2	Arginase-2: involved in immunity and amino acid metabolism
AST	Aspartate aminotransferase catalyzes the interconversion of aspartate and α -ketoglutarate to oxaloacetate and glutamate. The ratio AST/ALT is applied as biomarker for liver health
AT	Adipose tissue
ATB ^{0,+}	Amino acid/carnitine transporters
ATGL	Adipose triglyceride lipase, catalyzes the initial step in triglyceride hydrolysis in adipocyte and non-adipocyte lipid droplets (Zimmermann et al. 2004)
ATP	Adenosine triphosphate, an organic compound and hydrotrope that provides energy to drive many processes in living cells
Autophagy	Natural, regulated, destructive mechanism of the cell that disassembles unnecessary or dysfunctional components
B cells	Together with T cells are recognized as a fundamental organizing principle of the adaptive immune system in all vertebrates (Cooper 2015)
BCAA	Branched chain amino acid
Brockmann body	Principal site of insulin synthesis in teleost fishes; composed of a collection of islet tissues corresponding to the islets of Langerhans of mammals (Hine and Martin 2016)
Brush border	A region of surface epithelium that possesses densely packed microvilli (\rightarrow microvillus), rather like the bristles of a brush. This greatly increases the surface area of the epithelium and facilitates the absorption of materials. Brush borders are found in the convoluted tubules of the kidney and in the lining of the small intestine (Hine and Martin 2016)
BW, bwt	Body weight
C3	Complement component 3, a protein of the immune system, central in the activation of the complement system; a connecting link between innate and acquired immunity (Sahu and Lambris 2001)
CaA	Caprylic acid
Caspase-3...12	Members of the cysteine-aspartic acid protease (caspase) family: Sequential activation of caspases plays a central role in the execution phase of cell apoptosis

CAT	Catalase: enzyme that catalyzes the dismutation of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2)
CCAAT box	A distinct pattern of nucleotides with GGCCAATCT consensus sequence that occur upstream by 60–100 bases to the initial transcription site. The CAAT box signals the binding site for the RNA transcription factor, and is typically accompanied by a conserved consensus sequence
CFU	Colony forming units
Claudins	Transmembrane proteins that are the most important components of the tight junctions
CMP	Cytidine monophosphate
CNS	Central nervous system
comm.	Commercial
Complement component 3	Often simply called complement C3 or C3, is a protein of the immune system; central in the complement system and innate immunity. C3 plays a central role in the activation of complement system (Sunyer and Tort 1995)
Complement factor B	An acute phase protein increasing during inflammation
Complement system	Part of the immune system that enhances (complements) the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promotes inflammation, and attacks the pathogen's cell membrane. It is part of the innate immune system
COX-2	Cyclooxygenase forming prostanoids from PUFAs
CPT1	Carnitine palmitoyltransferase 1, a mitochondrial enzyme responsible for the formation of acylcarnitines by catalyzing the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A to l-carnitine. The carnitine palmitoyltransferase system is an essential step in the β -oxidation of long-chain fatty acids
CTR	Calcitriol, increasing the uptake of calcium from the gut into the blood
CuZn-SOD	→SOD1
CXCR4	C-X-C chemokine receptor type 4; pro-inflammatory chemokine

CYP17-II	Cytochrome P450 c17II; a monooxygenase affecting growth, gonad differentiation and development and other reproductive traits of fish
CYP1A	Cytochrome P450, family 1, subfamily A, is involved in phase I xenobiotic and drug metabolism
CYP24A1	Cytochrome P450 24A1: involved in steroid metabolism
CYP2M1	Cytochrome P450 2 M1: involved in xenobiotic metabolism
Cytokine	Cell signaling protein
DAA	Dispensable amino acids which can be synthesized de novo from α -keto acids in the TCA-cycle or through transamination
DAF-16	Ortholog of the FOXO family of transcription factors in the nematode <i>C. elegans</i> ; it is the primary (but not the only) transcription factor required for lifespan extension
DAH	Days after hatch
DDGS	Distillers' dried grain with solubles
DE	Digestible energy
DGAT2	Diacylglycerol O-acyltransferase 2, catalyzes the final reaction in the synthesis of TG (TAG)
DGGE	Denaturing gradient gel electrophoresis; method for identifying genes from natural ecosystems
DHA	Docosahexaenoic acid, ω 3 fatty acid with the chemical formula $C_{22}H_{32}O_2$
Dicer	A key initiative protein of the RNA interference (RNAi) pathway: (=endoribonuclease Dicer) an enzyme cleaves double-stranded RNA (dsRNA) and pre-microRNA (pre-miRNA) into short double-stranded RNA fragments called small interfering RNA
DM	Dry matter
DNMT	DNA methyltransferase
dpf	Days past fertilization
dph	Days past (after) hatch
dpi	Days past (post) infection
DR	Dietary restriction
dsRNA	Double-stranded (ds)RNA, central in RNAi
dw	Dry weight
Dysbiosis	Microbial imbalance or maladaptation
EAA	Nutritionally essential amino acids

EF-1 α	Eukaryotic translation elongation factor 1 alpha responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome
EFA	Essential fatty acid; EFA requirements vary qualitatively as well as quantitatively among different animal species (Bell and Tocher 2009)
EIF4E1C	Eukaryotic translation initiation factor 4E family member 1c, is involved in the insulin signaling pathway and RNA transport
Elovl1	FA elongase elongates saturated and monounsaturated C ₂₀ –C ₂₆ acyl-CoAs
Elovl2	FA elongase elongates C ₂₀ –C ₂₂ polyunsaturated acyl-CoAs
Elovl3	FA elongase elongates saturated and unsaturated C ₁₆ –C ₂₂ acyl-CoAs
Elovl4	FA elongase for the synthesis of ULCFAs (C \geq 26)
Elovl5	FA elongase elongates C ₁₈ –C ₂₀ polyunsaturated acyl-CoAs
Elovl6	FA elongase elongates C ₁₂ –C ₁₆
Elovl7	FA elongase elongates saturated and unsaturated C ₁₆ –C ₂₂ acyl-CoAs
EPA	Eicosapentaenoic acid, ω 3 fatty acid with the chemical formula C ₂₀ H ₃₀ O ₂
ER	Endoplasmic reticulum
FA	Fatty acid
FAA	Food anticipatory activity
fAA	Free amino acid
FABP, FABP2a2	Fatty acid-binding proteins, involved in fatty acid metabolism
FADS1	Δ^5 desaturation; missing in fishes (Monroig et al. 2018)
FADS2	Fatty acid desaturase enabling Δ^6 , Δ^5 , and Δ^4 desaturation
FAS	Fatty acid synthase
FBPase	Fructose-1,6-bisphosphatase: converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis and the Calvin cycle
FCE	Feed conversion efficiency
FCR	Feed conversion rate: efficiency with which the bodies of livestock convert animal feed into the desired output
FE	Feed efficiency = weight gain/feed consumed
FER	Feed efficiency ratio
FI	Feed intake

FM	Fishmeal
FO	Fish oil
FOS	Fructooligosaccharides
FoxOs	Forkhead box proteins, a family of transcription factors regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity
G6Pase	Glucose-6-phosphatase: hydrolyzes glucose-6-phosphate into a phosphate group and free glucose. It is central in \rightarrow glycogenesis and completes the final step in \rightarrow gluconeogenesis and therefore plays a key role in the homeostatic regulation of blood glucose levels (Nordlie and Sukalski 1985)
GABA	γ -aminobutyric acid, a non-proteinogenic amino acid: inhibitory neurotransmitter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase, catalyzes one step of glycolysis; recent discovery: involvement in several non-metabolic processes, including transcription activation, initiation of apoptosis (Tarze et al. 2006)
GDH	(Also GLDH) Glutamate dehydrogenase
Germ-free animals	Animals that have no microorganisms living in or on them
GF	Germ free, gnotobiotic
GFP	Green fluorescence protein
GH	Growth hormone that activates AgRP, thus, increasing appetite; is opposed by the hormone leptin; furthermore, as pleotropic hormone GH is involved in growth, stress response, energy homeostasis, reproduction
GHR (a, b)	Growth hormone receptor (a, b); proteins that are a transmembrane receptor for growth hormone
GHRH	Growth hormone-releasing hormone: a releasing hormone of growth hormone (GH) that stimulates GH production and release by binding to the GHRH Receptor (GHRHR) on cells in the anterior pituitary
GHRL	Ghrelin: G rowth H ormone R elease I nducing: the “hunger hormone” is a peptide hormone regulating appetite and the distribution and rate of use of energy. Ghrelin acts as orexigenic hormone
GI	Gastrointestinal (tract)
GIFT	Genetically improved farmed tilapia by conventional breeding

GK	Glucokinase, a membrane-bound glucose sensor, facilitating phosphorylation of glucose to glucose-6-phosphate
Glucagon	A peptide hormone, produced by alpha cells of the pancreas. It works to raise the concentration of glucose and fatty acids in the bloodstream and is considered to be the main catabolic hormone of the body
Gluconeogenesis	Synthesis of glucose from certain noncarbohydrate carbon substrates, such as pyruvate and lactate
GLUT-1 . . . n	Glucose transporters 1 . . . n
Glycemia	Presence, or the level, of glucose in the blood
Glycogen	Multibranched polysaccharide of glucose that serves as a form of energy storage in animals (Nelson and Cox 2005)
Glycogenesis	The formation of glycogen from monosaccharides via glucose-6-phosphate (Berg et al. 2015)
Glycogenolysis	The biochemical breakdown of glycogen to glucose (Berg et al. 2015)
Glycolysis	Sequence of reactions that metabolizes one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP (Berg et al. 2015)
GMP	Guanosine monophosphate (monophosphate)
Gnotobiotic	→ Germ free
GO	Gene ontology
GOS	Galactooligosaccharide
GOT	Glutamate oxalate transaminase or aspartate transaminase (⇒AST)
GPT	Glutamate pyruvate transaminase →ALT
GPx, GPX	Glutathione peroxidase
GR	Glutathione reductase
Gr	Growth rate
GS	Glycogen synthase, key enzyme in glycogenesis, the conversion of glucose into glycogen
GSH	Glutathione
GST	Glutathione transferase
H3K4, H3K27	Histones
HAMP	Hepcidin, a regulator of iron metabolism
HCD	High carbohydrate diet
Hepcidin	A key regulator of the entry of iron into the circulation with antimicrobial property
HK	Hexokinases: enzymes phosphorylating hexoses, forming hexose phosphate

HNF4 α	Hepatocyte nuclear factor 4 α , a protein implicated in the metabolism of cholesterol, FAs, and AAs (Hayhurst et al. 2001)
homeoviscous adaptation	The homeostatic process that regulates the viscosity of membrane lipids by specific membrane phospholipids and LC-PUFAs to counteract the tendency of cell membranes to become rigid at low temperatures or high salt contents (Ernst et al. 2016)
hpf	Hours past fertilization
hps	Hours post stimulation
HSD	High starch diet
HSFs	Heat shock factors: transcription factors regulating the expression of heat shock proteins
HSI	Hepatosomatic index = (liver weight/body weight) \times 100
HSL	Hormone-sensitive lipase which hydrolyzes diacylglycerol to monoglycerides and free fatty acids (Zimmermann et al. 2004)
HSP90	Heat shock protein 90 (atomic mass approximately 90 kDa), a chaperone protein assisting other proteins to fold properly, stabilizing proteins against stress; it also canalizes phenotypic variability
HSPs	Heat shock proteins: family of proteins produced by cells in response to exposure to stressful conditions. Many HSPs perform chaperone function
HUFA	Highly unsaturated fatty acids: here defined as FAs with ≥ 20 carbons and ≥ 3 double bonds, such as arachidonic acid (ARA; 20:4 ω 6), eicosapentaenoic acid (EPA; 20:5 ω 3), and docosahexaenoic acid (DHA; 22:6 ω 3) (Bell and Tocher 2009)
HYP	Hypothalamus
Hypoglycemia	Unusually low concentration of sugar in the blood
i.p.	Intraperitoneal (injection): injection of a substance into the peritoneum (body cavity)
IFN1, IFN- α	Type I interferon in fishes, equivalent to mammalian and avian interferon- α , involved in innate immune response against viral infection; in contrast to INF- α , IFN1 is present as a single gene
IFN- γ	Interferon- γ , or type II interferon: a cytokine critical for innate and adaptive immunity against viral, some bacterial and protozoal infections
ig; ig ⁺	Immunoglobulin; immunoglobulin positive
IGF-1...3, or IGF-I...III	Insulin-like growth factors 1...3, hormones similar in molecular structure to insulin

IGF1ra, IGF1rb	Insulin-like growth factor 1 receptors a and b
IgM, IgM _H	Immunoglobulin M, immunoglobulin M heavy chain
IL-1	Interleukin 1: family of 11 cytokines, inducing a complex network of pro-inflammatory cytokines
IL-10	Interleukin 10: an anti-inflammatory cytokine
IL-11	Member of the IL-6-type cytokine family. Overexpression of <i>il-11</i> is associated with a variety of cancers and may provide a link between inflammation and cancer in mammals (Xu et al. 2016)
IL-12	Interleukin 12: interleukin produced in response to antigenic stimulation, a T cell-stimulating factor, plays a role in the activities of natural killer cells. It is a regulator of cell-mediated immune responses and provides immune defense against parasites, viruses, and intracellular bacteria
IL-15	Interleukin 15: cytokine inducing cell proliferation of natural killer cells against infections
IL-17	Interleukin 17: pro-inflammatory cytokine family produced by T-helper cells and is induced by IL-23; can be induced in hemocytes after bacterial challenge
IL-1 β	Interleukin 1 β : a pro-inflammatory cytokine, involved in cell proliferation, differentiation, and apoptosis
IL-2	Interleukin 2: cytokine regulating the activity of white blood cells; part of the defense against microbial infections
IL-21	Interleukin 21: cytokine regulating cells of the immune system, including natural killer cells and cytotoxic T cells that can destroy infected cells
IL-22	Interleukin 22: member of the IL-10 superfamily
IL-3	Interleukin 3 promotes growth and reproduction of virtually all the different types of committed stem cells (Guyton and Hall 2005)
IL-4	Interleukin 4 anti-inflammatory cytokine, has many biological roles, including the stimulation of activated B- and T-cell proliferation
IL-5	Interleukin 5 stimulates B cell growth and increases immunoglobulin secretion
IL-6	Interleukin 6 acting as both pro-inflammatory cytokine and anti-inflammatory myokine
IL-8	Interleukin 8: a pro-inflammatory cytokine

IMP	Inosine monophosphate
iNOS	Inducible nitric oxide synthase
IU	International units: the amount of a drug, hormone, vitamin, enzyme, etc. that produces a specific effect as defined by an international body and accepted internationally
IκB	Inhibitor factor κB: protein complex that controls transcription of DNA, cytokine production, and cell survival
JNK	c-Jun N-terminal protein kinases are a mitogen-activated protein kinase family, and responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. For instance, JNK1 is involved in apoptosis, neurodegeneration, cell differentiation and proliferation, inflammatory conditions, and cytokine production mediated by AP-1 (activation protein 1) (Oltmanns et al. 2003)
Keap1	Kelch-like-ECH-associated protein1, a stress sensor protein
Keap1-Nrf2	Major regulator of cytoprotective responses to oxidative and electrophilic stress
KEGG	Kyoto Encyclopedia of Genes and Genomes, a database resource for understanding high-level functions and utilities of the biological system
LAB	Lactic acid bacteria
LauA	Lauric acid
LCFA	Long-chain fatty acid: fatty acid with 13–22 C atoms
LC-PUFA	Long-chain polyunsaturated fatty acid
LDLR	Low-density lipoprotein receptor: mediates the endocytosis of cholesterol-rich LDL and thus maintains the plasma level of LDL
LEAP	Liver-expressed antimicrobial peptides
LEAP-2a, LEAP-2b	Liver-expressed antimicrobial peptides 2
LiA	Lipoic acid
LIN	Often also abbreviated as LA, linoleic acid, a fatty acid: 18:2ω6
LPO	Lipid peroxidation
LPS	Lipopolysaccharides (also lipoglycans, endotoxins): large molecules consisting of a lipid and a polysaccharide; found in Gram-negative bacteria and cyanobacteria, and elicit strong immune responses in animals

Lysozyme	Glycoside hydrolase, an enzyme that damages bacterial cell walls
LYZ	Lysozyme
MAPK	Or MAP kinase is a type of protein kinase that is specific to the amino acids serine and threonine (i.e., a serine/threonine-specific protein kinase). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock, and pro-inflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis (Pearson et al. 2001)
MCFA	Medium-chain fatty acid, fatty acid with 6–12 C atoms
Melatonin	N-acetyl-5-methoxy tryptamine, a hormone that anticipates the daily onset of darkness; hormone affecting the modulation of wake/sleep patterns
MHC1..2 (also MHCII)	MHC (major histocompatibility complex) class 1: part of immunity, antigen presentation, important in initiating immune responses
Microbiota	Resident microbial communities in fishes and invertebrates
microRNA, miRNA, miR	Non-coding RNAs, about 22 nucleotides, functioning in RNA silencing and posttranscriptional regulation of gene expression
<i>microvilli</i>	Hairlike microscopic cellular membrane protrusions that increase the surface area for diffusion found, especially in secretory and absorptive cells. They constitute the brush borders observed in electron micrographs (Hine and Martin 2016)
MLCK	Myosin light chain kinase, a key regulator of tight junction barriers, important in the mechanism of contraction in muscle
Mn-SOD	→SOD2
MOS	Mannan oligosaccharides
MR	Methionine restriction
mTOR (MTOR)	Mechanistic (formerly mammalian) target of rapamycin; a protein that binds to rapamycin; thereby regulating cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription
mucins	Family of high molecular weight, heavily glycosylated proteins; a key component in most gel-like secretions, serving functions from lubrication to cell signaling to forming chemical barriers (Marin et al. 2007)

MUFA	Monounsaturated fatty acid containing one ethylenic bond
MYD88	Myeloid differentiation factor 88, a pivotal signaling component of the innate immune response, serving as an adaptor for the interleukin 1 receptor and the Majority of TLRs (Takeda and Akira 2005)
MyoD	One myogenic regulatory (differentiation) factor
Myogenin	(= myog, myogenic factor 4) Transcription factor involved in the coordination of skeletal muscle development or myogenesis and repair
Myokine	Cytokine produced and released by muscle cells (myocytes) in response to muscular contractions
Myostatin	A myokine: protein produced and released by myocytes inhibiting muscle cell growth and differentiation, member of the TGF- β superfamily
Na ⁺ /K ⁺ -ATPase	Sodium–potassium adenosine triphosphatase central in ion homeostasis. For every ATP molecule that the pump uses, three sodium ions are exported and two potassium ions are imported; involved in controlling perturbations of Na ⁺ and K ⁺ homeostasis during apoptosis (Panayiotidis et al. 2006)
NAD	Nicotinamide adenine dinucleotide
NEAAs	Nutritionally nonessential amino acids
Nesfatin-1	Appetite-reducing (anorexic) peptide
NF- κ B	Nuclear transcription factor kappa-B, responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized low-density lipoprotein, and bacterial or viral antigens. Incorrect regulation of NF- κ B has been linked to cancer, inflammatory, and autoimmune diseases, septic shock, viral infection, and improper immune development (Perkins 2007)
NO \cdot	Nitric oxide radical
NPC1L1	A transmembrane protein involved in intestinal cholesterol absorption (Hall et al. 1995)
NPY	Neuropeptide Y: with slight variations, a neurotransmitter in the brain and in the autonomic nervous system of animals; one of the strongest orexigenic signals
NRF2	Nuclear factor erythroid 2-related factor 2: master regulator of the antioxidant response, which is important for the mitigation of oxidative stress (Nguyen et al. 2009)
NSP	Nonstarch polysaccharides

NSPase	Nonstarch carbohydrases enzyme complex
OA	Oleic acid (18:1 <i>cis</i> -9), a monounsaturated fatty acid
Occludin	Integral plasma membrane protein located at the tight junctions
Orexigen	Drug, hormone, or compound that stimulates appetite
OTU	Operational taxonomic units
OX	Orexin, a neuropeptide that regulates arousal, wakefulness, and appetite
Oxidative burst	→Respiratory burst
Oxidative stress	States where the balance between generation and elimination of ROS is disturbed in favor of the generation of ROS
P38	P38 mitogen-activated protein kinase; is activated by a variety of cellular stresses (Cuadrado and Nebreda 2010)
P53	Tumor protein P53, which prevents cancer formation, functions as a tumor suppressor
PA	Phytic acid
PAMPs	Pathogen-associated molecular patterns
PAse	Phytase cleaving phytic acid
PBS	Phosphate-buffered saline solution
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase: central in gluconeogenesis. It converts oxaloacetate into phosphoenolpyruvate and carbon dioxide (Chakravarty et al. 2005)
PEPT1...2	Oligopeptide transporters (members of the solute carrier family 15) encode solute carriers localized to the brush border membrane of the intestinal epithelium and mediate the uptake of di- and tripeptides
PER	Protein efficiency ratio: weight gain divided by intake of feed protein
PFK-1	6-phosphofructo-1-kinase: one of the most important regulatory enzymes of glycolysis; converts fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP
PG	Peptidoglycan
PI	Protease inhibitor
PI3K/AKT/mTOR signaling	Intracellular signaling pathway important in regulating the cell cycle, directly related to cellular

	quiescence, proliferation, cancer, and longevity. PI3K activation phosphorylates and activates AKT (protein kinase B), localizing it in the plasma membrane (King et al. 2015)
PIT	Pituitary or hypophysis, an endocrine gland. Its hormones help control: growth, blood pressure, and certain functions of the sex organs, thyroid glands, metabolism, and reproduction
PK	Pyruvate kinase: catalyzes the final step of glycolysis, the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one molecule of ATP (Gupta and Bamezai 2010)
PL	Phospholipid: class of lipids that are a major component of all cell membranes
poly I:C	Polyinosinic-polycytidylic acid, an immunostimulant interacting with the toll-like receptor 3 (TLR3)
POMC	Proopiomelanocortin, a precursor polypeptide which cleavage gives rise to several peptide hormones. POMC neuron stimulation results in satiety (Mineur et al. 2011)
PPARs	Peroxisome proliferator-activated receptors; nuclear receptor proteins functioning as transcription factors. They are central in the regulation of cellular differentiation, development, and metabolism (carbohydrate, lipid, and protein)
PPAR α	Peroxisome proliferator-activated receptor α , ligand-activated transcription factor; key regulator of lipid metabolism
PPAR γ	Peroxisome proliferator-activated receptor γ ; regulates fatty acid storage and glucose metabolism (Jones et al. 2005)
Preproinsulin	Primary translational product of the <i>ins</i> gene. Preproinsulin is a proinsulin molecule with a signal peptide attached to its N-terminus. It is a biologically inactive precursor to the biologically active endocrine hormone insulin and has to be activated in two steps
proPO	Prophenoloxidase: part of the major innate defense system in invertebrates via melanization of pathogens and damaged tissues. The activated form, phenoloxidase facilitates the melanization of pathogens and damaged tissues

PRP	Pattern recognition protein
PRRs	Pattern recognition receptors
PSMs	Plant secondary metabolites
PUFA	Polyunsaturated fatty acid containing two or more ethylenic bonds, such eicosapentaenoic acid (EPA, 20:5n-3 or 20:5ω-3)
PWG	Percent weight gain
PXR	Pregnane X receptor, a nuclear transcription factor central in xenobiotic biotransformation
PY	Peptide Y, a NPY-related peptide
pyloric ceca	Organ with fingerlike projections, located near the junction of the stomach and the intestines. Its function is not entirely understood, but it is known to secrete enzymes that aid in digestion, may function to absorb digested food, or do both
Pyruvate kinase	Enzyme involved in the last step of glycolysis. It catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one molecule of ATP (Gupta and Bamezai 2010)
RA	Retinoic acid, a metabolite of vitamin A (retinol) that mediates the functions of vitamin A required for growth and development
Rapamycin	Also sirolimus; chemical immune suppressant
RAR	Retinoic acid receptor: type of nuclear receptor which can also act as a transcription factor
Respiratory burst	Rapid production and release of reactive oxygen species
Retinol	Vitamin A
RNS	Reactive nitrogen species (intermediates): antimicrobial molecules derived from nitric oxide (•NO) and superoxide (•O ₂ ⁻) via enzymatic activity
ROS	Reactive oxygen species: chemical reactive molecules containing oxygen, such as peroxides, superoxide, hydroxyl radical, and singlet oxygen (→electrophilic stress and oxidative stress)
RXR	retinoid receptors: nuclear receptors that bind to retinoids; when bound to a retinoid, they act as transcription factors (Rowe 1997)
S6K1	Ribosome protein S6 kinase 1: phosphorylates residues of the S6 ribosomal protein; kinase activity of this protein leads to an increase in protein synthesis and cell proliferation

SAH	<i>S</i> -adenosyl-l-homocysteine: amino acid derivative, intermediate in the synthesis of cysteine and adenosine; SAH is formed by the demethylation of <i>S</i> -adenosyl-l-methionine (SAM)
SAM	<i>S</i> -adenosyl-l-methionine: involved in methyl group transfers
SB	Sodium butyrate, a salt of the short-chain fatty acid butyric acid (C ₄ H ₈ O ₂)
SBM	Soybean meal
SCAP	SREBF chaperone essential in maintaining and controlling de novo synthesis of cholesterol
SCFA	Short-chain fatty acid, also referred to as volatile fatty acid (VFA): fatty acid with 2–6 C atoms
secretagog	Substance causing other substances to be secreted
SFA	Also abbreviated SAFA: saturated fatty acid; fatty acids without C=C double bonds
SGR	Specific growth rate
SOD	Superoxide dismutase: enzyme that catalyzes the dismutation (=disproportionation) of superoxide ($\cdot\text{O}_2^-$) radicals into ordinary molecular oxygen (O ₂) and hydrogen peroxide (H ₂ O ₂)
SOD1; SOD2	Cu-Zn-superoxide dismutase = superoxide dismutase 1; Mn-superoxide dismutase = superoxide dismutase 2
SREBP1, 2	Sterol regulated element-binding proteins-1, 2, transcriptional activator required for lipid homeostasis; SREBPs are master regulators of lipid homeostasis (Eberlé et al. 2004); its expression is regulated by insulin (Ferré and Foufelle 2010)
T cell	T lymphocyte: type of lymphocyte central in cell-mediated immunity (→B cells)
TB	Tributylin, a specific triglyceride naturally present in butter made of three butyric acid and glycerol
TET enzymes	Family of ten-eleven translocation (TET) methylcytosine dioxygenases. They are instrumental in DNA demethylation. 5-Methylcytosine is a methylated form of the DNA base cytosine that often regulates gene transcription and has several other functions in the genome (Wu and Zhang 2017)
TFAs, tFAs	Total fatty acids
TG, TAG	Triglyceride or triacylglycerol: ester derived from glycerol and three fatty acids

TGF- β	Transforming growth factor β : multifunctional cytokine. The TGF- β superfamily includes endogenous growth-inhibiting proteins with, for instance, anti-inflammatory function
TH	Tyrosine hydroxylase
TJ	Tight junction
TLRs	Toll-like receptors, a class of proteins that play a key role in the innate immune system by recognizing molecules that are broadly shared by pathogens. Once pathogen microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs, which activate immune cell responses
TLR3	Toll-like receptor 3, a member of the toll-like receptor family of pattern recognition receptors of the innate immune system
TLR9	Toll-like receptor 9, a pro-inflammatory cytokine
TNF- α	Tumor necrosis factor α : a pro-inflammatory cytokine, able to induce fever, apoptotic cell death, cachexia, inflammation and able to inhibit tumorigenesis. Moreover, involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation
TOR	Target of rapamycin: highly conserved, nutrient-sensitive protein kinase, a central controller of protein synthesis, cell growth, cell proliferation, cell motility, cell survival, autophagy, transcription, and aging under nutrient availability (\rightarrow AMPK)
ULCFA	Ultra long-chain fatty acid, fatty acid with 26 or more carbons
UMP	Uridine monophosphate
USFA	Unsaturated fatty acids possess one or more C=C double bonds
VA	Vitamin A
VAT	Visceral AT
VD	Vitamin D
WG	Weight gain
WSSV	White spot syndrome virus
ZO-1, ZO-2	Zonula occludens-1, -2, tight junction proteins ZO-1, ZO-2: proteins located on a cytoplasmic membrane surface of intercellular tight junctions;

	together with the claudin group of proteins, they are the main component of the tight junctions
β -Defensins	Antimicrobial peptides implicated in the resistance of epithelial surfaces to microbial colonization
β -Oxidation	Catabolic process by which fatty acid molecules are broken down in the mitochondria in eukaryotes to generate acetyl-CoA, which enters the citric acid cycle, and NADH and FADH ₂ , which are coenzymes used in the electron transport chain
Σ SAA	Sum of sulfur-containing amino acids

Major Microbial Disease Agents of Farmed Aquatic Animals¹

Aeromonas hydrophila Hemorrhagic septicemia, motile aeromonas septicemia, redsore disease, fin rot

Aeromonas salmonicida Furunculosis (particularly in salmonids), disease that causes septicemia, hemorrhages, muscle lesions, inflammation of the lower intestine, spleen enlargement, and death in freshwater fish populations

Aeromonas veronii Wound infections, diarrhea, or sepsis

Edwardsiella tarda* + *E. ictaluri Edwardsiellosis; infected fishes show abnormal swimming behavior, including spiral movement and floating near the water surface; varying clinical signs: loss of pigmentation, exophthalmia, opacity of the eyes, swelling of the abdominal surface, petechial hemorrhage in fin and skin, and rectal hernia (Park et al. 2012)

Flavobacterium psychrophilum “Coldwater disease,” risk of spinal deformities (Madsen et al. 2001)

Flavobacterium columnare Columnaris, saddleback disease

Photobacterium damsela* ssp. *piscicida Skin lesions, i.e., ulcers, formed, particularly in the region of the pectoral fin and caudal peduncle

Pseudomonas aeruginosa Skin darkening, scales detachment, fin necrosis, exophthalmia cataract/trachoma, and blindness, hemorrhaging on the body surface

Spiroplasma eriocheiris Tremor disease in Chinese mitten crab (*Eriocheir sinensis*)

Staphylococcus aureus Eye disease, jaundice

Streptococcus agalactiae Exophthalmia, hemorrhagic septicemia, meningoencephalitis, multiple necrotic foci

¹From Austin and Austin (2016), amended.

- Streptococcus iniae*** Acute septicemia, meningoencephalitis, streptococcosis/streptococcosis
- Vibrio alginolyticus*** Eye disease, septicemia
- Vibrio anguillarum*** Vibriosis
- Vibrio cholerae*** Ascites disease, septicemia
- Vibrio harveyi*** Common pathogen to many marine fishes and invertebrates. Diseases include eye lesions, gastroenteritis, vasculitis, luminous vibriosis, and “black gill disease.” Notable as a cause of luminous vibriosis in shrimps.
- Vibrio parahaemolyticus*** Pathogen mainly of shellfish, notably penaeids; role as fish pathogen highly debated (Austin and Austin 2016)
- Vibrio splendidus*** Vibriosis
- Yersinia ruckeri*** Enteric redmouth, salmonid blood spot; mostly restricted to salmonids

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