Dietary Supplements for the Health and Quality of Cultured Fish

Edited by
H. Nakagawa, M. Sato and D.M. Gatlin III

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Edited by

Heisuke Nakagawa

Professor Emeritus, Hiroshima University, Higashi-hiroshima, Japan

Minoru Sato

Professor, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

and

Delbert M. Gatlin III

Professor, Department of Wildlife and Fisheries Sciences and Faculty of Nutrition, Texas A&M University System, College Station, Texas, USA

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CABI Head Office
Nosworthy Way
Wallingford
Oxon OX10 8DE
UK

Tel: +44 (0)1491 832111
Fax: +44 (0)1491 833508
E-mail: cabi@cabi.org
Website: www.cabi.org

CABI North American Office
875 Massachusetts Avenue
7th Floor
Cambridge, MA 02139
USA

Tel: +1 617 395 4056
Fax: +1 617 354 6875
E-mail: cabi-nao@cabi.org

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Contents

Contributors

Becker, Klaus, PhD, *Professor, Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim (480), D 70593 Stuttgart, Germany; E-mail: nugent@uni-hohenheim.de.*

Francis, George, PhD, *Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim (480), D 70593 Stuttgart, Germany; E-mail: frgeorge@uni-hohenheim.de.*

Gatlin III, Delbert M., PhD, *Professor, Department of Wildlife and Fisheries Sciences and Faculty of Nutrition, Texas A&M University System, College Station, TX 77843-2258, USA; Tel: (+1) 979-847-9333, Fax: (+1) 979-845-4096; E-mail: d-gatlin@tamu.edu.*

Ishikawa, Manabu, PhD, *Research Associate, Faculty of Fisheries, Kagoshima University, Kagoshima 890–0056, Japan; Tel: (+81) 99-286-4180, Fax: (+81) 99-286-4184; E-mail: ishikawa@fish.kagoshima-u.ac.jp.*

Kaushik, Sadasivam J., PhD, *UMR NuAGe, INRA-IFREMER – University Bordeaux 1, 64310 Saint-Pée-sur-Nivelle, France; Tel: (+33) 559-51-5999, Fax: (+33) 559-54-5152; E-mail: kaushik@st-pee.inra.fr.*

Kirchner, Séverine, PhD, *Department of Pharmacology and Physiology, UMDNJ, New Jersey Medical School, Newark, NJ 07103, USA; E-mail: kirchnse@ umdnj.edu.*

Koshio, Shunsuke, PhD, *Professor, Faculty of Fisheries, Kagoshima University, Kagoshima 890-0056, Japan; Tel: (+81) 99-286-4182, Fax: (+81) 99-286-4184; E-mail: koshio@fish.kagoshima-u.ac.jp.*

Li, Peng, PhD, *Department of Wildlife and Fisheries Sciences and Faculty of Nutrition, Texas A&M University System, College Station, TX 77843-2258, USA.*

Maita, Masashi, PhD, *Professor, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan; Tel: (+81) 3-5463-0544, Fax: (+81) 3-5463-0552; E-mail: mmaita@s.kaiyodai.ac.jp.*

Montgomery, W. Linn, PhD, *Professor, Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA; Tel: (+1) 520-523-7505, Fax: (1) 520-523-7500; E-mail: Linn.Montgomery@nau.edu.*

Nakagawa, Heisuke, PhD, *Professor emeritus, Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima 739–8528, Japan; Home address: 881-2, Ohsawa, Saijo, Higashi-hiroshima 739-0034, Japan; Tel. and Fax: (+81) 82-425-1524; E-mail: naka1524@enjoy.ne.jp.*

Nakano, Toshiki, PhD, *Assistant Professor, Graduate School of Agricultural Science, Tohoku University, Sendai 981–8555, Japan; Tel: (+81) 22-717-8738, Fax: (+81) 22-717-8739; E-mail: nakanot@bios.tohoku.ac.jp.*

Panserat, Stéphane, PhD, *UMR NuAGe, INRA-IFREMER – University Bordeaux 1, 64310 Saint-Pée-sur-Nivelle, France; E-mail: panserat@st-pee.inra.fr.*

Sakamoto, Fumio, *Executive Managing Director, Kagoshima Industrial Trading Co. Ltd, Kagoshima 892-0821, Japan; Tel: (+81) 99-226-7681, Fax: (+81) 99-224-6140; E-mail: info@ktrade.co.jp.*

Sato, Minoru, PhD, *Professor, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan; Tel: (+81) 22-717-8736, Fax: (+81) 22-717-8739; E-mail: msato@bios.tohoku.ac.jp.*

Satoh, Shuichi, PhD, *Professor, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan; Tel: (+81) 3-5463-0557, Fax: (+81) 3-5463-0553; E-mail: ssatoh@s.kaiyodai.ac.jp.*

Takeuchi, Toshio, PhD, *Professor, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan; Tel: (+81) 3-5463-0545, Fax: (+81) 3-5463-0545; E-mail: take@s.kaiyodai.ac.jp.*

Preface

Fish are excellent sources of protein and lipids, especially unsaturated fatty acids which have various merits for human health. The anxiety associated with livestock meat from outbreaks and spread of some diseases, and expanding knowledge of the dietary benefits of fish suggest that fish products will become increasingly important among the world's food resources.

Advancements in fish nutrition have progressed considerably with regard to growth and feed efficiency, such that mass production of several species of cultured fish has been well established for at least two decades. In general, the quality and quantity of various nutrients in diets have been assessed in terms of fish survival and growth; both are strongly related to the availability (digestibility) and sufficiency of major organic macronutrients (e.g. carbohydrate, protein, lipid, without regard for the specific identity of the individual building blocks of these substances). Recently, however, much concern has arisen over the quality of fish flesh and health of fish in aquaculture. In response, a variety of feed supplements have been investigated with respect to their effects on health and quality of cultured fish, and some of the findings have been confirmed through practical use by fish farmers.

This book addresses current information on the effects of non-macronutrients such as micronutrients and other efficacious substances from plants, animals and bacteria, with regard to quality and health of cultured fish. Reports of the nutritional merits of various substances are based on evidence available in the published scientific literature or from yet unpublished data presented at scientific meetings or acquired as personal communications from researchers. Although some feed supplements have been used in fish culture without scientific evidence to support their efficacy, the book focuses on substances that have been experientially assessed by fish culturists or proven to have beneficial effects in mammals.

The first two chapters discuss how to evaluate and assess the quality, health and disease resistance of cultured fish. Systems for postharvest evaluation of fish products have been well established in many countries. Fish culturists, however, also require techniques for pre-harvest evaluation and control of quality; techniques that will produce healthy and high quality fish by ensuring proper metabolism and physical activity through balanced nutritional conditions. The required techniques are likely to be complex because the assessment of 'quality' may involve independent assessments of such factors as palatability, flavour, tissue firmness, colour (skin and muscle) and chemical composition (protein and lipid). Reports summarized here have similarly relied on a variety of approaches to evaluate physiological conditions, including haematology, serology, enzymology, biochemical composition, histological observation, disease resistance, stress resistance and physical activities.

Clearly, what consumers find desirable in cultured fish products may vary depending on many aspects including fish species, as well as human factors such as culture, customs and cooking method. None the less, some standardization of quality assessment based on worldwide scope might be possible.

The next four chapters of the book address essential nutrients. Diets should include sufficient quantities of all essential nutrients to enable maximum growth and feed utilization. Needs for essential nutrients such as amino acids, fatty acids, vitamins and minerals have been scientifically established in many fish species, and appropriate amounts of them are included in commercial diets. In addition to levels essential to support optimal growth performance, some of these nutrients exert a secondary influence on physiological functions and health such as metabolism, physical vitality and disease resistance. Adequate levels of some dietary supplements may further enhance health and carcass quality.

Chapters 7 to 12 deal with the feed supplements originating from microorganisms, plants and animals. Some of these substances are involved in regulation of metabolism and physical activities in fish. The actions of these substances are not completely understood, although findings from experimental studies have already led to their application in fish culture. Collectively, these chapters were concerned with how to improve health condition and quality, in terms of biochemical and physiological parameters, from the viewpoint of fish culturists and consumers.

Nutrigenomics presented in Chapter 13 would be one of the new directions for prospective fish nutrition to use in evaluating fish quality. The application of new technologies and approaches would stimulate further research to confirm the mechanisms of action of feed supplements. The economic aspects of marketing and security of feed supplements is included in the last chapter, Chapter 14. Consumers' attitudes about quality of cultured fish include concerns for safety and economic factors such as cost. The extra costs used to improve quality achieved during fish culture must be balanced against consumers' acceptance, and fish culture techniques must overcome consumers' worries.

We believe the information in this volume will serve fisheries scientists, fish culturists, feed engineers and students as a useful manual. We also hope that this volume will expand knowledge of various aspects of fish culture technology.

The volume partly consists of articles published in a book entitled *Micronutrients and Health of Cultured Fish* (Japanese edition) supervised by the Japanese Society of Fisheries Science (JSFS). The Japanese edition was presented as the proceedings of a symposium held in Tokyo, Japan, in April 2003. We proposed to publish an English edition that would be appreciably larger in scope, with expanded text, tables, figures and references, compared to the Japanese edition. So, this volume was published with the approval of the committee of JSFS.

Finally, we wish to thank all of the contributors for their excellent cooperation and Tim Hardwick of CABI for his encouragement.

Dr Heisuke Nakagawa
Dr Minoru Sato
Dr Delbert M. Gatlin III

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1 Evaluation of Quality in Cultured Fish

HEISUKE NAKAGAWA

Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima 739-8528, Japan

Mass production techniques in fish culture have been well established over the last two decades with regard to elevating growth and feed efficiency. However, very little work has been done on fish quality, which might influence market value. Criteria used to evaluate the quality of cultured fish would include: meat quality, durability of freshness, colour (meat and skin), metabolism, disease resistance, stress response, and physical activity besides growth and feed utilization. Factors concerning growth performance are essential and should not be disregarded in relation to evaluating quality of cultured fish. The fish quality, including carcass quality and physiological condition, should be evaluated in fish with maximum growth performance.

The feeding activity, which is influenced by dietary composition, rearing density, rearing environment, stressful conditions, disease, etc., influences growth and feed efficiency. At present, fish culturists may not take improvement of fish quality seriously because in the current system high quality fish does not always equate to high market value. However, internationalization of markets for marine products may demand higher product quality in the near future. This chapter summarizes possible indices of quality control in cultured fish relative to feed supplements. A lot of feed supplements, which are not essential nutrients, are used in fish culture. Although an adequate use of some may give positive effects on harvested products, excessive use of some supplements may give negative effects. Such effects of various supplements on cultured fish will be covered in Chapters 3 to 12.

1 Evaluation of Carcass Quality and Taste

The criteria for sensory evaluation of fish meat vary according to fish species, nationality, cooking and season. As primary factors dominating

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carcass quality, quantitative and qualitative properties of the reserved lipid and protein composition cannot be disregarded. A comparison of them between wild and cultured fish show that cultured fish are generally characterized by high lipid content and tenderness compared to wild fish. Protein composition influences hardness of meat and maintenance of freshness.

Sensory evaluation of the feed supplement *Spirulina* was determined by free amino acids (Liao *et al*., 1990; Watanabe *et al*., 1993) and proximate composition (Nandeesha *et al*., 1998). In yellowtail fed a diet supplemented with *Chlorella*-extract, sensory evaluation such as taste and texture improved (Nakagawa *et al*., 1985).

1.1 Muscle protein

Muscle protein is an important factor affecting physical characteristics such as carcass quality. Low strength of cultured fish meat is due to a weak Z-line in muscle fibres, compared to wild fish. The strength of the Z-line may be improved by exercise during the rearing period. Strength of muscle protein can be evaluated by the methods of Lavéty and Love (1972) and Ando *et al*. (1991, 1992). Ando *et al*. (1992) pointed out muscle firmness related to density and the arrangement of collagen fibrils in the pericellular connective tissue. While the stromal fraction in meat, which contains collagen, contributes to stiffness in raw fish meat, heating during cooking tenderizes the meat by solubilizing the collagen. Vitamin C is well known as a cofactor in hydroxylation of proline to hydroxyproline in collagen synthesis. As observed in sensory evaluation, the feeding of *Spirulina* and tea catechin elevated the stromal fraction, meat firmness and taste of red sea bream *Pagrus major* (Mustafa *et al*., 1994). These results agreed with studies of Liao *et al*. (1990) carried out in striped jack *Pseudocaranx dentex*. As polyphenols have synergistic effects on vitamin C metabolism (Bai and Gatlin, 1992; Nakagawa *et al*., 2000), supplementation of polyphenols might improve carcass quality (Tanimoto *et al*., 1993; Nakagawa *et al*., 2000).

Protein sythesis activity can be assessed by a combination of RNA/DNA ratio, as a parameter of protein synthesis, and acid protease activity, as that of protein degradation (Mustafa *et al*., 1995). Yone *et al.* (1986) evaluated the effects of algal supplements on dietary protein and carbohydrate, using levels of plasma free amino-N and blood sugar.

1.2 Lipids and lipid metabolism

Surplus energy is reserved in the muscle, liver, bone and adipose tissue as lipid reserves, mainly triglycerides. The site of lipid deposit is dependent on fish species. Excessive lipid accumulation in non-edible parts does not benefit consumers and is a great loss of feed energy. Lipid content, lipid class composition and fatty acids also may influence taste and tenderness of fish.

Triglycerides are elemental and important factors in the taste of meat, but relationships between meat quality and fatty acid composition is not clear.

Lipid content in the muscle and adipocytes is generally higher in cultured fish than wild fish because of overfeeding and low physical movement. Muscle lipid content highly influences taste, but high lipid or low lipid content is not always good. Accordingly, controlling lipid content should be one important factor in quality control, but depression of lipid levels should not be accompanied by depression of growth and feed efficiency. The lipid content is dependent upon the balance between lipogenesis and lipolysis. The reserved lipids derived from the surplus energy should be preferentially utilized as an energy source. However, reserved lipids accumulated under nutrient imbalance are consequently deposited as celoid and lipofustin, which cannot be used for energy. The utility of the reserved lipids can be assessed by short-term starvation. Constitutional change by starvation reveals lipolysis activity; the reserved lipids should be primarily mobilized to energy prior to conversion of muscle protein in response to energy requirements. If energy is supplied from muscle protein in place of reserved lipids during food shortage, the consumption of muscle protein causes much body weight loss. Also this reduction of muscle protein can result in depression of physical activity and high mortality. Lipolysis activity of the adipocytes can be evaluated by *in vitro* analysis (Nematipour *et al*., 1990). Lipoperoxide has been analysed to evaluate the effect of supplements (Bai and Gatlin, 1992; Nakagawa *et al*., 1997; Ji *et al*., 2003b).

Mobilization of reserved lipids as an energy source might depend on dietary history. Body weight loss caused by short-term starvation, which could be reduced by improvement of nutritional condition, was used as indicator of metabolism (Nakagawa *et al*., 1993). Lipid accumulation could be controlled by fortification of vitamin C (Ji *et al*., 2003a, b) and algae (Nakagawa, 1985; Mustafa and Nakagawa, 1995). Om *et al*. (2003b) used the starvation test to examine lipolysis activity by fortification of highly unsaturated fatty acids (HUFA), which are essential for marine fish for proper growth performance and physiological activity. As lipogenesis and lipolysis activities are affected by fatty acid composition of accumulated lipid (Jezierska *et al*., 1982), controlling this fatty acid composition may be a key to influence lipid metabolism.

Carnitine is involved in β-oxidation of long-chain fatty acids. Biosynthesis of carnitine hydroxylation reactions requires vitamin C as a cofactor. Carnitine accumulated by vitamin C fortification stimulated lipolysis activity (Miyasaki *et al*., 1995; Nakagawa *et al*., 2000). Accordingly, ascorbate, carnitine and lipolysis enzyme activity would be useful indicators to evaluate lipolysis activity.

Serum constituents and enzyme activity have been analysed to assess lipid metabolism affected by supplements (Nakagawa *et al*., 1982, 1985, 2000; Mustafa *et al*., 1997). Serum constituents, such as total lipid, non-esterified fatty acids, non-protein nitrogen and amino nitrogen were used to evaluate physiological condition by Nakagawa *et al*. (1985).

2 Colour of Muscle and Skin

The colour of muscle and skin is an important factor to directly affect market value. Muscle colour is mainly composed of myoglobin, haemoglobin, carotenoids and melanin. The deposition of myoglobin and carotenoids can be controlled by micronutrients. The carotenoids deserve consideration as useful antioxidants which may prevent problems linked to oxidative stress, in addition to their effects as pigments. Skin of ayu *Plecoglossus altivelis* (Mori *et al*., 1987) and striped jack (Okada *et al*., 1991) could be pigmented by carotenoids included in *Spirulina*. Skin colour abnormality of flatfish may be solved by consideration of nutritional aspects in the broodstock and larvae.

3 Vitality and Stress Response

Liver function can be evaluated by the recovery time from alcohol anaesthesia (Hilton and Dixon, 1982). The method was applied to evaluate the effect of dietary algae (Nakagawa *et al*., 1992; Nakagawa, 2004), lauric acid (Ji *et al*., 2005), chitin (Om *et al*., 2003a), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Om *et al*., 2003b) in red sea bream and black sea bream *Acanthopagrus schlegeli*.

Pancreatic function can be evaluated by the glucose tolerance test. Serum glucose secretion was controlled by *Chlorella*-extract supplementation to the diet of ayu (Nakagawa *et al*., 1992).

Some stressful conditions, such as oxygen shortage, handling, high rearing density and air-dipping, are more or less inevitable during the fish culture process. Tolerance to hypoxia was assessed by the method of Nakagawa *et al*. (1984) in which fish are placed in a closed container filled with oxygen-saturated water and are kept there for some time. The oxygen tension and the number of fish that cannot maintain a normal upright orientation can be monitored. The method was employed to evaluate the effect of algae (Nakagawa, 2004) and chitin (Om *et al*., 2003a). Sensitivity to air-dipping is defined as follows: fish are exposed to air on a net for a while and returned to oxygen-saturated water. The recovery time from the succumbed condition is compared. The recovery time was shortened by dietary HUFA (Om *et al*., 2003b), lauric acid (Ji *et al*., 2005), algae (Nakagawa, 2004) and vitamin C (Ji *et al*., 2003b), showing the supplements had improved physiological condition. Response to hypoxic stress was evaluated in seabream *Sparus aurata* fed diets supplemented with vitamin C (Henrique *et al*., 1998).

Salinity tolerance was used to evaluate the effect of feed supplements on fish larvae (Furuita *et al*., 1999; Kolkovski *et al*., 2000). Li *et al*. (1998) used crowding stress in channel catfish *Ictalurus punctatus* fed a diet fortified with vitamin C. Some stressors such as temperature increase, low salinity and low dissolved oxygen were used to evaluate the effect of phospholipids (Tago *et al*., 1999).

4 Behaviour

Various rearing conditions such as high density and inadequate nutrition influence the behaviour of fish. Schooling behaviour was used to evaluate dietary effects of vitamin C in ayu (Koshio *et al*., 1997) and yellowtail *Seriola quinqueradiata* (Sakakura *et al*., 1998), and HUFA in Pacific threadfin *Polydactylus sexfilis* (Masuda *et al*., 1998, 2001).

5 Disease Resistance

A potential concern of fish culturists is reduced resistance to bacterial and viral infections which could be caused by inadequate rearing conditions and/or malnutrition. As parameters of disease resistance, leucocyte number, phagocytosis, spontaneous haemolytic activity, complement activity, bactericidal activity, cortisol, lysozyme activity and other immune responses have been employed. One of the most convincing methods to compare disease resistance is a challenge test by microbes.

The preventative effects of supplements on disease resistance have been investigated by examining growth performance, mortality and blood properties (Nakagawa *et al*., 1981, 1986).

Phagocytosis, antibody response, leucocyte number, complement activities and bactericidal activities were used to access the effect of *Ulva* supplementation on disease resistance in red sea bream (Sato *et al*., 1987). The immunostimulating effects of dietary alginate fed to Atlantic salmon *Salmo salar* were confirmed using a challenge test and examining haemolytic activities (Nordmo *et al*., 1995). Kiron *et al*. (1993) and Inoue *et al*. (1998) confirmed the effect of dietary zinc on natural-killer activity of leucocytes in rainbow trout. Li *et al*. (1998) examined the effect of vitamin C on disease resistance of channel catfish by exposing them to *Edwardiella ictaluri*. Effects of carotenoids on biodefence mechanisms were confirmed by lysozyme activity, phagocytosis and complement in rainbow trout (Amar *et al*., 2001).

6 Preservation and Freshness

Freshness is one of the most important factors in eating raw fish, especially in Japan. Freshness is likely to be retained for longer in wild fish compared to cultured fish. Post-mortem changes play an essential role in fish quality. The processes of rigor mortis, autolysis, tenderization and deterioration could be prolonged by dietary and rearing conditions. The fragmentation of myofibrils that occurred during post-mortem was examined by morphological observation of fish meat (Tokiwa and Matsumiya, 1969). The process of tenderization of muscle can be controlled by certain feed supplements. Feeding enough vitamin E prevents storage instability as measured by the thiobarbituric acid value (TBA) of meat during postharvest storage (O'Keefe and Noble *et al*., 1978).

7 Reproductivity

Quality of eggs and larvae is primarily affected by the nutritional condition of the broodstock. Commonly used indices of egg and larval quality are: total egg production, number of spawns, mean egg number, egg diameter, floating egg ratio, hatching rate, viability and malformation rate. Dietary supplementation of broodstock and larval stages with amino acids, vitamins, fatty acids and carotenoids have been examined (Alava *et al*., 1993; Akiyama *et al*., 1996; Furuita *et al*., 1998, 1999; Emata *et al*., 2000; Kolkovski *et al*., 2000). However, excessive doses of these supplements might give negative effects.

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2 Fish Health Assessment

MASASHI MAITA

Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan

1 Importance of Nutrition on Fish Health

1.1 Background

Infectious disease is a major contributor to economic loss in intensive fish culture. Some antibiotics have been used to reduce death due to bacterial infections. Recently, food-importing countries, such as the EU, the USA and Japan, require exporting countries to meet various food sanitation controls. Many consumers are aware that residues of antibiotics and other chemotherapeutants in fish flesh are an important risk for human health. To produce safe farmed fish, it is necessary to prevent outbreaks of fish diseases without using antibiotics. Vaccination has come into wide use for disease prevention, but the effectiveness of vaccination decreases depending on fish condition. In addition to good management and vaccination schedules, nutritional prophylaxis will ensure beneficial disease control in farming systems.

Diet should both achieve rapid fish growth and maintain health. Health maintenance based on adequate nutrition and feeding practices has acquired greater importance in intensive fish culture. The efficiency of diet should be evaluated not only by growth but also by the impact on fish health. Therefore, suitable indices are needed for evaluating dietary efficiency linked to fish health.

Since major disease outbreaks typically occur when the balance between the fish (host), the etiologic agent and the environment is upset (Snieszko, 1974), the maintenance of fish health should lead to reduced risks of disease outbreaks. External factors, such as quality of diet, management of feeding and environment, and internal factors such as inherited quality would affect the health condition of fish. It is reasonable to assume that the levels and ratios of available dietary nutrients may influence the susceptibility of fish to

 Dietary Supplements for the Health and Quality of Cultured Fish (eds H. Nakagawa, M. Sato and D.M. Gatlin III)

diseases (Blazer and Wolke, 1984). Nutritional strategies that could be adopted to manage fish health include adjustment of specific nutrition levels in the diet, manipulation of nutritional condition through feeding regimes and administration of non-nutrient immunostimulants in the diet.

1.2 Fish health and nutritional studies

Adequate nutrition has long been acknowledged as crucial for maintaining animal health and disease resistance. Various immune deficiencies have been reported in humans and animals that do not receive sufficient amounts of essential nutrients (Waagbø, 1994). Evidence supporting the importance of nutrients in maintaining normal immune function and disease resistance is increasing. This research first appeared during the early 1980s (Blazer, 1982), but gained momentum only during the 1990s. Landolt (1989), Blazer (1992), Lall and Oliver (1993), Waagbø (1994), Sealey and Gatlin III (1999) have all published reviews in this area of research. Much of the early work was focused on vitamins C and E, but more recently fatty acids and amino acids are also being studied.

A schematic drawing depicting the relationship between the amount of nutrients in a diet and animal health is shown in Fig. 2.1. The health of fish fed on diets with excess or inadequate amounts of certain nutrients will decline. These health effects are recognized as nutritional diseases, sufficient to cause mortality in the fish. Dietary supplements fed within safety limits will result in beneficial effects for the animals.

Specific nutrient requirements are usually determined under standard, well-defined and favourable environmental conditions. However, such ideal conditions are not always present in commercial farms, where animals are often stressed, confronted with pathogens or other unfavourable environmental conditions. Under these conditions, the requirements for

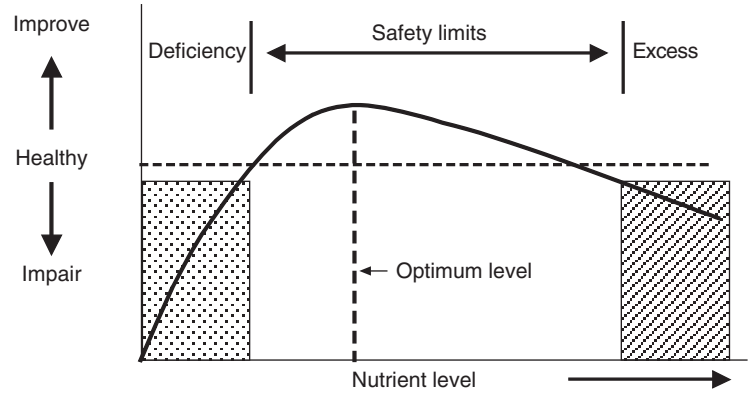

Fig. 2.1. Schematic drawing of the relationship between the level of a certain nutrient in a diet and animal health.

certain nutrients are likely to increase, due to greater activity of the immune system and physiological activity. In addition to changes in nutritional requirements, physical damage of digestive tracts, infection with parasites and decreased absorption due to the physical aspects of the diet may cause malnutrition. The deterioration of nutritional status will lead to the loss of disease resistance in fish (Blazer, 1992). Benedich (1988) observed a decline in various immune functions prior to the observation of symptoms of nutrient deficiency in mammals. Thus, it is obvious that insufficient nutrients result in animals susceptible to infectious disease.

2 'Fish Health' and Health Assessment

Fish culturists can become aware of abnormalities in fish through changes of body colour, abnormal swimming, increases in rate of mortality, reduction of appetite, etc. However, in the early stages of impaired health, fish may be easily infected with pathogens even if their appearance is normal. These fish that have a higher susceptibility to infectious disease should be recognized as 'compromised hosts' and the loss of disease resistance should be suspected at the earliest opportunity in order to either prevent or reduce the severity of epizootics. It is therefore important to establish evaluation procedures and indices of health condition for fish in order to detect the 'compromised hosts'. Besides sensitivity and precision, ease of application, rapidness and cost-effectiveness are also required for the methods of fish health assessment.

Various parameters have been used to assess the physiological changes or health condition of fish due to the effects of nutrients. Indices of fish health condition are also necessary to understand how fish health is affected by diet and to enhance disease resistance through dietary modification.

Health indices should correlate with factors that accelerate mortality due to infectious diseases. It is considered that various factors, such as imbalance of nutrients, quality of diet and feeding regimes may affect the function of various organs such as the liver and/or cause malnutrition, eventually accelerating mortality.

3 Methods of Fish Health Assessment

Fish health can be assessed using morphological, haematological and immunological examination as well as experimental disease challenge.

3.1 Morphological examination

Evaluation of fish health condition by observations and measurements of external characteristics and internal organs is rather simple. The fish health

condition profile (HCP) was originally developed for trout fish hatcheries in Utah (Goede, 1988). Novotny and Beeman (1990) reported on the health assessment of juvenile Chinook salmon by HCP. According to the report, abnormality of the thymus and increased abdominal fat were affected by rearing density. However, correlation between those abnormalities and disease resistance of fish was not obvious. While scored observations, scoring selections, and descriptions for each category of the HCP have been shown in detail (Goede, 1988), the external or internal observations are rather subjective. The effects of nutrients on the HCP are also unknown. Thus, evaluation of fish health by morphological examination is rather limited in sensitivity and precision.

3.2 Blood examination

Measurement of peripheral blood parameters and plasma constituent levels generally constitute blood examination. The advantage of blood examination is that it is easy to measure using commercial kits or equipment, and several samples can be analysed in a short period. In addition, it generally can be carried out without killing the fish. Therefore, blood examination is a reliable way of monitoring the health condition of fish.

Various factors including differences in species, age, sex, water quality, water temperature and handling methods may contribute to variability in haematological and biochemical data that is difficult to interpret. For this reason it is difficult to compare results from different studies or set 'normal ranges' and it has been suggested that reference intervals for each set of culture conditions may need to be determined.

Haematology is used as an index of fish health status in a number of fish species to detect physiological changes following different stress conditions such as exposure to pollutants, diseases, metals, hypoxia, etc. (Blaxhall, 1972; Duthie and Tort, 1985). Haematological characteristics such as haematocrit value (Ht), haemoglobin concentration (Hb) and red blood cell counts (RBC) also have been examined to evaluate the requirement of certain dietary micronutrients, and the quality of feed or feeding strategies. Mean corpuscular volume (MCV), the average size of erythrocytes, is calculated as (Ht $\times$ 10)/RBC. Mean corpuscular haemoglobin (MCH), the weight of haemoglobin in the average RBC, is determined as (Hb $\times$ 10)/RBC. Mean corpuscular haemoglobin concentration (MCHC), the relationship between the size of erythrocytes and their haemoglobin content, is calculated as (Hb $\times$ 10)/Ht. These blood indices (MCV, MCH and MCHC) need to be analysed together with erythrocyte morphology, because they provide complementary information.

It is well known that deficiency of various vitamins, minerals and malnutrition (starvation), as well as other feed-related factors often cause anaemia in fish (Smith *et al.*, 1974; Plumb *et al.*, 1986; Soliman and Wilson, 1992; Mohamed, 2001). A hypochromic microcytic anaemia, where the Ht,

Hb and MCV in the blood are reduced below normal, has been observed in fish fed iron-deficient diets (Sakamoto and Yone, 1978; Gatlin and Wilson, 1985). A macrocytic anaemia that showed marked reduction in Ht, RBC and an increase in MCV of red blood cells also was observed in Indian catfish, *Heteropneustes fossilis*, deprived of pyridoxine (Mohamed, 2001). The development of macrocytic anaemia that is defined by an increase of MCV has been reported for channel catfish (Lim and Lovell, 1978), rainbow trout (Hilton *et al*., 1978) and hybrid tilapia (Shiau and Jan, 1992) fed ascorbic acid deficient diets.

Decreases in Ht, Hb and RBC below the normal ranges are all signs of anaemia. Anaemic fish are sensitive to certain secondary pathogens and show a decreased tolerance to oxygen depletion (Piacentini *et al*., 1989; Haney *et al*., 1992; Rios *et al*., 2005). Maita *et al.* (1996) suggested that anaemia caused reduced ability to produce energy in the gill and kidney of Coho salmon and thus reduced tolerance to secondary stressors. Thus, haematological examination of fish to diagnose anaemia is recommended as a general and rather simple means of health assessment.

3.2.1 Parameters of blood smears

Observations obtained from examination of blood smears are also able to provide much information about the physiological status of fish. For example the percentage of erythroblasts, structural abnormality of erythrocytes, composition of leucocytes and leucocyte counts can be obtained from blood smears. Blood smears are commonly air-dried and stained using the May–Grünwald–Giemsa method and the proportion of each cell type is determined. Total number of leucocytes is estimated in relation to the number of red blood cells (obtained with a haemocytometer), and the proportion of each cell type observed in the blood smear.

Erythroblast abundance is observed as a result of the acceleration of erythropoiesis in different physiological conditions (Ueda *et al*., 2001). Decreases of erythroblast and lymphocyte counts were observed in starved *Hoplias malabaricus* (Rios *et al*., 2005), indicating that reduced erythropoiesis was caused by malnutrition. Rainbow trout deprived of folic acid have been shown to enucleate erythrocytes or possess erythrocytes with segmented or fragmented nuclei (Smith, 1968; Kawatsu, 1975). Appearance of poikilocytes (degenerating and segmented red blood cells) in blood smears provides evidence of nutrient imbalance or insufficient nourishment, and these malformed red blood cells may be removed from circulation, keeping the fish anaemic (Rios *et al*., 2005).

The primary consequence of a low lymphocyte count is immuno-suppression, resulting in an increased susceptibility to disease (Wedemeyer *et al*., 1990). Malnutrition could be a factor leading to a low lymphocyte count as mentioned above. Circulating monocytes/macrophages (represented as a percentage of total leucocytes) was significantly elevated in tilapia fed bacterial-derived β-1,3 glucan (Cain *et al*., 2003). Klinger *et al.* (1996) found that dietary lipid may affect not only the thrombocyte count, but also the

function of these cells. Lee *et al.* (2004) reported that leukocyte numbers were increased and survival was significantly improved by dietary supplementation of maca tuber meal in rainbow trout alevins.

3.2.2 Parameters of clinical biochemistry

The levels of different constituents in plasma have been used as indices for evaluating the physiological and health condition of fish. While examination of the blood may be useful for research on nutrient requirements, new diet ingredients, additives, etc., immunological indices, however, may serve as better indicators when examining the effects of immunostimulants because blood parameters do not easily reflect alteration. Among the various plasma components, the levels of plasma total protein, urea nitrogen, glucose, total cholesterol and triglyceride, as well as activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST; former GOT), and alanine aminotransferase (ALT; former GPT) seem to be useful indices for health assessment in fish.

Plasma total protein level has been measured frequently as an indicator of physiological condition in the studies of fish nutrition (Smith *et al*., 1974; El-Mowafi *et al*., 1997; Lygren *et al*., 1999; Bagni *et al*., 2000; Nakagawa *et al*., 2000; Farhangi and Carter, 2001; Watanabe *et al*., 2001; Harikrishnan *et al*., 2003). Total protein in plasma is the most stable component, and few dietary factors have been reported to affect the levels in fish. Decreases of plasma total protein accompanied with anaemia were reported in fish influenced by the effects of a low fishmeal diet (Takagi *et al*., 2001) or supplementation of gossypol (Yildirima *et al*., 2003). In these cases, as body moisture and protein contents were also lowered in fish showing low plasma total protein levels, a decrease in plasma total protein levels might be accounted for by blood dilution and disturbance of protein metabolism. On the other hand, Dügenci *et al*. (2003) reported that plasma total protein levels in rainbow trout fed diets supplemented with some medicinal plants were significantly elevated. In this case, it is considered that plasma immunoglobulin content should be measured.

The levels of glucose, urea nitrogen, total cholesterol and triglyceride have been reported to reflect the nutritional status of several fish species. Significant decreases in urea nitrogen and total cholesterol levels, and increases in triglycerides were observed in yellowtail starved for a short period (approximately 10 days) (Maita, unpublished data). Although plasma triglycerides and glucose showed a positive correlation with average daily food intake, total cholesterol levels did not show a significant correlation with average daily food intake in European sea bass (Kavadias *et al*., 2003). The positive influence of feeding rate on plasma cholesterol concentration was reported for sea bass (Lemaire *et al*., 1991) under laboratory conditions. On the other hand, in adult farmed Atlantic salmon, plasma total cholesterol levels showed a negative correlation with feeding rate (Sandnes *et al*., 1988). It is well known that the level of plasma glucose is also a good indicator of a stress response in fish.

Plasma urea nitrogen level of rainbow trout was elevated by an increase of dietary arginine (Kaushik *et al*., 1988) because of the increase in arginine catabolism through the urea cycle. In Coho salmon affected by kidney disease, elevation of plasma urea nitrogen indicated the loss of renal function (Wedemeyer and Ross, 1973).

It is well known that increases in AST and ALT activities indicate injury of liver cells caused by various chemicals or lipid peroxidation. Elevated plasma ALP activity measured in sea bass corresponded to an inflammatory reaction of the bile ducts induced by the diet (Lemaire *et al*., 1991). It was reported that enzymatic activity of plasma AST increased in a linear fashion, as acclimation temperature increased; however, ALP activity was not related to acclimation temperature in rainbow trout (Miller *et al*., 1983).

3.2.3 Correlation between plasma constituent level and disease resistance

It is considered that indices of plasma biochemistry to assess fish health condition should be selected based on the relationship between the levels in plasma and disease resistance. Maita *et al*. (1998a, b) have attempted to correlate some blood parameters and mortality due to the bacterial infection in yellowtail *Seriola quinqueradiata* in order to select suitable indices for fish health assessment.

Table 2.1 shows plasma constituent levels in a normal fish population reared under common conditions. Yellowtail were marked individually by placement of a PIT tag in their peritoneal cavity and plasma component levels were measured prior to artificial challenge, and in surviving and dead fish after challenge. Plasma cholesterol levels of fish that died following artificial infection with *Lactococcus garvieae* were significantly lower than that of surviving fish. Significant differences between surviving and dead fish were not observed in other plasma constituents. There was a significant correlation between mortality due to the artificial infection with *L. garvieae* and plasma cholesterol level ($r = -0.951$, $P < 0.01$). The mortality of the fish with low plasma cholesterol levels (lower than 250 mg/100 ml)

Table 2.1. Comparison of plasma constituent levels in yellowtail following artificial infection with *L. garvieae*.[a,b]

	Survived (n = 23)	Dead (n = 11)
Body weight (g)	349 ± 48	345 ± 55
Total cholesterol (mg/100 ml)	274 ± 51	238 ± 36[c]
Triglyceride (mg/100 ml)	72 ± 35	63 ± 29
Alkaline phosphatase (IU/L)	137 ± 28	145 ± 31
Total protein (g/100 ml)	2.7 ± 0.3	2.5 ± 0.4
Glucose (mg/100 ml)	110 ± 26	110 ± 28
Urea nitrogen (mg/100 ml)	10.2 ± 1.6	9.6 ± 2.1

[a] Data shown are mean values ± standard deviation.
[b] Fish were marked individually by PIT tag in their peritoneal cavity.
[c] Significant difference between two categories by *t*-test ($P < 0.05$).

was significantly higher than that of the fish with high cholesterol levels (higher than 275 mg/100 ml) ($P < 0.05$). In the case of rainbow trout, a significant correlation ($r = -0.924$, $P < 0.01$) was established between the plasma cholesterol level and the mortality due to the artificial infection with *Vibrio anguillarum*. These results suggest that fish with a low plasma cholesterol level had lowered disease resistance compared to fish with a high plasma cholesterol level, and the 'compromised host' could be identified by the levels of their plasma cholesterol.

An experiment was conducted to examine the effects of spoiled sardine and oxidized oil on disease resistance in yellowtail. Experimental diets were prepared as follows: a combination of frozen sardine and fresh oil (group 1); frozen sardine and oxidized oil (group 2); spoiled sardine and fresh oil (group 3); and spoiled sardine and oxidized oil (group 4). The effect of spoiled sardine was observed by comparing groups 1 and 3 and the effect of oxidized oil was observed by comparing groups 1 and 2. Table 2.2 shows the mortality due to natural and artificial infection with *L. garvieae* and the plasma constituent levels of yellowtail fed the moist diets. The mortality due to both natural infection and artificial infection with *L. garvieae* in fish fed spoiled sardine (groups 3 and 4) was higher than that in fish fed frozen sardine (groups 1 and 2). Fish fed spoiled sardine and oxidized oil had reduced disease resistance. A significant decline in plasma total cholesterol and urea nitrogen levels was observed in fish fed spoiled sardines. It would be suspected that fish health condition would be worsened by the decrease in plasma cholesterol and urea nitrogen. In group 2, mortality due to natural infection was similar to the control group, however, mortality due to the artificial infection was increased. This suggests that fish fed oxidized oil would be latently fragile. The effects of oxidized oil were observed in elevation of plasma triglyceride and glucose levels.

Table 2.2. Mortality and plasma constituent levels[a] of yellowtail fed various moist diets.[b]

	Group 1	Group 2	Group 3	Group 4
Mortality (%)[c]	4	6	13	11
Mortality (%)[d]	5	30	35	35
Total cholesterol (mg/dl)	218 ± 60	195 ± 15	163 ± 23	142 ± 42
Triglyceride (mg/dl)	70 ± 33	122 ± 25	93 ± 28	107 ± 25
Alkaline phosphatase (K-U)	0.7 ± 0.2	0.3 ± 0.2	0.3 ± 0.3	0.8 ± 0.3
Total protein (g/dl)	3.2 ± 0.1	3.7 ± 0.2	3.0 ± 0.4	3.2 ± 0.4
Glucose (mg/dl)	86 ± 3	104 ± 6	92 ± 5	150 ± 85
Urea nitrogen (mg/dl)	26.8 ± 4.7	20.7 ± 2.4	16.9 ± 3.3	14.5 ± 3.5

[a] Data shown are mean values ± standard deviation for five fish.
[b] Mash : Sardine : Oil = 47.5 : 47.5 : 5; Experimental diets were: Group 1, a combination of frozen sardine and fresh oil; Group 2, frozen sardine and oxidized oil; Group 3, spoiled sardine and fresh oil; and Group 4, spoiled sardine and oxidized oil.
[c] Mortality due to natural infection with *L. garvieae*.
[d] Mortality due to artificial infection with *L. garvieae*.

In cases where plasma cholesterol levels were lowered by various external factors, such as impaired diet quality, raw sardine feeding in yellowtail (Nakagawa *et al*., 1984) and supplementation of excessive vitamin E in ayu, *Plecoglossus altivelis* (Maita and Lee, 2001), there was a corresponding increase in mortality due to natural infection with pathogens. In red sea bream reared in high-density conditions, the level of plasma cholesterol was significantly lowered and the mortality due to Iridovirus infection was increased (Tanaka and Inoue, 2005). These studies all demonstrate that a reduction in the plasma total cholesterol level is linked to impaired disease resistance in fish.

In yellowtail, feeding of a non-fishmeal diet caused anaemia, hypocholesterolemia and a decrease in disease resistance (Maita *et al*., 1998a). It was suggested that the cause of malnutrition due to the feeding of the non-fishmeal diet was a deficiency of taurine (Goto *et al*., 2001).

The plasma constituent levels and mortality due to natural infection with pseudotuberculosis were compared between two groups of yellowtail fed a non-fishmeal diet manufactured under different processing conditions. Although the compositions of the two experimental diets were identical, diet 1 was manufactured by a large-sized twin-screw extruder, and diet 2 was made by a small-sized single-screw extruder (Fig. 2.2). The two experimental groups were reared in neighbouring net cages and each group were fed the experimental diet at the same feeding rate. Fish fed diet 2 had a shorter gut retention time, significantly lower plasma total cholesterol levels, while levels of triglycerides, urea nitrogen and alkaline phosphatase activity were significantly higher than those of fish fed diet 1 (Table 2.3). These changes suggest that fish fed diet 2 were malnourished compared to fish fed diet 1. It is stressed that these results were obtained at the time when there were no differences in growth performance or mortality between the two groups of fish. Mortality due to natural infection with pseudotuberculosis in fish fed diet 2 was significantly higher than that

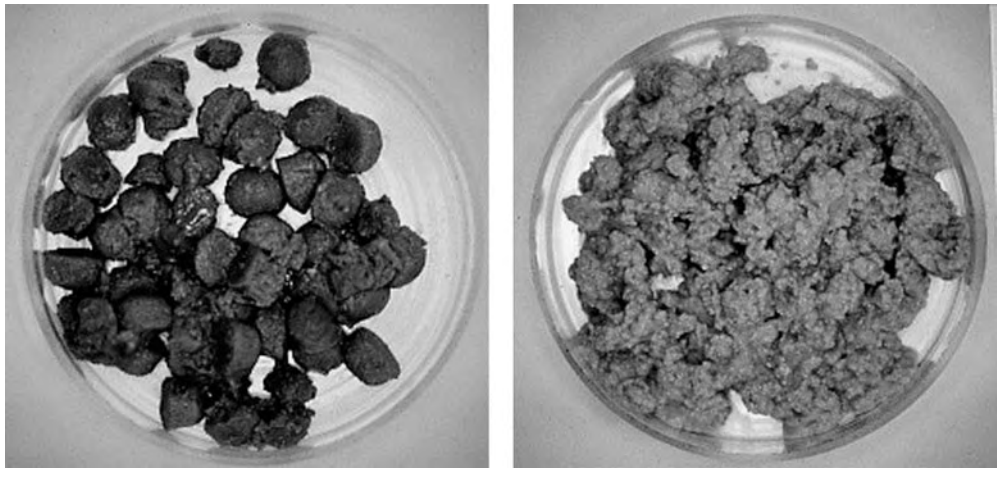

Fig. 2.2. Appearance of stomach contents 5 hours after feeding.

Table 2.3. Comparison of plasma constituent levels in yellowtail fed experimental diets with different gut retention times.[a]

	Diet 1 (n = 5)	Diet 2 (n = 5)
Condition factor (%)[b]	14.8 ± 0.6	15.0 ± 0.7
Total cholesterol (mg/dl)	240 ± 25	193 ± 16[c]
Triglyceride (mg/dl)	81 ± 20	133 ± 26[c]
Alkaline phosphatase (IU/L)	137 ± 12	168 ± 17[c]
Total protein (g/dl)	3.6 ± 0.1	3.4 ± 0.2
Glucose (mg/dl)	173 ± 33	153 ± 30
Urea nitrogen (mg/dl)	20.6 ± 1.2	23.9 ± 1.2[c]

[a] Data shown are mean values ± standard deviation.
[b] Condition factor (%) = body weight (g)/{fish length (cm)} 3 × 100.
[c] Significant difference between two categories by t-test (P < 0.01).

of fish in the other treatment (Fig. 2.3). Apparently, nutrients were not absorbed sufficiently because of the shorter retention time in the stomach and thus the fish were suffering from malnutrition and were more susceptible to pathogens.

Another experiment was conducted to examine the effects of supplementing taurine and cholesterol in non-fishmeal diets on haematological parameters and disease resistance at the end of 30 and 60 days. The diets were as follows: a control fishmeal diet (FM), a non-fishmeal diet (N), and non-fishmeal diets supplemented with cholesterol (C), or with taurine (T), or with both cholesterol and taurine (CT). The levels of Ht in the control and CT group were significantly higher than that in the other groups after 30 days feeding. After 60 days feeding, the level of Ht in group T was similar to the control and the CT group (Fig. 2.4). Cumulative mortality upon artificial infection with *L. garvieae* in the control and CT groups was significantly

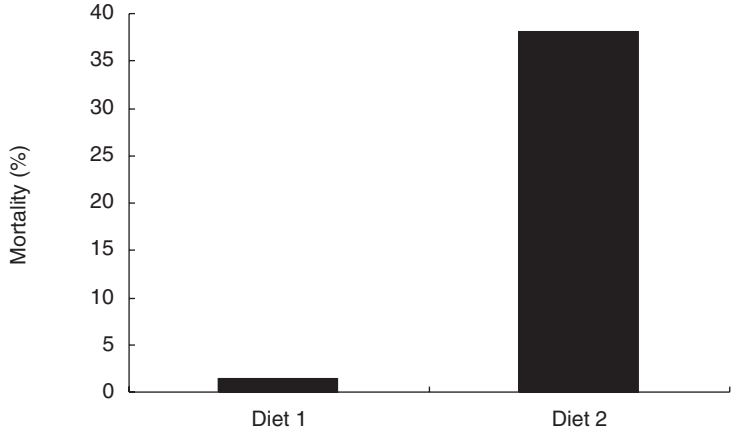

Fig. 2.3. Mortality due to natural infection with pseudotuberculosis in yellowtail fed experimental diets that had different gut retention times.

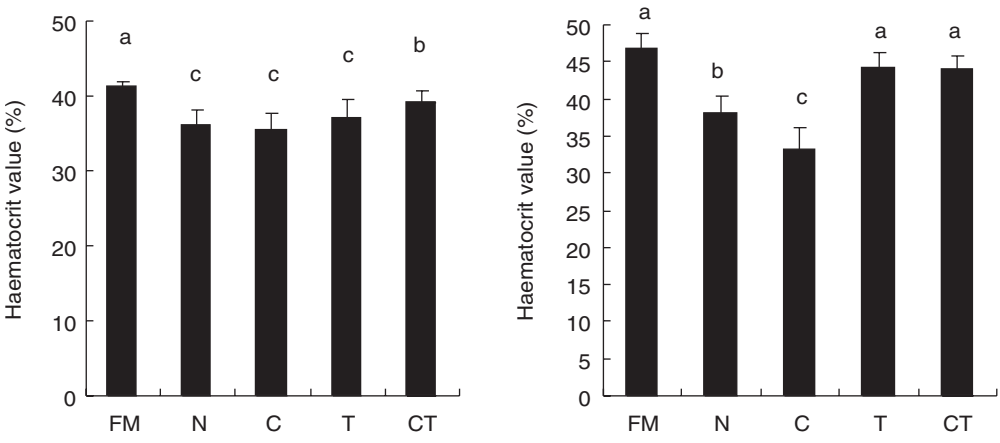

Fig. 2.4. The effects on haematocrit value of supplementing taurine and cholesterol in non-fishmeal diets. Data are shown as means and standard deviation for six fish in each group. FM, control diet containing fishmeal; N, non-fishmeal diet; C, non-fishmeal diet supplemented with cholesterol; T, non-fishmeal diet supplemented with taurine; CT, non-fishmeal diet supplemented with both cholesterol and taurine. Different superscripts are significantly different at $P < 0.05$.

lower compared to other groups at day 30; however, mortality in the control, T and CT groups was significantly lower than that of groups N and C in which Ht level remained low (Fig. 2.5). Mortality due to artificial infection was decreased only by both supplementation of cholesterol and taurine at day 30, but improved by the supplementation of taurine alone at day 60. The handling easily killed the fish belonging to groups N and C. Plasma cholesterol levels were increased by supplementation of dietary cholesterol or taurine, which improved lipid metabolism (Fig. 2.6). These results suggest that anaemia is one of the pathophysiological changes that may decrease disease resistance, and dietary taurine and cholesterol may improve lipid metabolism. It is well known that cholesterol is metabolized to bile salts, and bile salts conjugate with taurine and is excreted in the bile. It is considered that feeding a non-fishmeal diet (i.e. deficiency of dietary taurine) could derange lipid metabolism and the derangement may be related to a decrease in disease resistance.

Elevation of plasma cholesterol is observed to accompany steatosis. Accumulation of excessive fat is correlated with an alteration of lipid metabolism, secondary to a deficiency in essential fatty acids (Lemaire *et al.*, 1991). Satoh *et al.* (1996) reported that disease resistance of yellowtail was lowered by an excess dose of dietary lipid. In these cases, increased plasma cholesterol levels do not reflect increased disease resistance.

Changes in plasma cholesterol level as affected by various factors of dietary origin are divided into several patterns in Fig. 2.7. The plasma cholesterol level is affected by dietary cholesterol (exogenous cholesterol) and synthesized cholesterol in the liver (endogenous cholesterol). Pattern A is lowered plasma cholesterol level caused by a shortage of dietary

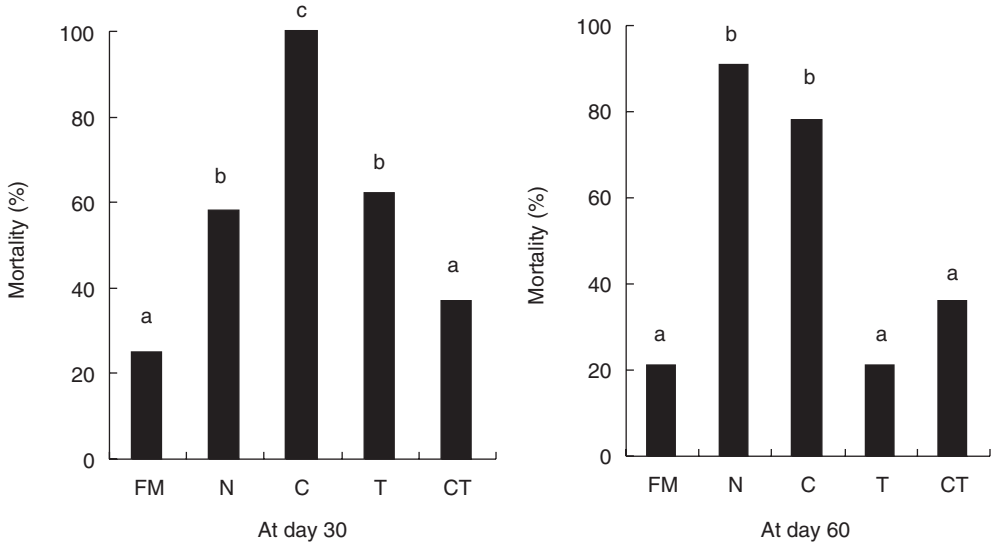

Fig. 2.5. The effects of supplementing taurine and cholesterol in a non-fishmeal diet on mortality due to artificial infection with *L. garvieae.* Different superscripts are significantly different at $P < 0.05$. Codes used are as described in Fig. 2.4.

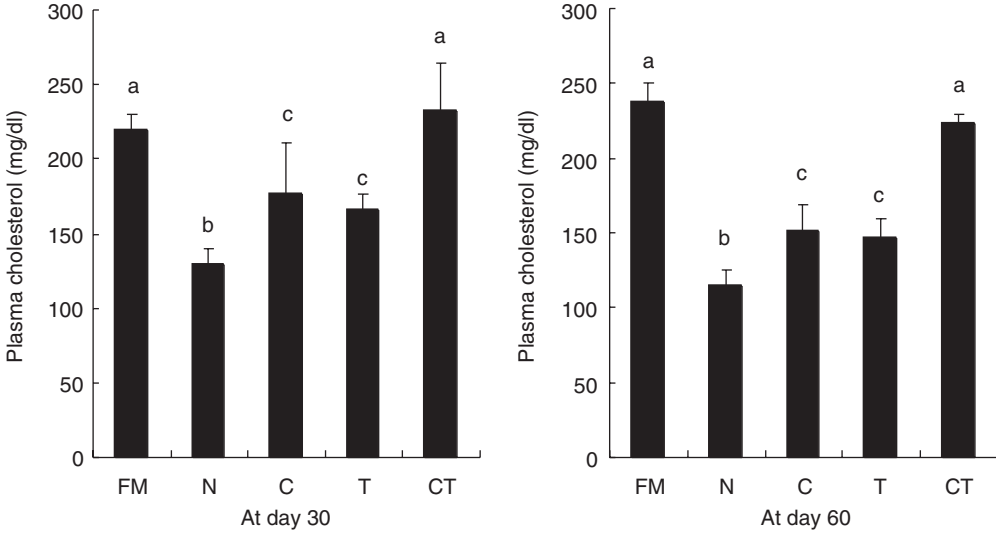

Fig. 2.6. The effects on plasma cholesterol levels of supplementing taurine and cholesterol in a non-fishmeal diet. Different superscripts are significantly different at $P < 0.05$. Codes used are as described in Fig. 2.4.

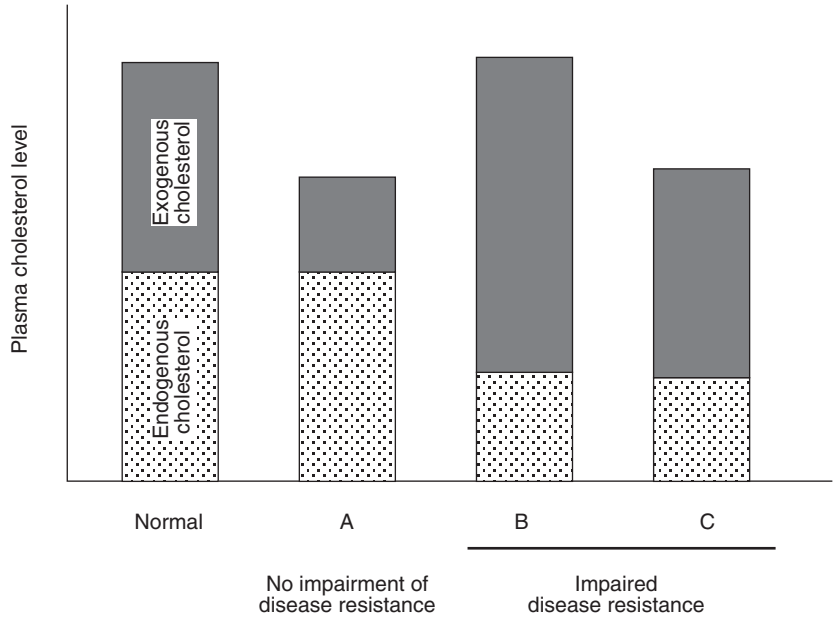

Fig. 2.7. Correlation between the level of plasma cholesterol and fish health condition.

cholesterol; however, endogenous cholesterol is maintained at a normal level. Pattern B is observed in the case of an excess dose of dietary cholesterol. The plasma cholesterol level is maintained at a normal level; however, endogenous cholesterol decreases. Pattern C indicates that low plasma cholesterol levels are caused by a decline in cholesterol synthesis, while fish are fed a normal diet. Fish belonging to patterns B and C would have impaired disease resistance.

3.3 Examination of immune function

As fish are primitive vertebrates they possess both non-specific defence mechanisms, such as phagocytosis, that are characteristic of invertebrates, as well as specific humoral and cellular responses mediated by lymphocytes, as in higher vertebrates (Table 2.4). They rely more on their non-specific defences, primarily the skin and mucus. When they encounter a pathogen, cellular mechanisms like phagocytosis are initiated, aided by humoral factors like complement and lysozyme. There also are other cells such as the natural killer cells and several other soluble factors that are involved at different stages. If the non-specific defences are not sufficient to ward off the infectious agent, then disease will develop leading to the induction of specific defence mechanisms. The cells functioning here are macrophages that are antigen-presenting cells and T-lymphocytes that participate in cell-mediated immunity and B-lymphocytes that are antibody producers and protect the fish for a certain period. These components of the

immune system in fish influence some responses that fish nutritionists have attempted to monitor in order to describe the role of dietary nutrients in immunity.

A number of researchers have investigated the relationship between nutrients and the immune responses of fish. Various responses of the immune system have been measured to evaluate their functions. However, the methodologies adopted in each study have not always been standardized so that the results cannot be compared and occasionally results from different studies are contradictory. The methodology used to study immune function in fish has been covered by Stolen *et al*. (1990). Table 2.5 summarizes the different immunological assays used in nutritional research and their sensitivity, variability and ease of use from a practical point of view.

The leucocytes used for examination of immunological parameters are usually collected from peripheral blood or head kidney by density gradient

Table 2.4. Immunological defence mechanisms in fish.

Components	Non-specific	Specific
Natural barriers	Skin and mucus Lysozyme	
Cellular defence mechanisms	Phagocytes	Macrophages
	Non-specific cytotoxic cells	Lymphocytes (T and B cells)
Humoral defence mechanisms	Complement	Antibodies
	Lysozyme	Cytokines
	Cytokines	

Table 2.5. Immunological assays that have been employed in nutritional studies on fish indicating their level of suitability.

Assay	Function	Sensitivity	Variability	Ease of use
Non-specific responses				
Phagocytic ability	Phagocytosis	Good	Wide	Fair
Oxidative burst	Phagocytosis	Excellent	Medium	Fair
T and B cell mitogenesis	Lymphocyte differentiation	Fair	Wide	Fair
Blastogenesis (RIA)[a]	Lymphocyte differentiation	Good	Medium	Difficult
Natural killer activity (RIA)	Lymphocyte or neutrophil function	Good	Wide	Difficult/Fair
Complement activity	Lysis of bacterial cell membrane, chemotaxis of phagocytes, splitr	Good	Wide	Fair
Lysozyme	Mucopolysaccharides from bacterial cell wall	Good	Medium	Fair
Specific responses				
Melanomacrophage centre	Haemopoesis	Good	Medium	Moderate
Passive haemolytic plaque assay	Antibody production	Good	Narrow	Moderate
ELISA	Antibody titre	Excellent	Narrow	Difficult

[a] RIA, Radio Immuno Assay.

centrifugation (Secombes, 1990; Rowley, 1990; Jeney *et al*., 1997; Sealey and Gatlin, 2002). As the band lying in each density interface is enriched in different leucocyte populations, optimum density should be established and chosen to obtain the appropriate cell population.

Phagocytes are the most important cells in non-specific cellular mechanism and are also helped by several soluble factors such as complement and lysozyme. It is well known that fish treated with immunostimulants show increased phagocytosis as well as respiratory burst activity. Both phagocytosis and respiratory burst are affected by several factors including the nutrients, temperature and pathogens. Fish phagocytic cells are able to engulf bacteria and kill them by generating a superoxide anion (O_2^-) and its derivatives such as hydrogen peroxide (H_2O_2) and hydroxyl-free radicals (OH^-) in the process known as respiratory burst. These reactive oxygen intermediates have potent bactericidal activities. Phagocytic activity (PA) and phagocytic index (PI) are determined by various procedures with minor modifications (Seeley *et al*., 1990; Yoshida *et al*., 1993; Bhatia *et al*., 1994). Congo red stained yeast cells, latex beads, fluorescent latex beads and opsonized zymosan have been used as materials, which are engulfed by the cells. The relatively small differences in phagocytic rates may be a result of the type of material used and the length of time the cells are incubated. Bactericidal activity of phagocytic cells is measured by two methods (detection of extracellular and intracellular activities). The intracellular superoxide anion production is often determined using the procedure of Secombes (1990) or Anderson and Siwicki (1995). Dügenci *et al*. (2003) measured the reduction of ferricytochrome *c* to determine levels of extracellular O_2^-. The reactive oxygen intermediates (ROIs) produced by stimulated phagocytes is also quantified using an automatic photoluminometer (Lygren *et al*., 1999; Kim *et al*., 2002). The chemiluminescent response measures the respiratory burst activity of phagocytic cells in which oxygen is converted into ROIs. Flow cytometry is an effective technique recently applied to examine phagocytosis (Thuvander *et al*., 1987; Chilmonczyk and Monge, 1999) and respiratory burst activity (Ortuño *et al*., 2000; Moritomo *et al*., 2003).

Non-specific cytotoxic cells (NCCs) in fish have been reported to resemble natural killer (NK) cells in mammals (Graves *et al*., 1984). NCCs from peripheral blood and head kidney of fish have been found to possess spontaneous cytotoxic activity against a mammalian cell line (Hinuma *et al*., 1980; Evans *et al*., 1984; Morita *et al*., 1989), protozoans (Graves *et al*., 1985) and certain cultured tumour cells (Moody *et al*., 1985). In rainbow trout, the occurrence of NCCs has been reported by Sakai (1984) and later found responsible for non-specific killing of virus-infected cells (Yoshinaga *et al*., 1994). The activity of the NCCs from the head kidney of rainbow trout has been reported to be influenced by the essential fatty acids and minerals such as zinc and manganese (Kiron *et al*., 1993; Inoue *et al*., 1998). The ^{51}Cr release and lactic acid dehydrogenase (LDH) release assays are general methods used for measuring the cytotoxic activity of the NCCs.

Lysozyme is an important enzyme in blood that actively lyses bacteria and an increased level of this enzyme has been considered to be a natural protective mechanism in fish (Ingram, 1980). Lysozyme has an antibacterial activity by attacking the peptidoglycan in the cell wall of bacteria, predominantly Gram-positive bacteria, thereby causing lysis and stimulation of phagocytosis of bacteria by phagocytic cells (Ellis, 1990). Neutrophils are thought to be the source of lysozyme, and the enzyme appears to be much more bactericidal than lysozyme of higher vertebrates (Ellis, 2001). Lysozyme levels in biological samples (plasma, serum, mucus) are typically assayed based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (*Micrococcus lysodeikticus*) that can be measured turbidimetrically (Siwicki and Anderson, 1993), or on an agarose plate (Osserman and Lawlor, 1966).

The Alternate Complement Pathway (ACP) activity is very high in fish serum as compared with mammals (Yano, 1996), suggesting that this pathway is very important as a defence mechanism of fish (Ellis, 2001). Measurement of serum haemolytic complement activity has been accomplished with the microtitre plate technique as described by Ingram (1990). Nutrients such as lipids, vitamin E, vitamin C, carotenoids, etc. have been found to modulate the complement activity in fish (Blazer and Wolke, 1984; Li and Lovell, 1985; Montero *et al*., 1998; Pearce *et al*., 2003; Amar *et al*., 2004).

Chemotaxis is the major mechanism for defending the host against infection and injury (Imhof *et al*., 1990). Lim and Klesius (1997) determined chemotaxis in channel catfish by a modification of the lower-surface method of Boyden (1962) as described by Klesius and Sealey (1996).

The proliferative response of head kidney lymphocytes was determined by the MTT [3-(4,5-Dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] colorimetric assay method described by Mosmann (1983) as modified for use with fish lymphocytes by Siwicki *et al*. (1996). Total plasma immunoglobulin (total Ig) levels in serum have been determined by a colorimetric assay (Anderson and Siwicki, 1995). To measure the potential killing activity of head kidney macrophages, the method of Rook *et al*. (1985) as modified by Siwicki and Anderson (1993) has been used.

3.4 Experimental challenge test

Because current methodology to comprehensively investigate immunity and disease resistance of fish is still limited, an effective biomarker for disease resistance of fish has been difficult to identify (Li and Gatlin III, 2006). Recently, Aoshima *et al*. (2005) reported that increased non-specific defence responses *in vitro* do not always reflect increased disease resistance. Therefore, the ultimate integrated responses of various immune mechanisms should be tested with a properly planned and standardized artificial challenge with an infectious agent.

The majority of reports have described increased resistance to mainly bacterial infections in the disease challenges. However, a few reports have

shown the effectiveness of nutrients on the resistance to parasitic and viral pathogens. There are some reports indicating dietary modification or feed additives may affect the resistance of fish to challenge with infectious hematopoietic necrosis (IHN) virus (LaPatra *et al*., 1998). Burrells *et al*. (2001) reported beneficial effects of dietary nucleotides when challenging salmonids with infectious salmon anaemia virus, *V. anguillarum* and a rickettsial organism (*Piscirickettsia salmonis*).

One of two different methods are usually adopted for a challenge with a pathogenic organism and these are a waterborne challenge or intraperitoneal injection. It seems that waterborne challenge more closely resembles natural infection in comparison with intraperitoneal challenge. If fish are immersed in aquaria containing a pathogen, and each group of fish is then returned to their respective aquarium, at least three replicate tanks per treatment should be used because mortality may vary considerably among the replicate tanks.

Immersion (bath) challenge, in which experimental fish are immersed in water containing a certain amount of bacteria for a specified period of time, has often been used as a means of providing a waterborne challenge. The disadvantage with this method is that the fish may be affected by handling stress. An alternative method is cohabitation of experimental fish (recipients) with diseased fish (donor) in the same tank. Fukuda *et al*. (1997) adopted a method in which a fixed bacterial suspension was introduced into a tank by use of a peristaltic pump. These two means of challenge will minimize handling stress during exposure to the pathogen.

The pathogenicity of the disease-causing agent and dose should be adjusted to detect differences in disease resistance of fish. If fish are overexposed to pathogenic organisms, their cumulative mortality may be elevated regardless of their health condition. Barros *et al*. (2002) examined the LC_{50} (lethal concentration which causes 50% mortality in exposed fish) of *Edwardsiella ictaluri* prior to experimental challenge to determine the optimum bacterial cell concentration to use. This is a reasonable method to determine the effective dose of pathogen.

Hypothetically, an experimental challenge that more closely mimics natural infection should provide a truer indication of a fish's disease resistance. Lygren *et al*. (1999) conducted an experimental challenge by the following protocol. Fish were marked according to dietary group using a Panjet (Wright Dental Group, Kingsway West, UK). Fish from each tank were randomly selected and moved to the challenge facilities. The fish were redistributed in three tanks with all the dietary groups represented in equal numbers. After an acclimatization period of 1 week, the fish were challenged using 12 cohabitants/tank injected intraperitoneally with 0.2 ml of a 2.1×10^5 colony-forming units (CFU)/ml suspension of *Aeromonas salmonicida* ssp. This protocol would closely mimic natural infection and eliminate variation among replicate tanks, but would preclude continuing to feed experimental diets during the disease exposure.

Regarding the effects of glucan on disease resistance, increases in non-specific resistance have been demonstrated in response to challenge

infections with *V. anguillarum*, *Vibrio salmonicida* or *Yersinia ruckeri* (Robertsen *et al*., 1990), *V. anguillarum* and *V. salmonicida* (Raa *et al*., 1992) and *A. salmonicida* (Nikl *et al*., 1993; Siwicki *et al*., 1994). However, no such benefit has been shown against *A. salmonicida* and *V. salmonicida* (Dalmo *et al*., 1998), *Enterococcus* species in turbot (Toranzo *et al*., 1995) or *V. anguillarum* in turbot (Ogier de Baulny *et al*., 1996). The results of previous studies on the effect of ascorbic acid on disease resistance in channel catfish are not consistent (Lim *et al*., 2000). In addition, information on the effects of ascorbic acid on disease resistance is also contradictory to that of salmonids. Thus, the differences between the results of those studies may be related to differences in species, strain, size and nutritional status of the fish used, pathogenicity of the bacteria and/or methods of disease challenge. Therefore, optimization and standardization of these methods should be carefully established. The proper procedures for a challenge test in the field of fish nutrition may be different from that in fish pathology. A standardized protocol for experimental challenge suitable for the studies of fish nutrition and a data validation system among laboratories should be developed in order to evaluate disease resistance in fish.

The size and sexual maturity of the experimental fish as well as the stressors of the rearing system affect the observations discussed in this section. Hence these points have to be given due consideration in interpreting the results from any study. It is difficult to evaluate fish health condition by a single indicator because the individual indicator may vary in sensitivity and precision. In addition, each indicator often shows wide individual variation among fish. A combination of measuring several parameters as well as using a standardized challenge test is recommended to assess fish health more comprehensively (Wedemeyer and McLeay, 1981; Sandnes *et al*., 1988).

4 Maintenance of Fish Health by Effective Feeding Regimes

The relationship between nutrition and fish health requires careful attention be paid to the effects of feeding regimes on fish health. Kim and Lovell (1995) first reported the effect of restricted feeding on disease resistance in channel catfish. They showed that plasma antibody titre and the survival rate in young fish (1 year old) following artificial infection with *E. ictaluri* were significantly higher in fish fed every day than those fed every other day or not fed at all. No differences, however, were observed between the fish fed every day and every other day in adult fish (2 years old). According to another report, it is suggested that humoral and cellular defences are not affected by starvation but disease resistance was improved in catfish (Okwoche and Lovell, 1997). In larval and juvenile Japanese flounder and red sea bream, excessive feeding caused lethal histopathological alterations, and increased mortality (Mobin *et al*., 2000, 2001). Self-feeding systems reduce stress and boost immune defences, as indicated by an increase in anticoagulant titre and lymphocyte counts (Endo *et al*., 2002).

Management of fish health by improving nutritional status and adopting effective feeding regimes will become more important in the future. However, many problems remain to be solved.

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3 Vitamins

SHUNSUKE KOSHIO

Faculty of Fisheries, Kagoshima University, Kagoshima 890-0056, Japan

1 Introduction

Vitamins are required to prevent disease, promote growth and maintain health of aquatic animals, leading to the quality improvement of cultured species. Since vitamins are not synthesized or synthesized in insufficient quantities to meet the requirements of the aquatic animal body, they should be supplemented in aquafeeds. Recommended dietary concentrations of vitamins for maximum growth and to avoid signs of deficiency have been established in several aquatic species (NRC, 1993; Kaushik *et al*., 1998). However, there are very limited data available on the requirements and/or recommended values in terms of improving health and quality of aquatic species. Recently, several studies have demonstrated that over supplementation of certain vitamins, particularly vitamins C and E, improved the stress tolerance, immunological response and disease resistance of aquatic animals (Lim and Webster, 2001; Lim *et al*., 2001).

This chapter reviews the recent progress of studies on the dietary supplement of vitamins concerning the health and quality of cultured aquatic species.

2 Improved Immune Responses and Disease Resistance with Vitamin Intake

Several studies are available on determining dietary vitamin requirements for immunological responses in aquatic animals (Dabrowski, 2001; Lim and Webster, 2001). It is likely that some vitamins have immunostimulant-like properties that activate the immune systems in aquatic animals. The cofactors necessary for the proper function of immune systems are also provided when animals take vitamins. It has been reported that the vitamin

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requirement for promoting aquatic animal health is, in general, much greater than that for maintaining normal conditions for growth. However, more detailed studies are required to draw decisive conclusions in this area since the vitamin requirement or optimal dietary dosage of vitamins can be influenced by many factors, making it difficult to determine the universal validity. Therefore, the detailed conditions applied in feeding trials should be provided when the requirement was determined or suggested for the particular species. This chapter mainly reviews the recent studies on the positive effects of vitamins for aquatic animal health.

2.1 Effect of vitamin C intake on enhancement of immune responses and disease resistance

Although the conclusion on the effectiveness of vitamin C for promoting aquatic animal health is varied, there is much evidence that supports the idea that vitamin C intake in several fish species enhances immune responses, stress and disease resistance (Table 3.1).

Table 3.1. Reported levels of dietary vitamins for enhancing immune responses, disease and stress tolerance in fish species after 1990.[a]

Species (scientific name)	Vitamin	Dietary content (mg/kg diet)	Effective trial duration (days)	Improved parameters	Reference
Marine fish					
Atlantic salmon (*Salmo salar*)	Vitamin A	60 (astaxanthin)	315	*Aeromonas salmonicida*	Christiansen *et al.*, 1995
		15	120	Serum antibody, bactericidal activity to *A. salmonicida*, antiprotease activity	*Thompson et al.*, 1994
	Vitamin C	2750	182	Complement, *A. salmonicida*, lymphokine	Hardie *et al.*, 1991
		2980	72	Antibody	Erdal *et al.*, 1991
		4000	168	Complement, lysozyme, antibody, *A. salmonicida*	Waagbø *et al.*, 1993
	Vitamin E	800	140	Phagocytosis	Hardie *et al.*, 1990
Gilthead sea bream (*Sparus aurata*)	Vitamin A	150 and 300 (retinol acetate)	7 or 14	Respiratory burst	Cuesta *et al.*, 2002
		300	14 or 28	Leucocyte myeloperoxidase	
		50 or 150	28 or 42	Leucocyte myeloperoxidase	

Table 3.1. *Continued*

Species (scientific name)	Vitamin	Dietary content (mg/kg diet)	Effective trial duration (days)	Improved parameters	Reference
	Vitamin C	3000	14 42 56	Phagocytic activity Haemolytic complement activity Respiratory burst	Ortuno *et al.*, 1999
	Vitamin E	1200 (α-tochopherol acetate)	30 to 45	Haemolytic activity, phagocytosis	Ortuno *et al.*, 2000
		600	28	Cytotoxic activity of leucocytes	Cuesta *et al.*, 2001
		1800	14	Cytotoxic activity of leucocytes	
	Combined vitamins C and E	3000 and 1200	30	Respiratory burst of phagocytes	Ortuno *et al.*, 2001
		3000 and 1200	14	Blood glucose level after physical disturbance, crowding with anaesthesia, air exposure	Ortuno *et al.*, 2003
Japanese flounder (*Paralichthys olivaceus*)	Vitamin C	10	120	Survival after freshwater exposure	Panganiban *et al.*, 2004
		50	120	Haematocrit after low salinity stress	
		500	120	Red blood cell counts	
Japanese parrot fish (*Oplegnathus fasciatus*)	Vitamin C	3000	112	Late deficiency sign after low oxygen stress Higher vitamin C level in plasma, kidneys and gills under low oxygen stress Better growth under low oxygen stress	Ishibashi *et al.*, 1992
Red spotted grouper (*Epinephelus coioides*)	Vitamin C	750	50	HSP70[b], mucus secretion	Tomino, 2003
Turbot (*Scophthalmus maximus*)	Vitamin C	800 to 1200	127	Phagocytosis, lysozyme	Roberts *et al.*, 1995
Yellow tail (*Seriola quinqueradiata*)	Vitamin C	390	60	Survival after air exposure, low salinity	Koshio *et al.*, 2002 Tomino, 2003

Continued

Table 3.1. *Continued*

Species (scientific name)	Vitamin	Dietary content (mg/kg diet)	Effective trial duration (days)	Improved parameters	Reference
Freshwater fish					
Channel catfish (*Ictalurus punctatus*)	Vitamin C	25 to 50	70 (21 for challenge)	*Edwardsiella ictaluri*	Li *et al*., 1998
	Vitamin E	240	120	Macrophage intracellular superoxide anion	Wise *et al*., 1993b
		2500	180	Phagocytic index	Wise *et al*., 1993a
Japanese eel (*Anguilla japonica*)	Vitamin C	27 to 645	56	Bactericidal activity, serum protein	Ren *et al*., 2005
Golden shiner (*Notemigonus crysoleucas*)	Combined vitamins C and E	98 to 222 and 38	119	Alternative complement activity	Chen *et al*., 2004
Indian major carp (*Cirrhinus mrigala*)	Vitamin C	1000	120	*Aeromonas hydrophila*	Sobhana *et al*., 2002
Rainbow trout (*Oncorhynchus mykiss*)	Vitamin C	1000 or 4000	14	Complement, chemiluminescence	Verlhac *et al*., 1996
		1000	14	Macrophage oxidative burst activity, pinocytosis, lysozyme, complement, antibody titres	Verlhac *et al*., 1998
	Vitamin E	295	80	Phagocytosis for leucocytes	Clerton *et al*., 2001
		100	140	Serum complement activity	Pearce *et al*., 2003
	Combined vitamins C and E	2000 and 800	240	Proliferation of lymphocytes, macrophage oxidative burst activity, bacterial and virus disease	Wahli *et al*., 1998

[a] The data for crustaceans are excluded here but are included in the text.
[b] HSP70, heat shock protein 70 family.

2.1.1 Marine species

In Atlantic salmon *Salmo salar* high amounts of vitamin C intake could improve the immunological responses such as complement, antibody and lysozyme as well as the resistance to bacterial challenge in fish (Erdal *et al*., 1991; Hardie *et al*., 1991; Waagbø *et al*., 1993). Ortuno *et al*. (1999) demonstrated in the study of gilthead sea bream *Sparus aurata* that when fed a diet containing 3000 mg vitamin C/kg diet, the phagocytic activity, haemolytic complement activity and respiratory burst were enhanced 14

days, 42 days and 56 days after feeding, respectively. Serum lysozyme and phagocytic capacity of kidney and spleen cells were enhanced by feeding diets containing vitamin C from 800 to 1200 mg/kg in a study of juvenile turbot *Scophthalmus maximus* (Roberts *et al*., 1995).

Although studies are very limited, in recent years there have been a few studies on the effectiveness of vitamin C for marine shrimp health. Four different types of vitamin C derivatives, such as L-ascorbyl-2-sulphate (C2S), L-ascorbyl-2-polyphosphate (C2PP), L-ascorbyl-2-monophosphate-Na (C2MP-Na) and L-ascorbyl-2-monophosphate-Mg (C2MP-Mg) with adequate or high dietary levels, were compared by evaluating total haemocyte count (THC), superoxide anion production ratio (O^{2-}) and phenoloxidase (PO) activity. It was found that significantly higher THC, O^{2-}, PO were obtained in shrimp fed diets supplemented with adequate and high levels of vitamin C than shrimp fed vitamin C-free diets, regardless of the vitamin C sources. However, different vitamin C derivatives affected the immune responses in different ways (Lee and Shiau, 2002).

2.1.2 Freshwater species

Although conflicting results have been demonstrated in the study of channel catfish *Ictalurus punctatus*, low levels of dietary vitamin C were as effective as high dose levels for normal growth, stress response, and disease resistance (Li *et al*., 1998). Indian major carp (mrigal) fed vitamin C supplemented diets (1000 mg/kg diet) exhibited lower mortality rates when challenged with *Aeromonas hydrophila* than those fed a non-vitamin C supplemented diet (Sobhana *et al*., 2002). In rainbow trout, *Oncorhynchus mykiss*, the complement activation and chemiluminescence response of macrophages were enhanced when the fish were fed a diet containing 1000 or 4000 mg vitamin C/kg together with glucans (Verlhac *et al*., 1996) and at a level of 1000 mg, oxidative burst, pinocytosis, lysozyme activity, complement and antibody titres were enhanced compared to fish fed a vitamin C-free diet (Verlhac *et al*., 1998).

We also found in the study of Japanese eel *Anguilla japonica* (Ren *et al*., 2005) that juvenile eels fed diets containing more than 27 mg vitamin C/kg showed a higher bactericidal activity of serum than the fish fed the diets containing 3 mg and 10 mg/kg. Furthermore, the inclusion of 645mg/kg or more increased the haematocrit, haemoglobin, total serum protein value and liver and brain vitamin C concentrations.

2.2 Effect of vitamin E intake on enhancement of immune responses and disease resistance

Vitamin E is often supplemented in aquafeeds because of its antioxidant properties. Therefore, there are many studies determining the optimal vitamin E level in the diets for minimizing oxidation. On the other hand, due to the popularity of high-fat diets, the role of vitamin E on fish health is

considered to be very important, resulting in several studies concerning vitamin E and fish health, stress and disease resistance in recent years.

2.2.1 Marine species

Ortuno *et al*. (2000) demonstrated in the study of gilthead sea bream that a moderate level of dietary vitamin E (1200 mg/kg diet) stimulated the non-specific immune system such as haemolytic activity and phagocytosis after 30 days of administration. They suggested that lower or higher vitamin E concentrations than mentioned above might not be so effective due to an imbalance in the vitamin E ratio with other antioxidants. Another study on gilthead sea bream indicated that after 2 and 4 weeks of feeding, the natural cytotoxic activity was significantly enhanced by feeding diets containing 1800 mg and 600 mg vitamin E/kg diet, respectively (Cuesta *et al*., 2001).

2.2.2 Fresh water species

In rainbow trout, altered dietary levels of vitamin E modulated the phagocytosis of gut leucocytes when fed at 295 mg vitamin E/kg diet, and the authors suggested that the effect of a diet containing vitamin E seems to be greater on the local intestinal response than on the systemic response such as the head kidney (Clerton *et al*., 2001). Pearce *et al*. (2003) also indicated that serum complement activity was enhanced by feeding a moderate level of vitamin E (100 mg/kg) but did not seem to improve further at 1000 mg vitamin E/kg diet.

2.3 Combined effects of vitamins or other nutrients on enhancement of immune responses and disease resistance

In recent years, more studies have begun to deal with the combined effects of nutrients on aquatic species. This is definitely a necessary approach to understand realistic vitamin requirements since nutrients interact in various metabolic pathways. For example, it is widely accepted that vitamin C spares vitamin E by regenerating it from the radical form. Although information is still limited, some studies on the combined effects of vitamins C and E for enhancing immune responses and the resistance to stress and disease have become available in recent years (Table 3.1).

Ortuno *et al*. (2001) demonstrated that a diet containing 3000 mg vitamin C/kg together with 1200 mg vitamin E/kg enhanced the respiratory burst of phagocytes, and they suggested a synergetic effect between the two vitamins. The interactive effect of vitamins C and E on alternative complement activity and the percentage of thrombocytes was reported in the study of golden shiner *Notemigonus crysoleucas*, and the best combination was 98 to 222 mg vitamin C together with 38 mg vitamin E per kg diet (Chen *et al*., 2004). In the study of rainbow trout (Wahli *et al*., 1998), the combination of high dietary doses of vitamins C and E significantly

stimulated lymphoproliferation and macrophage oxidative burst activity when compared with fish fed a low level of both vitamins. In disease resistance experiments, the best survival rates in trout infected with viral haemorrhagic septicaemia were achieved with diets containing both vitamins at a high level, or at least one at a high and the other at a low level. The best survival of fish exposed to *Ichthyophthirius multifiliis* was obtained with diets high in one and low in the other, or high in both vitamins.

The interactive effects of dietary vitamin C and copper (Cu) were also demonstrated in the study of marine shrimp *Penaeus monodon* (Lee and Shiau, 2003). The authors suggested that increased dietary vitamin C level improved haemocyte respiratory burst response and growth, and prevented tissue Cu accumulation in shrimp when fed the diet containing a high dietary Cu level. Lopez *et al*. (2003) studied the interactive effects of vitamin C and β 1–3 glucan on immunological responses such as blood cells and prophenoloxidase (ProPO) of marine shrimp *Litopenaeus vannamei*. They found that glucan was degraded in the digestive gland by β-glucanases to produce energy; whereas vitamin C was used to improve animal health, enhancing the general metabolism of shrimp.

2.4 Effectiveness of other vitamins on aquatic animal health

Compared to studies with vitamins C and E, there are very limited studies on the effects of other vitamins on aquatic animal health. Cuesta *et al*. (2002) demonstrated that vitamin A (retinol acetate) plays an important role in the gilthead sea bream non-specific cellular immune system due to its antioxidant properties, showing that respiratory burst activity was enhanced in fish fed the diet containing retinol acetate of 150 and 300 mg/kg diet. Furthermore, as feeding period increased, leucocyte myeloperoxidase activity was even enhanced at lower levels of vitamin A such as 50 or 150 mg/kg diet. In Atlantic salmon, serum antibody, bactericidal activity to *Aeromonas salmonicida* and antiprotease activity were enhanced by feeding a diet containing 15 mg vitamin A/kg diet (Thompson *et al*., 1994).

3 Effect of Dietary Vitamin Intake on Enhancement of Stress Tolerance in Aquatic Animals

In aquaculture conditions, animals are often under stress resulting from transport, crowding, handling and deteriorating water quality. Therefore, it is critical to establish means of reducing stress in animals to allow them to relax under given culture conditions. Feeding nutrients such as vitamins would be one of the ways to minimize stress to aquatic animals. There are limited studies on the effects of vitamins on the enhancement of stress tolerance in aquatic animals except for studies on ascorbic acids and because of this only the studies related to vitamin C are reviewed here.

It was reported that feeding high amounts of vitamin C was beneficial for reducing the effects of physiological stress in fish (Dabrowski, 2001; Lim and Webster, 2001). On the other hand, studies on short-term acute stress with common carp and channel catfish showed no correlation between dietary vitamin C levels and serum or plasma cortisol concentrations (Dabrowska *et al*., 1991; Davis *et al*., 1998; Li *et al*., 1998). In a study of Atlantic salmon, the responses to acute confinement stress, as measured by plasma glucose concentration, were not affected by dietary vitamin C levels (White *et al*., 1993). However, Ishibashi *et al.* (1992) indicated that Japanese parrot fish *Oplegnathus fasciatus* under low dissolved oxygen stress survived well and grew faster when fed a diet containing 300 mg of vitamin C. Furthermore, plasma, kidney and gill vitamin C contents in fish fed the same level of vitamin C were higher than those in control fish. Gilthead sea bream fed a vitamin C-free diet showed a higher concentration of plasma glucose 3 hours after hypoxia stress, and a higher concentration of plasma cortisol 9 hours after hypoxia stress than fish fed diets containing vitamin C (Henrique *et al*., 1998).

A recent study on gilthead sea bream (Ortuno *et al*., 2003) also demonstrated that increased stress indicators such as blood glucose concentration were lowered by feeding the vitamin C and/or E supplemented diet (3000 mg/kg vitamin C and 1200 mg/kg vitamin E, respectively) compared to those obtained from fish fed the control diet (100 mg/kg of both vitamins) after 2 weeks. The authors have suggested that vitamins C and E interfere with tertiary stress responses such as immunosuppression, and those vitamins also protect leucocyte functions. The suggestion was also made in the study of gilthead sea bream that vitamin C and/or E seemed to prevent a stress-related immuno-suppression, and the protective role of vitamin E against stress appeared to be greater than that of vitamin C (Montero *et al*., 1999).

We conducted several trials with marine species on investigating the effects of vitamin C on improvement of stress tolerance, where parameters such as heat shock protein, mucus secretion, air exposure and low salinity tolerance were evaluated. In a trial of juvenile yellow tail *Seriola quinqueradiata* using L-ascorbyl-2-monophosphate Na-Ca (Koshio *et al*., 2002; Tomino, 2003), dry diets with six levels (0, 10, 50, 90, 390 and 820 mg/kg) of ascorbic acid (AA) were fed to juveniles for 60 days. The stress tolerances against low salinity and air exposure were the highest in fish fed 390 mg AA/kg diet. In a trial of the red spotted grouper *Epinephelus coioides* higher levels of heat shock protein 70 family and mucus secretion were recorded in fish fed the vitamin C supplement diets (Panganiban *et al*., 2004).

A stress trial was also conducted using larval and postlarval kuruma shrimp *Marsupenaeus japonicus* in my laboratory (Moe *et al*., 2004, 2005). Different concentrations of two types of vitamin C derivatives (L-ascorbyl-2-monophosphate-Na-Ca and L-ascorbyl-2-monophosphate-Mg) were supplemented in microbound diets, and fed to larval kuruma shrimp from the zoea1 stage until they reached the postlarval stage. The tolerance against formalin was significantly poorer in larvae fed the vitamin C-free

diet than other vitamin C fed groups. Comparing the different dietary concentrations, there was no significant difference in stress resistance among 10, 100, 1000 mg vitamin C/kg diet groups for both types of derivatives. In the postlarval trial, test diets containing vitamin C from 0 to 1700 mg/kg diet were fed for 30 days, and the tolerance to osmotic and formalin stresses were examined. This study concluded that a dietary level of more than 800 mg vitamin C was needed to ensure high resistance to stressful conditions.

The interactive effects of vitamin C and β-glucan after salinity stress have also been evaluated for marine shrimp, *L. vannamei* (Lopez *et al*., 2003). The authors found that after salinity shock, blood cell numbers increased in shrimp fed supplemental glucans and decreased in shrimp fed supplemental vitamin C. On the other hand, ProPO decreased in all shrimp after the salinity shock. The authors suggested that after the salinity stress, shrimp fed glucans could synthesize cells and ProPO, whereas in shrimp fed vitamin C, blood cells were utilized to respond to the stress.

Combined effects of dietary vitamin C and astaxanthin (AX) on osmotic shock were investigated using *P. monodon* (Merchie *et al*., 1998). An increase in dietary vitamin C from 100 to 3400 mg/kg resulted in a concomitant drop in mortality after an osmotic shock. In shrimp fed the diet containing 810 mg AX/kg, the beneficial effect of extra dietary vitamin C levels was not found. An increase in the dietary AX for shrimp fed comparable vitamin C levels resulted in a significant drop in the stress index. The authors concluded that an increased resistance to salinity shock was shown in association with supplementation of high dietary AA or AX levels.

4 Concluding Remarks

It is possible to enhance stress and disease resistance, and therefore aquatic animal health, by feeding aquafeeds containing higher vitamin concentrations than those required for normal growth. However, when determining optimal dietary concentrations of vitamins the following factors should be considered and defined:

1. Confirmation of vitamin type and function.
2. Ration size.
3. Feeding method (continuous or interval).
4. Timing of administration.
5. Feeding duration.
6. Rearing or culture condition.
7. Health status of target species.
8. Genetic history of target species.

Recently, it was reported that fish behaviour such as schooling and tilting was affected by vitamin C intake (Koshio *et al*., 1997; Sakakura *et al*., 1998; Koshio, 2001). Because these normal behaviour patterns are demonstrated in wild specimens, they are considered to be reliable

indicators for determining the quality of artificially raised species in captivity. The studies above suggest that dietary vitamin C content is very important in producing healthy fish.

Proper application of vitamins in aquafeeds can be one of the most safe and useful strategies to not only increase aquaculture production but also sustain the aquaculture industry because the application of antibiotics is very limited, and food safety is a major concern for human food consumption.

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4 Amino Acids, Peptides

TOSHIO TAKEUCHI

Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan

More than 200 kinds of amino acid occur in nature, however, only 20 primary amino acids are incorporated into body proteins. Among them, ten amino acids are essential amino acids (EAA) for fish and shellfish in that they cannot be synthesized and must be provided in the diet. In vertebrates, most of the EAA or their derivatives also function as hormones and neurotransmitters. This chapter summarizes recent topics concerning the effects of amino acids and their derivatives on fish and shellfish.

1 Classification and Role

1.1 Amino acids and their derivatives

Table 4.1 shows the main physiologically active substances derived from amino acids (Abe, 1995). Histidine is decarboxylated to histamine (a primary amine) which stimulates gastric acid secretion. Histamine can become a toxic substance called gizzerosine which is the main cause of stomach erosion. Tryptophan is hydroxylated and decarboxylated to serotonin (a neurotransmitter), and melatonin (a secondary amine) which can synchronize reproduction performance with photoperiod. It also can be decarboxylated to nicotinamide (a kind of water-soluble B vitamin) and converted further into nicotinamide adenine dinucleotide (NAD). Lysine is decarboxylated to cadaverine (a primary amine), and is converted to be a constituent of so-called vitamin B_T – carnitine (a quaternary amine) via methylation. Serine is methylated to choline (a water-soluble vitamin). Taurine is synthesized from methionine and cysteine via oxidation. Furthermore, tyrosine is converted into thyroxine (T_4), a well-known thyroid hormone, via iodination and polymerization. It is converted further to a well-known black pigment – melanin. Thus, amino acids not only serve

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Table 4.1. Main physiologically active substances derived from amino acids.

Precursor amino acids	Reaction	Physiologically active substance
Methionine, cysteine	Oxidation	Taurine
Tryptophan	Hydroxylation and decarboxylation	Serotonin and melatonin
	Decarboxylation	Nicotinamide
Histidine	Decarboxylation	Histamine
Lysine	Decarboxylation	Cadaverine
	Methylation	Carnitine
Tyrosine	Iodination and polymerization	Thyroxine
Serine	Methylation	Choline

as constituents of proteins and sources of energy, but also can be converted into important biochemically active substances *in vivo*.

1.2 Peptides

Many dipeptides, tripeptides and polypeptides (tens of amino acids bonded together) have physiological activities, such as creatinine (two amino-acid residues), glutathione (three amino-acid residues), plasmakinine (nine or ten amino-acid residues), angiotensin (seven to ten amino-acid residues), etc. Insulin is well known as a peptide hormone.

2 Effect of Taurine on Fish

Taurine is synthesized from methionine, a sulphur amino acid, after methionine is oxidized to cysteine (Fig. 4.1). But taurine is an essential nutrient for cats, monkeys and infants (Hayes *et al*., 1975, 1980; Knopf *et al*., 1978; Hageman and Schmidt, 1987). In these animals, the rate of taurine synthesis is slow due to the low activity of the cysteinesulphinate decarboxylase (CSD) enzyme, by which cysteine sulphinate is decarboxylated to hypotaurine – the precursor of taurine. In fish, it has been recently clarified that taurine is also an essential nutrient for larva and juvenile of red sea bream *Pagrus major* and Japanese flounder *Paralichthys olivaceus*. Furthermore, dietary supplementation with taurine has been found to be effective in alleviating the condition of green liver in adult yellowtail *Seriola quinqueradiata* and red sea bream, as well as the condition of non-spawning in broodstock yellowtail fed diets containing a low content of fishmeal. In contrast, rainbow trout *Oncorhynchus mykiss* and tilapia *Oreochromis niloticus* can synthesize taurine from methionine. Therefore, the effect of dietary supplementation with taurine on fish is complex, varying with species and growth stages as related to their ability to synthesize taurine. The mechanism of *in vivo* synthesis of taurine in fish, as well as the effect of dietary supplementation with taurine on larval, juvenile, adult and broodstock fish is summarized below.

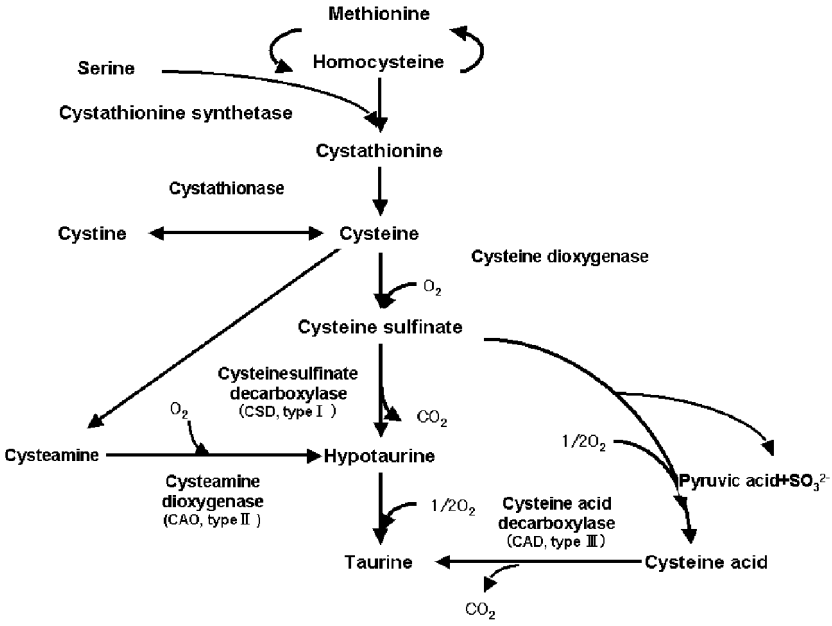

Fig. 4.1. Metabolic pathway of sulphur amino acids.

2.1 Larvae and fingerlings

It was found that the taurine content in fertilized eggs does not decrease as other free amino acids during the embryonic development of artificially produced yellowtail and Japanese flounder larvae and juveniles, but the taurine content after hatching decreased rapidly during the rotifer feeding period (Matsunari *et al*., 2003a). Although the taurine content increased to a certain extent during the *Artemia* feeding period, it decreased again when larvae were fed commercial diets (Takeuchi, 2001; Takeuchi *et al*., 2001). The pattern of taurine content in the larvae after hatching is similar to that of docosahexaenoic acid (DHA), which is necessary for the growth of larvae and juveniles for the promotion of their activity (Takeuchi, 1991). Moreover, the taurine content in the whole body of artificially reared larvae and juveniles is significantly lower than that in the wild fish (Fig. 4.2) (Takeuchi *et al*., 2001; Matsunari *et al*., 2003b). In contrast, no difference was found in the histidine content, which is rich in muscle of yellowtail, between cultured fish and wild ones. The difference stems from the taurine content in live foods and in the diets that are utilized in artificial seed production. Precisely, the taurine content in rotifers is 80–180 mg/100 g (dry basis), 600–700 mg in *Artemia*, and 200–520 mg in commercial diets; widely different from that in wild plankton (1200 mg) and in mysids (2900 mg) (Table 4.2) (Takeuchi, 2001; Matsunari *et al*., 2003b). Therefore, the higher taurine content in wild fish, compared with artificially produced fish, can be attributed to their consumption of zooplankton. This suggests that

Table 4.2. Taurine content in live foods and in commercial diets (mg/100 g dry weight).

Rotifers	Taurine enriched rotifers	*Artemia*	Natural Copepoda	Frozen Copepoda	Mysids	Commercial micro-particulate diet	Low fishmeal content diet (5% fishmeal + 5% krill)	Experimental fishmeal diet (50% fishmeal)	Crumble diet	Commercial diet (Φ 22 mm)	Commercial diet (Φ 4.3 mm)	Commercial diet (Φ 16 mm)
80–180	300–1250	600–700	1200	460	2900	440	200	520	380	450	460	440

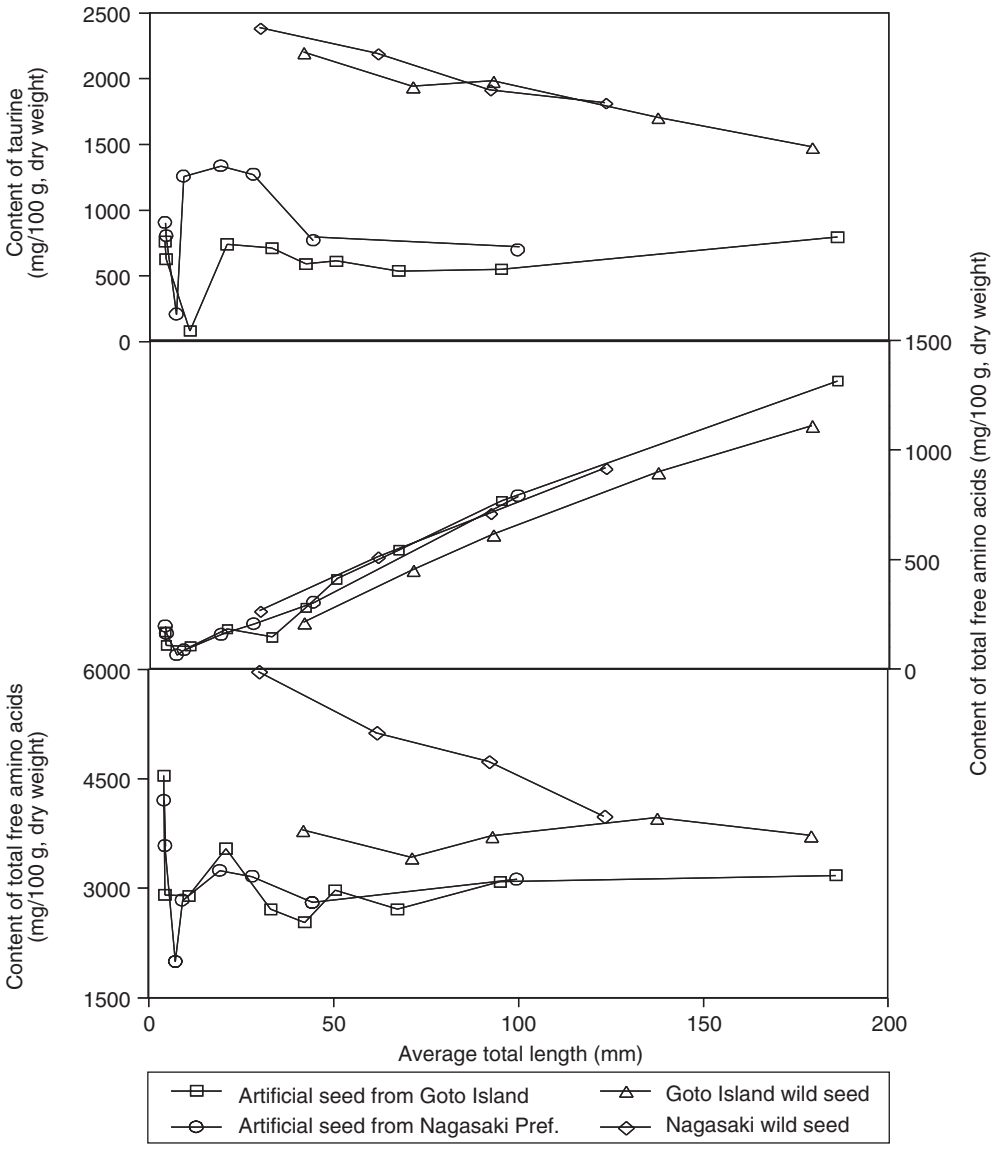

Fig. 4.2. Comparison of free amino acid contents in whole body of larval and juvenile yellowtail.

differences in taurine intake may affect growth and quality of artificially produced fish.

As mentioned above, the taurine content in rotifers (which are widely used in artificial larval fish production) is significantly lower than plankton and mysids. Recently, with the advance in the development of taurine enrichment material for rotifers, the taurine content in rotifers can be enriched to 1250 mg/100 g (dry basis) by means of supplementing 800 ppm

taurine in the rotifer medium tank (Takahashi *et al*., 2003). When Japanese flounder and red sea bream larvae were fed these taurine-enriched rotifers, their growth performance and survival rate, as well as survival rate in a starvation-activity index (SAI) assay, were found to be improved. Accumulation of taurine in the body of fish fed the taurine-enriched rotifers also was observed (Chen *et al*., 2004, 2005). Thus it is clear that rotifers can readily accumulate low molecular taurine in the body. Bivalves such as scallops also show the same trend (described below). In our other experiments of feeding juvenile Japanese flounder with mysids, compared with commercial diets, significant differences were found in juvenile growth and feed efficiency (Seikai *et al*., 1997). Furthermore, a significant effect of the abundant taurine in mysids was observed (Park *et al*., 1997; Takeuchi, 2001) and we found that juvenile Japanese flounder and red sea bream require taurine at 1500–2000 mg/100 g, much more than the current taurine content of 300–400 mg/100 g in commercial diets (Park *et al*., 2001). In addition to growth and feed efficiency, body skin colour was found to be improved in red sea bream that were fed diets with increased taurine content (Akimoto *et al*., 2002). Furthermore, trials were conducted in feeding Japanese flounder and common carp diets containing water-washed fishmeal in which taurine was largely removed. Japanese flounder showed poor growth and low feed efficiency (Kim *et al*., 2003). Moreover, abnormal feeding behaviour was observed (Kim *et al*., 2005). By contrast, no negative effect of this water-washed, taurine-reduced fishmeal was observed in the growth performance of carp (unpublished data). Why do the requirements of taurine differ in different species of fish?

2.2 Mechanism of taurine synthesis

The major pathway for taurine synthesis from methionine in mammals is believed to involve the transformation of methionine to cystathionine by cystathionine synthetase, the transformation of cystathionine to cysteine by cystathionase, the oxidation of cysteine to cysteine sulphinate, the decarboxylation of cysteine sulphinate to hypotaurine and further oxidation of hypotaurine to taurine (Worden and Stipanuk, 1985). It is well known that rainbow trout have the above physiological ability for taurine synthesis. However, recently Yokoyama *et al*. (2001) reported that CSD activity differs among species, and the activity in Japanese flounder, yellowtail, and bluefin tuna was very low compared with rainbow trout (Table 4.3). Moreover, cysteamine dioxygenase (CAO) activity differs markedly between fish species, which is another pathway of taurine synthesis (Goto *et al*., 2001). Therefore, it is suggested that some kinds of fish have a low activity to synthesize taurine, the same as in the cat. In fact, there was no increased taurine level in the muscle and liver of Japanese flounder fed a cystine-supplemented diet. Cystathionine contents of muscle and liver from fish fed a control and cystine-supplemented diets were much higher than those of fish fed taurine-supplemented diets (Park *et al*., 2002).

Table 4.3. The activity of cysteinesulphinate decarboxylase (CSD) and cysteamine dioxygenase (CAO) in liver of several species of fish and mammal.

	CSD activity[a]		CAO activity[b]	
Species	BW[c] (g)	n mol hypotaurine min/mg protein (25°C)	BW (g)	n mol/min/mg protein (35°C)
Freshwater fish				
Tilapia	86	0.56	NA[d]	NA
Bluegill	NA	NA	25–40 (30–70)[e]	3.06 (0.66)[e]
Rainbow trout	41	0.55	141–167 (121–167)	0.09 (0.14)
	307	0.67		
Carp	372	0.01	72–162 (102–164)	0.86 (0.44)
Ayu	NA	NA	68–90 (73–91)	1.85 (1.88)
Marine fish				
Largescale blackfin	194	2.55	NA	NA
Japanese flounder	0.8–12.6	0.26–0.39	300–1000 (600–3000)	0.78 (0.89)
Red sea bream	1071	0.25	200–1000 (1000–1500)	2.04 (2.61)
Yellowtail	99 and 3900	trace and 0.01	NA (800–900)	NA
Bluefin tuna	138	trace	NA	NA
Mammal				
Mouse	28	4.25	NA (37–43)	NA (1.74)
Rat	NA	1.80	NA	NA

[a] Yokoyama *et al.* (2001).
[b] Goto *et al.* (2001).
[c] BW, body weight.
[d] NA, not analysed.
[e] Goto *et al.* (2002).

On the other hand, high levels of serine, not cystathionine, were observed in yellowtail fed a low taurine diet (Matsunari *et al*., 2005).

In contrast, CSD activity is high in some freshwater fish, such as rainbow trout and tilapia. For example, muscle of tilapia *O. niloticus* contained taurine in spite of tilapia being fed solely on *Spirulina* (containing no taurine) for 30 weeks. It is supposed that taurine may be synthesized from methionine in tilapia (Takeuchi *et al*., 2002). In fact, normal maturation was observed in tilapia fed solely on *Spirulina* throughout three generations, and juveniles of good quality were produced (Lu and Takeuchi, 2004). In contrast, CSD activity in carp is extremely low. No decrease was found in carp fed diets made of low-taurine fishmeal. Therefore, this suggests that another pathway of taurine synthesis occurs in carp. In addition to the cysteine sulphinate route (type I), Goto (2002) found that there are at least two other routes, the route of creating hypotaurine from cysteamine (cysteamine route, type II) and the route of direct decarboxylation of cysteine acid to taurine (cysteine acid route, type III). Thus he determined that bluegill *Lepomis macrochirus* use type I (the same as rat, mouse and dog), ayu (*Plecoglossus altivelis*) type II, red sea bream types

II and III (the same as chicken), while yellowtail and Japanese flounder, the same as human and cat, are unable to biosynthesize taurine. Since the enzyme activity of all synthesis types was found to be low in carp, it is supposed that biosynthesis of taurine may occur in another organ than the liver. It was also pointed out that CSD and cysteine acid decarboxylase (CAD) are the same enzyme in red sea bream (Goto *et al*., 2003). In addition, rainbow trout was found to excrete taurine in the urine when it was fed taurine-enriched diets, while no taurine excretion was found in Japanese flounder even though it was fed diets with a high taurine content of 1900 mg/100 g (Park *et al*., 2002). In consideration of these phenomena, further intensive studies on the biosynthesis and metabolic routes of taurine are necessary.

2.3 Adult fish

Nowadays, domestic fishmeal production in Japan has decreased sharply due to the decrease of sardine production. Supply of fishmeal has become unstable, since it is necessary to depend on imports. Accordingly, research on alternative protein sources as substitutes for fishmeal, the main ingredient of many fish feeds, has been accelerated. In the process of that research, it was found that the colour of livers in yellowtail and red sea bream fed diets containing low or no fishmeal changed to green, the so-called 'green liver' syndrome. Soybean protein concentrate (SPC), and corn gluten meal were used as a substitute for fishmeal, but these vegetable protein feedstuffs hardly contain taurine. Takagi (2002) observed decreased growth and feed efficiency, as well as a high incidence of 'green liver' in yellowtail and red sea bream fed diets containing SPC as the main ingredient. In contrast, growth was improved and the 'green liver' symptom was cured in fish fed diets supplemented with taurine at a similar level to that of fish fed diets containing fishmeal as the main protein ingredient. It was concluded that the 'green liver' caused by feeding the low- or non-fishmeal diets may be due to the fact that erythrocytes become frail and thereby induce haemolysis because of the deficiency of taurine. As a result, the content of biliverdin (a bile pigment) increases and becomes excessive in the liver causing the symptom of 'green liver' (Takagi *et al*., 2003). Another reason indicated was that the poor feeding of the low- or non-fishmeal diets may enhance myxosporean parasitism in the bile duct, and therefore the bile duct is blocked, and bile is unable to be released (Maita *et al*., 1997).

2.4 Broodstock

Recently, we reported the effect of taurine on broodstock of yellowtail (Hamada *et al*., 2003; Matsunari *et al*., 2003a). Yellowtail were fed broodstock diets supplemented with taurine 3 months before spawning,

and compared with broodstock fed a control diet (containing 40% fishmeal) with no supplemental taurine. It was found that the broodstock could not spawn when fed diets containing taurine lower than 700 mg/100 g. Thus this suggests that it is necessary to supplement broodstock diets with taurine. Furthermore, it was found that eggs of all experimental groups had almost the same content of taurine. It was considered that broodstock of yellowtail may transfer taurine into eggs selectively. No difference was found among the experimental groups in survival under the non-feeding condition in a SAI assay. All of these results suggest that taurine may have an effect on broodstock during the process of egg maturation. Further intensive studies are necessary.

3 Effects of Peptides on Fish

This section describes the effects of di- and tripeptides and their enzymatically hydrolysed substances especially on larvae and juveniles.

3.1 Di- and tripeptides

It has been clarified that several species of di- and tripeptides have effects as feeding attractants or on growth improvement in juvenile red sea bream (Kanazawa *et al*., 1991). Some kinds of peptides were combined in microdiets and were fed to larval and juvenile red sea bream for 30 days. Compared with the control group fed a diet containing no supplements, fish in groups fed Ala-Pro, Ala-Ser, Ala-Gly-Gly, Asp-Phe methyl ester, Ala-Phe and Ala-Pro-Gly showed greater total length and body weight, while no difference was found in groups fed Ala-Val, Pro-Ala, and Ala-Asp.

Glutathione, containing glutamic acid, glycine and cystine (an intermediary metabolic substance in the process of the production of taurine from methionine), is a tripeptide which usually occurs in the reduced form. It is well known that the reduced form of glutathione is effective in eliminating active oxygen (hydrogen peroxide and superoxide anion) and lipid peroxides, protecting membranes of liver cells and red blood cells, preventing side effects of medicine, and detoxifying. It is supposed that stress builds up easily in fish cultured in cages on account of overcrowding and fluctuations in water temperature and dissolved oxygen, and oxidation stress becomes severe when fish are medicated, or fed diets containing a large amount of fish oil (which is easily oxidized due to its highly unsaturated fatty acids). It has been verified in practical culture that glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) (criteria for yellow fat disease) were improved, and mortality caused by *Streptococcicosis* was decreased in adult yellowtail, red sea bream and white trevally *Pseudocaranx dentex* when they were given 20 mg/kg body weight/day of glutathione via the diet (Kubono, 2000).

3.2 Substances of enzymatic hydrolysis

Polypeptides of molecular weight from 700 to several thousands can be produced via enzymatic hydrolysis of soybeans and casein. Soybean protein was enzymatically hydrolysed into soybean peptides by protease and these soybean peptides were fed to larval and juvenile flounder (total length 15.2 mm) in the form of microdiets for 30 days. Growth of fish in the group fed a diet in which these soybean peptides replaced 20% of casein was superior to the other groups (Kanazawa *et al*., 1990). In another experiment, larval sea bass at 19 days after hatching were fed microdiets in which protease-hydrolysed fishmeal was substituted for regular fishmeal. Fishmeal was hydrolysed into amino acids (molecular weight < 1000), dipeptides, tripeptides, and short-chain peptides hydrolysed from polypeptides (< six residues). Fish in the groups fed diets with 20% and 40% short-chain peptides showed superior growth and survival (Zambonino-Infante *et al*., 1997).

Milk protein, roughly classified into milk casein and milk serum protein, can be enzymatically hydrolysed into milk protein peptides by various proteolytic enzymes. These milk protein peptides are broadly utilized in human nutrition as supplements for sports, infant and clinical nutrition applications as well as for food property improvement (Horii *et al*., 1995). We developed a new type of microdiet, which is based on a combination of peptides and fatty acid-calcium salts, for larval Japanese flounder from first feeding. The peptides were obtained from enzymatically hydrolysed casein, with molecular weights of about 30,000 Da (C700) and 1000–2000 Da (C800), respectively. It was found that fish fed a mixture of different molecular weight peptides C700 and C800 in a microdiet were superior in growth and survival to fish fed C700 only (Takeuchi *et al*., 2003). Moreover, leaching of C800 increased rapidly when the percentage of C800 was increased. This leaching would not only lower the nutritional value of microdiets, but also cause deterioration of the rearing water quality (Fig. 4.3). Investigations into the effects of these various peptides on growth of larval and juvenile fish are not only useful for clarifying nutritional requirements for protein (especially amino acids), and the absorption mechanisms in larval fish, but also are a promising and effective means for the intensive development of microdiets.

4 Other Compounds

Amino acids, peptides, and their related compounds have both positive and negative effects on fish and shellfish. Below is a summary of compounds which have become the focus of attention in recent nutritional research.

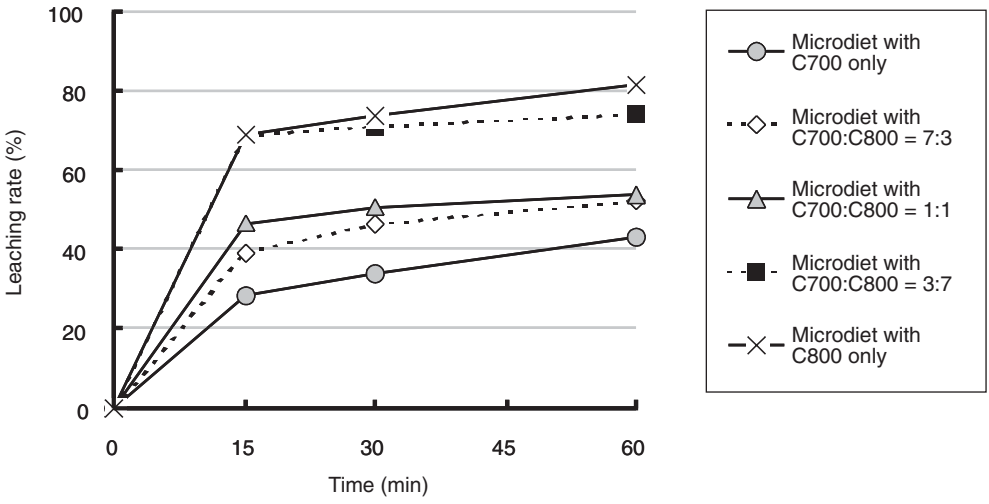

Fig. 4.3. Leaching rate of micro-particulate diets.

4.1 Tryptophan

Tryptophan has two metabolic routes: one is a serotonin biosynthesis route, in which tryptophan is converted to serotonin and melatonin and accumulates in the central nervous system and acts on the regulation of sleeping, appetite, secretion of growth hormone and prolactin. Another is the kynurenine route, in which tryptophan is transformed into acetyl-CoA and NAD. Trout and salmon show vertebral deformity on account of tryptophan deficiency. This correlates closely with a low level of serotonin. It was clarified that vertebral deformity could be prevented when the dietary content of serotonin was kept at 100–130 mg/100 g (Akiyama *et al.*, 1986, 1989).

4.2 Lactoferrin

Milk of mammals contains various kinds of factors inhibiting infection, one of which is known as lactoferrin. Lactoferrin is an iron-containing glycoprotein (red colour, molecular weight 80,000). It is known that lactoferrin is effective in fish (red sea bream, goldfish, ayu, etc.) by increasing mucus secretion to prevent parasitic infection, increasing immunity by activating their phagocytic cells, natural killer cells, lectins and lysozyme, as well as decreasing the level of cortisol (a rough indicator of stress) in plasma (Kakuta *et al.*, 1996, 1998). In kuruma prawn *Marsupenaeus japonicus*, growth and stress tolerance could be improved when they were fed a dietary supplement of lactoferrin at 700–1000 ppm (Koshio *et al.*, 2000).

4.3 Histamine

Lean fish such as sardine and saury *Cololabis saira* contain high levels of histidine. A rapid and large increase in the histamine content is observed during decomposition. Histamine leads to hypersecretion of gastric acid and an allergic reaction. Moreover, heat treatment of histamine produces gizzerosine, and gizzerosine causes the symptom of gizzard erosion in poultry. Cadaverine, is another amine compound well known as a decomposition substance from gizzerosine rather than histamine. Commercial diets manufactured from heat-treated fishmeal that contain histamine have been found to potentially cause abnormality in the gastric wall of rainbow trout (Watanabe *et al*., 1987).

Recently, fishmeal containing over 1000 ppm histamine has been imported into Japan, causing concerns over potentially adverse influences on yellowtail. However, no adverse effects were found on the growth of yellowtail fed the diets containing 1500 ppm histamine and 0.6 ppm gizzerosine, respectively (Watanabe *et al*., 2000). Moreover, it was confirmed that histamine did not accumulate in muscle of the yellowtail fed the diets containing 740 ppm histamine and 0.4 ppm gizzerosine for 126 days (Takeuchi, 2002). Histamine is a food hazard according to Hazard Analysis and Critical Control Point (HACCP) regulations, and consideration of its potential accumulation in imported products is necessary.

4.4 Carnitine

It is well documented that carnitine is required for the oxidation of long-chain fatty acids by mitochondria. Also, carnitine, by modulating the coenzyme A (CoA)/Acyl-CoA ratio, affects carbohydrate and lipid catabolism as well as the rate of the Kreb's cycle. In recent years, carnitine has attracted the interest of many investigators due to the potential of its positive effects on growth and lipid metabolism in fish. Indeed, there is evidence that carnitine has a growth-promoting effect on sea bass (Santulli and D'Amelio, 1986), African catfish (Torreele *et al*., 1993) and red sea bream (Chatzifotis *et al*., 1995, 1996). It also has been shown that carnitine can reduce the lipid content of liver and muscle in sea bass (Santulli and D'Amelio, 1986), as well as viscera and fillet lipid in Atlantic salmon (Ji *et al*., 1996). However, contrary to the above observations, carnitine did not cause any observable effects on the growth of rainbow trout (Rodehutscord, 1995; Chatzifotis *et al*., 1997) and Atlantic salmon (Ji *et al*., 1996), nor did it reduce the lipid content of the whole body of trout (Rodehutscord, 1995) or muscle and liver lipid of red sea bream (Chatzifotis *et al*., 1995, 1996). The carnitine content of animal tissues depends on the physiological state of the animal and is influenced by environmental conditions (e.g. temperature, short-term lack of food, etc.). The carnitine content in red sea bream was monitored under three different temperatures (Fig. 4.4). An increase in the amount of long chain acylcarnitine was observed for the low temperature

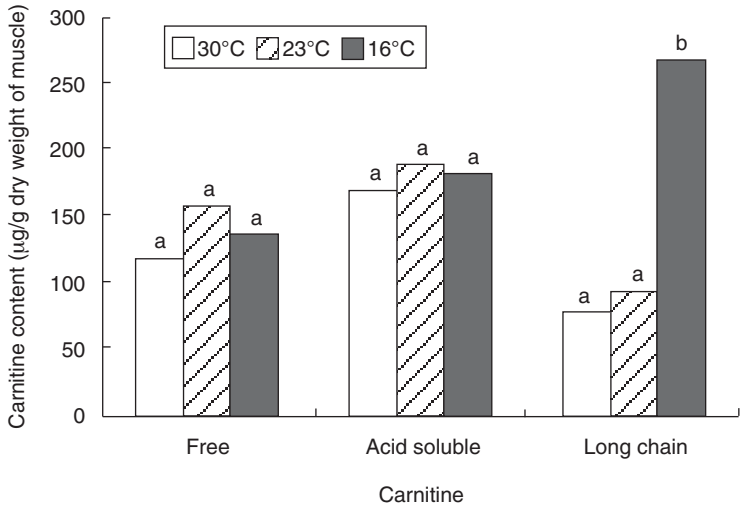

Fig. 4.4. Carnitine content in muscle of red sea bream reared under different temperatures. Different letters indicate significant differences between groups ($P < 0.05$).

treatment. The effect of carnitine on the growth of fish and accumulation in muscle may be species specific and may depend on environmental conditions.

4.5 Thyroxine (T_4)

Flounder larvae show a symmetrical body structure until the onset of metamorphosis. During metamorphosis, one of the eyes migrates from one side of the body to the other and other asymmetrical changes occur at the same time. These morphological changes cause a gradual shift in the body axis. A concomitant asymmetric differentiation occurs on the skin pigmentation pattern after the metamorphic climax, rendering a brownish coloration on the ocular side of the body while the blind side becomes white. The appearance of unpigmented juveniles renders them more susceptible to predation when released into the wild, besides lowering their market value. It is therefore necessary to understand the mechanism of this colour anomaly and establish methods for its effective prevention. It is well known that histological changes in thyroid-hormone stimulating cells and in the thyroid gland have been observed during the metamorphosis of Japanese flounder. Recently, Yoo *et al.* (2000) found that a very high occurrence of abnormal pigmentation (up to 90%) was characteristic of fish treated with T_4 (10 mM) from E to F stage, when the content of the hormone in the body was at a maximum level, suggesting that the mechanism of pigmentation strongly depends on thyroid hormones.

4.6 Incorporation of amino acids in shellfish

Pearl oyster *Pinctada fucata martensi*, Japanese oyster *Crassostrea gigas* and giant scallop *Patinopecten yessoensis*, similarly to rotifers, can accumulate free amino acids from seawater into their body when glycine, taurine and glutamine acid, known as taste components, were directly supplemented into seawater at a density of 2.7 mmol/l (200–400 ppm). Measurement of their incorporation showed that pearl oyster and Japanese oyster were superior in incorporating glycine. Furthermore, in the investigation of osmotic tolerance of oysters, when transferred into fresh water from seawater, it was found that glycine-enriched oysters showed a higher survival (Takeuchi and Endo, 2004). This suggests that glycine is effective as an energy source. In contrast, scallops were superior in incorporating taurine. It was clarified that the incorporation of free amino acids as dissolved nutrients in different bivalves is species-specific. Therefore, direct supplement of free amino acids to shellfish could easily enhance their activity, and may be an effective measure of preserving the quality of shellfish.

5 Conclusion

In summary, amino acids and their related compounds – peptides and peptide hormones – have a wide variety of effects on living organisms, such as improvement of growth and survival, enhancement of hepatic function, reduction of stress, elimination of peroxide, etc. Their effects and mechanisms have not been completely clarified to date; further research progress in this field can be expected. It is important to further elucidate the species-specific taurine requirements of fish and shellfish and delineate underlying mechanisms for those requirements. In addition, more research should be conducted to further characterize the utilization of amino acids as 'direct supplements' by shellfish, and continue development of peptide-based microdiets, as well as determine the disease-preventing contribution of peptides.

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5 Lipids

MANABU ISHIKAWA

Faculty of Fisheries, Kagoshima University, Kagoshima 890-0056, Japan

1 Nutritional Value of Lipids and Essential Fatty Acids

1.1 Nutritional value of lipids

In recent years, as a result of a shortage of fishmeal supplies, research has been developed to find alternative protein sources to mimimize the amounts of fishmeal in aquafeeds. In addition, new processing technology has made it possible to use high lipid levels in aquafeeds for some species. For example, aquafeeds containing more than 20% of dietary lipid are now available in the market for red sea bream *Pagrus major* and yellowtail *Seriola quinqueradiata*.

Since fish oils in Japan are mostly imported from foreign countries, their price and production figures are dependent upon the wild catch of these oil-yielding species. Although Takeuchi *et al.* (1978) suggested possible use of palm and lard lipids for carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* when the optimal amounts of dietary essential fatty acids were provided, the studies on fish oil replacement are still limited compared to those on fishmeal replacement. Aoki (1999) reported in the study of yellowtail that growth and feed efficiency ratio were not affected by test diets containing palm and lard oils with a 50% replacement level of fish oil. Moreover, peroxidizability index, which is an index for rates of oxidized lipid production obtained from fatty acid composition of muscle and liver, was lower in the diet and muscle of fish fed a diet containing palm oil and/or lard, indicating they may be able to suppress oxidation. A study on the effects of vegetable oils on gilthead sea bream (*Sparus aurata*) health was conducted using soybean oil, rapeseed oil, linseed oil or a blend of those oils (Montero *et al*., 2003). Feeding dietary vegetable oils for a long period did not affect lysozyme or neutrophil activity. However, the inclusion of soybean oil reduced both serum

alternative complement pathway activity and head kidney phagocytic activity. Inclusion of rapeseed oil reduced phagocytic activity. Fish fed vegetable-oil-containing diets showed different patterns of stress response, especially those fish fed the linseed oil diets that showed a significant increase in plasma cortisol level after stress. Montero *et al.* (2003) concluded that 60% of fish oil could be replaced by a blend of different vegetable oils without affecting gilthead sea bream health. However, if a single vegetable oil is used to replace 60% of fish oil, fish health can be affected in terms of immunosuppression or stress resistance.

1.2 Essential fatty acids

1.2.1 Nutritional evaluation of essential fatty acids

Studies on essential fatty acids (EFAs) for aquatic animals started in the late 1960s. Linoleic, linolenic and/or both in freshwater species, and n-3 highly unsaturated fatty acids (HUFAs) such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) in marine species have been reported to be essential, respectively. In larval studies, not only development performance but also resistance to stress is used for the evaluation of EFA requirements. Such stress tests include: air exposure for red sea bream (Izquierd *et al*., 1989), Pacific cod *Gadus macrocephalus* (Zheng *et al*., 1996), yellowtail (Furuita *et al*., 1996b), dorado *Coryphaena hippurus* (Kraul *et al*., 1993) and Japanese parrot fish *Oplegnathus fasciatus* (Kanazawa, 1993); high salinity exposure for Japanese flounder *Paralichthys olivaceus* (Furuita *et al*., 1999), milkfish *Chanos chano*s (Gapasin *et al*., 1998) and Asian sea bass *Lates calcarifer* (Dhert *et al*., 1990); and low salinity, dissolved oxygen and raising water temperature for red sea bream (Kanazawa, 1997) and Japanese flounder (Tago *et al*., 1999). To determine the optimal EFA effects, fish species, developmental stage, measured parameters and test conditions should be clarified (Dhert *et al*., 1992; Kitajima, 1993; Takeuchi, 1997).

1.2.2 EFA requirements

Dietary requirements (based on dry units) of n-3 HUFAs during the rotifer feeding stage are reported to be 3.5% for red sea bream (Izquierd *et al*., 1989), 3.0% for Japanese parrot fish, 1.2 to 3.2% for European turbot *Scophthalmus maximus* (Le-Milinaire *et al*., 1983), and 0.9 to 1.7% for blue crab *Portunus trituberculatus* (Takeuchi *et al*., 1999). During the *Artemia* feeding stage, n-3 HUFA requirements have been reported as 1.0 to 1.6% for red sea bream and Japanese flounder (Furuita *et al*., 1996a, 1999); and 1.4 to 2.6% for yellowtail (Furuita *et al*. 1996b), striped jack *Pseudocaranx dentex* (Takeuchi *et al*., 1996) and Pacific cod (Zheng *et al*., 1996). In most cases, the effectiveness is greater for DHA than EPA. The typical deficiency signs for n-3 HUFAs are, for example, lower survival rates in all species, deformity of

vertebrae in red sea bream, abnormal pigmentation in Japanese flounder, and gill deformity in milkfish *C. chanos* (Gapasin and Duray, 2001). More details on fatty acid requirements have been reviewed in the studies of Watanabe and Kiron (1994) and Takeuchi (1997).

1.2.3 Effectiveness of arachidonic acid as an EFA

It has been reported that arachidonic acid (AA) is deeply involved in the formation of eicosanoids and immunity of mammals. Marine fish cannot synthesize AA as they lack the enzymes needed to elongate and saturate EFAs. As a result, several studies (Zheng *et al*., 1996; Furuita *et al*., 1998; Ishizaki *et al*., 1998) on the effects of AA were conducted with Japanese flounder, Pacific cod and yellowtail. These studies demonstrated that effectiveness of AA is not as great as other EFAs such as EPA and DHA. Since it was reported in the study of turbot (Sargent *et al*., 1989) that AA was specifically incorporated in phosphotidylinositol, some studies have been carried out using marine species in Europe (Castell *et al*., 1994; Bell and Sargent, 2003). When juvenile turbot were fed test diets containing coconut oil supplemented with DHA and AA, fish fed a diet containing 1% AA grew and survived similarly to those fed a fish oil-based diet. Furthermore, growth and survival were improved in the study of gilthead sea bream, when fed test diets containing 1% AA and 1.8% AA, respectively (Bessonart *et al*., 1999). Koven *et al*. (2001) also demonstrated that tolerance to handling and salinity fluctuation in gilthead sea bream was improved when AA was supplemented in diets.

In many cases the effectiveness of AA seems to be influenced by the ratio of DHA and EPA in a diet or live feed. Therefore, when studies of fish oil replacement are conducted it is important to take into account the ratios of DHA/EPA or AA in diets.

2 Physiological Effect and Accumulation in Tissues of HUFAs

2.1 Physiological effect of HUFAs

Tocher and Mackinlay (1990) examined uptake of DHA by the body tissues and organs of turbot using radio-labelled DHA and demonstrated that orally administered DHA was incorporated into the polar lipid fraction of the brain. In addition, Masuda *et al*. (1999) confirmed that DHA was mainly incorporated into the brain and eyes in yellowtail fed *Artemia* enriched with ^{14}C labelled DHA. In red sea bream, orally administered DHA was also accumulated in the polar lipid fraction of the eyeball and brain (Tago and Teshima, 2002b). Ishizaki *et al*. (2000, 2001) demonstrated that yellowtail larva fed oleic acid did not show normal schooling behaviour, and the cerebellum, which controls equilibrium, was reduced in volume compared to those of larva which were fed *Artemia* enriched with EPA or DHA. The capacity of the cerebellum of larval flatfish fed EPA or DHA-enriched

Artemia was bigger than that of larva fed non-enriched *Artemia*, suggesting that EFAs play an important role for the development of a brain. Administered DHA was incorporated into the polar lipids of cell membranes and improved the tolerance of environmental change in the cell membrane and nerve centre, and it is supposed to reduce the influence of environment change to the nervous system.

HUFAs also have been shown to affect non-specific immune responses like phagocytosis or non-specific cytotoxic cell activity in rainbow trout (Kiron *et al*., 1993), and these activities were influenced by lipid sources and amounts of HUFAs (Puangkaew *et al*., 2004).

2.2 Potential modification of DHA and EPA contents in fish as accessed by pharmacokinetics

Pharmacokinetics (PK) is the method used to determine the time course movement of a drug or substance in the body from the time it is administered, orally or by intravenous injection, until it is excreted. In this method, variations in the concentration of the substance in the blood or serum are measured over the time course, and blood concentration–time curves are converted to mathematical models to calculate parameters like area under the curve (AUC) of blood concentration–time, mean retention time (MRT) of substance, and maximum blood concentration (C_{max}), to evaluate the utilization of the substance (Uno *et al*., 1997; Uno, 2000). Tago and Teshima (2002a) compared the biochemical availability (BA) of the three forms of EPA, free fatty acid (FFA) type, ethyl ester (EE) type and phosphatidylcholine (PC) type, in the flatfish using ^{13}C labelled EPA which is one of the stable isotopes of carbon. They reported that BA of EPA differed according to the form of ^{13}C-EPA, and availability of EE type was lower than availability of FFA type and PC type. These results also supported the report of Geurden *et al.* (1997) on sea bass and turbot which showed high absorption of n-3 HUFAs in phospholipids in comparison with the EE type. In addition, ^{13}C-EPA concentration in the plasma was increased by increasing the dose of EPA, suggesting that that the EPA concentration in the plasma could be controlled by regulating the dose of EPA in diets. Similar results were obtained using ^{13}C labelled fatty acid (DHA composition about 35.8% of total fatty acids) in red sea bream juveniles, and the DHA concentration in the plasma could be estimated from the dose of DHA (Tago and Teshima, 2002b). When the nutrient concentration in plasma changes depending on the dose in the diet, the dosage schedule could be adjusted based on a PK parameter thus regulating the nutrient concentration in the plasma.

3 Utilization of Lipids

3.1 Phospholipids

Phospholipids (PLs) are indispensable for the normal growth and survival of larval fish, and have been shown to improve growth and prevent deformity in flatfish (Kanazawa, 1993), ayu *Plecoglossus altivelis* (Kanazawa *et al*., 1981), Japanese striped knifejaw *Oplegnathus fasciatus* (Kanazawa, 1993) and prawn *Marsupenaeus japonicus* (formerly *Penaeus japonicus*; Kanazawa *et al*., 1985). The absorption of free fatty acids was improved by supplementation of PLs, and it has been suggested that PLs must play an important role in lipid metabolism of the larval stage (Coutteau *et al*., 1997; Geurden *et al*., 1998a, b; Hadas *et al*., 2003). However, there is still very little information about the mechanism of PL metabolism in aquatic animals available. With PLs such as a soybean lecithin (SBL) and chicken egg lecithin, Kanazawa (1993) examined the effect of growth and survival in flatfish, and demonstrated that PLs improved growth and survival. SBL was superior to other PL sources. Furthermore, the phosphatidyl choline (PC) fraction from SBL provided the highest growth in flatfish among PC, phosphatidyl inositol (PI) and phosphatidyl ethanolamine (PE).

Geurden *et al*. (1998a) reported on the effect of soybean PLs on larval carp *C. carpio* fed experimental diets containing peanut oil, and the PC fraction from the PL improved growth and PI improved survival rate. PI was also effective for the prevention of malformation in larval carp, which is the same as in ayu larvae.

From these studies, PC or PI containing a HUFA moiety in the sn-2 position showed a high efficacy as the PL sources in fish, but the mechanism of use is still not clear. Recently, to clarify the mechanism of PC in lipid metabolism, several research studies were conducted using chemically synthesized PC with different fatty acids. Tago *et al*. (1999) synthesized the PC with a DHA or EPA moiety (DHA-PC, EPA-PC) and determined the growth and tolerance to environment change stress in flatfish. They found that DHA-PC was more effective than EPA-PC and triglyceride containing DHA for increasing tolerance to low salinity, low dissolved oxygen levels and raised water temperature.

Samples *et al*. (1999) examined the influence of adding fatty acids to a medium on the expression of heat shock protein 70 (HSP70) mRNA in white blood cells of rainbow trout. They demonstrated that expression of mRNA was increased with addition of fatty acids after stress brought about by raised temperatures. The expression of mRNA with HUFA supplementation such as DHA or AA was higher than oleic acid, and addition of 1,25-dihydroxy vitamin D_3, an inhibitor of phospholipase A_2, repressed the expression of mRNA. During temperature stress the cell membrane protein was denatured, and phospholipase A_2 influenced PL in the cell membrane and released DHA from the PL. It is supposed that tolerance to stress improved and the released DHA performed a signal transmission of HSP70 and the HSP70 was used for the restoration of the membrane protein.

3.2 Triglycerides

Triglycerides and PLs that have a polyunsaturated fatty acid such as EPA or DHA moiety can be made available by the development of lipid synthesis technology such as enzymatic synthesis (Hosokawa *et al*., 1993, 2000), chemical synthesis (Tago *et al*., 1999) and biosynthesis using alga or microorganisms (Hayashi *et al*., 2001, 2002). Hayashi *et al*. (2001) succeeded in the synthesis of triglycerides containing the fatty acids in a *Chlorella* cell by using controlled culture conditions. Such synthesized lipids could be used for the enrichment of live feed. In addition, it is an effective lipid source for the elucidation of lipid metabolism in aquatic animals, because of the highly purified lipids available.

3.3 Medium chain fatty acids and medium chain triglycerides

Hirazawa *et al*. (2000) examining the prevention of *Heterobothrium okamotoi*, a parasite on tiger puffer *Takifugu rubripes*, used short chain fatty acids (two to four carbons) and medium chain fatty acids (six to ten carbons). Medium chain fatty acids prevented attack by *Heterobothrium*, but octanoic acid (OTA, capric acid) with eight carbons was most effective among the medium chain fatty acids. However, Hirazawa *et al*. (2001) also reported that the prevention of the parasite deteriorates at high water temperature. The problem of parasites in tiger puffer culture is serious, therefore safe and eco-friendly prevention treatments are expected. The mechanism of action of OTA in fish tissues still needs to be understood and effective dosage levels need to be established.

Triglycerides containing medium chain fatty acids are absorbed by a different pathway from those containing long chain fatty acids and are used for improvement of pancreatitis or biliary atresia in mammals (Harrison *et al*., 1973). In aquatic animals, the effects of medium chain triglycerides (MCT) on growth and lipid accumulation were determined in ayu (Nematipour *et al*., 1989; Mustafa *et al*., 1991; Nakagawa and Kimura, 1993) and tilapia *Oreochromis niloticus* (Nakagawa and Kusunoki, 1990). MCT are utilized as energy sources in fish and depressed lipid accumulation in muscle and organs. These results suggest that supplementation of the optimum ratio of MCT and fish oil improves fish quality.

3.4 Conjugated fatty acids

Conjugated fatty acids in mammals are reported to have the following functions: improvement in lipid metabolism, anti-tumour activity, anti-arteriosclerosis, anti-diabetes and physiological functions such as immunity reinforcement. However, there are few studies into the effects of conjugated fatty acids in aquatic animals. Yasmin and Takeuchi (2002) determined the effect of linoleic acid (LA) and conjugated linoleic acid (CLA) on growth

and body lipid composition of tilapia juveniles (average weight 3.8 g) using four kinds of diets (LA/CLA: 8/0, 6/2, 4/6, 0/8). Replacement of the part of LA with CLA decreased the lipid content of muscle and liver, especially the triglyceride content, and growth of the fish also decreased.

4 Concluding Remarks

Dietary lipid has been shown to have various effects on fish including influences on growth, fatty acid composition and immune responses. Further developments in this area are needed, in particular clarification of physiological function and metabolism of lipids in aquatic animals. Regarding the quality of fish, several studies have reported the effects of dietary lipid sources on the odour, taste and storage stability of fresh fish. However, limited information about the relationship between lipid sources and flavour of fish muscle is available. Further studies on the effects of the dietary lipids on flavour and volatile aroma in fish flesh are expected.

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6 Minerals

SHUICHI SATOH

Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan

1 Introduction

Studies on the nutritional requirements of fish, feed technology and production of aqua feeds are proceeding very rapidly as a result of the appearance of new technology for aqua feed manufacture and increased global production of aquaculture products. Aqua feeds have improved tremendously by using very sophisticated manufacturing systems, such as single- or twin-screw extruders. Aqua feeds produced by these systems are of high quality and are readily available to fish, however, the precise nutrient requirements of aquatic organisms are not supposed to change even though feed manufacturing systems have improved. Nevertheless, these techniques might change the character of certain feed constituents such as minerals. Thus the optimum supplemental levels of these should be re-discussed according to their stability and availability.

Compared to the studies on macronutrients, such as proteins, lipids and carbohydrates, studies on micronutrients, such as minerals, are less advanced. However, there are a number of recent investigations on minerals that are remarkable and general information on mineral nutrition, such as requirements or deficiency signs, are reviewed in several books and papers (NRC, 1983, 1993; Watanabe *et al*., 1988, 1997; Hilton, 1989; Lall, 1989; Davis and Gatlin, 1991).

Fishmeal is still a main ingredient in many aqua feeds and is a rich source of minerals, however, landing of fish for fishmeal cannot satisfy the increased demand resulting from recent increases in aquaculture production. Thus, many aqua feeds contain plant feedstuffs or by-products. Mineral bioavailability in the feed ingredients varies and inhibitors of mineral absorption, such as tricalcium phosphate and phytate, may exist as well. It is important to increase the availability of minerals in aqua feeds and decrease the influence of inhibitors. The immune responses of

 Dietary Supplements for the Health and Quality of Cultured Fish (eds H. Nakagawa, M. Sato and D.M. Gatlin III)

terrestrial animals are known to be influenced by dietary minerals, especially trace elements, and some responses have been observed in fish as well. In this chapter we will review recent advances in micronutrients, namely recent information on availability, interactions and new sources of minerals.

2 Mineral Requirements of Fish

Minerals are required for normal life processes, and all animals, including fish, need these inorganic elements. Fish may derive these minerals from the diet and also from ambient water. The mineral nutrition of fish has been fairly well investigated and some of the findings have been highlighted in reviews by Watanabe *et al*. (1988, 1997), Hilton (1989), Lall (1989) and Steffens (1989). Although minerals are required by animals in small amounts (Table 6.1), they are absolutely necessary for normal growth. If excess amounts of the elements are ingested and assimilated, toxicity may develop. Therefore, the animal maintains a delicate balance of the various minerals in the body by coordinating their uptake, storage and excretion.

The dietary mineral requirements of the main cultured fish species are shown in Table 6.1. The amounts of minerals required vary depending on the kind of mineral, however, generally there is little variation in the amount required of a particular mineral among different species. The differences in requirements are much smaller than those of vitamins. Also the differences between freshwater fish and marine fish are very small. However, optimum minimum supplemental levels of minerals do vary due to many factors, such as digestive systems and the form the minerals are in and whether they are available to fish in this form.

The biological availability of a mineral from the diet is marked by the efficiency with which the body utilizes it. It varies depending on the feedstuffs and the composition of a diet. Several factors influence bioavailability and these include the level and form of minerals, interacting compounds, the physiological and pathological condition of the fish, waterborne mineral concentrations and the species under consideration. Among these factors, those related to the chemical state of the mineral are important because the element may assume different molecular forms, valence states and ligands when ingested from different dietary constituents.

3 Inhibitors of Mineral Availability

Calcium (Ca), phosphorus (P), tricalcium phosphate (a complex of Ca and P that exists in hard tissues of fishmeal) and phytic acid (exists in plant protein sources) are well known as inhibitors of zinc availability to animals.

Table 6.1. Mineral requirements of the main cultured fish species as determined in a semi-purified diet.

Mineral (weight of mineral/weight of diet)	Yellowtail	Red sea bream	Atlantic salmon	Rainbow trout	Japanese eel	Common carp	Channel catfish	Tilapia
Macro minerals								
Ca (mg/g)	R[a]	N[b]	0.3	1	2.7	N	4.5	R
P (mg/g)	6.7	6.8	6	7–8	5.8	6–7	4.2–4.5	4.5–6
Mg (mg/g)	R	R	0.1–0.5	0.5–0.7	0.4–0.7	0.4–0.5	0.4–0.7	0.4–0.7
K (mg/g)	NT[c]	R		0.6	NT	NT	R	NT
Micro minerals								
Fe (mg/kg)	60~160	150	60	60	170	150	30	NT
Zn (mg/kg)	20	R	20	15–30	(80–100)[d]	15–30	20	20
Mn (mg/kg)	R	R	7.5–10	12–13	R	12–13	2–3	12
Cu (mg/kg)	NT	N	R	3	(5)	3	5	3~4
I (mg/kg)	NT	N	R	0.6–0.8	(5~50)	NT	NT	NT
Se (mg/kg)	NT	NT	R	0.2–0.4	0.3–0.5	R	0.25	NT

[a] R, required; [b] N, not required; [c] NT, not tested; [d] values in parentheses are determined with fishmeal-based diets.

Hardy and Shearer (1985) and Satoh *et al*. (1987) investigated the zinc (Zn) antagonistic effect of tricalcium phosphate, by employing semi-purified diets containing different levels of tricalcium phosphate. High absorption of Zn and manganese (Mn) noted in the tricalcium phosphate-free diets decreased markedly as levels of tricalcium phosphate increased in the diet. In a related study with common carp, it was also found that the availability of Zn was affected by dietary tricalcium phosphate, but the ill-effects on mineral utilization were less pronounced than in rainbow trout. This was because the tricalcium phosphate was not dissolved in the intestine of carp as it has less gastric juice compared to rainbow trout (Satoh *et al*., 1992a).

Further experiments were performed on rainbow trout to clarify which portion of tricalcium phosphate, Ca or P, inhibits Zn availability (Porn-Ngam *et al*., 1993; Satoh *et al*., 1993, 1997a). Mono-, di- and tri-basic phosphates of Ca, sodium (Na) and potassium (K) incorporated in casein based diets were tested. The di-basic calcium phosphate-containing diet performed better than the others because it had an almost equal proportion of Ca and P. Excess dietary P unbalanced the Ca/P ratio, not only depressing growth, but also affecting the availability of Zn and Mn. There was a drop in the content of these minerals in the body and vertebrae. Satoh *et al.* (1997a) conducted a similar feeding experiment with rainbow trout fed fishmeal based diets. Fishmeal was deboned and then restored with the removed portion of Ca or P. Fish fed the diet restored in the P portion showed a similar performance to those on the original fishmeal based diet. However, fish fed the diet restored with the Ca portion showed much better growth and mineral status. Thus they suggested that P in tricalcium phosphate from fishmeal might primarily inhibit Zn availability, and that the existence of Ca in an appropriate proportion to P in the diet might improve the absorption and retention of Zn. Porn-Ngam *et al*. (1993) showed that a 1:1 ratio of Ca to P in a high-level phosphorus diet was optimum. Satoh *et al.* (1996) concluded that the available P content should be kept at less than 1.5% in a rainbow trout diet to avoid reduction of Zn bioavailability. Also the formulation should maintain a Ca:P ratio close to unity when 1.8–2.4% of P is derived from dietary ingredients such as fishmeal and meat bonemeal.

Phytic acid present in plant protein sources like soybean meal, cotton seed meal or rape seed meal strongly chelates divalent minerals, such as Zn and Mn, to form insoluble phytates in the intestinal lumen, thereby lowering Zn and Mn availability. Spinelli *et al*. (1983) reported the presence of phytic acid in the feed has been linked to reduced growth of rainbow trout. Therefore, commercial diets containing elevated amounts of fishmeal and phytic acid require higher Zn contents. Richardson *et al*. (1985) fed semi-purified diets containing various levels of calcium phosphate, Zn and sodium phytate to chinook salmon and noted that a high dietary phytic acid content (25.8 g/kg) depressed fish growth and feed performance. Satoh *et al.* (1989) observed that the elevation of phytic acid level from 1.1 to 2.2% in channel catfish *Ictalurus punctatus* diets containing 50 mg Zn/kg lowered growth performance, apart from decreasing the Zn content in the vertebrae.

Gatlin and Phillips (1989) also reported the negative influence of phytate (1.5%) on Zn bioavailability to channel catfish in a purified diet.

Spinelli *et al*. (1979) using soybean meal based diets found reduced growth and high mortality in rainbow trout which were attributed to the presence of phytate and/or other compounds in soybean meal affecting the bioavailability of certain minerals including Zn. With increases in dietary soybean meal, the gain in fish body weight decreased. When the diet contained 30% soybean meal, body weight was comparatively low, even when Zn was supplemented at 40 mg/kg (Satoh *et al*., 1997b). The effect of dietary Zn deprivation was more pronounced at the higher dietary inclusion of soybean meal (levels included 0, 15, 30%) as indicated by the comparatively low content of Zn and Mn in the whole body. An interaction between Zn and phytic acid in soybean meal may be the reason for the low bioavailability of Zn. Later it was found that heat treatment of soybean meal by extrusion at 150°C improved growth performance of the diet. The original content of 1.3% phytic acid in the soybean meal was reduced to less than 1%, thereby enhancing Zn availability.

4 Availability of Minerals in Fishmeal to Marine Fish

It was reported that young red sea bream *Pagrus major* (50–150 g), a major aquaculture species in Japan, require P and iron (Fe) at levels of 6.8 mg/g and 150 mg/kg, respectively (Sakamoto and Yone, 1978a, b) even in a semi-purified diet, and that trace element supplementation of casein based diets is not essential when aluminium (Al), Zn, Mn, copper (Cu), cobalt (Co) and iodine (I) exist at levels exceeding 2 mg/kg, 24 mg/kg, 18 mg/kg, 5 mg/kg, 4 mg/kg and 0.1 mg/kg, respectively (Sakamoto and Yone, 1978c). On the other hand, yellowtail *Seliola quinqueradiata*, another major aquaculture species in Japan, require P, Fe and Zn at levels of 6.7 mg/g, 60–160 mg/kg and 20 mg/kg, respectively (Shimeno, 1991). Recently, Minoso *et al*. (1999) reported that milkfish *Chanos chanos* also require P, Fe and Zn when they were fed casein based diets. However, when experiments were conducted to investigate the influence of un-supplementation with some minerals to a fishmeal based diet for middle-sized red sea bream and yellowtail no effect was observed (Furuichi *et al*., 1992). And the influence of un-supplementation of minerals was observed only in puffer fish *Takifugu rubripes* which does not have a stomach.

Satoh *et al*. (1998, 2001) reported that very small juvenile red sea bream, yellowtail and Japanese flounder *Paralichthys olivaceus* showed poorer performance when they were fed fishmeal based diets without supplemental minerals. Namely, juvenile red sea bream and yellowtail fed the fishmeal based diet without supplemental Zn, magnesium (Mg) or P showed poorer growth and lower mineral contents than those on the diet with supplemental minerals.

5 Absorption of Minerals from Feed Ingredients

The difficulties in determining absorption or availability of minerals from feed ingredients and diets per se are discussed in a review by Lall (1989). Riche and Brown (1996) reported that there was true absorption of P from 14 kinds of feedstuffs fed to juvenile rainbow trout. Apparent P availability values ranged from 19.5 to 50.5% for fishmeals and were as high as 30.7% for the plant protein sources. True P absorption values were increased, and ranged from 21.5 to 55.4% for fishmeals and 9.7 to 48.4% for the plant feed ingredients.

Supplementation of plant protein sources with phytase significantly increased apparent P absorption values from 46.2 to 75.6%. Yamamoto *et al.* (1997) also reported apparent absorption of six kinds of minerals from several protein sources for fingerling rainbow trout (Table 6.2). They noted that absorption of P in fishmeal, soybean meal, corn gluten meal and malt protein flour were 37, 14, 1.6 and 36%, respectively, and that those of Mg were also 55, 24, 0 and 37%, respectively. As for trace elements, it is very interesting that absorption of Zn was relatively high from all feed ingredients, especially from soybean meal where apparent absorption was above 70%. Also absorption of copper (Cu) was quite high from plant proteins, from approximately 40 to 80%. However, absorption of Mn was much lower than that of Zn from any of the feed ingredients. Apparent P absorption values from soybean meal and corn gluten meal were quite different from those of Riche and Brown (1996). These differences show the difficulty of this kind of study.

Riche and Brown (1996) also reported a very interesting phenomenon, namely that apparent P absorption was at its maximum when experimental diets contained levels of P at or near the most recently published requirement for trout (Ketola and Richmond, 1994) and was lower when dietary content of P was higher or lower than the requirement. Satoh *et al.* (2002) also determined P absorption from eight kinds of feed ingredients by rainbow trout during the growing stages. They found that P absorption from fishmeals by rainbow trout showed a slight decrease as body weight increased. On the other hand, an increase in the absorption from plant protein sources, such as soybean meal and corn gluten meal, was observed

Table 6.2. Apparent absorption of minerals (%) from fishmeal, soybean meal, corn gluten meal and malt protein flour by rainbow trout (Source: Yamamoto *et al.*, 1997).

Mineral	Fishmeal	Soybean meal	Corn gluten meal	Malt protein flour
P	37.0	14.1	1.6	35.6
Mg	54.9	24.2	0	37.2
Fe	0	19.3	26.0	7.9
Zn	30.5	74.1	47.7	38.1
Mn	0	14.6	10.0	11.4
Cu	4.6	77.7	63.6	39.0

during the growing stages. Moreover, the absorption from soybean meal was quite improved by extrusion cooking.

6 Effect of Organic Trace Elements

Recently, many organic trace elements were applied to terrestrial animal feed. Trace elements chelated with a protein or an amino acid have been reported to improve mineral bioavailability in poultry and lambs (Aoyagi and Baker, 1992; Herry *et al*., 1992a, b; Wedekind *et al*., 1992). Hardy *et al*. (1987) reported that rainbow trout fed a Zn-amino acid-chelate had a higher level of body Zn than fish fed zinc sulphate or zinc sulphate EDTA in low Ca/P diets, but not in high Ca/P diets. Gomes and Kaushik (1993) reported no differences in Zn concentration in plasma, whole body or viscera of rainbow trout fed zinc sulphate or zinc methionine. Apines *et al*. (2001) also reported that no differences were observed in growth, whole body and vertebrae Zn content of rainbow trout fed diets supplemented with zinc sulphate, zinc sulphate methionine or amino acid-chelated Zn. On the other hand, the fish receiving organic Zn showed higher activity of alkaline phosphatase in their blood than those fed the diet supplemented with zinc sulphate. Maage and Berge (1997) compared the effect of zinc gluconate with zinc sulphate as a Zn source in Atlantic salmon *Salmo salar*. They reported no differences in growth, mortality, feed conversion and whole body Zn content between the different Zn forms. No researchers have observed any effect of organic Zn compounds on growth in salmonids. However, in rainbow trout supplementation with organic Zn compounds is expected to increase disease resistance, but further research is necessary to establish the effect of organic Zn compounds on rainbow trout.

Research in channel catfish showed that zinc methionine had approximately 300 to 600% relative bioavailability compared to zinc sulphate for channel catfish fed either a purified or an all-plant practical diet (Paripatananout and Lovell, 1995). However, Li and Robinson (1996) reported that the bioavailability of zinc sulphate and zinc methionine to channel catfish was equivalent and that zinc protenate is not as highly available to the fish.

Other organic elements were also studied with channel catfish and salmonids. Lorentzen *et al*. (1994) compared the effects of supplementing a fishmeal based diet with sodium selenite or selenomethionine in Atlantic salmon, however, there were no significant differences in activity of the selenium (Se)-containing enzyme, glutathione peroxidase between Se forms. On the other hand, Wang and Lovell (1997) compared the bioavailability of Se from sodium selenite, selenomethionine and selenoyeast in channel catfish, and reported that the relative bioavailability values of selenomethionine and selenoyeast compared to sodium selenite were 336% and 269% for growth, and 147 and 149% for glutathione peroxidase activity. Lim *et al*. (1996) also compared the bioavailability of Fe from iron methionine and iron sulphate in channel catfish, and reported

that iron methionine and iron sulphate were equally effective in preventing anaemia.

7 Dietary Dependent Immunosurveillance

Elements of natural immunity serve as the first line of an organism's defence against pathogens. It includes the internal defensive cells, which are comprised of phagocytes and natural-killer cells (NKs). Kiron *et al*. (1993) reported the role of Zn in rainbow trout reared for varying periods in different experiments. The immune response was recorded by measuring the release of ^{51}Cr from labelled P815 mouse mastocytoma target cells after 8 hours' incubation. Leucocytes isolated from the head kidney of experimental fish were employed as effector cells. The activity was low when the diets were deficient in Zn. Inoue *et al*. (1998) also reported recovery from derangement of natural killer-like activity of leucocytes due to Zn and Mn deficiency in rainbow trout by the oral administration of these elements. When the fish were fed the diets for 1 year, the NK activities in trace element-deficient fish were lower than those recorded in control fish. Leucocytes of fish that had been transferred to a diet with sufficient trace elements after a year continued to exhibit low cytotoxicity for about 8 weeks, but by 16 weeks they had returned to normal levels. Fish that continued to be fed the trace element-deficient diets until the end of this experiment showed the same lower level of cytotoxicity as noted at the end of the first year feeding term. Zn and Mn evidently have a significant role in NK activity of fish.

8 Amino Acid-chelated Trace Elements and the Immune Response

As mentioned above, dietary supplementation of trace elements has been achieved through the use of inorganic salts. However, because of low bioavailability of elements from this source, continuous efforts have been made to improve their utilization. Also, the mineral status of an animal is significant to its health as it can influence its susceptibility to infectious diseases. For these reasons, organic sources of trace elements, such as amino acid-chelated trace elements, are widely used in animal nutrition. Recent studies reported that amino acid-chelates are a promising source of trace elements with better bioavailability to improve growth and tissue mineral deposition.

Apines-Amar *et al*. (2004a) reported the effect of amino acid-chelated trace elements on enzyme activities related to immune responses in rainbow trout. A feeding experiment was conducted to elucidate the effect of the chelate in practical diets. Three diets were formulated: Diets 1 and 2 contained the same concentration of trace elements either from the sulphate or amino acid-chelate, while Diet 3 contained trace elements from the

amino acid-chelate at half the concentration provided in the other two diets. Each diet was fed to rainbow trout juveniles for 15 weeks. Growth of fish in the full concentration of amino acid-chelate group was higher though not significantly different from the rest. Similar results were also observed for the haematocrit level and alkaline phosphate activity. Further, DNA polymerase, CuZnSOD and cytochrome C oxidase expression in amino acid-chelate groups were higher compared to the sulphate group.

In another study of Apines-Amar *et al*. (2004b), an infection trial with bacterial pathogens was carried out to determine the effect of amino acid-chelated trace elements on disease resistance. Two trials with rainbow trout were conducted using *Streptococcus* sp. The fish were fed a diet supplemented with trace elements either from the sulphate or amino acid-chelate and were injected with *Streptococcus* sp. The cumulative mortality was lower in the amino acid-chelate group compared to the sulphate group. Serum lysozyme and complement activity as well as plasma total immunoglobulin levels tended to be higher in the amino acid-chelate group. Serum haemagglutinating antibody titres were also significantly higher in the amino acid-chelate group.

9 Recommended Levels of Minerals in Fish Diets

As described above, availability of minerals in fish feed is influenced by many factors, namely forms of supplemental sources, existence of inhibitors or feed ingredients that influence bioavailability. Among the minerals, P is the most important mineral to fishes. Excess amounts of P per se inhibit bioavailability of other minerals, especially Zn. Therefore dietary P content, strictly speaking the available P content, in feed should be regulated at the level of P requirement, not above 1.5%. Moreover, P supplementation should be achieved by using calcium phosphate or potassium phosphate, since it was reported that an overdose of sodium phosphate induced high mortality in rainbow trout (Satoh *et al*., 1993). If available P content is regulated, the minimum levels of recommended available minerals for most fish species are as shown in Table 6.3.

Table 6.3. The minimum levels of recommended available mineral supplements.

Mineral	Available amount (mg/kg)
Mg	7500
Fe	150
Zn	40
Mn	20
Cu	3
Se	0.3
I	0.1

It is not easy to determine the available mineral content in a fish diet. Only the fractionation method for determining available phosphorus content in diets for carp and rainbow trout has been reported by Satoh *et al.* (1992b, 1997c). As for other minerals, simple methods for determining available amounts have not reported.

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7 Microorganisms

TOSHIKI NAKANO

Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

1 Introduction

It is well known that aquaculture is very important to world food production. In fact, approximately 30% of world fishery production was based on aquaculture in 2001 (FAO, 2003), when aquaculture production, excluding aquatic plants, reached around 38 million tonnes by weight (US$55.7 billion by value). More than 80% of the total world aquaculture production is performed in Asia in countries such as China (26.1 million t), India (2.2 million t), Indonesia (0.9 million t), Japan (0.8 million t) and Thailand (0.7 million t). World market demand for high quality products has stimulated much of the growth in aquaculture, especially for salmonid, marine shrimp and shellfish species.

In aquaculture facilities, cultured animals are exposed to many kinds of stress and the chances of catching infectious diseases are high. Hence, it is thought that supplying feed ingredients or effective supplements (e.g. micronutrients), probiotics, vaccines, as well as proper diagnostics and suitable treatments such as antibiotics is required for successful aquaculture (Meyers, 1994; Fletcher, 1997; Morgan and Iwama, 1997; Nakano and Takeuchi, 1997c; Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Sakai, 1999; Verschuere *et al*., 2000a; Olafsen, 2001; Irianto and Austin, 2002; Nakano, 2003, 2006; Nakano *et al*., 2003; Burr and Gatlin, 2005; Vine *et al*., 2006).

Recently, it has been recognized that prevention of disease is more important than medical treatment. In aquaculture, antibiotics have been used to prevent disease and to improve feed efficiency for a long time. However, there are many problems associated with the use of antibiotics in aquaculture, such as the residue of the antibiotics left in the tissues of cultured animals following treatment, the generation of antibiotic-resistant bacteria and an imbalance in the normal beneficial intestinal microflora. Accordingly, part of disease prevention must come from a proper diet

 Dietary Supplements for the Health and Quality of Cultured Fish (eds H. Nakagawa, M. Sato and D.M. Gatlin III)

including functional constituents, other than essential nutrients. The Food and Agriculture Organization (FAO) recognizes that research on development and application of immunostimulants, non-specific immune enhancers and probiotics are required to control aquacultural disease (Subasinghe, 1997). Unfortunately, the study of probiotics use in aquaculture is limited to date (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000a; Irianto and Austin, 2002; Nakano, 2003, 2006; Burr and Gatlin, 2005; Vine *et al*., 2006). Recently, consumers have been demanding safe food with a reduced risk of chemical residues (Sakamoto, 2003). Functional feed ingredients have been screened from natural products and might be safe for cultured animals and humans who consume them.

This chapter begins with a review of the possible biological and nutritional actions on rainbow trout (*Oncorhynchus mykiss*) of red yeast *Phaffia rhodozyma* containing astaxanthin (ASX) as its predominant carotenoid. Carotenoids are unique fat-soluble natural pigments, which have many kinds of biological activities in animals (Bendich and Olson, 1989; Matsuno and Miki, 1990; Sueki, 1991; Ito, 1992; Bendich, 1993; Bertram, 1993; Meyers, 1993, 1994; Miki, 1993, 2003; Olson, 1993a, b; Tomita, 1995; Nishino *et al*., 2002; Nakano, 2003, 2006; Nakano *et al*., 2003, 2005; Matsuno, 2004). ASX, particularly, has attracted considerable interest and is expected to have economic value and to play a major role in the aquaculture of salmonids. The chapter then provides an overview of the studies on probiotics in aquaculture.

2 Effect of Red Yeast as a Functional Feed Supplement on Salmonids

2.1 General properties of carotenoids

Carotenoids, which are usually yellow to red isoprenoid polyene fat-soluble pigments, are widely distributed in nature. Carotenoids that often give a specific colour to organisms are found in all plants (including algae), animals, bacteria, yeast, fungi and plankton. More than 650 kinds of carotenoids have been identified, and about 40 carotenoids are commonly found in daily foods (Matsuno, 1993, 2004; Nishino *et al*., 2002; Miki, 2003; Nakano *et al*., 2003). Carotenoids are classified into five groups: carotenes (e.g. α-carotene, β-carotene, γ-carotene, lycopene and phytoene); xanthophylls (e.g. zeaxanthin, lutein, canthaxanthin, ASX, tunaxanthin, echinenone); homo-carotenoid (e.g. bacterioruberin); apo-carotenoid; and nor-carotenoid (Sueki, 1991). The most common carotenoids contain 11 conjugated double bonds, though the number can vary from three to 13 double bonds (Olson, 1993a).

Figure 7.1 shows typical carotenoids that have been found in aquatic animal tissues. All photosynthetic organisms can produce carotenoids *de novo*. However, most animals, including salmonids are unable to perform *de*

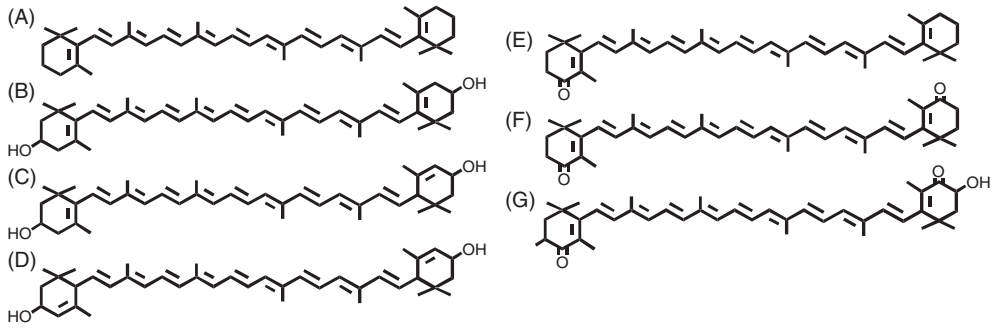

Fig. 7.1. Typical carotenoids in aquatic animal tissues. (A) β-carotene, (B) zeaxanthin, (C) lutein, (D) tunaxanthin, (E) echinenone, (F) canthaxanthin and (G) astaxanthin.

novo synthesis of carotenoids (Liaaen-Jensen, 1978; Matsuno and Hirao, 1989). The pink to red colour (salmon pink) in salmonid muscle is known to be due to carotenoids of dietary origin. This is because salmonids are incapable of biosynthesis of ketocarotenoid ASX (3,3′-dihydroxy-β, β-carotene-4,4′-dione), being unable to oxidize 3,3′-dihydroxy carotenoids (Matsuno, 1993; Meyers, 1994). On the other hand, goldfish and carp can oxidize zeaxanthin and produce ASX (Hata, 1978; Matsuno, 1993). In nature, fish have to obtain carotenoids through carotenoid-containing organisms in the aquatic food chain (Matsuno, 1993; Meyers, 1994). Accordingly, wild salmonids obtain carotenoids by ingesting prey crustaceans such as shrimp and copepods. The predominant carotenoid found in crustaceans is ASX. In crustaceans such as prawns, an oxidative pathway has been suggested for metabolizing dietary carotenoids, including β-carotene and zeaxanthin to ASX. Prawns can also directly deposit ASX in their tissues (Matsuno, 1993; Meyers, 1994). Carotenoids often occur as protein complexes of free, mono- and diesters in the crustacean exoskeleton. These carotenoid-protein complexes are known to be a carotenoprotein and are usually green, blue, blown, purple and red varieties to match their environment (Liaaen-Jensen, 1978; Hata, 1991). It is postulated that oxygenated carotenoids serve as an intercellular oxygen reserve (energy-generating intercellular compound) in crustaceans (Liaaen-Jensen, 1978; Karnaukhov, 1990; Meyers, 1993, 1994). Carotenoids might supply energy in cells under anoxia when the normal function of mitochondria is inhibited. Hence, the crustaceans could adapt and survive under anoxic conditions that might occur in pond cultures. It is also suggested that the tolerance of marine invertebrates to environmental pollution is correlated with level of carotenoids in their tissue (Karnaukhov, 1990). ASX is found in a free unesterified form in salmonid muscle and in an esterified form in the skin and ovaries. In salmonid muscle, it has been suggested that one β-ionone ring of ASX might form two hydrogen bonds, which bind to a hydrophobic binding site on the surface of actomyosin. Accordingly, ASX can combine more strongly to actomyosin than do other

carotenoids (Henmi *et al*., 1989; Storebakken and No, 1992). More recently, one of the components of muscular protein, α-actinin, has been found to be the only myofibrillar protein correlating significantly with ASX binding in Atlantic salmon (Matthews *et al*., 2006).

Attention has been given to the biological activities of ASX, other than its role in muscle pigmentation, and certain other kinds of carotenoids in fish. This is because it has been suggested that some carotenoids show biological activities in mammals (Bendich and Olson, 1989; Matsuno and Miki, 1990; Sueki, 1991; Ito, 1992; Bendich, 1993; Bertram, 1993; Miki, 1993, 2003; Olson, 1993a, b; Tomita, 1995; Nishino *et al*., 2002; Nakano *et al*., 2003; Matsuno, 2004). These activities have been classified into three categories: functions, actions and associations (Bendich and Olson, 1989; Olson, 1993b). The functions of carotenoids are thought to be the essential roles that they play (e.g. protection against light-induced damage, vitamin A formation and coloration related to sexual processes). The actions might be physiological or pharmacological responses to the carotenoids (e.g. reduced free radical formation, reduced mutagenesis and increased immune responsiveness). The associations are mainly of an epidemiological nature and have been considered as correlations between carotenoids and physiological and/or pharmaceutical phenomena, which might display a causal relationship (e.g. reduced lung cancer incidence, possible reduction in cancerous conditions and possible reduction in atherosclerosis). In mammals, carotenoids are thought to affect reproduction, the immune system, the endocrine system and certain diseases such as cardiovascular disease and cancer. Many kinds of natural carotenoids, for example ASX, lutein, zeaxanthin, fucoxanthin, lycopene and phytoene, often show preventive activities against carcinogenesis and mutagenesis (Bendich, 1993; Bertram, 1993; Olson, 1993a, b; Omenn, 1996; Gradelet *et al*., 1998; IARC, 1998; Nishino *et al*., 2002). Accordingly, there is considerable research into the cancer chemopreventive actions of carotenoids.

Many researchers have speculated about the biological functions of carotenoids in salmonids and other cultivated aquatic animals (Liaaen-Jensen, 1978; Karnaukhov, 1990; Matsuno and Miki, 1990; Ito, 1992; Miki, 1993, 2003; Meyers, 1993, 1994; Tsushima *et al*., 1997; Kiron and Maita, 2003; Nakano 2003, 2006; Nakano *et al*., 2003, 2005; Matsuno, 2004). It has been suggested that carotenoids can enhance both specific and non-specific immune responses. Carotenoids and even xanthophylls have also been known to act as a precursor of vitamin A (provitamin A), to stimulate growth, and to improve egg quality and reproduction in fish (Matsuno and Miki, 1990; Katsuyama, 1993; Miki, 1993). When high concentration ASX diets were fed to salmon, both high growth and survival rates were observed (Ito, 1992; Meyers, 1993). The high level of ASX in salmonid eggs would have high potential provitamin A activity during the initial feeding period of growth. It has been suggested that the function of ASX in eggs is that it has a role in protecting the eggs against light. Such a photoprotection role is thought to be one of the most common functions of carotenoids in nature. ASX might protect biological membranes from oxidative injury

caused by UV light and other oxidants. In general, eggs of aquatic organisms are thought to be sensitive to light. ASX in broodstock has also been found to improve spawning and hatching rates (Ito, 1992; Meyers, 1993; Miki, 2003).

2.2 Properties of red yeast *P. rhodozyma* and effect of red yeast on healthy fish

Farmed salmon must obtain carotenoid pigments from their food as mentioned above. Feed is supplemented with ASX and canthaxanthin (β, β-carotene-4,4′-dione; CAX) to impart the desired muscle coloration. This is because one of the most important factors affecting consumer acceptance of salmon is the distinctive red colour (the salmon-pink colour) of their muscle. Accordingly, a variety of sources of dietary carotenoids have been tested for coloration of fish, and a great amount of data is available on this subject (Storebakken and No, 1992; Mori, 1993; Meyers, 1993; Higgs *et al*., 1995; Nakano *et al*., 1995b, 2003; Hertrampf and Piedad-Pascual, 2000a; Baker and Gunther, 2004; Matsuno, 2004). The major sources of carotenoids generally used in aquaculture are as follows: both synthetic ASX (Carophyll pink) and synthetic CAX (Carophyll red) manufactured by Hoffmann La Roche (Basle, Switzerland), red yeast *P. rhodozyma*, green microalga *Haematococcus pluvialis*, krill *Euphausia superba*, alga *Spirulina* spp., crustacean meal and marigold flowers (Meyers, 1993; Mori, 1993; Higgs *et al*., 1995; Hertrampf and Piedad-Pascual, 2000a; Nakano *et al*., 2003; Matsuno, 2004). ASX is deposited in the muscle of salmon more efficiently than CAX due to preferred absorption in the digestive tract as well as muscle deposition (Foss *et al*., 1987; Torrissen, 1989). Trout are observed to utilize dietary free ASX around 1.5 times more efficiently than CAX (Foss *et al*., 1987).

The genera of yeasts that produce carotenoids are *Cryptococcus*, *Rhodotorula* and *Sporidiobolus*. The predominant carotenoids of those yeasts are β-carotene (Simpson *et al*., 1971). The heterobasidiomycetous yeast *P. rhodozyma* was first isolated during the 1970s from deciduous trees in mountainous regions of Japan and Alaska, and was found to be a uniquely coloured yeast. This is because few species of microorganisms produce ASX in nature. This red yeast can produce free ASX, which has been reported to be a 3R, 3R′-isomer and surpasses 80% total carotenoids (Andrewes *et al*., 1976; Johnson and Lewis, 1979). The concentration of ASX in cultured wild red yeast is 30–800 mg/g dry matter of yeast (Hertrampf and Piedad-Pascual, 2000a). Free ASX is deposited in fish muscle more efficiently than esterified ASX (Storebakken and No, 1992; Higgs *et al*., 1995). The fermentation industry has been researching mutant strains of this yeast for maximum production of ASX (Meyers, 1993; Tangeras and Slinde, 1994; Hertrampf and Piedad-Pascual, 2000a; Nakano, 2003). The mutant strain is able to produce 2000–3000 mg ASX/kg yeast in 4–7 days (Hertrampf and Piedad-Pascual, 2000a). Recently, a new commercial preparation of *P. rhodozyma* which can

produce high concentrations of ASX became available from KI Chemical Co. and Kyowa Hakko Kogyo Co. in Japan.

In general, the yeast's cell wall is not easily digested in the gastrointestinal tract of fish (Hertrampf and Piedad-Pascual, 2000b). Nakano *et al.* (1995b) treated the cell walls of red yeast with alkali and/or milling, such that cell walls were almost destroyed by these procedures. The muscle pigmentation of rainbow trout fed with treated red yeast was found to be very desirable. The concentration of ASX in the muscle of fish fed treated red yeast was comparable to that of the fish fed synthetic ASX.

2.3 Effect of red yeast *P. rhodozyma* on oxidative stressed fish and fingerling fish

Many kinds of stress, especially oxidative stress, lead to an increased risk of several diseases, for example mutations and inflammation in both mammals and fish (Inoue, 1992; Ames *et al.*, 1993; Spector, 1995; Nakano and Takeuchi, 1997b; Sawa *et al.*, 2002). Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion radical, hydrogen peroxide and lipid peroxides (LPOs), are thought to be strong oxidants that occur in the body with increases in stress (Inoue, 1992; Ames *et al.*, 1993; Spector, 1995; Nakano and Takeuchi, 1997a, b; Sawa *et al.*, 2002). The resulting ROS attack almost all components of cells and finally cause fatal damage in the tissues (Inoue, 1992; Spector, 1995; Nakano and Takeuchi, 1997b; Sawa *et al.*, 2002). In both mammals and fish, insufficient ingestion of nutritional antioxidants would increase susceptibility to stressors and diseases (Nakano and Takeuchi, 1997b; Nakano *et al.*, 1999a, b, 2004; Nakano, 2003).

It is known that fish oil used in fish culture contains plenty of highly unsaturated fatty acids (HUFAs). The oxidized HUFAs contain LPOs and are thought to lead to oxidative stress in fish (Nakano and Takeuchi, 1997a, b; Nakano *et al.*, 1999a). Cultured fish also have many occasions to be exposed to stressors in nature, such as pollutants, thermal shock, diseases, handling and crowding (Barton and Iwama, 1991; Barton, 1997; Nakano and Takeuchi, 1997b; Iwama *et al.*, 1999; Basu *et al.*, 2001; Nakano *et al.*, 2002). Nutritional muscular dystrophy and haemolysis, which are major farmed fish diseases, might be catalysed by oxidative stress (damage) to tissue (Nakano and Takeuchi, 1997b). Aerobic organisms have both enzymatic and non-enzymatic defensive systems against ROS-induced oxidative stress (Nakano, 1995; Nakano and Takeuchi, 1997b, c; Nakano *et al.*, 2004). In the course of studies on the defences of fish against oxidative stress (Murata and Yamauchi, 1989; Nakano *et al.*, 1992a, b, 1993, 1995a, 2004; Nakano, 1995), it has been accepted that improvement in the defensive potential of farmed fish is important. Hence, several studies have been done to strengthen the fish's abilities to combat stress by the administration of micronutrients (antioxidants), such as α-tocopherol, ascorbic acid and polyphenolic compounds (Takeuchi, 1985; Watanabe, 1990; Fletcher, 1997; Nakano and Takeuchi, 1997c; Koshio, 2003; Sato, 2003;

Nakano *et al.*, 2004). ASX has been reported to have potent antioxidant activity other than bioactivity for animals, and the activity is much stronger than that of α-tocopherol under certain conditions (Matsuno and Miki, 1990; Sueki, 1991; Ito, 1992; Miki, 1993, 2003; Baker and Gunther, 2004). Some carotenoids show dramatic radical trapping activity at low oxygen pressure in most tissues under physiological conditions (Burton and Ingold, 1984). Hence it seems the action of carotenoids, particularly ASX, on oxidative stress in fish is positive.

The effects of both synthetic ASX and ASX-rich red yeast *P. rhodozyma* on oxidative states in rainbow trout have been studied (Nakano *et al.*, 1995b, 1997, 1999a, b, 2003, 2004; Nakano, 2003, 2006). For example, the liver, plasma and red blood cells (RBCs) from fish fed the ASX-supplemented diet were observed to have a significantly higher level of α-tocopherol and carotenoids than those from control fish fed a non-ASX diet. On the other hand, ASX significantly decreased LPO levels in the tissues. Plasma LPO is considered to be a sensitive indicator of tissue damage derived from oxidative stress and a trigger which causes several diseases in the body (Hata, 1986). Dietary red yeast *P. rhodozyma* and ASX were found to decrease the level of LPOs in the serum of healthy trout (Nakano *et al.*, 1995b, 2004). Similar phenomena also were observed in the serum from oxidative-stressed trout (Nakano *et al.*, 1999a). Most LPOs are found in lipoproteins that contain highly susceptible lipids, such as triglycerides, cholesterol and phospholipids (Hata, 1986). Dietary ASX and α-tocopherol are found to exist in the lipoproteins of fish serum (Ando, 1993; Tokuda, 1994) along with many kinds of circulating small molecular antioxidants (Tokuda, 1994; Nakano and Takeuchi, 1997b; Nakano *et al.*, 2004). Accordingly, ASX, α-tocopherol and circulating antioxidants might act synergistically to protect lipoproteins from oxidation. Carotenoids are thought to be located in membranes, which contain a large amount of HUFAs and thus increase the membrane's mechanical strength (Palozza and Krinsky, 1992; Britton, 1995). In phospholipid model membranes, singlet oxygen was observed to be effectively scavenged by α-tocopherol and some carotenoids (Fukuzawa, 1998; Fukuzawa *et al.*, 1998; Fukuzawa and Tokumura, 2001). Consequently, membrane-bound antioxidants such as α-tocopherol and ASX protect the membrane lipids and proteins in the tissues such as liver and RBCs from oxidation. Susceptibility to lipid peroxidation in the liver of trout fingerlings fed ASX was also decreased by red yeast administration (Nakano *et al.*, 1999b). Fingerling trout are unable to accumulate ASX in the muscle (Storebakken and No, 1992). Nakano *et al.* (1999b) could not also detect ASX in muscle of fingerlings. As a result, in fingerling rainbow trout fed red yeast, the effective antioxidative compounds of red yeast are thought to be metabolized and protect the tissue lipids from oxidation. The alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) activities, which are useful indices for diagnosing liver function, were lower in the serum of fish fed ASX than those of control fish (Nakano *et al.*, 1995b, 1999a). In addition, the hepatosomatic index (HSI) of fish fed the diet supplemented with red yeast

was significantly lower than that of control fish (Nakano *et al*., 1995b). The red yeast, therefore, might have HSI-decreasing factors. Such HSI-decreasing factors seem to be specific for red yeast as brewer's yeast *Saccharomyces cerevisiae* administration did not decrease the HSI of trout. The liver is known to be a major metabolic organ for ASX (Storebakken and No, 1992). Dietary ASX shows improvements in the histology of the fish liver structure (Segner *et al*., 1989) and also in the quality of fish eggs (Matsuno and Miki, 1990; Meyers, 1993). Membrane damage and cell death were observed in cultured rainbow trout cells, when cells were given reactive oxygen-induced oxidative stress. On the other hand, ASX could dramatically inhibit membrane damage to the cell, and increase cell viability, when the cell was stressed (Nakano *et al*., 2004, 2005). Hence, red yeast improves liver function, and further, strengthens the protective abilities and maintains membrane dynamics of the tissue against oxidative stress. A similar phenomenon was observed in the RBCs of mice administrated β-carotene (Nakagawa *et al*., 1996).

Yeast is known to contain vitamins, macro and trace minerals, protein and fat, which should give the product an added value as a nutritional source (Sanderson and Jolly, 1994; Tangeras and Slinde, 1994; Hertrampf and Piedad-Pascual, 2000b). The glucans and nucleotides in yeast are expected to stimulate the immune system of fish (Tangeras and Slinde, 1994; Sakai, 1999; Hertrampf and Piedad-Pascual, 2000c; Sakai *et al*., 2001; Takahashi *et al*., 2001; Li and Gatlin, 2003; Wago, 2003). ASX was reported to improve the survival rate and to stimulate the immune response of fish such as rainbow trout, yellowtail and red sea bream (Matsuno and Miki, 1990; Meyers, 1993; Kiron and Maita, 2003; Nakano *et al*., 2003; Matsuno, 2004). In addition, many kinds of natural carotenoids such as ASX, fucoxanthin and phytoene have shown preventive activities against oxidative stress-induced disease such as cancer (IARC, 1998; Nishino *et al*., 2002). The possible biological effects of red yeast on rainbow trout are summarized in Table 7.1. Red yeast should have an effect not only on improvement of pigmentation of muscle but also on fish health. Hence, red

Table 7.1. Possible biological effects of *P. rhodozyma on* rainbow trout.

Improvement of muscle coloration
Modulation of liver function
Decrease of hepatosomatic index
Decreases of both alanine aminotransferase (GPT) and aspartate aminotransferase (GOT)
Improvement of lipid metabolism
Improvement of antioxidative defence system
Decrease of oxidative stress
(Suppression of lipid peroxide generation in tissues)
(Decrease of susceptibility to lipid peroxidation in liver)
Enhancement of immunity
Improvements of both egg quality and embryonic mortality
Provitamin A activity

yeast has many biological and beneficial properties and is required for a source of ASX making it an ideal supplement for aquacultural diets.

3 Probiotics in Aquaculture

3.1 Definition of probiotics

In general, it is not easy to define the term probiotics. This is because the term has been defined in several ways, depending on the effects on health and well-being of mammals (Salminen *et al.*, 1999). Many kinds of dysfunctions in the gut are thought to be caused by imbalances of intestinal microflora. The mechanistic actions of probiotics were investigated for their effect on intestinal microflora (Salminen *et al.* 1999). Accordingly, probiotics were first defined as live microbial feed supplements (viable microbial cultures) that improve the intestinal microbial balance (gut microflora) of the host animal (Fuller, 1989). Probiotics might prevent and correct microbial dysfunction of the host. However, in most cases, it is difficult to specify the improvement of intestinal microbial balance of the host animal. Accordingly, Tannock (1997) proposed that probiotics were living microbial cells administered as dietary supplements for improvement of the host's health.

Recently, probiotics have been defined as components of microbial cells or products from microbes that beneficially affect the health and immune system of the host (Salminen *et al.*, 1998, 1999; Kitazawa, 1999; Mitsuoka, 2002; Benno, 2003; Nakano, 2003, 2006; Saito, 2003). This definition for probiotics implies they do not necessarily need to be viable (Salminen *et al.*, 1998, 1999). Non-viable forms of probiotics may show beneficial effects on the health of the host. Probiotics, therefore, also include fermented substances such as fermented milk 'yogurt'. Other than probiotics, functional foods, which have been investigated recently, include two categories, prebiotics and biogenics (Gibson and Roberfroid, 1995; Kitazawa, 1999; Mitsuoka, 2002; Nakano, 2003, 2006). Prebiotics are recognized as a non-digestible food ingredient that promotes the growth of beneficial intestinal microbes such as bifidobacteria and depress the increase of harmful intestinal microbes. Prebiotics including oligosaccharides (fructo-oligosaccharides, xylo-oligosaccharides, galacto-oligosaccharides and others), dietary fibre and bifidogenic growth stimulator (BGS), may improve the intestinal condition of the host. On the other hand, biogenics are defined as food ingredients that modulate several functions of the body such as immunity, lipid metabolism, blood pressure and ageing. Biogenics, such as bioactive peptides, polyphenolic compounds, vitamins, carotenoids and HUFAs, should not affect the intestinal microflora rather they should affect body functions directly. Figure 7.2 summarizes the relationship between probiotics, prebiotics and biogenics (Isolauri *et al.*, 1998; Salminen *et al.*, 1999; Kitazawa and Saito, 2002; Mitsuoka, 2002; Benno, 2003; Saito, 2003).

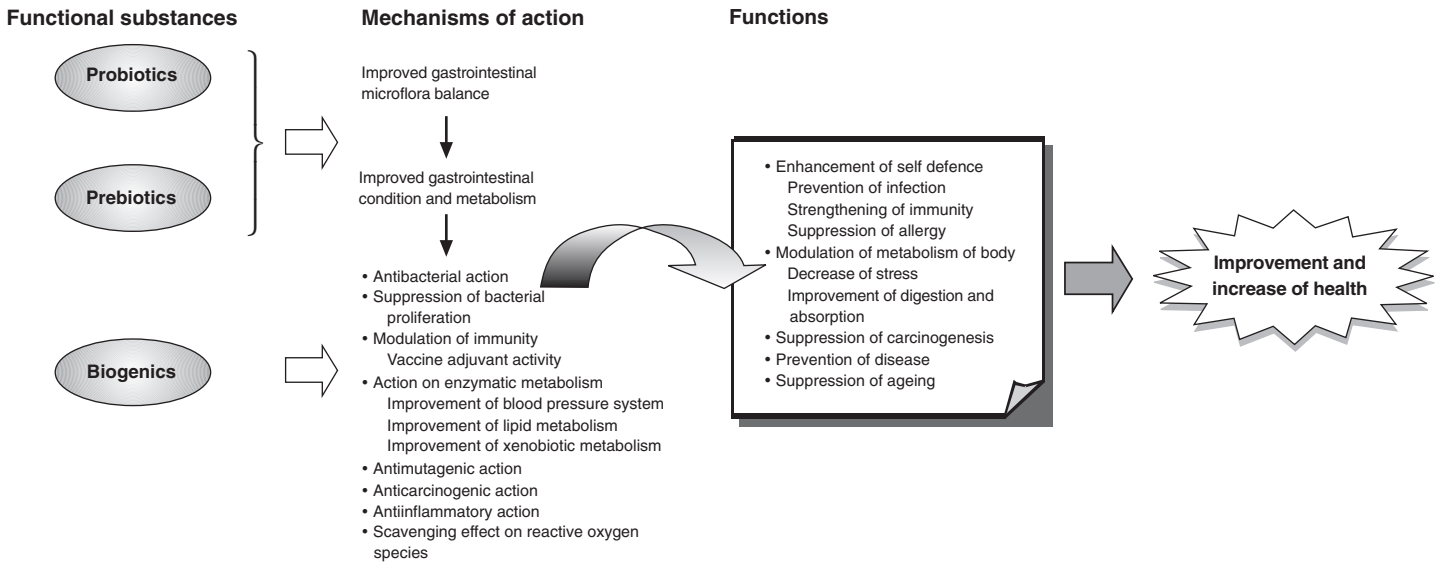

Fig. 7.2. Proposed mechanism of action and functions of probiotics, prebiotics and biogenics.

3.2 General properties of lactic acid bacteria

Many references on probiotic bacteria are available regarding improvement of the health of humans and domestic animals. Especially, recently there has been interest in the use of lactic acid bacteria (LAB) as probiotics. LAB are known to include several genera, such as *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus* and *Vagococcus*. These genera consist of Gram-positive, non-spore forming cocci or rods that produce lactic acid as the major metabolic product (homofermentative strains) or as a significant component in a mixture of metabolic products (heterofermentative strains) (Salminen *et al*., 1998; Mitsuoka, 2002; Benno, 2003). Bifidobacteria are often included with LAB, although they are phylogenetically distinct from other members of the group (Salminen *et al*., 1998). LAB have been mainly detected both in the mucous membranes of animals and in some common foods (e.g. yogurt, salami sausages and pickles).

A healthy human gastrointestinal tract contains a large and diverse population of microorganisms (Mital and Garg, 1995; Mitsuoka, 2002; Benno, 2003). For example, the number of species that comprise intestinal microflora in adult humans might be over 100. Intestinal microflora consist of *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Welsh*, *Escherichia*, *Staphylococcus*, *Pseudomonas*, *Bacteroides*, *Eubacterium*, *Streptococcus* and so on (Mitsuoka, 2002; Benno, 2003). *Lactobacillus* and *Bifidobacterium* are beneficial intestinal bacterium species and predominant members of the normal microflora. Many kinds of probiotic LAB have been used to modulate mammalian intestinal disorders, which are thought to be related to disturbances of microflora in the intestine and inflammation of the intestinal mucosa (Salminen *et al*., 1996; Mitsuoka, 2002; Benno, 2003; Saito, 2003). To have the probiotic effects, LAB generally resist gastric acidity, adhere to intestinal cells and thus colonize the intestine (Salminen *et al*., 1998; Mitsuoka, 2002; Benno, 2003; Saito, 2003).

The desirable established and postulated properties of LAB for mammals are summarized in Table 7.2 (Salminen *et al*., 1998, 1999). It is important to estimate both the functional properties (survival, adherence and stimulation of immunity) and technological properties (growth in food,

Table 7.2. Established and postulated properties of lactic acid bacteria (LAB) for mammalian health.

Suppression of growth of harmful bacteria
Prevention of diarrhoea
Reduction of symptoms of lactose intolerance
Decrease of faecal mutagenicity
Modulation of immunity
Prevention of intestinal cancers
Prevention of superficial bladder cancer
Prevention of *Helicobacter pylori*-induced gastritis and ulcers

viability and stability) of probiotics. LAB have been observed to exhibit nutritional and therapeutic effects such as modulation and stimulation of immune responses and antimutagenic properties.

The many desirable properties of probiotics indicate species specificity. This is because probiotic bacteria might adhere to specific epithelial cells of the intestine. There are species-to-species differences in the survival ability of probiotic bacteria in the stomach (where there is high acidity due to gastric acids) and small intestine (where there is a high concentration of bile components). Accordingly, probiotic bacteria are desired to show good survivability, in both the product (feed) and digestive tracts.

3.3 Probiotic bacteria in aquaculture

In aquatic animals, intestinal microorganisms interact with the environment in a similar way as they do in terrestrial animals (Holzapfel *et al.*, 1998). The ecosystem is supposed to be shared with the fish (host) and microorganisms in the aquatic environment (Verschuere *et al.*, 2000a). However, additional considerations are required if the definition of probiotics described by Fuller (1989) is applied in aquaculture. This is because the interaction between host and microbe is often qualitatively and quantitatively thought to be very different in terrestrial host animals and aquatic animals. Although the activity of microbes might be limited in terrestrial host animals, microbes in the aquatic environment can live and affect many host tissues such as the gills, skin, surface mucus and intestinal tract (Harris, 1993). Aquatic animals are also surrounded by pathogens and the concentration of pathogens is thought to easily reach high densities around the animal (Moriarty, 1998). Accordingly, fish health might be more easily affected by microorganisms in the aquatic environment compared to terrestrial animals affected by microorganisms in their environment. It has been revealed that the microorganisms that exist in the aquatic environment affect the composition of the gut microflora (Cahill, 1990). Many species of intestinal microorganisms, which might survive and proliferate in the intestinal tract of fish, are thought to come from the environment and/or diet (Cahill, 1990; Hoshino, 2003; Hagi *et al.*, 2004; Sugita, 2004; Burr and Gatlin, 2005).

Probiotic research in aquaculture was focused in the beginning on juvenile fish. Recently, more attention is being given to both eggs and larvae of fish and crustaceans (Gatesoupe, 1999; Hansen and Olafsen, 1999; Gomez-Gil *et al.*, 2000; Verschuere *et al.*, 2000a; Olafsen, 2001; Irianto and Austin, 2002; Vine *et al.*, 2006). Unlike terrestrial mammals, aquatic animals usually spawn their eggs in the external environment. The surface of the egg is attacked by many species of bacteria under natural conditions and ambient bacterial colonization may have a probiotic role. Conversely mammals are able to get an important part of their initial bacterial colonization through contact with their mother. In addition, the immune system of newly hatched larvae is usually not sufficiently developed and

initially they are not thought to have a microflora in the intestine or on the surface of the gills and skin. The primary microbial conditions in the early larval stages might be partly affected by the quality of the surrounding water (Hansen and Olafsen, 1999; Verschuere *et al*., 2000a; Vine *et al*., 2006) as fish often ingest surrounding microorganisms with their feed and water. Accordingly, fish are said to be exposed to many species of microorganisms in the aquatic environment and the quality of water (ambient water) is an important rearing condition. Bacteria are active on both the gills and the skin of fish in the aquatic environment. Many species of probiotic bacteria could be incorporated into the host fish from the environment and some research has been conducted into which probiotic bacteria, such as LAB, could be used as a biological conditioning and control tool (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000a; Irianto and Austin, 2002; Wago, 2003; Burr and Gatlin, 2005; Nakano, 2006; Vine *et al*., 2006). However, LAB are not thought to be the predominant species in the microflora of the larval intestine (Ringø and Gatesoupe, 1998; Hansen and Olafsen, 1999; Gomez-Gil *et al*., 2000; Olafsen, 2001; Sugita, 2004; Vine *et al*., 2006). When a high concentration of LAB is administered to juveniles, the proportion of LAB in their intestinal microflora rises. However, unfortunately the added LAB strains are observed to be lost from the intestine within a few days after administration (Ringø and Gatesoupe, 1998). Furthermore, LAB isolated from salmonid intestines did not improve the survival rate of Atlantic salmon fry against pathogens (Gildberg *et al*., 1997). This suggests that the induction of artificial LAB dominance in larvae, especially in the developmental stages, is not easy. The proliferation of probiotic bacteria in the gastrointestinal tract might be affected by age and health conditions of the host. It is important to use the fish's normal dominant gastrointestinal microflora species as the selected probiotic microbial species.

The microflora condition of the gastrointestinal tract in fish is thought to be different from that in mammals (Cai *et al*., 1999; Gatesoupe, 1999; Tanaka, 2000; Hoshino, 2003; Hagi *et al*., 2004; Sugita, 2004; Burr and Gatlin, 2005). Fish have many more chances to encounter invading microorganisms via the mouth compared to terrestrial animals. In addition, it is known that the composition of the microflora of the gastrointestinal tract in fish is affected by environmental conditions, especially water temperature (Sugita *et al*., 1989; Cai *et al*., 1999; Hoshino, 2003; Al-Harbi and Uddin, 2004; Hagi *et al*., 2004; Sugita, 2004). In the fish intestine, it has been difficult to detect beneficial and specific microorganisms. Accordingly, the candidate probiotics must be able to reach the specific area of the gastrointestinal tract where their probiotic effects can be expressed. Several researchers have proposed the main possible actions of probiotics in aquaculture (Salminen *et al*., 1998; Verschuere *et al*., 2000a; Irianto and Austin, 2002; Sugita *et al*., 2002; Sugita, 2004; Burr and Gatlin, 2005; Nakano, 2003, 2006; Vine *et al*., 2006).

It is known that bacterial adhesion to mucus and the wall surfaces of the gut and other tissues are important for the initial stage of pathogenic infection (Krovacek *et al*., 1987). Accordingly, one probiotic effect should be

competition with pathogens for adhesion sites, that is, adhesion receptors. In mammalian studies, the mechanism of bacterial adhesion to tissue has been thought to be both specific (adhesion molecules on the surface of adherent bacteria or specific receptor molecules on epithelial cells of the intestine) and non-specific (Salminen *et al*., 1996). There are several bacterial species isolated from fish intestines that are ideal probiotic candidates as they are able to adhere to and grow on intestinal mucus where pathogenic bacteria usually grow (Olsson *et al*., 1992; Joborn *et al*., 1997; Hoshino, 2003).

Microorganisms often release chemicals which show bactericidal and/or bacteriostatic effects on other microbial populations. For example, antibiotics, bacteriocins, lysozymes, protease, hydrogen peroxide, organic acids and ammonia are often observed to be produced by microorganisms. Especially, LAB produces bacteriocins, which suppress the growth of other bacteria.

Competition for nutrients might play an important role in the intestinal microflora. In the aquacultural environment, the microbial ecosystem is dominated by heterotrophs competing for organic substances as both carbon and energy sources. The selected bacteria compete with pathogens for chemical substances and available energy (Verschuere *et al*., 1999, 2000b). It is well known that almost all microorganisms require iron for their growth. The requirement for iron is reported to be especially high for many pathogens compared with harmless bacteria (Verschuere *et al*., 2000a). Siderophores which are ferric ion-specific chelating substances are found in harmless bacteria (Gatesoupe, 1997). Hence, siderophore-producing bacteria can be used as a probiotic 'biological modulator' to scavenge free iron and to suppress the growth of pathogens.

Some species of probiotic bacteria which are orally administered have been shown to enhance the immune response of the mammalian host (Holzapfel *et al*., 1998; Isolauri *et al*., 1998). In fish and shrimp, several specific components of bacteria cells, such as DNA motifs and glucans, are observed to stimulate the host immune response (Anderson, 1992; Sakai, 1999; Jorgensen *et al*., 2001; Sakai *et al*., 2001; Takahashi *et al*., 2001; Wago, 2003). However, the relationship between probiotics and host immunity are still not well characterized. The beneficial actions of probiotic bacteria taken by fish and shellfish on the immune system should be elucidated.

Many bacterial species have been found to affect microalgae (Munro *et al*., 1995; Fukami *et al*., 1997). For example, the growth of red tidal microalgae such as *Pavlova* sp. is suppressed by several species of bacteria (Munro *et al*., 1995). Accordingly, the interaction between bacteria and phytoplankton should be considered when probiotic bacteria are added to a culture environment. Several species of nitrifying bacteria have been used to control the level of ammonia in water. For example, biological filters that use aerobic nitrifying bacteria have been readily employed to improve water conditions in aquariums and other water recirculating systems (Perfettini and Bianchi, 1990). However, the improvement of water quality in large-scale areas such as fish-culture ponds by the addition of probiotics might not be easy (Rengpipat *et al*., 1998).

The various detailed mechanisms of action associated with probiotic use in aquatic animals have not been clearly elucidated. Hence, when probiotics are applied in aquaculture, the possible properties and effects mentioned above should be considered.

3.4 LAB in aquaculture

Most probiotics used in aquaculture belong to LAB (e.g. *Lactobacillus* and *Carnobacterium*), *Vibrio* (e.g. *Vibrio alginolyticus*), *Bacillus* and *Pseudomonas* (Verschuere *et al*., 2000a; Irianto and Austin, 2002; Nakano, 2003, 2006; Burr and Gatlin, 2005). More than 15 commercial products containing several probiotic bacteria such as *Bacillus* spp., *Bifidobacterium* spp. and *Lactobacillus acidophilus* have been developed and are available in the Japanese fishery market. The specific probiotic strains are added on their own to feed or in combination with other bacteria. About 11 bacterial strains (e.g. strains of *Enterococcus*, *Clostridium*, *Bacillus*, *Bifidobacterium* and *Lactobacillus*) are officially approved for domestic animal feed as a probiotic supplement by the Japanese Ministry of Agriculture, Forestry and Fisheries, but of these commercial products, *Bacillus cereus* (toyoi strain) is the only strain which is approved specifically to supplement feed for fish in Japan. *B. cereus* toyoi is characterized as a Gram-positive and spore-forming species and the spore of this species can tolerate conditions of high acidity, high alkalinity and high temperatures. The promotion of feed conversion and weight gain, resulting from improvement of digestion and absorption, has been observed in some fish species which were administered *B. cereus* toyoi (Tanaka, 2000).

Several LAB species found in the intestine of healthy fish are able to produce inhibitory compounds which act on pathogens, such as antifungal peptide bacteriocins, hydrogen peroxide and organic acid (Sugita *et al*., 1989; Ringø and Gatesoupe, 1998; Cai *et al*., 1999; Hoshino, 2003; Hagi *et al*., 2004; Sugita, 2004; Burr and Gatlin, 2005; Vine *et al*., 2006). In mammals, DNA motifs derived from LAB genomes have been reported to activate the host immune system (Kitazawa, 1999; Kitazawa and Saito, 2002; Saito, 2003). Bacterial DNA motifs also stimulate the immune response of fish (Jorgensen *et al*., 2001; Tassakka and Sakai, 2002, 2003). Other than DNA motifs, live LAB cells administered orally have been observed to positively affect the aquatic animal defence system against pathogens (Byun *et al*., 1997; Gildberg *et al*., 1997; Gatesoupe, 1999; Verschuere *et al*., 2000a; Irianto and Austin, 2002; Nikoskelainen *et al*., 2003; Panigrahi *et al*., 2004, 2005; Burr and Gatlin, 2005; Vine *et al*., 2006). The components of microbial cells such as glucans and lipopolysaccharides, stimulate the immune response of animals (Anderson, 1992; Tangeras and Slinde, 1994; Sakai, 1999; Hertrampf and Piedad-Pascual, 2000c; Takahashi *et al*., 2001; Irianto and Austin, 2002; Wago, 2003). Accordingly, probiotics might be used to improve vaccinations. Several effects of prebiotics such as oligosaccharide on intestinal LAB in fish have also been determined (Burr and Gatlin, 2005).

However, the results regarding the effects of both probiotics and prebiotics on the health of aquatic animals in the literature is not well characterized, therefore, both *in vitro* and *in vivo* studies concerning applications of probiotics and prebiotics in aquaculture should be continued.

4 Conclusions and Perspectives

Many substances mentioned in this chapter are found in nature, so they should be safe for fish and consumers. In the aquaculture industry, cultured fish often receive stress from the environment and have many chances to become diseased, necessitating use of antibiotics or other medication. However, the use of antibiotics raises anxiety over antibiotic levels in the tissues of cultured animals and the generation of antibiotic-resistant bacteria. On the other hand, the use of probiotics in aquaculture should be regarded as a milder supplement therapy for fish and an environmentally (ecologically) friendly method of aquaculture. This is because probiotic treatment, 'probiotic therapy', is based on modulation of the microbial ecosystem in the host (Gatesoupe, 1999; Koga, 2002). If probiotics can effectively stimulate host immunity, the action of probiotic therapy should be regarded as an oral vaccination. Probiotics are usually demanded to modulate target functions and reduce the risk of disease (Salminen *et al*., 1998, 1999). In addition to probiotics, the red yeast *P. rhodozyma* has been shown to improve survival rate of fish larvae (Scholz *et al*., 1999) as well as serve as a source of ASX.

The influence of consuming a diet supplemented with such probiotics and prebiotics on the intestinal microflora of fish is still largely unknown (Gatesoupe, 1999; Verschuere *et al*., 2000a; Irianto and Austin, 2002; Nakano, 2003, 2006; Burr and Gatlin, 2005; Vine *et al*., 2006). In addition, typically single strains of probiotic bacteria have been used for aquacultural research (Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000a; Irianto and Austin, 2002; Nakano, 2003, 2006; Burr and Gatlin, 2005; Vine *et al*., 2006), but the actions of mixtures of probiotic bacteria, 'bacterial consortium', on host animals need to be further studied. Thus further research should be conducted to evaluate interactions between probiotics, prebiotics and the microbiological communities of the host animals. It would also be desirable to develop another analytical method to characterize the gastrointestinal microflora of cultured fish. For example, molecular approaches and tools based on the detection and identification of genetic components in microorganisms, such as 16S ribosomal DNA (16S rDNA), should be useful for monitoring microflora (Salminen *et al*., 1999; Verschuere *et al*., 2000a; Benno, 2003).

It is anticipated that ideal functional feed supplements, which are of benefit to the health and well-being of cultured animals, will be developed by further investigation of microorganisms, especially probiotics.

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8 Terrestrial Plants

MINORU SATO

Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan

1 Introduction

It is widely known that consuming fish is good for human health. Following outbreaks and spread of bovine spongiform encephalopathy (BSE), foot-and-mouth disease and avian influenza in many countries, an avoidance of livestock meat has resulted in increased seafood consumption, especially in Europe and the USA. The demand for marine products, especially fish, will surely increase worldwide hereafter. Control of wild fish resources for sustainable utilization and progress in fish culture are therefore indispensable.

Aquaculture can be a very productive use of resources, with the amount of food produced per hectare being considerably higher than with livestock rearing. Resource availability and use have allowed the sector to grow more than three times faster than terrestrial farm animal meat production (FAO, 2006). In contrast to capture fisheries, in aquaculture it is feasible to control fish harvesting and fish quality and feeding various compounds or natural supplements, including terrestrial plant materials, enhances quality control in cultured fish.

Numerous terrestrial plant materials or plant extracts have been used in fish farming for various purposes. When plant materials are added at comparatively high levels to feed, they are expected to provide proteins, lipids, carbohydrates and energy as well as binding together the feedstuff (Belal and Al-Dosari, 1999; Carter and Hauler, 2000; Glencross *et al*., 2002; Hossain *et al*., 2002; Borlongan *et al*., 2003; da Silva *et al*., 2003; Richter *et al*., 2003; Stone *et al*., 2003; Fotedar, 2004). On the other hand, when small quantities of certain plant powders or extracts are added as supplements to diets, it is expected that they will maintain or activate fish health, feed efficiency, growth, attraction activity, disease resistance and stress resistance (Sato and Takeuchi, 1996; Hossain *et al*., 2001; Afuang *et al*., 2003; Lee *et al*.,

 Dietary Supplements for the Health and Quality of Cultured Fish (eds H. Nakagawa, M. Sato and D.M. Gatlin III)

2004; Rao *et al*., 2004). Moreover, interest in the addition of supplements has increased as it is believed that they may improve the product (fish) quality for the consumer, for example with respect to freshness, fat content, colour, odour, taste, texture and quality persistence during distribution which is important to ensure product safety (Sato and Takeuchi, 1996).

Plant species, plant parts and the type of additive product used for aquaculture are quite varied as shown in Table 8.1 (Sato, 2003). Plant materials are used frequently because they are usually inexpensive, easy to obtain as natural raw or cultivated material, there is a long history of human usage as Chinese or herbal medicines, and there are intensive demands for recycling wastes from plant processing (Sato and Takeuchi, 1996; Boscolo *et al*., 2002; Deka *et al*., 2003).

Although there are many instances when raw plant materials or plant extracts are used as supplements for aquaculture, there are few scientific data available concerning this use. This chapter describes the effects of the addition of stevia *Stevia rebaudiana*, green tea *Camellia sinensis*, banana *Musa* genus, maca *Lepidium meyenii*, *Lepidium peruvianum*, herbs such as kugija *Lycium chinense*, *Achyranthes aspera* and some other plants including mushroom, as feed supplements for aquaculture.

2 Effects on Growth and Feeding

Fish growth is influenced in a broad sense by nutritional and environmental factors. Plant supplements affect fish growth by influencing factors of digestibility, feed efficiency and also feed attraction.

2.1 Effects on growth

2.1.1 Maca Lepidium peruvianum

Maca is grown just below the glaciated slopes of the Peruvian Andes. Maca was first used by the Incas more than 3000 years ago for energy and endurance and is still commonly traded as a food and folk medicine in Peru today (Zheng *et al*., 2000; Li *et al*., 2001).

Table 8.1. Plant species, parts and additive products used for aquaculture.

Species of plant	Part of plant	Type of additive product
Annual plants	Leaf	Meal
Perennial plants	Stem	Extract
Bush	Root	Refined substance
Tree	Fruit/seed	Charcoal
Bamboo	Husk	Wood vinegar compound
(Mushroom)		(By-product of charcoal production)

Effect of maca tuber meal was tested by two consecutive experiments using rainbow trout (Lee *et al*., 2004). In the first experiment with rainbow trout alevins (0.096 ± 0.002 g), starter diets were offered from first feeding until 15 weeks. High protein content (approx. 60%) semi-purified starter diets supplemented with 0%, 5%, 10% or 15% maca tuber meal (control, M-5, M-10 and M-15, respectively) were used. The second feeding trial was conducted with rainbow trout juveniles (1.56 ± 0.02 g) fed one of three diets (control, M-15 and a commercial diet) for 8 weeks. In the first experiment, fish fed M-10 and M-15 diets exhibited significantly higher growth rates than the other dietary groups. The second experiment showed a higher growth rate in the M-15 group compared with the control and the commercial diet. The findings of the study suggest that a maca tuber meal inclusion of at least 5% improves growth rate and feed utilization of rainbow trout alevins and juveniles (Lee *et al*., 2004).

2.1.2 Fruits and seeds

A feeding trial was carried out to evaluate nutrient digestibility and transit velocity in tambaqui *Colossoma macropomum* (1627 ± 112.8 g) fed two species of fruits, jauari *Astrocaryum jauari* and embauba *Cecropia* sp. and two species of seeds, munguba *Pseudobombax munguba* and seringa barriguda *Hevea spruceana*, incorporated in a reference diet. In the reference diet, 55% of the yellow maize grain was replaced, in equal proportions, by ground meal prepared from the jauari and embauba fruits, and from munguba and seringa barriguda seeds. Fruit and seed inclusion in the diet significantly altered (having a negative effect) the nutrient composition and the digestibility coefficient of all experimental diets. Diet composition also showed a significant effect on the gastrointestinal transit time (da Silva *et al*., 2003).

2.1.3 Dhanincha Sesbania aculeate

The effect of purified alcohol extract (saponin) from dhanincha seeds on the growth and feed utilization of common carp *Cyprinus carpio* (6.32 ± 0.02 g) has been reported (Hossain *et al*., 2001) based on an 8-week feeding trial conducted in a recirculation system at 27 ± 0.2°C. Six isonitrogenous and isoenergetic diets were formulated. Diet 1 was used as the control and diet 2 contained 0.3 g/kg quillaja bark saponin. Diets 3 to 6 contained 0.3 to 2.4 g/kg alcohol extract from dhanincha seeds. On the basis of the observed growth rate, metabolic growth rate, food conversion ratio, protein efficiency ratio, protein productive value and energy retention, only the fish fed diet 6 containing 2.4 g/kg alcohol extract from dhanincha seed had significantly lower growth performance and feed utilization compared to the other diets. However, the alcohol extracts significantly reduced the muscle and plasma cholesterol levels of fish fed diets 5 and 6. Fish fed diet 6 had significantly higher whole-body moisture content with concomitant decrease in lipid content and hence the significantly lowest total gross energy. There was no

significant ($P > 0.05$) difference between the whole-body crude protein and ash content of fish fed different experimental diets. The results of the study showed that purified alcohol extract (saponin) from dhanincha seed at 0.3, 0.5 and 1.2 g/kg dietary inclusion did not affect the growth and feed utilization but at 2.4 g/kg inclusion level it significantly reduced growth and feed utilization of common carp.

2.1.4 Red clover powder Menoflavon

Menoflavon is the commercial name of red clover powder sold by Melbrosin (Vienna, Austria). The effects of the red clover extract on growth, body composition and survival of African catfish *Clarias gariepinus* was studied (Turan and Akyurt, 2005). Three diets were prepared containing different concentrations (25, 50 and 75 mg/kg) of red clover and these were used for 120 days. The final weights of red clover-treated groups were significantly different from each other and the control group. The best growth was observed in the group receiving the 75 mg/kg diet. Fish fed with all levels of red clover had higher weight gains than the control. The highest values of the specific growth rate and the food conversion rate were 1.14 ± 0.01 and 2.26 ± 0.01, respectively, for the 75 mg/kg diet group. During the red clover administration period, the survival ranged from 97.78% for the 50 mg/kg group to 95.56% for all other groups. Protein contents of the dosage groups were significantly different from the control ($P < 0.05$). The highest value of protein content (19.97%) was observed for the 75 mg/kg diet. Lipid and ash contents were significantly affected by the red clover levels in the diets. The highest lipid content was in the 75 mg/kg diet (4.67%), and the highest ash content was 1.39% for the 25 mg/kg diet (Table 8.2).

2.2 Effects on feeding

2.2.1 Spices

The feeding attraction activities of water-extracts from spices for adult oriental weatherfish *Misgurnus anguillicaudatus* and juvenile yellowtail *Seriola quinqueradiata* were studied (Harada, 1990). Among 30 specimens tested in 28 species of spices, allspice *Pimento officinalis* and ten others were attractive for oriental weatherfish, and allspice and 14 others for yellowtail. Above 60% of the attractive specimens for both fish were odorous spices. Furthermore, seven spices were attractive for both fish and these were allspice, caraway *Carum carvi* and cardamon *Elettaria cardamomum* of the odorous spices, white pepper *Piper nigrum* as the only acrid spice, and garlic *Allium sativum*, onion *Allium cepa* and savory *Satureia hortensis* in the odour-corrective group. Especially strong attractants were caraway for oriental weatherfish, and cumin *Cuminum cyminum* for yellowtail. The attraction activities of caraway, cumin and allspice clearly depended on the concentration used.

Table 8.2. The effects of different concentrations of dietary red clover on survival, weight gain, specific growth rate, feed efficiency and the chemical composition of the whole body of African catfish *C. gariepinus* fed for 120 days at a water temperature of 25 ± 1°C (Source: Turan and Akyurt, 2005).[1]

Red clover (mg/kg)	Weight gain[2] (g)	Specific growth rate[3] (%)	Food conversion ratio[4]	Survival (%)	Chemical composition[5] (%)			
					Moisture	Crude protein	Crude lipid	Ash
0	106.48 ± 0.80[a]	1.06 ± 0.01[a]	2.48 ± 0.01[b]	95.56 ± 1.11[a]	72.84 ± 0.19[a]	18.18 ± 0.09[a]	2.65 ± 0.05[a]	1.23 ± 0.01[a]
25	113.05 ± 3.12[b]	1.09 ± 0.02[a,b]	2.34 ± 0.06[a]	95.56 ± 2.22[a]	72.65 ± 0.15[a]	19.92 ± 0.03[b]	3.70 ± 0.04[b]	1.39 ± 0.01[d]
50	118.83 ± 1.45[b,c]	1.13 ± 0.01[b]	2.35 ± 0.02[a]	97.78 ± 1.11[a]	72.39 ± 0.25[a]	19.80 ± 0.05[b]	4.65 ± 0.04[c]	1.32 ± 0.01[c]
75	119.27 ± 0.75[c]	1.14 ± 0.01[b]	2.26 ± 0.01[a]	95.56 ± 1.11[a]	72.49 ± 0.11[a]	19.97 ± 0.05[b]	4.67 ± 0.04[c]	1.33 ± 0.01[c]

[1] Values are means ± standard error of triplicate with different superscripts in each column indicating significant differences ($P < 0.05$).

[2] Weight gain = final weight − initial weight.

[3] Specific growth rate = ([lnW2 − lnW1]/[T2 − T1]) × 100, where W1 and W2 are mean body weight at times when the first and second samples were taken (T1 and T2).

[4] Food conversion ratio = (dry feed intake/weight gain) × 100.

[5] Chemical composition data presented are on a wet basis and initial figures for chemical composition (%) are: moisture 75.45 ± 0.25; crude protein 17.83 ± 0.14; crude lipid 2.50 ± 0.02; ash 1.10 ± 0.03.

2.2.2 Fruit extracts

The attraction activities of fruit-flesh or fruit-rind water extracts were statistically estimated on the basis of exploratory behaviour in the oriental weatherfish (Harada and Miyasaki, 1993). Thirty-six forms of 25 species of fruits were tested: 22 of 13 in the *Rosaceae*, six of six in the *Rutaceae*, three of one in the *Vitaceae* and one each in five other families (*Actinidiaceae*, *Cucurbitaceae*, *Ebenaceae*, *Moraceae* and *Myricaceae*). Of the fruit-flesh tested, the attraction activity in the *Rosaceae* was high in eight forms: one apricot (adventitious seedling form), three cherries (*Napoleon biggreau*, hybrid and *Prunus cerasus* forms), one European plum (*Soldum* form), one nectarine, one peach (adventitious seedling form) and one strawberry. The attraction in the *Rutaceae* was high only in the Unshiu orange, but was low in two other forms of orange. The attraction in the *Vitaceae* was found to be low in only one grape form (the Delaware form). The attraction in the other five families was low in kiwi fruit (*Actinidiaceae*), persimmon (*Ebenaceae*) and myrica (*Myricaceae*). Of the fruit extracts tested, strawberry was found to be highest in attraction, though its attraction activity depended on the concentration. Of the fruit-rinds tested, the attraction in the *Rutaceae* was low only in the Unshiu orange.

2.2.3 *Stevia* Stevia rebaudiana

Preliminary experiments revealed that stevia has an attractant effect on the feeding of the adult oriental weatherfish (average body length, 9.8 cm) and the juvenile yellowtail (average fork length, 5.6 cm) (Harada *et al*., 1993). Furthermore it was observed that there were some individuals in the two test species which attacked the crumpled gauze containing the stevia extract.

The attraction index of an authentic sample of stevioside, which may be the effective component of the stevia, was estimated quantitatively using 60 individuals of oriental weatherfish, and 50 (in the final experiment) to 148 (in the initial one) yellowtail. After fractionation of the stevia extract it was assumed that the active component was stevioside. The attraction activity of the two species tested was closely correlated to the concentration of stevioside. Furthermore it was estimated that the attraction activity of the extract was nearly equivalent to that of the authentic sample of stevioside. These results indicate that the attraction activity of the stevia was mainly accounted for by that of stevioside.

2.2.4 Mushrooms

Attraction activities of 30 strains from 22 species of mushroom for the oriental weatherfish and the yellowtail were examined (Miyasaki and Harada, 2003). To some extent all the aqueous extractions of the mushrooms tested were attractive to the fish. Three kinds of mushrooms, ningyoutake *Albatrellus confluens*, kishimeji *Tricholoma flavovirens* and matsutake *Tricholoma matsutake*

attracted both fish, and the highest activity was observed in *Naematoloma sublateritium* for the oriental weatherfish and tsukuritake *Agaricus bisporus* for the yellowtail. The attraction activities of these mushrooms clearly depended on the concentrations tested.

2.3 Improvement of water quality

2.3.1 Yucca Yucca schidigera

Yucca is native to south-western deserts of the USA, Central America and the Caribbean. Saponin-free yucca extract reduced the ammonia and nitrate concentration of the water, providing a better water quality for the fish. The liquid saponin-free yucca extract has been added to the water at a level of 3.0 to 5.0 ppm. Saponin-free yucca powder is used in feed at level of 100 to 150 ppm (Hertrampf and Piedad-Pascual, 2000b).

3 Effects on Fish Health

The fish farming industry has grown rapidly and for the aquaculturist, an outbreak of disease is one of the greatest concerns. If such diseases can be managed effectively then potential losses will be limited. Terrestrial plants or their extracts may be used to this end.

3.1 Maca *Lepidium meyenii*

Supplementation of maca tuber meal in diets was confirmed to improve survival of rainbow trout *Oncorhynchus mykiss* alevins and juveniles (Lee *et al.*, 2004). Effects of maca tuber meal were tested in two experiments using rainbow trout. In the first experiment with alevins (0.096 ± 0.002 g), starter diets were offered from first feeding until 15 weeks. High protein content (approx. 60%) semi-purified starter diets supplemented with 0%, 5%, 10% or 15% maca tuber meal (control, M-5, M-10 and M-15, respectively) were used. The second feeding trial was conducted with juveniles (1.56 ± 0.02 g) fed one of three diets (control, M-15 and a commercial diet) for 8 weeks. Survival was significantly improved in the groups fed diets supplemented with maca tuber meal (60.0–69.2%) in comparison with the group fed the control diet (21.7%). In the second experiment, leucocyte numbers were increased by dietary supplementation of maca tuber meal. The findings of this study suggest that inclusion of maca tuber meal at a level of at least 5% improves immunity by increasing leucocyte numbers, and this in turn increases survival of rainbow trout alevins and juveniles.

3.2 *Achyranthes aspera*

Potentiation of antibody production in rohu *Labeo rohita* by the herbal plant, *A. aspera* (*Amaranthaceae*) has been confirmed by Rao *et al*. (2004). Indian major carp (200±17 g) were fed two types of diets, an experimental diet containing root extract (0.5%) of *A. aspera* as an ingredient and a control diet without the root extract. After 4 weeks of feeding, fish were immunized with chicken red blood cells. Antigen-specific antibody response, total serum globulin and RNA/DNA ratio of spleen were determined 4 weeks after immunization. Antigen-specific antibody and total serum globulin levels peaked on day 14 after immunization and gradually decreased towards day 28. Although haemagglutination antibody titers were always higher in the test group than the control group, the total serum globulin level was significantly higher only on days 14 and 21. A sequential relationship between the RNA/DNA ratio and protein level was found, as the RNA/DNA ratio reached a maximum level on day 7 and this was followed by a higher serum protein level on day 14 in both groups. The RNA/DNA ratio was significantly ($P < 0.05$) higher in the test group of fish than the control group on days 7 and 14. These results showed the immunostimulatory activity of the prepared diet containing root extract of *A. aspera*.

3.3 Other medicinal plants

Hundreds of medicinal herbs have been used for the purpose of disease treatment and immune enhancement for human beings and other animals including fish (Shimizu, 1982a, b; Hwang *et al*., 1999; Kwon *et al*., 1999; Choi *et al*., 2004). Practical usage for disease prevention and treatment were mentioned for 17 Japanese herbal plants (Shimizu, 1982a, b). Among them, 49 species of medicinal herbs were selected and tested for antibacterial activities against 19 strains of pathogenic bacteria, such as *Edwardsiella tarda*, *Vibrio* sp., *Lactococcus garvieae*, *Lactococcus raffinose*, *Streptococcus parauberius* and *Streptococcus iniae* (Choi *et al*., 2004). The obaeja *Galla rhois*, gaeonnamu *Rhus trichocarpa* and hwangleyon *Coptis chinensis* showed antibacterial activities on both Gram-negative and Gram-positive bacteria that are pathogenic to fish. The plant species youkgae *Cinnamomum cassia*, sangbaekpi *Mori cotex*, bogolji *Psoralea hair* and gamcho *Glycyrrhiza uralensis* showed very effective antibacterial activities on Gram-positive pathogens while jiyu *Sanguisorba officinalis*, aeyoeb *Artemisiae asiaticae* and yeonkyo *Forsythia koreana* showed very effective antibacterial activities on Gram-negative pathogens.

In order to investigate the immune response induced by dietary supplementation of herbal medicines on the Nile tilapia *Oreochromis niloticus*, fish were fed one of four different experimental diets supplemented with 2% ginseng *Panax ginseng*, 3% kugija *L. chinense*, 3% hasuo *Polygonum multiflorum* and 2% omija *Schizandra chinensis*, respectively, for 84 days. Changes in the non-specific immune responses during the feeding period

were investigated at 2, 4, 6, 8, 10 and 12 weeks. Average body weight of the Nile tilapia fed supplemented diets was heavier than the control group. Fish fed the diet supplemented with 3% kugija showed the best growth compared to the other test groups. Complement activity such as complete haemolytic activity (CH_{50}) and bactericidal activity against *Escherichia coli* tended to be increased by the supplementation of herbal medicines. The lysozyme activity of serum and adherent phagocyte activity were higher in fish fed diets supplemented with 3% kugija than the other test groups. Following experimental *E. tarda* infection all groups fed the herbal medicines appeared to have higher relative percentage survival rates than the control group. From these results, it can be concluded that the herbal medicines *P. ginseng*, *L. chinense*, *P. mulftiflorum* and *S. chinensis* might be used as diet additives to increase non-specific immune responses and resistance against bacterial fish diseases (Hwang *et al*., 1999; Kwon *et al*., 1999).

3.4 Banana

Banana is inexpensive, has high nutritional value and is the most popular tropical fruit all over the world. More than 300 kinds of banana are cultivated all over the world, and those in the genus *Musa* are the most famous. Banana contains antioxidants such as vitamin C, polyphenol, carotenoids, tocopherols and various other vitamins, as well as dietary fibre. Recently, the health enhancement effects of banana have become evident. Banana has been found to activate white blood cells, enhance production of tumour necrosis factor (TNF) and have a life-prolonging/healing effect in mouse implanted cancer cells (Murcia *et al*., 2001; Jang *et al*., 2002; Herraiz and Galisteo, 2003; Horiuchi, 2003; Lo *et al*., 2004; Patel *et al*., 2004).

Haemolysis complement activity was used as an index of disease resistance in serum of rainbow trout and red sea bream *Pagrus major* which were fed banana powder. Haemolysis complement activity (haemolysis ratio) was elevated in both rainbow trout and red sea bream fed banana powder; thus, it can be expected to enhance disease resistance activity of cultured fish (Horiuchi, 2003).

Liver function also is important in animals as it relates to the detoxification of chemicals including anaesthetics. The effect on liver function of banana powder was examined in red sea bream. Recovery (awakening) time was apparently shortened in red sea bream fed banana powder. Therefore the banana powder appeared to enhance liver function of red sea bream (Horiuchi, 2003).

3.5 Fermented vegetable product (Manda)

The stimulatory effect of fermented vegetable product (FVP; commercial name Manda, produced by Manda Fermentation Co. Ltd, Mihara, Japan)

upon the phagocytic and superoxide generation of leucocytes was studied in the Japanese flounder *Paralichthys olivaceus* (Ashida and Okimasu, 2005). It was found that the phagocytic activity of casein-induced intraperitoneal leucocytes was significantly increased ($P < 0.05$ or $P < 0.01$) by the addition of FVP at levels above 3 mg/kg body weight. Further analysis investigated the effect of FVP on superoxide generation in leucocytes measured by an *in vitro* cytochrome C reduction assay. It was found that reduced levels of FVP in assay samples had a profound effect on superoxide generation. FVP was also incorporated in commercial diets and fed to Japanese flounder for 4 weeks. The phagocytic activities and superoxide generation of peritoneal induced leucocytes were significantly higher ($P < 0.05$, $P < 0.01$) in fish fed the FVP supplemented diet than fish fed the control diet. FVP feeding in fish had a significantly higher ($P < 0.05$) activity of lysozyme than in the control fish.

3.6 Mushroom glucan

Immunostimulants are compounds which have the ability to activate cells of the immune system. They have included the following: bacterial products, products from mycelial fungi, yeast glucans, yeast nucleotides, soluble and particle bound β-1,3-glucans, immunostimulatory glycans, peptides from animal extracts, synthetic compounds and cytokines (Hertrampf and Piedad-Pascual, 2000a).

The protective effect of mushroom β-1,3-glucan, schizophyllan and scleroglucan, which are β-1,3-glucans derived from *Schizophyllum commune* and *Sclerotium glucanicum*, were evaluated for their ability to enhance protection against bacterial infection in yellowtail (Matsuyama *et al*., 1992). Intraperitoneal injections of the β-1,3-glucans (2–10 mg/kg) into fish 6 and 3 days prior to intraperitoneal challenge with *Streptococcus* sp. resulted in a significantly increased survival rate, but the injection did not enhance resistance against *Pasteurella piscicida*. In β-1,3-glucan-treated fish, an elevation of serum complement and lysozyme activity was observed in addition to an increase in the phagocytic activity of pronephros cells. This suggests that the β-1,3-glucans enhanced the resistance of yellowtail against *Streptococcus* sp. infection through the activation of the non-specific immune system.

4 Detoxification of Histamine in Feed

Fishmeal is a widely used protein feedstuff for cultured animals including fish. Raw materials for fishmeal production are mainly dark-meat fish such as anchovies, which contain large quantities of free histidine. Histidine is easily converted to histamine by decarboxylation in fish under inferior preservation conditions. Histamine is thermally stable and produces a toxic compound gizzerosine by heating, so the presence of histamine in fishmeal

is a concern. Histamine and gizzerosine in feed induce gizzard erosion in broiler chickens and may result in death by 'black vomit disease'. Also gastric erosion due to gizzerosine has been observed in mice. However, histamine in aquaculture feeds is thought to be harmless to fish because there are some reports that histamine in feed does not influence fish growth (Reyes-Sosa and Castellanos-Molina, 1995; Masumoto *et al*., 2000). The effect of histamine on fish is not clear and thus further detailed examination is needed.

4.1 Stevia extracts

Takahashi *et al*. (2001) reported on the preventative effect of stevia extract (commercial name: BMD Stevia extract, JBB Stevia Co. Ltd, Saitama, Japan) on gizzard erosion and ulceration of chicks induced by histamine. Stevia extract is a fermentation product made from a mixture of 80% stem powder and 20% leaf powder from stevia *S. rebaudiana*. The content of stevia extract is shown in Table 8.3 (Sato, 1995). The LD_{50} value of this extract is over 30 g/kg in rat.

The protective effect of stevia extract on the gastric mucosa of rainbow trout *O. mykiss* fed dietary histamine was confirmed by Shiozaki *et al*. (2004). Rainbow trout (average weight 12 g) were fed 1% dietary histamine for 4 weeks. Administration of dietary histamine to trout did not result in a reduction in growth rate or feed consumption but caused a gastric abnormality, for example exfoliation of the mucosal epithelium and atrophy of mucosal lamina propria. Stevia extract protected gastric tissue from histamine-induced damage. Pepsin activity in gastric fluid increased and liver α-tocopherol content was reduced after histamine treatment, but stevia treatment prevented these abnormalities. The results suggest that the stevia extract might protect the rainbow trout stomach from histamine toxicity.

Table 8.3. Ingredients of stevia extract (Source: Sato, 1995).

Component	Amount per 100 ml
β-carotene	23 mg
Vitamin A value	13 IU
Biotin	6.3 mg
Vitamin B^2	0.21 mg
Niacin	2.4 mg
Pantothenic acid	0.98 mg
Calcium	120 mg
Iron	1.3 mg
Potassium	2200 mg
Phosphorus	200 mg
Sodium	22 mg
Calories	47 kcal

Shiozaki *et al*. (2003) investigated the metabolic mechanisms of histamine detoxification by stevia extract in rainbow trout. They measured the levels of histamine catabolic enzymes, diamine oxidase and histamine N-methyl transferase in the tissues of the rainbow trout and detected diamine oxidase in the stomach, pylorus caeca and intestine, and histamine N-methyltransferase in the liver.

The contents of histamine and its metabolites were observed to change in the tissues of rainbow trout after oral administration of histamine. A large amount of imidazole acetic acid was observed in serum, kidney, liver and muscle. On the other hand, L-methyl histamine was observed only in the liver. Histamine and its metabolites, imidazole acetic acid and L-methyl histamine, were metabolized and diminished after 48 hours in all tissues. These results showed that histamine was easily metabolized to imidazole acetic acid by the intestinal diamine oxidase of rainbow trout. Okada *et al*. (2005) confirmed the protective effect against ethanol-induced damage to rat gastric mucosa by fermented plant extracts prepared from about 50 kinds of vegetables and fruits. The active compounds were identified as chlorogenic acid and caffeic acid. Both compounds were already known as antioxidants.

5 Effects on Stress Resistance

Various stressors, such as sorting, congestion, low oxygen levels, and sudden change of water temperature are often a burden to some cultured fish (Barton, 1997). Methods to enhance stress resistance in cultured fish have been requested by fish farmers.

5.1 Low oxygen stress

5.1.1 Stevia extract

To evaluate the effects of stevia extract on tolerance of low dissolved oxygen (DO) levels rainbow trout (5.6 ± 0.2 g) were fed one of four diets: a control or basal diet and diets supplemented with stevia extract at levels of 500, 1000 and 2000 ppm for 5 weeks. The basal group showed an average lethal time of 4.88 min and DO of 0.84 ml/l at the time of final death, while fish fed stevia extract at 2000 ppm showed an average lethal time extended to 6.57 min and a DO of 0.66 ml/l at the time of final death. It was, therefore, observed that addition of stevia extract produced a remarkable improvement in low oxygen tolerance of trout as shown in Fig. 8.1 (Sato and Takeuchi, 1996).

Nakagawa *et al*. (1984) found that there was a remarkable improvement in low oxygen tolerance of fish when they were fed a diet supplemented with powdered algae, but they did not identify the reason. It may be that the additive might improve the oxygen utilizing efficiency of fish by inducing changes in erythrocyte cell walls, increasing the haematocrit value and/or improving oxygen-binding capacity.

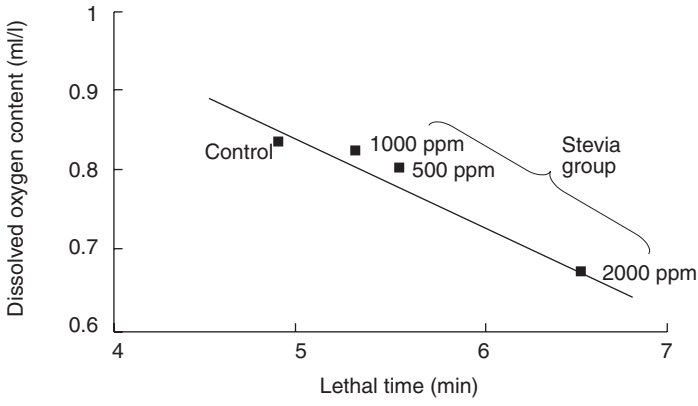

Fig. 8.1. The effect of stevia extract on low oxygen tolerance of rainbow trout.

5.1.2 Banana

Various stress resistance characteristics were studied by feeding banana powder to rainbow trout, ayu *Plecoglossus altivelis* and flounder. When banana powder was fed to fish, it was clear that stress tolerance to air exposure and low oxygen levels in water were enhanced. Tolerance of a sudden change of water temperature was also improved in flounder (Horiuchi, 2003).

5.2 Oxidative stress

The active attack of DNA and protein in a cell by oxygen may produce lipid peroxides such as strong toxic lipid hydroperoxides that cause cell and tissue injury. The lipid peroxides may cause inflammation, cardiovascular disease, cancer and ageing. Anti-oxidative enzymes such as superoxide dismutase, catalase and antioxidants such as uric acid and glutathione exist in the body and prevent some of the actions of active oxygen species, but it is generally not enough and other antioxidants, such as vitamin E and vitamin C, must be consumed (Nakano *et al*., 1993, 1995; Sato and Takeuchi, 1996).

5.2.1 Stevia extract

Xi *et al*. (1998a, b) found that a hot water extract of *S. rebaudiana* has anti-oxidative activity *in vitro* and Sato and Takeuchi (1996) demonstrated that the anti-oxidative activity of stevia stem was higher than that of stevia leaf. Various studies have been made into the *in vivo* anti-oxidative effect of stevia extract using rainbow trout (Sato *et al*., 1994; Fukui *et al*., 1996; Sato and Takeuchi, 1996).

Rainbow trout (average weight 5.6 g) were fed one of six diets including a diet containing non-oxidized oil (control) and five diets

containing oxidized oil, of which one was a control (oxidized oil control), three had stevia extract added as a powder at levels of 100, 500 or 1000 ppm, respectively, and one was supplemented with catechin at 500 ppm. The experimental diet was supplied to each group for 4 weeks. The oxidized oil control diet group exhibited a weight gain of 99%, while the stevia extract added groups increased in weight by as much as 167%, an even higher gain than that of the non-oxidized oil control group. Against 9.6 nmol/ml for the oxidized oil control diet group, the concentration of peroxide lipid (LPO) in the serum showed a remarkable decrease to 4.3 nmol/ml and 6.1 nmol/ml for the stevia extract treatments at 500 ppm and 1000 ppm, respectively (Fig. 8.2). These values for stevia extract added groups were very close to the result (4.5 nmol/ml) for the non-oxidized oil diet group, and it was proven that stevia extract contained an antioxidant component to hold back LPO in serum. The group with the 500 ppm addition of catechin, which is known to be an antioxidant contained in tea, exhibited 9.2 nmol/ml, a value very close to that of the oxidized oil control diet group, and any effect to limit serum LPO was not observed with catechin.

A second experiment was conducted to determine the effective fraction of stevia extract (Sato and Takeuchi, 1996). Eight fractions were prepared by chromatographic separation, namely five water eluted fractions (F-1–F-5), a 30% methanol eluted fraction (F-6), a 60% methanol eluted fraction (F-7) and a 100% methanol eluted fraction (F-8). As the experimental diets, whole stevia extract (as a solid matter at 2000 ppm) and stevia fractional material (equivalent to 2000 ppm in terms of stevia extract) were respectively added to the basal diet which contained 10% of oxidized oil. Rainbow trout (average weight 4.6 g) were fed each fractionated diet for 30 days.

As the result, the whole stevia extract group as well as the diet groups F-2, F-3, F-4, F-7 and F-8 showed significantly higher average weight at the end of the test than the oxidized oil control group, especially the groups F-4 and F-7 exhibiting higher average weight than the non-oxidized oil control

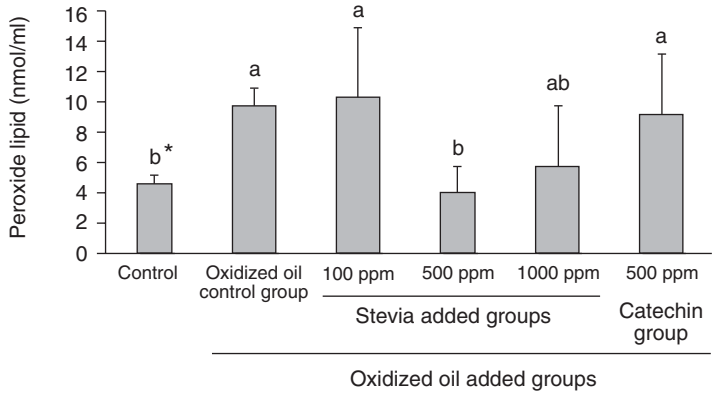

Fig. 8.2. The dietary effect of stevia extract on the concentration of peroxide lipid (LPO) in rainbow trout serum. *Columns with different letters are significantly different ($P < 0.05$).

group. Blood properties such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) did not show any significant difference, but LPO concentration of the whole stevia extract group as well as the diet groups F-4, F-5, F-6 and F-7 were observed to be significantly lower than the oxidized oil control group (Fig. 8.3). Diet groups F-4, F-5, F-6 and F-7 also had significantly lower thiobarbituric acid reactive substances in liver (TBARS) than the oxidized oil control group. The above results clarified that stevia extract contains an anti-oxidizing substance which limits LPO in the rainbow trout serum and TBARS in the liver.

Okada *et al*. (2005) confirmed the anti-oxidative activity in fermented plant extract (FPE) prepared from about 50 kinds of vegetables and fruits. They identified the antioxidants as chlorogenic acid and caffeic acid. Both compounds were also confirmed to have protective activity against gastric mucosal damage induced by ethanol in rats.

5.2.2 Green tea polyphenol (catechin)

Green tea made from the leaf of the plant *Camellia sinensis* (family name: *Theaceae*), is one of the most common drinks to be found around the world. Green tea contains high levels of polyphenol antioxidants, such as catechin, (-)-epicatechin, (-)-gallocatechin, (-)-epigallocatechin, (-)-gallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin gallate. The anti-oxidative activity of green tea polyphenols have been confirmed in *in vitro* experiments (Jung *et al*., 1994; Mohri *et al*., 1999).

The suppressive effects of green tea polyphenols on lipid oxidation in cultured fish meat also have been investigated (Ishihara *et al*., 2000). Moist pellets supplemented with different concentrations of green tea polyphenols (0, 0.02 and 0.2% (w/w)) were fed to young yellowtails for 4 weeks. Changes in peroxide value (POV), TBARS value and the metmyoglobin formation

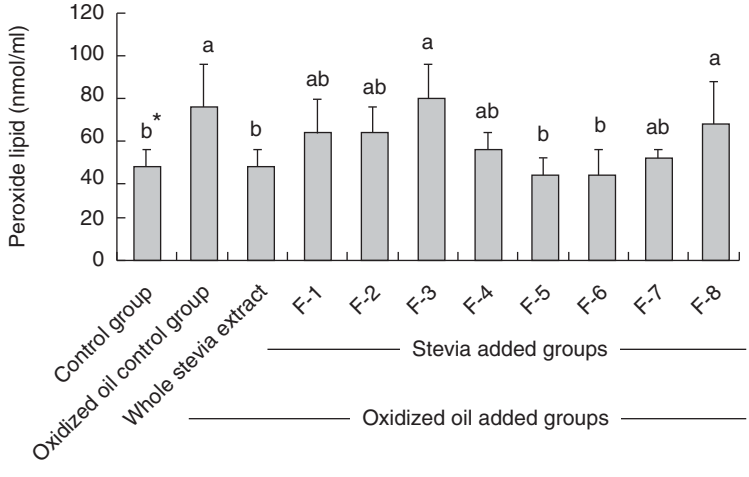

Fig. 8.3. The dietary effect of stevia fraction on the concentration of peroxide lipid (LPO) in rainbow trout serum. *Columns with different letters are significantly different ($P < 0.05$).

ratio in meat were measured during iced storage. An increase in all the measured values was suppressed by feeding green tea polyphenols. Feeding green tea polyphenols lowered the POV and TBARS value on the day when fish were killed. These results show that green tea polyphenols in the diet had a physiological anti-oxidative potential. Feeding green tea polyphenols for 4 weeks was more effective than 2 weeks, and also the feeding of 0.2% green tea polyphenols was more effective than feeding 0.02% green tea polyphenols. Therefore, it was suggested that green tea polyphenols were effective for maintaining freshness during transportation of cultured fish, and might contribute to the production of healthy cultured fish. Anti-oxidative and hepatoprotective effects of catechin have also been confirmed in rat (Byun *et al*., 1994).

5.2.3 Banana

There are various conventional methods for measuring active oxygen elimination ability such as the electron spin resonance method (ESR method) or cytochrome C reduction method, but a new chemiluminescence system for the measurement of reactive oxygen scavenging activity (the XYZ method) was proposed by Okubo and Yoshiki (2000). This method can identify the role of each substance in a reactive oxygen scavenging system as reactive oxygen species, hydrogen donors or mediators.

Measurements of the active oxygen scavenging activity of muscles from red sea bream fed a control diet or diet supplemented with banana powder were made using the XYZ method. Results suggested that the active oxygen scavenging activity of muscle was elevated in red sea bream fed with banana powder (Horiuchi, 2003).

6 Effects on Product Quality

Nowadays consumers are interested in the quality of cultured fish especially their freshness, lipid content, colour, odour, taste, texture, quality persistence during distribution and product safety (Sato and Takeuchi, 1996).

The lipid content of cultured fish is usually higher than that of wild fish because of over supplementation of lipids in the diets used and limited exercise. Controlling the lipid content of cultured fish by starvation and addition of natural products to diets has been investigated.

6.1 Leaf nutrient concentrate

Leaf nutrient concentrate (LNC) was produced using the leaves of *Brassica oleracea*, *Medicago sativa* and *Avena sativa.* The green plants were disintegrated by a hammermill and subsequently pressed in a single-screw press. The expressed green juice was treated with Silo-Ferm (a mixture of

Lactobacillus plantarum and *Pediococcus acidilacti*) to lower the pH to 4.0 to precipitate the proteins. The protein coagulum was separated, dried and used as a diet additive.

The effects of LNC in the diet of rainbow trout were investigated. Rainbow trout fed a diet supplemented with LNC did not have the characteristic delicate acidulous taste of fresh rainbow trout and the muscle lipid level was reduced with increased LNC in the diet (Johansson *et al*., 1991; Johansson 2001).

6.2 *Moringa oleifera* leaf meal

The growth performance of Nile tilapia fed raw moringa leaf or diets containing methanol extracts of the moringa leaf meal were evaluated (Afuang *et al*., 2003). Weight gain was reduced and lipid accretion decreased with increased inclusion of raw moringa leaves, and ash content increased. Dietary moringa methanol extracts did not affect growth or lipid and ash content of the body, but it did reduce protein accretion compared to a fishmeal control diet.

6.3 *Stevia rebaudiana*

An improvement in the carcass quality by addition of stevia extract (BMD stevia extract) to the diet at levels of 1000 and 2000 ppm was observed in rainbow trout, based on an increase in the oxidative stability of the fish. Also, the breaking load at the initial phase of biting increased and the strain value (%) at the breaking point in fish flesh became lower. These results meant that chemical and physical properties of fish flesh were improved by the addition of stevia extract to the diet (Sato, 2003).

6.4 Dhanincha *Sesbania aculeate*

The effect of purified alcohol extracts from dhanincha seeds on the body composition of common carp were reported (Hossain *et al*., 2001). Fish were fed various diets including a control diet and diets containing 0.3, 0.5, 1.2 and 2.4 g/kg alcohol extract from dhanincha seeds. The muscle and plasma cholesterol levels of fish fed diets containing 1.2 and 2.4 g/kg of extract were significantly reduced. Fish fed diets containing 2.4 g/kg of extract had significantly higher whole-body moisture content with a concomitant decrease in lipid content. There was no significant difference ($P > 0.05$) between the whole-body crude protein and ash content of fish fed different experimental diets.

6.5 Green tea extract, ground used tea leaves and tea polyphenols

Green tea is one of the most popular drinks in Japan. Associated with the manufacture of tea drinks, there is a large quantity of used tea leaf waste. In the used tea leaves, catechin and cellulose remain; therefore, there is an interest to recycle the used tea leaves.

The effects of green tea extracts and grounds on growth and lipid content in two types of cultured fishes, yellowtail and ayu, were examined. In yellowtai1, the accumulation of body lipids was suppressed by the supplementation of 3.6% green tea grounds and 0.7% green tea extracts in the diets. In ayu, it was suppressed by the supplementation of 1% green tea extracts in the diet, while the effect of tea grounds was not significant. The fish fed green tea extracts or tea grounds-supplemented diets commonly had decreased average body weights, but their condition factors were kept at the same level as fish fed the control diet (Kono *et al*., 2000).

Green tea polyphenols are used for flavour improvement and maintaining freshness of processed food. Green tea polyphenols exhibit antioxidant action and have been confirmed to have texture improvement effects on domestic animals. It is thought that these effects can be expected for cultured fish (Ishihara *et al*., 2000).

The texture improvement effect by feeding green tea polyphenols to young yellowtail was studied. The addition of green tea polyphenol at 0.02 and 0.2% to the diet did not have any negative influence on the growth of the fish. Extension of rigor mortis was induced by addition of polyphenols. Breaking load, which relates to muscle texture, however did not differ with polyphenol addition.

In the polyphenol-supplemented group an increase in the number of general and psychotrophic bacteria was apparently inhibited during the preservation of muscle in proportion to the level of polyphenols added. Production of ammonia, trimethylamine and dimethylamine was lowered in the polyphenol-supplemented group compared to the control group. This can be expected to increase quality.

As a feed supplement, catechin has been reported to be an antioxidant and hepato-protective agent that improves liver function and lipid metabolism in rats. Nakagawa *et al*. (2000) reported the effect of dietary catechin on vitamin C metabolism in young red sea bream. Total ascorbate concentration in the serum and liver were increased significantly in the catechin-fed groups relative to the control. Feeding with catechin suppressed non-essential fatty acids and total lipid in serum. Liver lipid also was depressed by feeding catechin. The collagen fraction soluble at 20°C was lower and the insoluble collagen fraction (not soluble at 70°C) was higher in the catechin-fed group. These results suggest that dietary supplementation with catechin improved vitamin C metabolism in young red sea bream.

The effects of leaf powder of tochu *Eucommia ulmoides*, a kind of tea, on the texture of eel *Anguilla japonica* muscle have been studied (Tanimoto *et al*., 1993). The muscle of eels fed tochu leaf powder was 1.8 times harder

than the control. The component analysis showed no difference in moisture, lipid or protein content between the muscles of the control and the tochu leaf-fed eels. Not only the extracted neutral fat but also the compound fat of the raw muscle of tochu leaf-fed eel and the control had similar lipid and fatty acid composition. However, there was a great difference between the tochu leaf-fed eel and the control concerning the amount of muscle protein stroma fraction, which mainly consisted of collagen. Microscopic observation showed that the perimysium and endmysium, which were the main components of the stroma fraction of the muscle of tochu leaf-fed eel, were firm and thick compared to those of the control. These findings suggest that the intake of tochu leaf powder hardened the muscle.

6.6 Banana

Occurrence of flesh juice in fish slices or sashimi gives a negative image to consumers during distribution. Horiuchi (2003) reported that flesh juice meat drip apparently decreased in a slice of rainbow trout fed diets containing 0.5% banana powder. He also reported the positive effect of banana supplement for delaying lipid oxidation and prolonging fish freshness. He supposed antioxidants such as polyphenols or carotenoids play some roles in these quality changes.

7 Antinutritional Factors

Aquaculturists sometimes find the results relating to the additive effects of supplements derived from plant material for cultured fish diets disappointing. Sometimes no effect or even negative effects were observed in cultured fish, despite *in vitro* experiments confirming that plant materials are biologically active. The lack of positive effects may be the result of low absorption in the fish intestine or the low stability of active components when they coexist with original feed ingredients.

In the case of green tea polyphenols, positive effects on human health have been well documented, but *in vitro* studies have shown that plant polyphenols generally bind to proteins. Green tea polyphenols significantly lowered the apparent digestibility of casein in rats. Green tea polyphenols have an inhibitory action on caecal fermentation, in particular, *n*-butyrate production, and they lower both protein digestion in the small intestine and the activity of microflora in the large intestine (Ohnishi *et al*., 2005).

It is also widely known that there are a number of antinutritional factors in terrestrial plants that are related to fish growth and health. These are listed in Table 8.4 (Guillaume and Merailler, 2001; Sato, 2003). So when using terrestrial plant materials, aquaculturists have to consider and take care about the species, amounts and pretreatment methods (milling, extraction) used. Also trials are needed to find ways to reduce antinutritional substances (Mukhopadhyay and Ray, 1999).

Table 8.4. Antinutritional factors in terrestrial plants (Source: Guillaume and Merailler, 2001; Sato, 2003).

Fibre	Saponin
Phytic acid	Gossypol
Protease inhibitor	Tannin
Amylase inhibitor	Alkaloid
Lectin	Fungal toxin
Glucosinolate compound	

However, application of supplements from terrestrial plants is useful for the improvement of growth, health and flesh quality of cultured fish. Concerning cultured fish, it is advantageous to be able to control the supply of fish to market and to control quality for consumers. In this respect, it seems necessary to test various plant materials and accumulate scientific data on such additives as supplements or other functional constituents.

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9 Algae

HEISUKE NAKAGAWA[1] AND W. LINN MONTGOMERY[2]

[1]*Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima 739-8528, Japan;* [2]*Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA*

1 Introduction

Micro-algae, especially *Chlorella* and *Spirulina*, are well known not only as human health food but also as fish feed supplements in Japan. Studies on the efficacy of algae as feed supplements for cultured fish began with *Chlorella*-extract and have expanded to include other micro-algae as well as macro-algae. Various algae are receiving attention as possible alternative protein sources for cultured fish, particularly in tropical countries, because of their relatively high protein content (especially for some micro-algae) and production rate. However, use of algae as a protein source seems to be less effective than fishmeal (Stanley and Jones, 1976; Hepher *et al*., 1979).

The use of algae as the sole protein source or supplement in fish feed sometimes results in malformation and impaired growth (Meske and Pheffer, 1978). The nutritional value of such supplements is generally evaluated in terms of growth and survival, with little attention paid to other physiological merits. Although dietary algae as feed supplements may be expected to improve growth and digestive efficiency of feed, the addition of small amounts of algae to the fish diet can produce considerable improvement of physiological condition, fish vitality, disease resistance, desired body composition and carcass quality (reviewed by Nakagawa, 1985; Mustafa and Nakagawa, 1995). Accordingly, some species of algae are now used as supplements in commercial diets.

A variety of fish ingest algae in nature, including some primarily carnivorous fish (e.g. rainbow trout; Leibfried, 1986). Regular ingestion of algae by wild fish suggests a positive function of dietary algae, although algae have not been regarded as important food organisms for many fish species. Some of this bias relates to a misunderstanding of the availability of various nutrients in algae and a lack of attention to the structural and biochemical characteristics of herbivorous fish. Generally, for example, herbivorous fish

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feed regularly throughout the day and, somewhat like horses, rely on relatively low assimilation efficiencies of large quantities of food rather than high efficiencies with a smaller ration (Horn, 1989; Nakagawa *et al*., 2002; Clements and Raubenheimer, 2005). This chapter describes the effects of dietary algae as feed supplements on physiological condition and fish quality in addition to growth performance, and suggests that better integration of recent information about nutrient supply in and digestion of algae may stimulate interest in use of algal products in fish culture.

2 Micro-algae

2.1 Micro-algae used as feed supplements

Micro-algae, important producers of complex organic substances from solar energy and carbon dioxide, support aquatic basal production as feed organisms for both zooplankton and larger herbivores. *Chlorella*, a unicellular green alga (Chlorophyta), went into mass production in 1964 and has developed as a human health food. Commercial production of the blue-green alga, *Spirulina* (Cyanobacteria) began in 1978. Scientific studies on other micro-algae as potential feed supplements lag behind studies of these two taxa. Both these micro-algae are characterized by high protein content (Table 9.1).

2.2 Effect of micro-algae as feed supplements

2.2.1 Physiological characteristics, feed utilization and growth

Nakagawa (1985) first described effects of *Chlorella*-extract supplementation on the physiological characteristics of cultured ayu (Osmeridae [previously Plecoglossidae]: *Plecoglossus altivelis*), an anadromous fish related to salmonids. The fish is one of the most popular and economically important freshwater fish in Japan. The primary foods of wild fish in rivers are benthic blue-green algae and diatoms (Bacillariophyta) that cover submerged cobbles and boulders. Ayu are also cultured widely on a prepared diet of fishmeal.

Micro-algal feed supplements affect physiological characteristics such as lipid metabolism, disease resistance, carcass quality and vitality in ayu as well as other species. A number of studies have been made into the effects of micro-algae as a feed supplement (Table 9.2). Effects of dietary *Chlorella* as a feed supplement have been assessed in yellowtail *Seriola quinqueradiata* (Carangidae) and eel *Anguilla japonica* (Anguillidae).

Effects of micro-algal supplements on growth and feed efficiency are mixed. Positive effects of supplementation with *Chlorella* and *Spirulina* meal (1–5%) have been reported for nibbler *Girella punctata* (Kyphosidae; Nakazoe *et al*., 1986) and striped jack *Pseudocaranx dentex* (Carangidae; Watanabe *et al*., 1990). Supplementation with *Spirulina* at 2–5% levels

Table 9.1. Chemical composition of *Chlorella* and *Spirulina* (Source: Maruyama and Nakagawa, 2003).

	Chlorella	*Spirulina*
Protein (%)	55–67	55–70
Lipid (%)	8–13	6–9
Carbohydrate (%)	10–20	15–20
Fibre (%)	18–23	2–4
Ash (%)	5–8	6–8
Moisture (%)	3–5	2–5
Chlorophyll (%)	1.5–4	0.8–2
Total carotenoids (mg/100 g)	200–300	200–400
Phycocyanin (%)	–	3.5–7
Ca (mg/100 g)	40–150	100–400
Fe (mg/100 g)	70–250	50–100
K (mg/100 g)	700–1400	1000–2000
Mg (mg/100 g)	100–350	200–300
Vitamin B_1(mg/100 g)	1–3	1.5–4
Vitamin B_2 (mg/100 g)	3–8	3–5
Vitamin B_6 (mg/100 g)	0.3–1.2	0.5–0.7
Vitamin C (mg/100 g)	25–100	–
Vitamin E (mg/100 g)	9–15	5–20
Inositol (mg/100 g)	150–450	40–100
Linoric acid (%)	1.4–2.2	0.86
α-linolenic acid (%)	1.2–2.9	–
γ-linolenic acid (%)	–	0.8–1.3

Table 9.2. Studies of the effects of micro-algae as a feed supplement for fish.

Fish (scientific name)	Algae (level)	References
Ayu (*Plecoglossus altivelis*)	*Chlorella*-extract (1–2%)	Nakagawa *et al.* (1981, 1983, 1984c), Nematipour *et al.* (1987, 1988, 1990)
	Scenedesmus sp. (15%)	Hirano and Suyama (1985)
Carp (*Cyprinus carpio*)	*Spirulina platensis* (2.5–100%)	Nandeesha *et al.* (1998)
	Spirulina sp. (50%)	Hirano and Suyama (1985)
Nibler (*Girella punctata*)	*Chlorella* (5%)	Nakazoe *et al.* (1986)
	Spirulina (5%)	Nakazoe *et al.* (1986)
Red sea bream (*Pagrus major*)	*Spirulina* (2–5%)	Mustafa *et al.* (1994a, b, c, 1995a, 1997), Nakagawa *et al.* (2000)
Eel (*Anguilla japonica*)	*Spirulina* (3%)	Kato (1992)
Striped jack (*Pseudocaranx dentex*)	*Spirulina* (5%)	Liao *et al.* (1990), Watanabe *et al.* (1990)
Yellowtail (*Seriola quinqueradiata*)	*Chlorella*-extract (0.5%)	Kato (1992), Nakagawa *et al.* (1982a, b, 1985)

produced pronounced effects on growth and feed efficiency of red sea bream (Pagridae: *Pagrus major*; Mustafa *et al*., 1994a, b, 1995a, b) without negative effects. However, diets supplemented with *Chlorella*-extract had no such effects on ayu (Nakagawa *et al*., 1981, 1983) or yellowtail (Nakagawa *et al*., 1982a, b). The conflicting evidence might be derived from collateral effects due to improvement of physiological condition by dietary algae. In the giant prawn *Macrobrachium rosenbergii*, dietary *Spirulina* elevated growth performance and feed utilization over ranges of supplementation of 5–20% of the diet (Nakagawa and Gómez-Díaz, 1995).

2.2.2 Carcass quality

There are great differences in body constituents between cultured and wild ayu (Suyama *et al*., 1977; Hirano and Suyama, 1983; Nakagawa *et al*., 1991). For example, the body lipid level of wild ayu is generally less than 3.4%, while that of cultured ayu is over 8.2% (Nakagawa *et al*., 1991). Triglycerides accumulate in muscle around the interneural bones (Hirano and Suyama, 1980) and in the abdominal cavity. In ayu culture during the 1980s, feed oil was employed abundantly to promote growth and spare dietary protein for growth, with an accompanying increase in body lipid levels (Takeuchi, 1978). Such cultured ayu were not desirable to consumers because of fattiness, lack of taste and bad odour. In addition, excessive feed oil supplementation caused obesity, induced various physiological disorders, and depressed market price. Nakagawa *et al*. (1983) found that 1% *Chlorella*-extract supplementation in ayu fed a diet containing 4% feed oil could reduce the muscle lipid level. The stored lipid is quantitatively and qualitatively altered under the influence of *Chlorella*-extract, and consequently becomes more similar to lipid of wild ayu (Fig. 9.1; Nematipour *et al*., 1987). Although the lipid content in the intraperitoneal

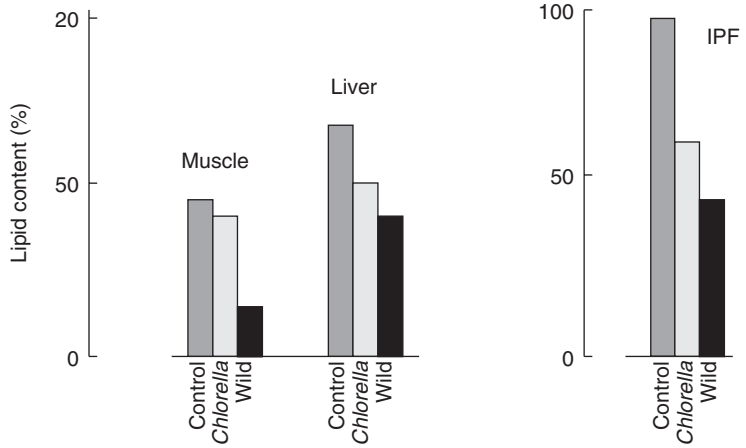

Fig. 9.1. Effect of dietary *Chlorella*-extract (1%) on lipid accumulation in ayu (Source: Nematipour *et al*., 1987). IPF, intraperitoneal fat body.

fat body (IPF) of the control group was almost 98%, supplementation with *Chlorella*-extract reduced the value to 66.7%.

Taste and lipid content closely correlate with each other, but the reduced acceptability of cultured ayu is not only due to the muscle lipid content, but also to muscle protein composition. Sensory evaluation rated the *Chlorella*-extract fed fish better in taste and texture than the control group (Nakagawa, 1985). The distinctive taste and odour of wild ayu relate to various free amino acids and other low molecular weight compounds. Low molecular weight nitrogen compounds are important determinants of the slightly bitter taste which is attributed to the natural dietary micro-algae. Suyama *et al*. (1977) and Hirano and Suyama (1980) related the taste of wild and cultured ayu to the distribution of nitrogen constituents. Specifically, anserine is largely responsible for the specific taste of wild ayu. Although total extractive nitrogen increased with *Chlorella*-extract supplementation in ayu, small differences in muscle anserine were not enough to explain the difference in taste (Nakagawa, 1985).

In yellowtail fed a diet supplemented with *Chlorella*-extract, carcass quality improved (Nakagawa *et al*., 1985). *Spirulina* supplementation to the diet of striped jack resulted in increased free lysine and histidine, consequently improving sensory evaluation (Liao *et al*., 1990). A suitable level of *Spirulina* intake (0.1 g/100 g body weight) improves flesh quality of marketable size striped jack (Watanabe *et al*., 1993).

Muscle protein composition is an important factor in carcass quality. Red sea bream fed a diet supplemented with *Spirulina* at a 2% level exhibited elevated protein synthesis, and the stroma (connective tissue) fraction was significantly increased (Fig. 9.2). The muscle protein composition measured by solubility approached that of wild fish (Mustafa *et al*., 1994c).

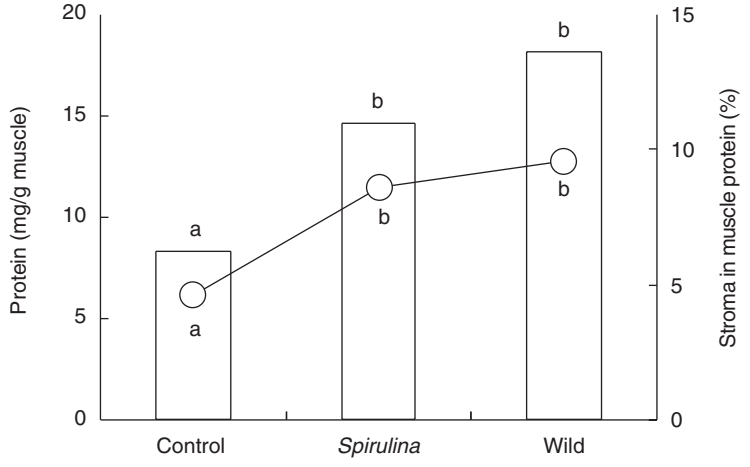

Fig. 9.2. Effect of *Spirulina* supplementation (2%) of diet on protein deposited in muscle (bars) and stroma fraction of muscle (circles) of red sea bream. Different letters are significantly different ($P < 0.05$).

Because the connective tissue includes collagen, the effect of *Spirulina* on muscle collagen composition was examined. Muscle collagen was fractionated into 20°C soluble, 70°C soluble and insoluble fractions. *Spirulina* increased total muscle collagen, as calculated from hydroxyproline content (Fig. 9.3); the collagen fractions soluble at 20°C and 70°C decreased, but the insoluble collagen increased (Nakagawa *et al*., 2000). Fish containing high collagen and high insoluble collagen fractions have firm raw meat texture.

Vitamin C is a cofactor in hydroxylation of proline to hydroxyproline during collagen synthesis. The effect of dietary *Spirulina* on muscle protein composition resembled that of another antioxidant, the flavonoid catechin. This suggests that effects of dietary algae on the muscle collagen fraction may be partly explained by improvement of vitamin C metabolism.

2.2.3 Lipid metabolism

There were significant changes in proximate composition of muscle and the nature of adipocytes in fish fed micro-algae. For the sake of assessing physiological condition of fish, we applied standard medical tests for blood parameters in ayu (Table 9.3). Serum albumin and serum lipid were significantly reduced by the *Chlorella*-extract (Nakagawa *et al*., 1985). Fish serum albumin is a lipoprotein and functions as a lipid-carrier protein. Serum lipoperoxide, measured by the thiobarbituric acid (TBA) method, tended to decrease. Lipoperoxide may be derived from metabolic endogenous oxidation and can cause various physiological disorders. The reduction of the value with dietary *Chlorella*-extract is most probably associated with improvement of lipid metabolism.

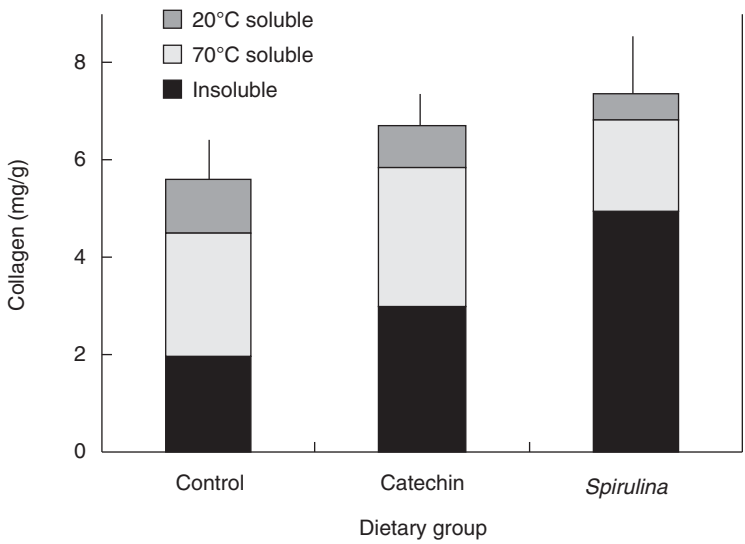

Fig. 9.3. Effect of *Spirulina* supplementation (3%) of diet on muscle collagen composition in red sea bream (Source: Nakagawa *et al*., 2000).

Table 9.3. Effect of *Chlorella*-extract on blood properties of ayu (Source: Nakagawa and Kasahara, 1985).

		Chlorella-extract	
Blood property[a]	Control	1%	2%
Haematocrit (%)	40.9	41.9	43.0
Haemoglobin (g/100 ml)	8.80	9.24	8.86
Serum protein (g/100 ml)	3.20	2.98	2.97
Serum albumin (g/100 ml)	2.58	2.42	2.11[b]
Serum lipid (g/100 ml)	1120	999[c]	899[b]
Serum lipoperoxide (nmol/l)	31.6	24.1	22.4[b]

[a] Serum constituents were expressed as the value in the blood.
[b] Significant at $P < 0.01$.
[c] Significant at $P < 0.05$.

Fish can resist prolonged starvation imposed by wintering and food shortage in a way that metabolizes reserve lipids before consumption of muscle protein. The utilization pattern of body constituents is not consistent during the course of starvation, but is responsive to feeding history. In fish reared on a nutritionally imbalanced diet, the muscle protein is preferentially mobilized prior to the reserve lipid for energy required during food shortage.

As tolerance to starvation is related to the mode of energy metabolism and is critical for survival, the mobilization of body constituents was compared in fish fed diets with or without algal supplementation. *Chlorella*-extract suppressed loss of body weight during starvation in ayu (Fig. 9.4). In addition, analysis of body constituents before and after starvation showed that muscle lipid decreased remarkably and consumption of muscle protein was suppressed in *Chlorella*-extract fed ayu. Mobility of stored energy, such as muscle lipid and intraperitoneal fat, may make a substantial contribution to energy supply. *Chlorella*-extract appeared to activate lipid utilization to energy prior to muscle protein consumption. The same phenomenon occurred in *Chlorella*-extract fed yellowtail (Nakagawa *et al*., 1985).

Dietary *Spirulina* at 2% of ration depressed triglyceride accumulation in the muscle and IPF in red sea bream, resulting in a triglyceride content somewhat similar to that of wild fish (Mustafa *et al*., 1994b). Depression of lipoperoxide and non-esterified fatty acids in the serum could be due to multiple effects of *Spirulina* and ascorbate. Activities of hepatic NADP-isocitrate dehydrogenase and arginase were significantly depressed by a combination of *Spirulina* and ascorbate (Mustafa *et al*., 1997). Carnitine, which requires vitamin C for its synthesis, is a key substance in fatty acid β-oxidation in mitochondria. *Spirulina* supplementation correlated with marked increases in some key substances for β-oxidation of fatty acids, including hepatic free carnitine and long-chain acylcarnitine (Fig. 9.5; Nakagawa *et al*., 2000). Hepatic carnitine palmitoyltransferase activity was markedly elevated, while glucose-6-phosphate dehydrogenase and fatty acid synthase activities were not influenced. Vitamin C deficiency depresses

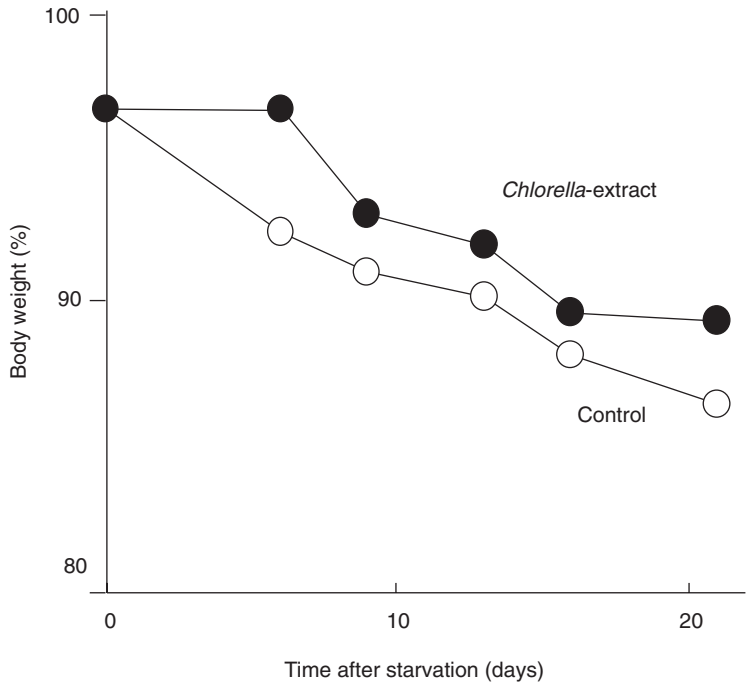

Fig. 9.4. Body weight loss during starvation of ayu fed a diet supplemented with 1% *Chlorella*-extract (Source: Nakagawa *et al*., 1984c).

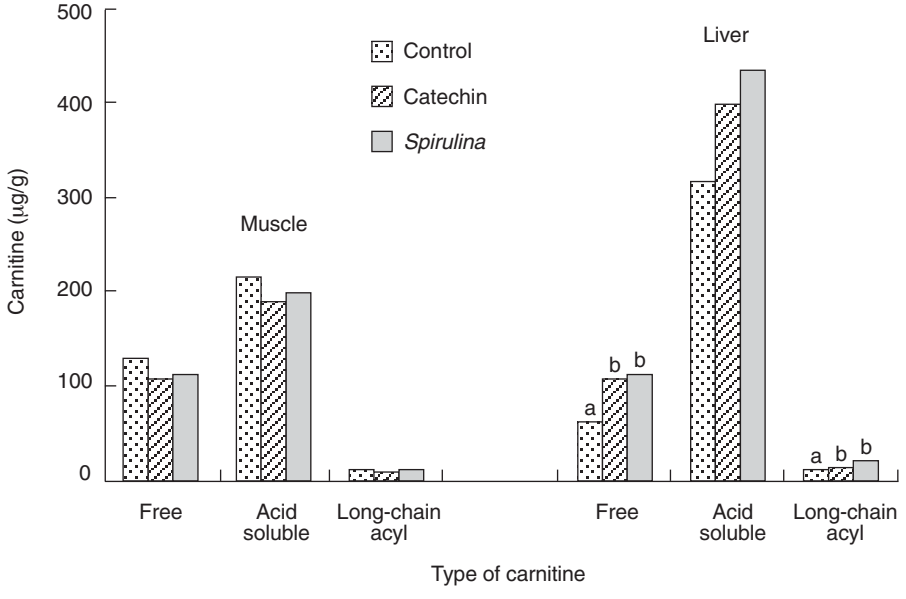

Fig. 9.5. Effect of *Spirulina* supplementation (2%) of diet on carnitine in red sea bream (Source: Nakagawa *et al*., 2000). Different letters on the bars represent significant differences ($P < 0.05$).

lipid utilization for energy and consequently deposition of oxidized lipids in the body. These results suggest improvement of vitamin C metabolism by dietary *Spirulina*. Carnitine was abundant in red sea bream fed a *Spirulina* supplemented diet. Similar to catechin supplementation, free carnitine and long-chain carnitine were significantly increased. The higher levels of carnitine can explain high lipolysis activity, and vitamin C was effectively supplied by both supplements (Fig. 9.6; Nakagawa *et al*., 2000). Stimulation of lipolysis activity can be explained by improvement of vitamin C incorporation with *Spirulina*.

The effect of *Chlorella*-extract on lipid metabolism was investigated by *in vitro* lipolysis in a cell free system of the IPF. The pooled IPF was homogenized in 0.25 M sucrose. Following centrifugation, the top and middle layers of homogenate were assayed for lipolytic activity. The lipolytic activity was measured as the amount of fatty acid liberated from the IPF homogenate in the presence of lipolytic hormones (adrenaline, noradrenaline, cortisol, glucagon and vasopressin). The results show that the lipolysis activity was superior in the *Chlorella*-extract fed group (Fig. 9.7; Nematipour *et al*., 1990). Fish starved for 10 days also showed high lipolytic activity in the group, demonstrating efficacy of the *Chlorella*-extract for activation of the reserved lipids as an energy source.

2.2.4 Vitality

Deteriorating water quality, handling and transportation are virtually impossible to avoid in fish culture. The response to these stressors may result in an increased energy demand and reduce the innate resistance of fish, and thus may consequently affect growth, survival and reproduction.

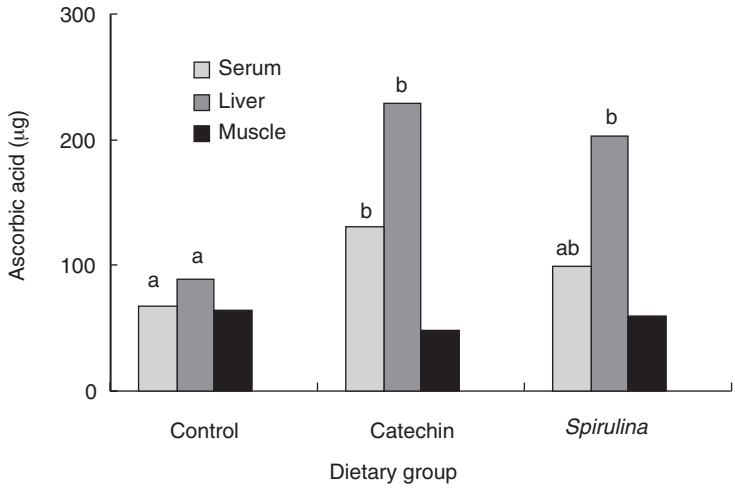

Fig. 9.6. Effect of *Spirulina* supplementation (2%) of diet on ascorbic acid incorporated into red sea bream (Source: Nakagawa *et al*., 2000). Different letters on the bars represent significant differences ($P < 0.05$).

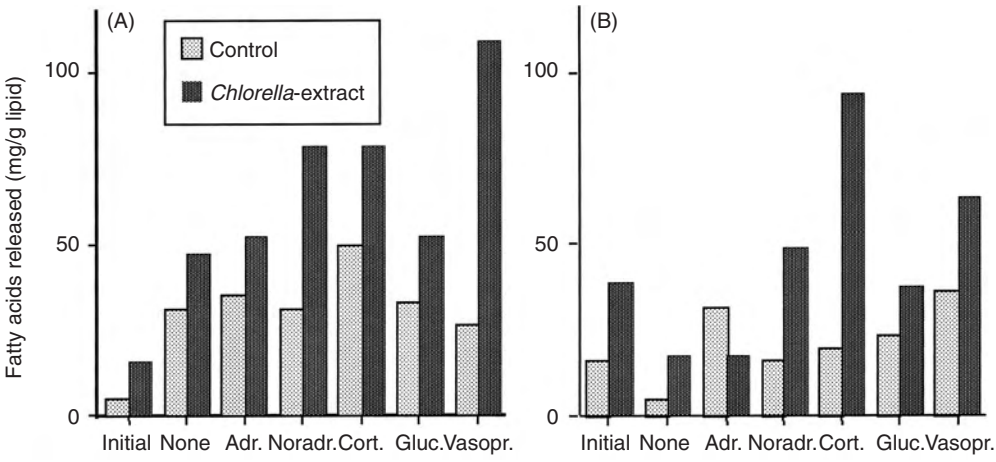

Fig. 9.7. *In vitro* lipolysis activity of the adipocyte of the intraperitoneal fat body (IPF) of ayu fed a diet supplemented with *Chlorella*-extract, (A) after the feeding experiment; (B) after 10 days' starvation (Source: Nematipour *et al*., 1990). The activity was defined as the free fatty acids liberated from the homogenate. Adr., adrenaline; Noradr., noradrenaline; Cort., cortisol; Gluc., glucagon; Vasopr., vasopressin.

As a stress response test, ayu were subjected to air-dipping (exposure in ambient air) for 2 min and returned to oxygen-saturated water. The response was monitored haematologically and serologically for a period of 20 min post air-dipping. Swelling of erythrocytes caused by hypoxic conditions elevated the haematocrit value. Reduced rates of increase in levels of haematocrit, serum glucose and serum lactate in the *Chlorella*-extract group indicated improved tolerance to the stressor (Nakagawa *et al*., 1983). Moderation of the rate of increase in haematocrit and serum glucose also was observed in *Chlorella*-extract fed yellowtail (Nakagawa *et al*., 1982a). The stressor exhibited weak effects on erythrocyte swelling in the *Chlorella*-extract group (Nakagawa *et al*., 1982b).

Tolerance of low oxygen levels in water was evaluated by keeping ayu in a closed container initially filled with oxygen-saturated water. The number of fish that failed to maintain normal orientation was tracked as an indicator of response to declining oxygen levels. The *Chlorella*-extract group was distinctly more tolerant of hypoxic conditions than the control group. The oxygen saturation at which 50% of fish failed to maintain normal orientation was 20% in the control group and only 8% in the *Chlorella*-extract group. After this experiment, the fish were immediately returned to a tank filled with oxygen-saturated water and their recovery and survival were monitored. Mortality after the experiment was 25% in the control group but only 10% in the *Chlorella*-extract group (Nakagawa *et al*., 1984c).

Liver function was defined by recovery time from anaesthesia, because the alcoholic anaesthetic, 2-phenoxyethanol, is detoxified in the liver (Hilton and Dixon, 1982). The *Chlorella*-extract reduced recovery time from the anaesthesia, indicating improved liver function in ayu (Nakagawa *et al*., 1992).

Pancreatic function was determined by a glucose tolerance test. Restoration of serum sugar to normal levels after glucose loading and rise of insulin in response to blood sugar occurred sooner in the *Chlorella*-extract fed ayu than in the control group (Nakagawa *et al*., 1992).

2.2.5 Disease resistance

Although dietary effects of *Chlorella* on disease resistance have been examined in mammals, little is known in fish. In mammals, *Chlorella* exerts a protective effect against hepatic damage induced by ethionine (Wang *et al*., 1979), physiological stress-induced apoptosis (Hasegawa *et al*., 2000), sarcoma (Vermeil and Morin, 1976) and opportunistic infections (Hasegawa *et al*., 1995). The elimination of *Escherichia coli* in organs of mice was enhanced by *Chlorella*-extract, an effect possibly related to the acceleration of superoxide generation and chemokinesis in polymorphonuclear leucocytes (Tanaka *et al*., 1986). In addition, the protection against *E. coli* infection was enhanced by systemic administration of *Chlorella*-extract. A host-mediated antiviral effect against murine cytomegalovirus infection was found in mice fed *Chlorella*-extract (Ibusuki and Minamishima, 1990). Hasegawa *et al*. (1990, 2000) traced substances effective for restoration of drug-induced leucocyte resistance against *E. coli* infection and reduction of stress-induced apoptosis to hot water extracts of *Chlorella*.

Chlorella effects on ayu were tested in a challenge test with *Vibrio anguillarum* (Nakagawa *et al*., 1981). The fish were immersed in the bacterial suspension for 5 min and their survival after immersion was recorded during the following 7 days. The survival of the *Chlorella*-extract fed group was 75%, and that of the control group was 55%. The infected fish showed characteristic symptoms of vibriosis; tumescene of the body surface, dermal ulcers, and haemorrhaging of the pectoral fin base. The organism isolated from dead ayu was positive in agglutination reaction for *V. anguillarum*. In addition, lymphocyte count was significantly increased by *Chlorella*-extract supplementation (Table 9.4; Nakagawa *et al*. unpublished data).

Over-crowded rearing of ayu causes a disease resembling ulcerative dermal necrosis of salmonids. This disease in ayu, termed 'chochin disease',

Table 9.4. Effect of *Chlorella*-extract supplementation on white blood cells of ayu.

No. (/10^4 RBC)	Control	*Chlorella*-extract 2%
Experiment I		
Lymphocyte	22.7 ± 12.8	36.9 ± 13.6[a]
Granulocyte	36.0 ± 34.8	32.0 ± 15.8
Experiment II		
Lymphocyte	10.0 ± 6.0	26.6 ± 8.4[b]
Granulocyte	10.2 ± 6.0	10.0 ± 7.1

[a] Significant at $P < 0.05$.
[b] Significant at $P < 0.01$.

appears to be due to social stress from high density rearing, high water temperature, and excessive dietary lipid. Reduction of stocking density can lead to recovery. Under high-density rearing conditions, almost all members of a control group were attacked by, and about 22% died from the 'chochin disease' (Nakagawa *et al*., 1981). The *Chlorella*-extract group had a depressed incidence of the disease (Fig. 9.8), and serum lipoperoxide level of survivors was lower in the *Chlorella*-extract group than the controls.

We recognize that the preventive action exerted by *Chlorella*-extract might involve some internal barrier to infectious disease, such as an inflammatory response and an increase in the number of phagocytes, rather than an immediate effect on the disease as an antibiotic substance. A well-regulated metabolism and balanced body constituents, both dependent on dietary history, could provide disease resistance.

2.2.6 Other effects

There is anecdotal evidence that feeding micro-algae (*Scenedesmus actus* and *Spirulina maxima*) to ayu has improved egg quality during egg formation (no scientific report).

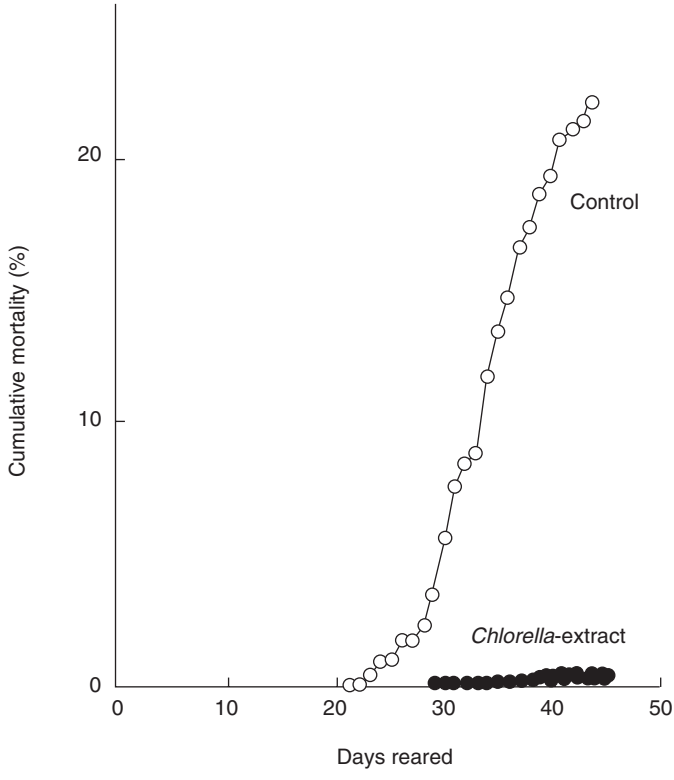

Fig. 9.8. Preventive effect of *Chlorella*-extract supplementation (1%) of diet on mortality caused by 'chochin disease' in ayu (Source: Nakagawa *et al*., 1981).

2.3 Negative effects

Although the replacement of fishmeal by *Chlorella* at less than 50% exerted positive effects (Higashi *et al*., 1972; Goto *et al*., 1974; Suzaki and Hataya, 1976), use of *Chlorella* as the sole protein source and supplement in ayu caused retardation of vitality and growth (Table 9.5). Although ayu can digest blue-green algae and diatoms in their natural environment, green algae in the gut appears to be hardly digested at all (Nakagawa *et al*., 2002). The positive effects of micro-algae as feedstuffs could be due to micronutrients in the algae.

Different types of algae produce different responses. Although macro-algae and *Chlorella*-extract supplementation improved responses to anaesthesia, air-dipping and low dissolved oxygen levels in black sea bream (Nakagawa *et al*., 1984b) and ayu (Nakagawa *et al*., 1984c, 1992), *Spirulina* supplementation impaired liver function, response to air-dipping and resistance to low dissolved oxygen in ayu (Nakagawa *et al*., 2003). Furthermore, recovery time from alcoholic anaesthesia was relatively slow in the 10% supplemented group compared to the 5% group; a similar trend in liver function was also found with resistance to air-dipping and low oxygen.

Responses to algal supplementation vary among species in ways that may not be predictable from studies focusing on a few model animals. The positive effects of dietary *Spirulina* in red sea bream implied the presence of certain substances as synergists of vitamin C (Mustafa *et al*., 1997; Nakagawa *et al*., 2000). However, the reduction of vitality observed in ayu also suggests the presence of substances in *Spirulina* that exert a negative influence on physiological functions. Nandeesha *et al*. (1998) reported positive effects of *Spirulina* used as the sole protein source on growth performance in carp *Cyprinus carpio*, without negative effects. As beneficial

Table 9.5. Effect of *Chlorella* as a protein source on growth of ayu.

	Control	*Chlorella*
Total diet given (kg)	1.63	1.43
Survival (%)	98.3	100
Biomass gain (g)	1285	685[a]
Feed efficiency (%)	78.6	47.9[a]
Protein efficiency ratio	1.64	1.03[a]
Body weight (g)	19.2 ± 3.2	17.8 ± 1.7[a]
Muscle ratio (%)[b]	39.1 ± 6.8	44.7 ± 5.0[a]
Hepatosomatic index	0.67 ± 0.10	0.70 ± 0.10
IPF ratio[c] (%)	3.0 ± 1.2	3.2 ± 1.1

[a] Both the mean and the standard deviation are significantly different from the control ($P < 0.05$).
[b] (Muscle weight/body weight) × 100.
[c] Intraperitoneal fat body ratio = (intraperitoneal fat body weight/body weight) × 100.

effects cannot be always expected in *Spirulina* supplementation, special care should be taken with use of micro-algae as a feed additive for cultured ayu and other species not tested for their individual response.

3 Macro-algae

3.1 Macro-algae used in diets

Many kinds of macro-algae or extracts from them are or have been used as human foods in East Asian and other countries (Table 9.6). Except for some species known to contain 'secondary compounds' (compounds generally not attributable to normal metabolic functions or involved in common metabolic pathways) often considered to be defences against herbivores,

Table 9.6. Genera of marine macro-algae used presently or historically as a human food in various locations around the world. In several cases (e.g. *Ulva*, *Laminaria*, *Fucus*) multiple species of a genus may have been used in the same or different geographic regions or time periods (Source: Compiled from Dawson, 1966; Madlener, 1977; Wikfors and Ohno, 2001; Burtin, 2003).

Cyanobacteria – blue-green algae	Chlorophyta – green algae
Nostoc	*Caulerpa*
	Chaetomorpha
	Codium
	Enteromorpha
	Monostroma
	Ulva
Rhodophyta – red algae	**Phaeophyta – brown algae**
Ahnfeltia	*Alaria*
Asparagopsis	*Analipus*
Bangia	*Ascophyllum*
Chondrus	*Chorda*
Eucheuma	*Cladosiphon*
Gelidiella	*Eisenia*
Gelidiopsis	*Fucus*
Gelidium	*Hizikia*
Gigartina	*Himanthalia*
Gloiopeltis	*Kjellmaniella*
Gracilaria	*Laminaria*
Halosaccion	*Macrocystis*
Hypnea	*Nemacystus*
Iridea	*Nereocystis*
Kappaphycus	*Petalonia*
Nemalion	*Pleurophycus*
Palmaria	*Postelsia*
Polyneura	*Sargassum*
Porphyra	*Scytosiphon*
Pterocladia	*Undaria*

almost all algae have the potential to be used as feed additives. This potential is difficult to assess, however. Although algae represent the world's third-largest aquacultured crop (behind fin fishes and molluscs; FAO, 2002), few of the algae used for human food (Table 9.6) have been studied for their possible use as food additives for fish. Others, for example purple laver *Porphyra* and sea lettuce *Ulva*, are available as fish feed supplements, but their use has not reached its potential because of cost and availability (Nakagawa, 2004). The costs of transportation and processing, as well as insufficient supplies of the algae for large-scale processing, normally preclude their use by feed producing plants. None the less, some macro-algae imported from foreign countries are widely used in fish diets and there are a number of studies into their effects (Table 9.7).

Here we focus on studies of cultured fish. However, because studies of fish in nature may provide culturists with important information about algal nutrients and their digestion, we provide occasional reference to relevant work on wild herbivores, omnivores and carnivores.

3.2 Effects of macro-algal meal as feed supplements

3.2.1 Growth and feed utilization

Early feeding trials with macro-algal meal predicted improvement of vitality, disease resistance and carcass quality. The addition of a very small amount of algal meal has produced a significant increase in the growth and feed utilization of a variety of fish such as: red sea bream (Mustafa *et al*., 1994a, 1995a, b); Japanese flounder *Paralichthys olivaceus* (Xu *et al*., 1993); yellowtail *S. quinqueradiata* (Hamauzu and Yamanaka, 1997; Hamauzu *et al*., 1999); ayu (Amano and Noda, 1985); rockfish *Sebastes schlegeli* (Yi and Chang, 1994); nibbler (Nakazoe *et al*., 1986); and snakehead *Channa striatus* (Hashim and Saat, 1992). The optimum feed efficiency and protein efficiency were attained in black sea bream when the supplementation level of *Ulva* sp. meal was 2.5–5.0% of the diet. Body weight loss of black sea bream during wintering was minimized with supplementation of *Ulva* meal at 2.5–5.0% of the diet (Nakagawa *et al*., 1993; Fig. 9.9). Growth of Japanese flounder *P. olivaceus* was maximized with *Ulva* at 2% of the diet (Xu *et al*., 1993; Fig. 9.10).

Algae also affect other growth indicators when used as dietary supplements. Supplementation with *Ascophyllum*, *Porphyra* and *Ulva* at 3–5% in prepared diets elevated muscle RNA/DNA ratio (protein synthetic activity) and suppressed acid protease activity (protein catabolism) in red sea bream, providing biochemical proof of growth (Mustafa *et al*., 1995a; Tables 9.8 and 9.9). Yone *et al*. (1986b) interpreted the effect on growth as due to an acceleration of nutrient absorption by dietary algae.

Table 9.7. Studies of the effects of macro-algae as a feed supplement for fish.

Fish (scientific name)	Algae (level)	References
Ayu (*Plecoglossus altivelis*)	*Monostroma nitidum* (2.5%)	Amano and Noda (1985)
Rockfish (*Sebastes schlegeli*)	*Undaria pinnatifida* (3–7%)	Yi and Chang (1994)
Nibler (*Girella punctata*)	*Ulva conglobata* (5%)	Nakazoe *et al.* (1986)
Black sea bream (*Acanthopagrus schlegeli*)	*Ulva* spp. (2.5–15%)	Nakagawa *et al.* (1984b, 1986, 1993)
Red sea bream (*Pagrus major*)	*Ulva*-extract (1%)	Nakagawa *et al.* (1984a)
	Ulva spp. (5%)	Mustafa *et al.* (1995a, b), Nakagawa and Kasahara (1986), Satoh *et al.* (1987), Xu and Hirata (1990)
	Undaria pinnatifida (5–10%)	Yone *et al.* (1986a, b)
	Ascophyllum nodosum (3–5%)	Mustafa *et al.* (1994a, 1995a, b), Nakagawa *et al.* (1997)
	A. nodosum (5–10%)	Yone *et al.* (1986a, b)
	Porphyra yezoensis (3–5%)	Mustafa *et al.* (1995a, b), Nakagawa (2004)
Snakehead (*Channa striatus*)	*Ulva* spp. (5%)	Hashim and Saat (1992)
	Sargassum spp. (5%)	Hashim and Saat (1992)
	Polycavernosa spp. (5%)	Hashim and Saat (1992)
	Grasilaria spp. (5%)	Hashim and Saat (1992)
Japanese flounder (*Paralichthys olivaceus*)	*Ulva pertusa* var. (2%)	Xu *et al.* (1993)
Yellowtail (*Seriola quinqueradiata*)	*U. pertusa* var. (3%)	Hamauzu and Yamanaka (1997), Hamauzu *et al.* (1999)
	Laminaria digitata (0.5%)	Nakagawa *et al.* (1985, 1986)

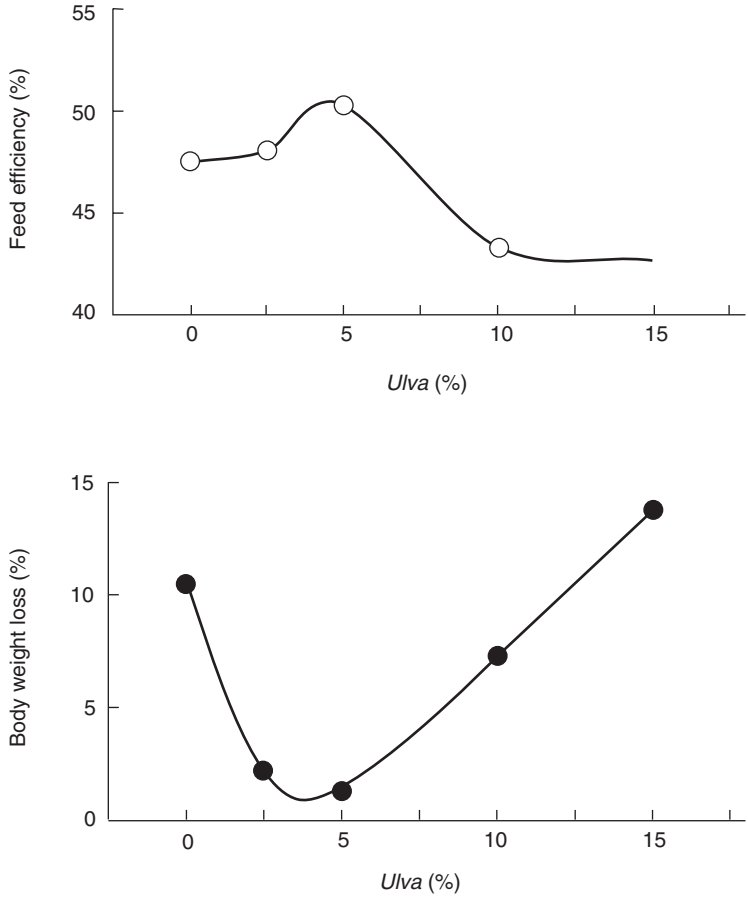

Fig. 9.9. Effect of various levels of *Ulva* meal supplementation of diet on feed efficiency (upper) and body weight loss after 150 days wintering (lower) in black sea bream (Source: adapted from Nakagawa *et al.*, 1993).

3.2.2 Carcass quality

Cultured fish have characteristically high lipid reserves compared to wild fish. Excessive lipid accumulation in cultured fish is a nutritional loss and induces deterioration of carcass quality. Furthermore, factors influencing carcass quality are lipid class composition, fatty acid composition, lipids deposition and distribution, muscle protein composition, muscle conformation, etc. Muscle lipid content does not always affect palatability of fish. Such factors are dependent on season, growing stage, sex and environment as well as food composition.

The addition of a small amount of algal meal to the fish diet can exert considerable effects on carcass quality. While micro-algae generally decrease accumulation of lipid in the muscle, macro-algae often induce an increase in muscle lipid. This is unexpected given the generally low lipid

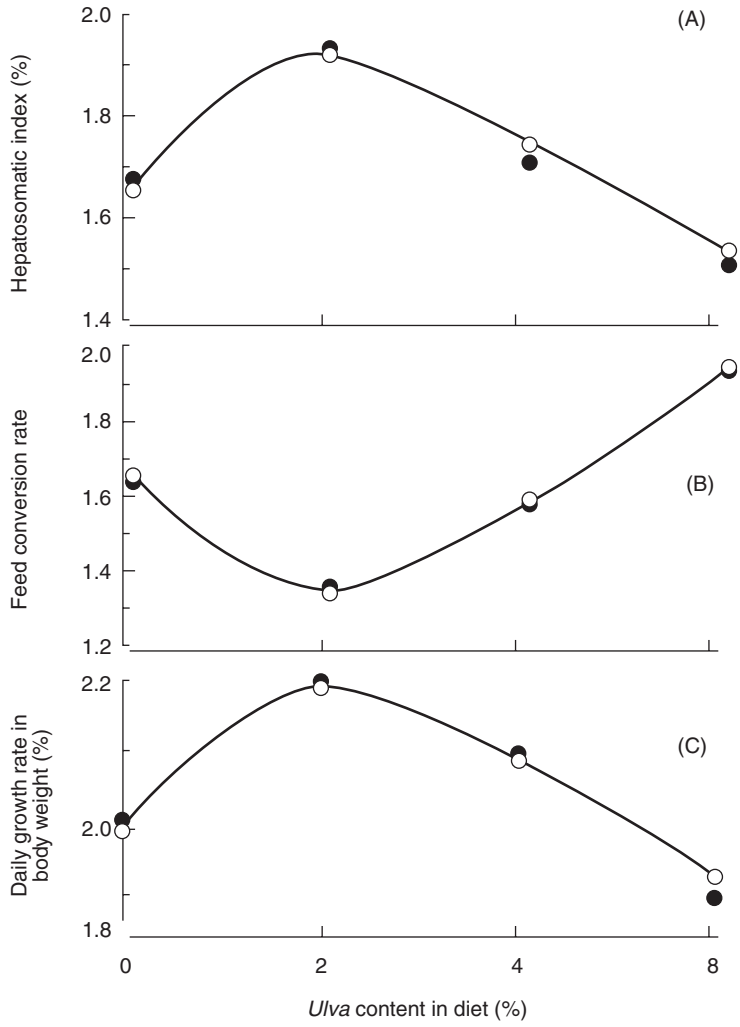

Fig. 9.10. Relation between supplement level of *Ulva* and hepatosomatic index (A), feed conversion rate (B) and daily growth rate in body weight (C) of Japanese flounder (Source: Xu *et al.*, 1993). The symbols ● and ○ represent tanks 1 and 2, respectively.

levels in macro-algae (Montgomery and Gerking, 1980; Wahbeh, 1997; Nelson *et al.*, 2002), suggesting that other compounds mediate the effect. While muscle lipid of a control group of young yellowtail was 1.6%, supplementation of *Laminaria digitata* at 1% increased muscle lipid to 5.2%. Similar tendencies for increased muscle lipid occurred with supplementation of *Undaria pinnatifida* and *Ascophyllum nodosum* in the diet of red sea bream (Yone *et al.*, 1986a, b; Nakagawa, 2004) and with *Ulva* in black sea bream (Nakagawa *et al.*, 1987). Sensory evaluation of fish meat

Table 9.8. Effects of macro-algae supplemented to diet on growth and feed efficiency of red sea bream (Source: Mustafa *et al.*, 1995a).[1]

	Zero year fish				One year fish		One year fish	
	Control	*Asco.*[2] 5%	*Porp.*[2] 5%	*Ulva* %	Control	*Porp.* 3%	Control	*Asco.* 5%
Food given (kg)	3.56[a]	3.78[b]	3.85[c]	3.80[b]	14.3	14.4	6.73	7.12
Survival (%)	77.8	84.0	87.8	84.3	100	98.0	98.8	100
Body weight (g)	13.2[a]	14.8[a,b]	17.2[b]	15.8[b]	214[a]	233[b]	214[a]	227[b]
Feed efficiency (%)	51.5	56.8	62.3	52.4	26.9[a]	29.8[b]	52.2[a]	55.5[b]
Protein efficiency ratio	1.31	1.48	1.60	1.36	0.88	0.95	1.07[a]	1.16[b]

[1] Values with different superscripts in the same line are significantly different ($P < 0.05$).
[2] *Asco.*, *Ascophyllum nodosum*; *Porp.*, *Porphyra yezoensis*.

Table 9.9. Effect of dietary algae on muscle constituents in red sea bream (Source: Mustafa *et al*., 1995a).[1]

	Zero year fish				One year fish	
	Control	*Porphyra*	*Ulva*	*Ascophyllum*	Control	*Porphyra*
Protein (mg/g)	175	178	172	175	168	174
RNA/DNA ratio	3.14[a]	4.17[c]	3.25[a]	3.72[b]	2.09	2.12
Protein/DNA	4.47[a]	5.03[b]	4.30[a]	5.04[b]	76.5	81.1
Acid proteinase	NA[2]	NA	NA	NA	15.5	7.3*

[1] Different superscripts on the same row indicate significant difference ($P < 0.05$);
* significantly different from control ($P < 0.05$).
[2] NA, not analysed.

showed that supplementation of macro-algae in the diet generally improved taste and quality as a whole.

3.2.3 Lipid metabolism and composition

The utilization patterns of energy reserves in fish are not consistent throughout different physiological states such as starvation, and are in part dependent on the rearing conditions and feeding history. The decrease in lipid reserves during starvation is mainly due to exhaustion of triglycerides. The reserved lipids accumulated in the presence of dietary algae would be actively mobilized as an energy source at such times, although lipid levels of algae tend to be low (e.g. 1–6% of dry weight; Montgomery and Gerking, 1980; Nelson *et al*., 2002; Burtin, 2003; Crossman *et al*., 2003) in macro-algae compared to some micro-algae (e.g. diatoms). Accumulated lipids in muscle are actively mobilized as an energy source in black sea bream (Nakagawa *et al*., 1987, 1993), red sea bream (Nakagawa and Kasahara, 1986; Nakagawa, 2004) and yellowtail (Nakagawa *et al*., 1985).

Dietary algal lipid, as well as carbohydrate, may spare protein for growth. For example, in the Grand Canyon, Arizona, a green alga *Cladophora glomerata* heavily epiphytized by oil-rich diatoms often constitutes > 50% of the gut contents of rainbow trout *Oncorhynchus mykiss*, normally a carnivore. When fed *Cladophora* with diatoms, trout digest > 80% of the algal protein and maintain or gain small amounts of weight (+0–0.3% body weight/day; Leibfried and Montgomery, unpublished). They lose weight (−0.3% body weight/day) when approximately 60% of the diatoms are removed. Sparing of protein by lipid occurs at low levels of dietary protein in a variety of other omnivorous or carnivorous fish (Company *et al*., 1999; Nyina-Wamwiza *et al*., 2005).

Lipid composition plays important roles beyond the simple supply of energy. Macro-algal lipids contain, for example, a wide variety of fatty acids, including long-chain polyunsaturates important to neural function and human health. For example, Nakagawa *et al*. (unpublished data) examined stomach contents of three species of Japanese surgeonfishes (Acanthuridae)

that feed exclusively on macro-algae. More than 30 commonly occurring fatty acids were identified, polyunsaturates averaged 35–45% of all fatty acids, and the most notable long-chain polyunsaturates (eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid) averaged 20–30%. Rates of fatty acid absorption appear to increase with carbon chain length and degree of unsaturation (reviewed in Clements and Raubenheimer, 2005), suggesting that fatty acids crucial to early development and nervous system health may experience enhanced rates of absorption.

Under imbalanced nutritional conditions and inadequate feeding regimes (over feeding, inadequate feeding frequency, etc.), muscle protein may be consumed in place of the reserved lipids. Fish reared under such conditions cannot endure food shortage. Consumption of muscle protein would also induce high body weight loss and high mortality. Supplementation of *Ulva* meal to a red sea bream diet (Nakagawa and Kasahara, 1986), or *Laminaria* meal to a yellowtail diet increased consumption of reserved lipids and suppressed muscle protein consumption (Nakagawa *et al*., 1985). Figure 9.11 shows schematic changes in body weight and proximate composition of black sea bream fed a diet supplemented with *Ulva* meal during 138 days' wintering in net cages (adapted from Nakagawa *et al*., 1987). These results indicate preferential consumption of the reserved lipids during food shortage.

As with *Spirulina* supplementation (Mustafa *et al*., 1997; Nakagawa *et al*., 2000), suppression of vitamin C degradation in the diets and acceleration of vitamin C absorption by dietary macro-algae may result in activation of reserved lipids for energy. Macro-algae often contain relatively high levels of vitamin C (Qasim and Barkati, 1985). Wild red sea bream and black sea bream ingest algae in nature, and the proportion of algae ingested increases before wintering. The ingestion of algae before wintering could help to activate reserved lipids for energy during the winter period.

3.2.4 Vitality

Stressors during fish rearing such as high rearing density, treatment, low dissolved oxygen, water pollution and nutritional imbalance can depress disease resistance. Figure 9.12 shows the effects of algae supplementation (*Ascophyllum nodosum*, *Porphyra yezoensis* and *Ulva* sp.) on liver function and vitality (low-oxygen condition and air-dipping) in red sea bream (Nakagawa, 2004). Recovery time from 30 min of anaesthesia with 2-phenoxyethanol, an indication of liver function, was shorter in the fish fed the algae-supplemented diet. Furthermore, fish fed the algae-supplemented diet recovered more rapidly than controls from 5 min of exposure to air. Resistance to low-oxygen levels was higher in algae-fed red sea bream as observed for: *P. yezoensis*, *Ulva* sp. and *A. nodosum* (Nakagawa *et al*. 1997; Nakagawa, 2004), in black sea bream *Ulva* sp. (Nakagawa *et al*., 1984b), and in rockfish *S. schlegeli* receiving *U. pinnatifida* (Yi and Chang, 1994). Many algal species are likely to improve vitality in a similar way because they contain a wide array of macronutrients, micronutrients and other compounds. For example, supplementing diets with dimethyl-β-propiothetin, a common

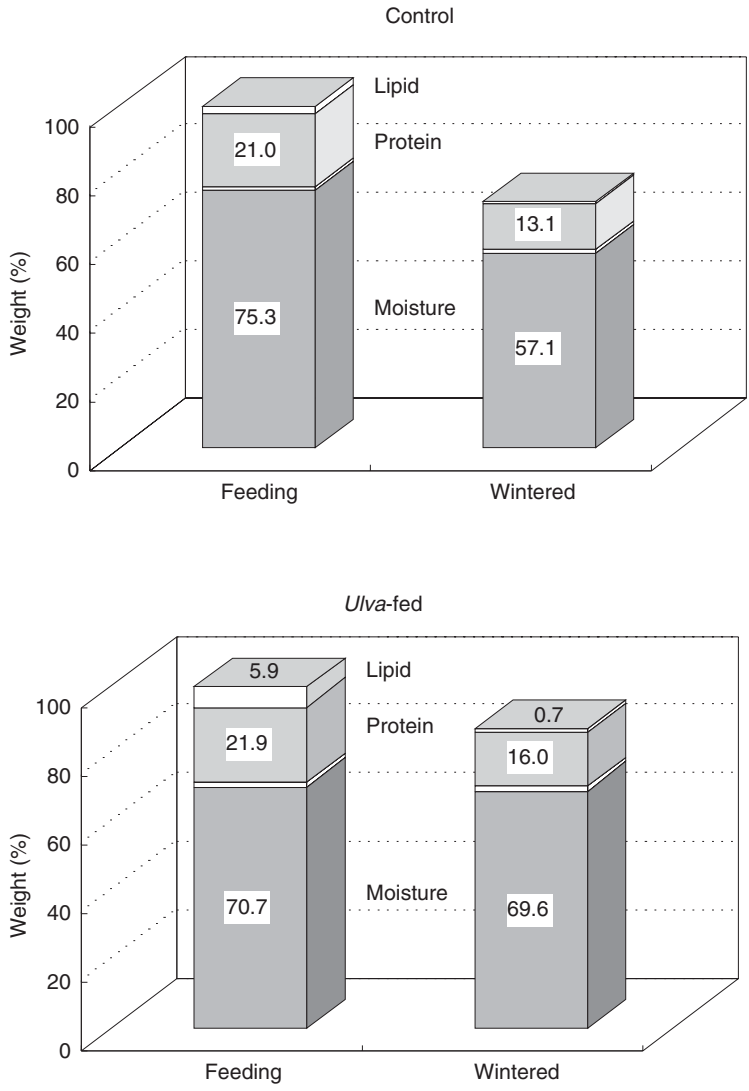

Fig. 9.11. Effect of *Ulva* meal (10%) as a supplement on muscle proximate composition of black sea bream (Source: adapted from Nakagawa *et al.*, 1987). The fish were wintered for 138 days without feeding in net cages.

compound in algae (White 1982), improved physical vitality in rainbow trout, goldfish *Carassius auratus* and carp (Nakajima, 1991a, b).

3.2.5 Disease resistance

Disease resistance is of great interest for fish culturists. Physiological morbidity caused by nutritional imbalance and stressors favour bacterial infection and parasitism. Supplementation of *Ulva* meal to a prepared diet at 5% elevated phagocytosis in black sea bream (Nakagawa, unpublished

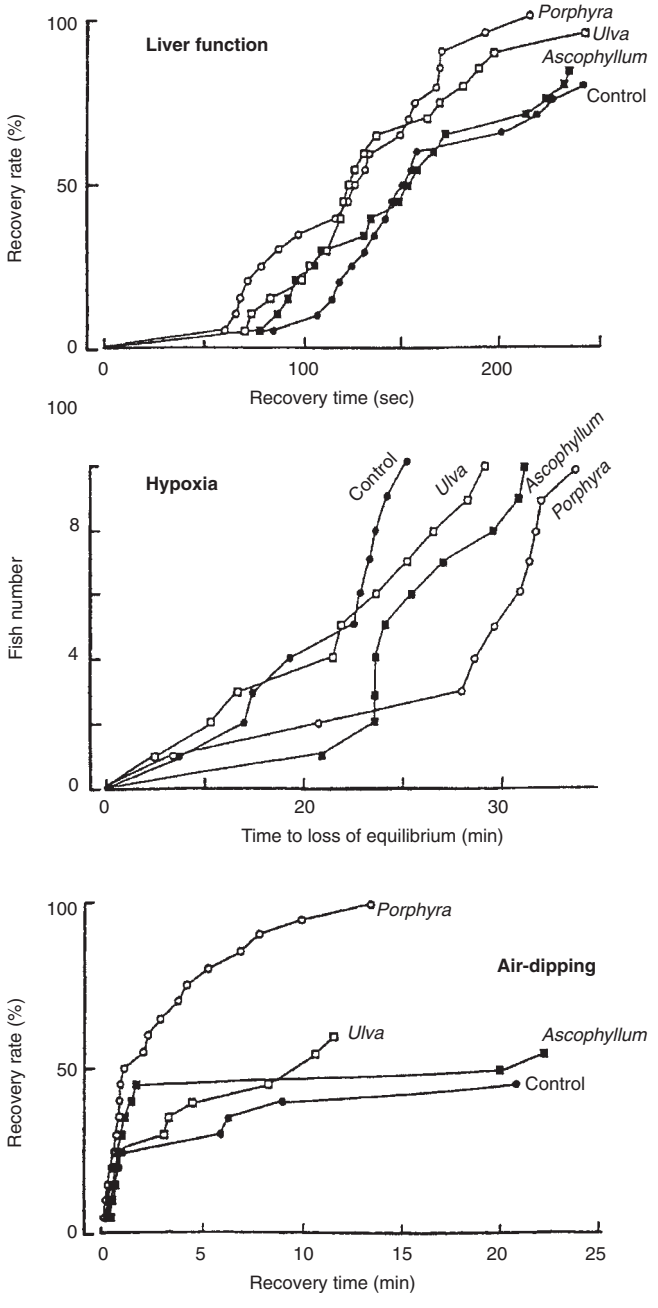

Fig. 9.12. Effect of various algae (5%) on liver function, hypoxia tolerance and air-dipping resistance in zero year red sea bream (Source: Nakagawa, 2004). Liver function: Recovery time was measured from anaesthetization with 1% of 2-phenoxyethanol for 30 min. Hypoxia tolerance: Fish were kept in a 5 l closed container filled with oxygen-saturated water. The number of fish that could not maintain a normal upright orientation was monitored. Air-dipping tolerance: Fish were exposed to air for 5 min and replaced in oxygen-saturated water. The number of fish that swam laterally along the bottom was monitored.

data). Satoh *et al*. (1987) examined effects of similar supplementation with *Ulva* on numbers of lymphocytes and granulocytes, agglutinin titre, and haemolytic and bactericidal activities in red sea bream (Figs 9.13 and 9.14).

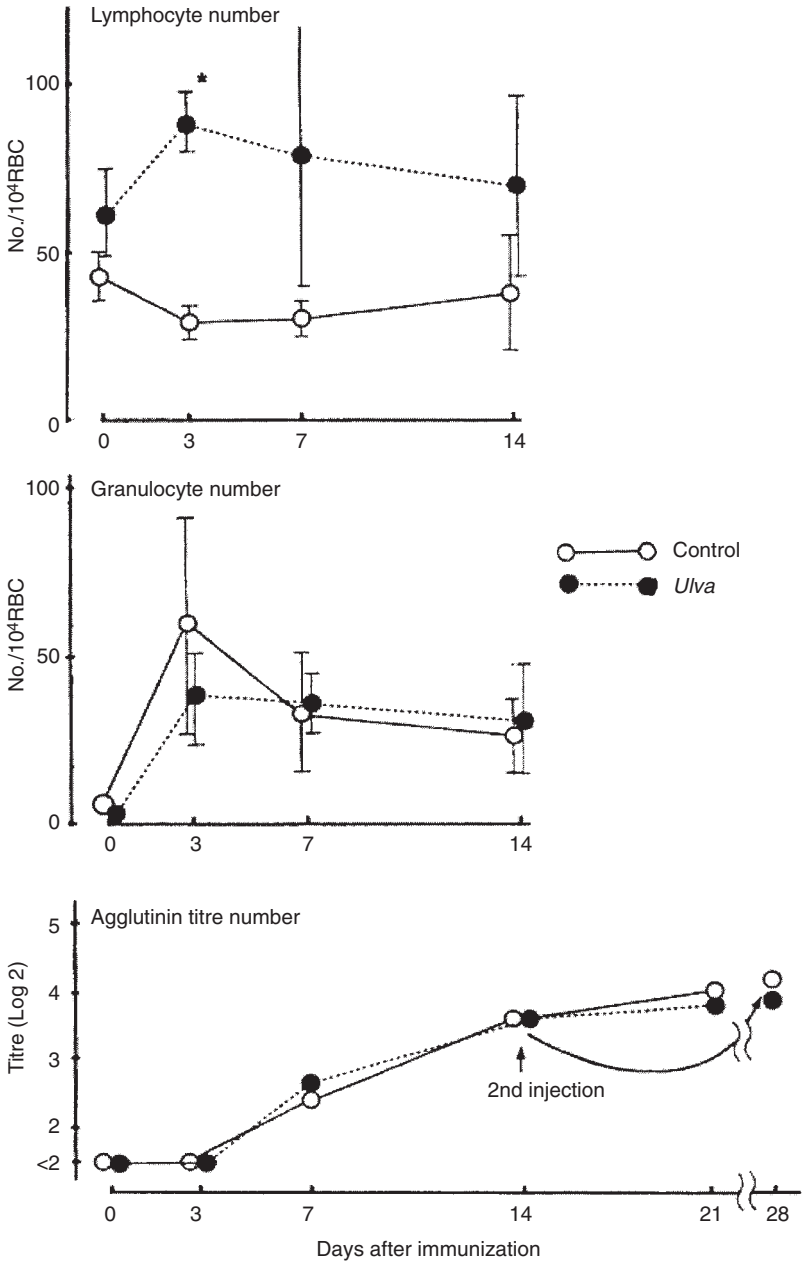

Fig. 9.13. Effect of *Ulva* meal supplementation (5%) on leucocyte number and agglutinin titre in immunized red sea bream (Source: Satoh *et al*., 1987). Results show mean ± SE; **P* < 0.001.

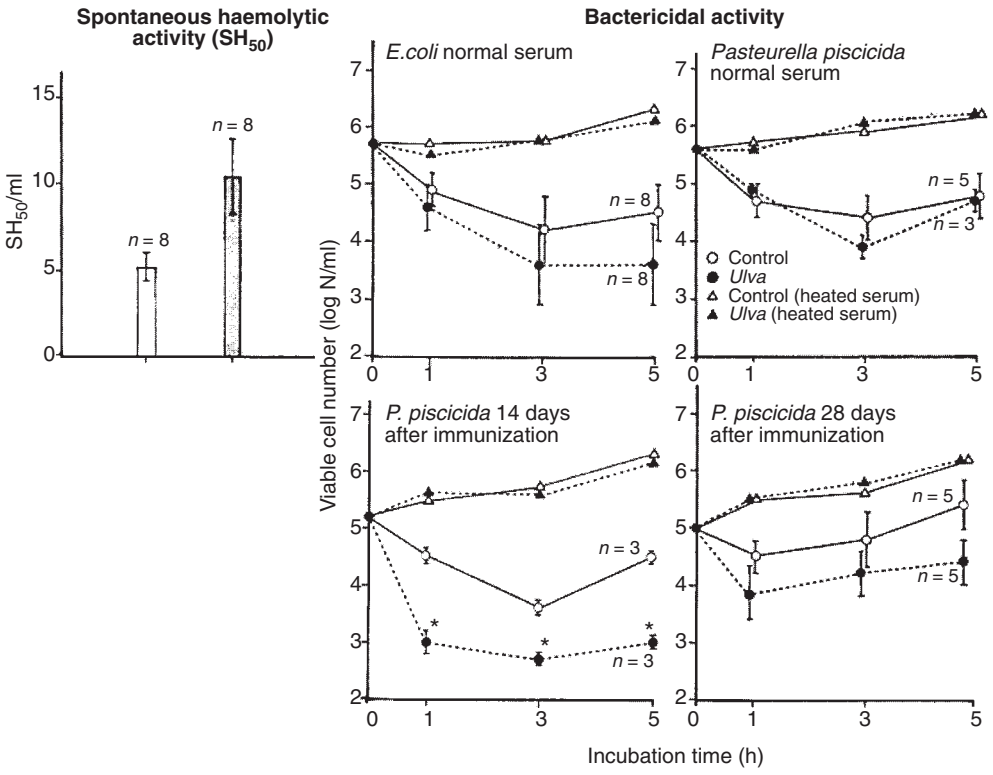

Fig. 9.14. Effect of *Ulva* meal supplementation (5%) on complement activities in red sea bream (Source: Satoh *et al.*, 1987). Results show mean ± SE; $^*P < 0.01$.

An effect was found only in lymphocyte number. In addition, spontaneous haemolytic and bactericidal activities were measured, but only bactericidal activity after immunization was enhanced.

In yellowtail, continuous feeding of sardine *Sardinops* sp. causes a nutritional disease that retards growth and causes high mortality, kidney malfunction and high serum lipoperoxide. Retardation of growth and high mortality was seen in the young yellowtail fed sand lance *Ammodytes personatus* and this was prevented by supplementation of 0.5% of *L. digitata* to sardine *Sardinops melanocticta* (Fig. 9.15; Nakagawa *et al.*, 1986). Simultaneous addition of a vitamin mixture enhanced the effect, suggesting a synergistic effect of algae and certain vitamins. Atlantic salmon *Salmo salar* fed on a diet supplemented with alginate had a high survival rate and complete haemolytic activity after challenge by *Aeromonas salmonicida* (Nordmo *et al.*, 1995). Other possible uses of dietary algae to reduce risk of disease need to be established in additional trials.

3.2.6 Constraints on use of macro-algae as food supplements

Many factors influence the chemical composition of macro-algae collected in nature, including taxonomic group, season, available nutrients, growing

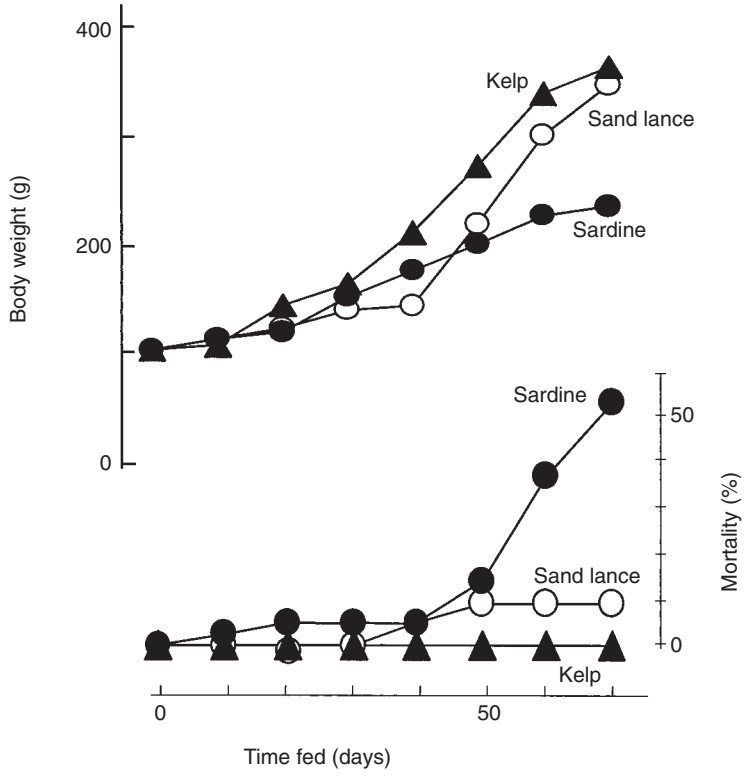

Fig. 9.15. Preventive effect of supplements on nutritional disease of sardine-fed yellowtail (Source: Nakagawa *et al.*, 1986). Kelp: sardine + *Laminaria* meal + vitamin mixture.

conditions, age, growth rate, location on the thallus and rates of grazing. Thus, detailed composition of supplements may be difficult to predict unless they derive from algae maintained in artificial culture. In nature, herbivorous fish may benefit from ingestion of many species of macro-algae (Montgomery, 1980; Montgomery *et al.*, 1989; references in Crossman *et al.*, 2003) as well as the micro- and macro-algae that grow epiphytically on the targeted algal foods. Such mixtures could dilute harmful compounds contained in some ingested algae while compensating for the absence or low levels of beneficial compounds in other species.

Algal meal or powder may contain natural products that interfere with the efficacy of beneficial compounds, inhibit digestion or otherwise serve defensive functions (Targett and Mitsui, 1979; Hay, 2001). Although toxic algae are becoming a serious problem in various fisheries (Noga, 1998), these are frequently planktonic micro-algae with rapid population growth rates that present difficulties when cultured fish cannot be isolated from them. Those used as human foods also are used as feedstuffs for fish, because they are macroscopic and normally grow attached to substrata, and they are likely to be easier to control than unicellular phytoplankton.

Digestive efficiency of whole-algae supplements may be reduced by the inability of many fishes to macerate the algal thallus and/or disrupt the walls of individual cells. Algae may be masticated by the action of pharyngeal teeth (minnows, cichlids, parrotfish) or gizzard-like stomachs (mullet, some surgeonfishes; reviewed in Clements and Raubenheimer, 2005), but many species lack these traits. Zemke-White *et al*. (1999) determined that low pH solutions solubilized unidentified compounds from a variety of macro-algae, damaged plasma membranes, and probably disrupted the integrity of algal cell walls (no structural changes were noted in transmission electron micrographs) sufficiently to allow penetration of digestive enzymes into cells. None the less, short gastric residence times, moderate gastric pH and relatively low ambient temperatures may not support extensive digestion for many fish.

4 Possible Beneficial Substances in Micro- and Macro-algae

Relatively few studies of fish nutrition address enhancement of physiological function, although the studies cited above reflect a variety of positive physiological benefits in various fish. The activation of physiological functions by algae also might be achieved through regulation of hormonal balance, however, information in this area is rather limited.

Many advantageous effects of dietary algae are derived from their contributions of minerals, dietary fibre, carotenoids, feeding attractants and vitamins, as well as synergistic effects with vitamins and antioxidants. The utility as a binder for prepared diets cannot be disregarded. Many physiologically active substances have been identified from algae and the following compounds have beneficial uses in fish nutrition. However, the efficaciousness of these substances and mechanisms are not well established.

4.1 Carotenoids

Carotenoids included in algae have the potential to exert positive effects. As the effects on pigmentation are described in the chapter on microorganisms (Chapter 7 this volume), the effect on pigmentation by algae was not included in this chapter. Brown algae are rich in fucoxanthin, β-carotene, violaxanthin, while common carotenoids in red algae include β-carotene, α-carotene, zeaxanthin and lutein. Green algal carotenoids are similar to those of higher plants, and include β-carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin and neoxanthin (reviewed in Burtin, 2003). The blue-green alga *S. maxima* is useful for pigmentation of cultured striped jack (Okada *et al*., 1991). Antioxidant properties of algal carotenoids may prevent problems linked to oxidative stress.

4.2 Minerals

A diet with inadequate mineral balance induces low disease resistance and abnormal metabolism. Algal minerals have the potential to overcome such effects. Minerals of macro-algae, for example, commonly constitute 8–40% of dry weight, are more concentrated than in many land plants and animal products, and contain all essential minerals and trace elements needed for human nutrition (reviewed in Ruperez, 2002). Although iodine is not stable in composed diets, organic iodine included in algae has been used in the treatment of human goitre and should be a physiologically active substance in the fish body.

4.3 Vitamins

Although algae contain many vitamins, including vitamins B_{12}, C and E (Burtin, 2003), there are no specific studies to define the contribution of algae as a vitamin source. However, inclusion of algae with certain vitamins might induce synergistic effects, as shown by Mustafa *et al*. (1997) and Nakagawa *et al*. (2000).

4.4 Polysaccharides

The beneficial effects of dietary fibre have been confirmed in fish as well as mammals. Various effects of binding agents included in algae on growth performance have been well established. Table 9.10 shows the effect of polysaccharides derived from macro-algae on growth rate and feed utilization in snakehead *C. striatus* (from Hashim and Saat, 1992). Carragenan-based diets exerted the best growth and feed efficiency by increasing the water stability of the diet. As these effects might not be solely attributed to the binding properties of the alga, further studies are needed to identify other mechanisms. High levels of soluble dietary fibre may slow, or serve as a barrier to, starch digestion by forming thick mucilages in the gut (Gofii *et al*., 2000). Effects of dietary alginate on disease resistance are confirmed in Atlantic salmon (Nordmo *et al*., 1995).

4.5 Other substances

In mammals, a water-soluble anti-tumour glycoprotein was identified from *Chlorella* (Noda *et al*., 1996, 1998). In fish, Liao *et al*. (unpublished) suggested an effect of γ-linolenic acid in *Spirulina* on suppression of excessive lipid accumulation. *Ulva*-extract increases muscle protein deposition in red sea bream (Nakagawa *et al*., 1984a). Dimethyl β-propiothetin, a precursor of dimethylsulphide which contributes to the typical odour of marine green algae, was reported to enhance growth and swimming activity of several

Table 9.10. Growth rate and feed utilization of *Channa striatus* fry fed diets containing different binders for 8 weeks[1] (Source: Hashim and Saat, 1992).

	Binder					
	Ulva meal	*Sargassum* meal	*Polycavernosa* meal	*Gracilaria* meal	Carrageenan	Wheat flour
Initial weight (g)	$0.24^{a,b}$	$0.22^{a,b}$	0.28^{c}	$0.27^{b,c}$	$0.24^{a,b}$	$0.26^{b,c}$
Final weight (g)	1.81^{c}	1.44^{b}	1.51^{b}	1.20^{a}	2.75^{d}	$1.30^{a,b}$
Weight gain (g)	1.57^{c}	1.22^{b}	1.23^{b}	0.93^{a}	2.51^{d}	$1.04^{a,b}$
Growth rate[2]	654^{d}	555^{c}	439^{b}	344^{a}	1046^{c}	4000^{b}
Total feed intake (g/fish)	2.70^{b}	$2.40^{a,b}$	$2.44^{a,b}$	2.17^{a}	3.48^{c}	$2.40^{a,b}$
Feed efficiency[3]	0.58^{b}	0.51^{b}	0.50^{b}	0.43^{a}	0.72^{c}	0.43^{a}
Protein efficiency ratio[4]	1.14^{b}	$0.99^{a,b}$	$0.99^{a,b}$	0.85^{a}	1.43^{c}	0.84^{b}
Survival (%)	88.0^{b}	77.0^{a}	92.0^{c}	91.0^{c}	94.7^{d}	85.0^{b}

[1] Mean of five replicate groups. Means with the same superscript in the same row are not significantly different ($P < 0.05$).
[2] Growth rate (%) = (final wt – initial wt)/initial wt $\times$ 100.
[3] Feed efficiency = wet weight gain (g)/amount of feed fed (g).
[4] Protein efficiency ratio = wet weight gain (g)/amount of protein fed (g).

fish (Nakajima, 1991a, b). The substance strongly stimulates the striking behaviour of goldfish, crucian carp *Carassius auratus cuvieri* and carp (Nakajima *et al*., 1989). Physiologically active substances such as lectin, aromatic amino acids, fatty acids, polysaccharides, nucleotides and polyphenol may also prove to be beneficial substances in algae. In rats, composed lipids, dietary fibre, polyunsaturated fatty acids and chlorophyll in micro-algae have been suggested to improve lipid metabolism. Antioxidative substances have been isolated from many kinds of micro- and macro-algae. A pancreatic lipase inhibitor was isolated from macro-algae by Bitou *et al*. (1999).

Tea catechin affects vitamin C metabolism. Red sea bream fed diets supplemented with *Spirulina* showed a high incorporation of vitamin C, a high carnitine accumulation and a high level of muscle collagen. These responses resemble the results of dietary catechin (Nakagawa *et al*., 2000) and may be explained by improvement of vitamin C metabolism. *Spirulina* protects against vitamin C degradation, as does catechin (Mustafa *et al*., 1997; Takii *et al*., 1999). The polyphenol content of *Spirulina* powder is less than 0.2%, and this antioxidant can improve vitamin C metabolism as seen in *Spirulina*, as well as collagen synthesis, disease resistance, improvement of lipid metabolism and physical vitality.

5 Present Status and Future Prospects

Analysis of feeding behaviour of wild fish demonstrated that many kinds of fish ingest algae as foods, even carnivorous fish. Accordingly, use of algae

as a feedstuff might aid normalization of physiological condition in cultured fish. As noted above, supplementation level on a commercial basis is limited by the cost of algal meal. In Japan, algae supplemented to commercial diets are mainly brown algae, *A. nodosum*, *L. digitata*, *Laminaria japonica*, *Laminaria hyperborean* and *Laminaria pallida* imported from the north Atlantic Ocean, *Ecklonia maxima* from South Africa, and *Lessonia nigrescens* and *Lessonia flavicanus* from Chile. At present, commercial diets for larval stages and growing stages of fish are supplemented with brown algae at 2% and 1% of diet, respectively.

As the main biochemical constituents and digestibility are different among different types of algae (Montgomery and Gerking, 1980), the effect of dietary algae will probably vary with the species of both algae and fish. Also, adequate levels may be variable depending on the feeding habits. In the carnivorous yellowtail, supplementation of 0.5–1.0% of *Laminaria* meal provided positive effects (Nakagawa *et al*., 1985, 1986).

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10 Chitin

HEISUKE NAKAGAWA

Graduate School of Biosphere Science, Hiroshima University, Higashi-hiroshima 739-8528, Japan

1 Introduction

Chitin, a polymer of glucosamine, is a major organic component in the exoskeleton of crustaceans. Chitin and chitosan (Fig. 10.1), which is obtained by deacetylation of chitin, are unbranched polymers of *N*-acetylglucosamine linked by β-1, 4 glycoside bonds. Crustaceans are important and often the major food organisms of larval fish and contain a considerable amount of chitin. The chitin content of various copepods that are food organisms for wild juvenile fish has been reported to range from 2.1–9.3% (average of 4.6%) dry weight (Table 10.1; Båmstedt, 1986). Table 10.2 shows chitin in the stomach contents of juvenile black sea bream. Chitin made up 3.6% (wet weight) of the stomach contents of 50-day old juvenile black sea bream (*Acanthopagrus schlegeli*; Om *et al*., 2003a), a value that decreased with growth. The chitin content of *Artemia* nauplii, a food organism for larval fish production, ranged between 0.19 and 0.34% (average of 0.26%) on wet weight basis (Nakagawa, unpublished data).

2 Effect on Growth Performance

The effects of chitin, chitosan and glucosamine as food supplements have been established in mammals. In rats, dietary chitin shows a strong inhibitory effect on fat digestion (Deuchi *et al*., 1994) and a synergistic effect with ascorbic acid (Kanauchi *et al*., 1994). However, the effects of these components as feed supplements for fish have not been examined as carefully as in mammals. The positive dietary effects of chitin in fish are summarized in Table 10.3. A chitin-supplemented diet improves growth in red sea bream (*Pagrus major*), Japanese eel (*Anguilla japonica*), yellowtail (*Seriola quinqueradiata*; Fig. 10.2; Kono *et al*., 1987a) and black sea bream (Fig.

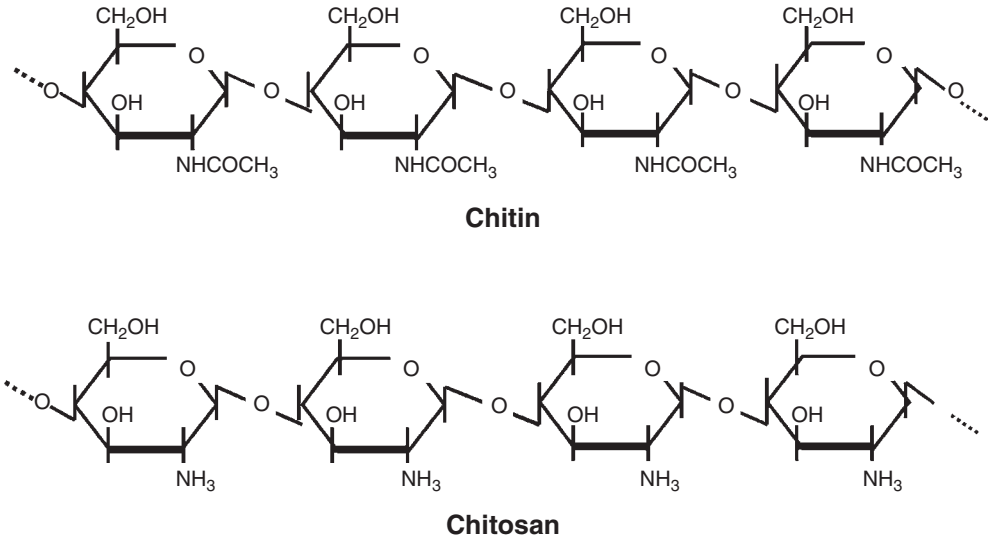

Fig. 10.1. Structure of chitin and chitosan.

Table 10.1. Chitin contents (expressed as percentage of dry weight) of marine copepods (Båmstedt, 1986).

Species	Percentage chitin	Reference
Bathycalanus princeps	6.7	Childress and Nygaard (1974)
Calanus cristatus	5.2	Ikeda (1971)
C. cristatus	3.5	Ikeda (1972)
Calanus finmaarchicus	5.1	Mayzaud and Martin (1975)
Calanus plumchrus	2.1	Ikeda (1972)
Chiridius armatus	5.7–7.4	Båmstedt (1980)
Eucalanus bungii bungii	2.7	Ikeda (1972)
Eucalanus norvegica		
female	3.7–6.4	Båmstedt (1980)
female	4.0	Orr (1934)
male	4.4–6.4	Båmstedt (1980)
male	5.0	Orr (1934)
E. norvegica C–V stage[a]	3.7–9.3	Båmstedt (1980)
	3.1	Orr (1934)
Gaussia princes	6.6	Childress and Nygaard (1974)
Total range	2.1–9.3	
Average value	4.6	

[a] C–V stage, copepodid 5th stage.

10.3; Om *et al.*, 2003a). Although dietary chitin improves growth and feed efficiency, chitosan appears to inhibit these responses. These results can be explained by the presence of high chitinase and the absence of chitosanase and cellulase activities in the stomachs of fish (Kono *et al.*, 1987a). Shiau and Yu (1998) found significant effects of dietary chitin supplementation at a 5%

Table 10.2. Chitin (wet weight) in the gut contents of wild black sea bream (Source: Om *et al.*, 2003a).

Days (range)	*n*	Body length (mm) (range)	Chitin (%) (range)
50 (43–55)	10	19.2 ± 0.2 (16–22)	3.64 ± 0.27 (3.46–3.89)
140 (129–151)	11	61.8 ± 0.1 (42–82)	0.70 ± 0.11 (0.67–0.73)
211 (187–232)	9	94.9 ± 0.2 (66–104)	1.15 ± 0.31 (0.97–1.39)

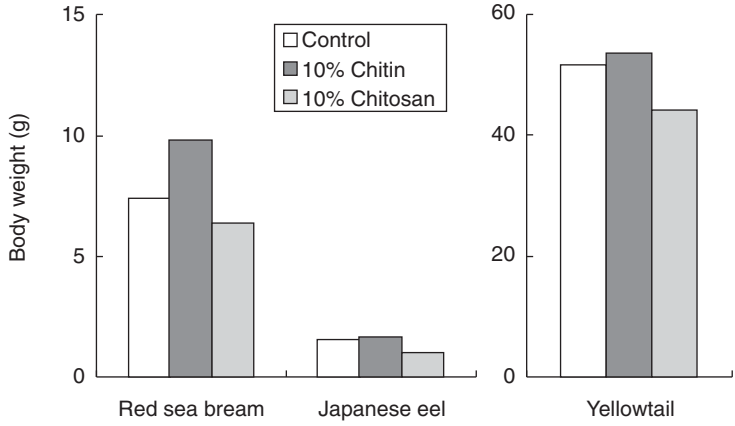

Fig. 10.2. Effects of chitin and chitosan as diet supplements on growth (Source: from data of Kono *et al.*, 1987a).

level in grass shrimp (*Penaeus monodon*), but chitosan decreased growth performance. As to the negative effects of chitosan, Bullock *et al*. (2000) indicated that soluble acidified chitosan in rearing water is highly toxic to rainbow trout (*Oncorhynchus mykiss*).

3 Effect on Metabolism

Responses related to lipid metabolism were significantly suppressed without negative effects on growth performance of black sea bream fed a purified diet supplemented with 10% chitin (Fig. 10.3). Dietary chitin significantly reduced the intraperitoneal fat body (IPF). Shrinkage of the adipocyte diameter of the IPF resulted in a reduction of the IPF ratio. While lipid accumulation in the muscle increased, lipid accumulation in the IPF was significantly reduced. As a result, total lipid accumulation was markedly depressed in chitin-fed fish. In addition, the diameter of lipid droplets in the liver also was reduced by chitin supplementation. As adipocyte diameter might be a useful indicator of lipolysis activity (Jocobson and Smith, 1972), the shrinkage of the adipocyte in the IPF and liver implies certain lipid metabolic changes. These phenomena would be caused by suppression of lipogenesis.

Improvement of growth with chitin supplementation can be explained by an acceleration of protein deposition. Significantly higher protein

Table 10.3. Positive effects of dietary chitin in fish and shrimp.

Inclusion level	Fish (scientific name)	Effects	References
10%	Red sea bream (*Pagrus major*)	Growth	Kono *et al.* (1987a)
10%	Japanese eel (*Anguilla japonica*)	Growth	Kono *et al.* (1987a)
10%	Yellowtail (*Seriola quinqueradiata*)	Growth	Kono *et al.* (1987a)
25–100 mg/kg	Gilthead seabream (*Sparus aurata*)	Innate immune system	Esteban *et al.* (2001)
10%	Black sea bream (*Acanthopagrus schlegeli*)	Growth, metabolism, vitality	Om *et al.* (2003a)
5%	Grass shrimp (*Penaeus monodon*)	Growth, feed efficiency	Shiau and Yu (1998)

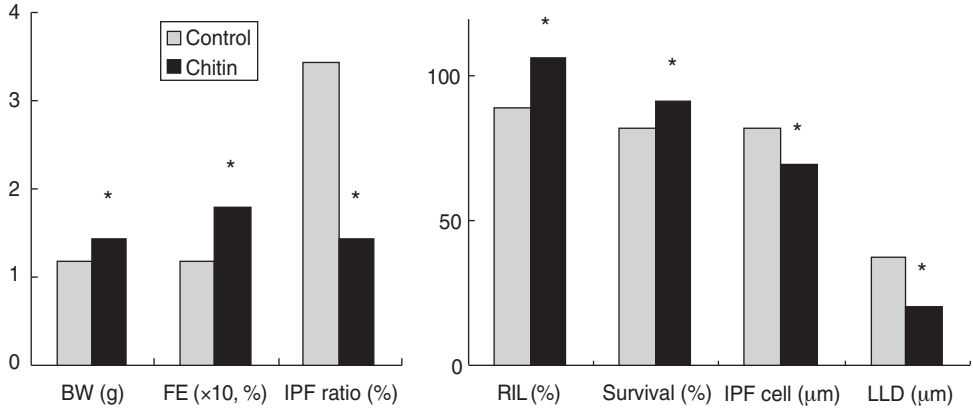

Fig. 10.3. Effects of 10% chitin in moist diet on biometric parameters of black sea bream (Source: modified from Om *et al.*, 2003a). BW, body weight; FE, feed efficiency; IPF ratio, intraperitoneal fat body ratio (fat body/body weight × 100); RIL, relative intestine length (intestine length/body length × 100); IPF cell, adipocyte diameter of intraperitoneal fat body; LLD, liver lipid droplet diameter. *Significantly different from control ($P < 0.05$).

deposition in the muscle and lower lipid in the liver occurred in chitin-fed black sea bream (Fig. 10.4; Om *et al.*, 2003a).

After that feeding experiment, the fish were then starved for 17 days to compare energy metabolism. Mortality, as well as consumption of both muscle protein, as reflected by loss of body weight, and IPF lipid were suppressed by dietary chitin consumption (Fig. 10.5; Om *et al.*, 2003a). Consequently, body weight loss during starvation was lower for chitin-fed fish than that of the control group. Despite the reduced loss of IPF ratio, the large drop in the IPF ratio compared to the drop in body weight implies that energy during starvation came from metabolism of IPF lipid prior to muscle protein consumption. Low lipid accumulation in the fish fed the chitin could be due to suppression of lipogenesis and/or activation of lipolysis, either of which could be suggested by preferential utilization of the lipid reserves during starvation.

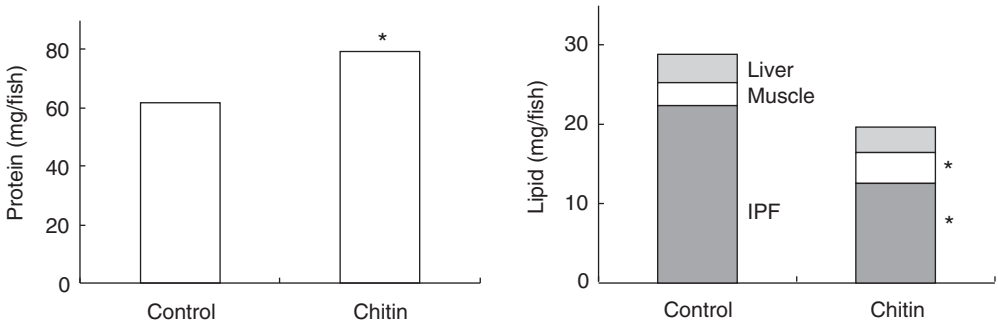

Fig. 10.4. Effects of dietary chitin on accumulation of muscle protein and whole lipid in juvenile black sea bream (Source: Om *et al.*, 2003a). *Significantly different from control ($P < 0.05$).

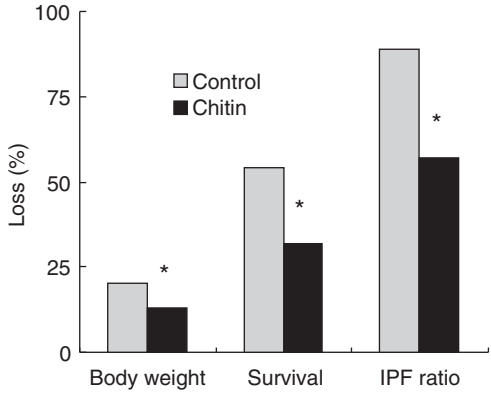

Fig. 10.5. Effects of dietary chitin on biometric parameters after 17 days of starvation (Source: Om *et al.*, 2003a). *Significantly different from control ($P < 0.05$).

4 Effect on Vitality

Figure 10.6 shows liver function and air-dipping resistance in black sea bream (Om *et al.*, 2003a). Liver function was evaluated by recovery time from 0.1% of 2-phenoxyethanol anaesthesia, because alcoholic anaesthetics are discharged through the liver (Hilton and Dixon, 1982). Liver function was slightly improved by dietary chitin. The recovery time after air-dipping (5 min) was significantly shortened by dietary chitin. During this treatment, 50% of the fish in the control group died, while all chitin-fed fish recovered within 300 s. The mechanism of how dietary chitin improves metabolism and vitality is unclear. The activation of vitality might be correlated to the oxygen-binding ability of the red blood cell.

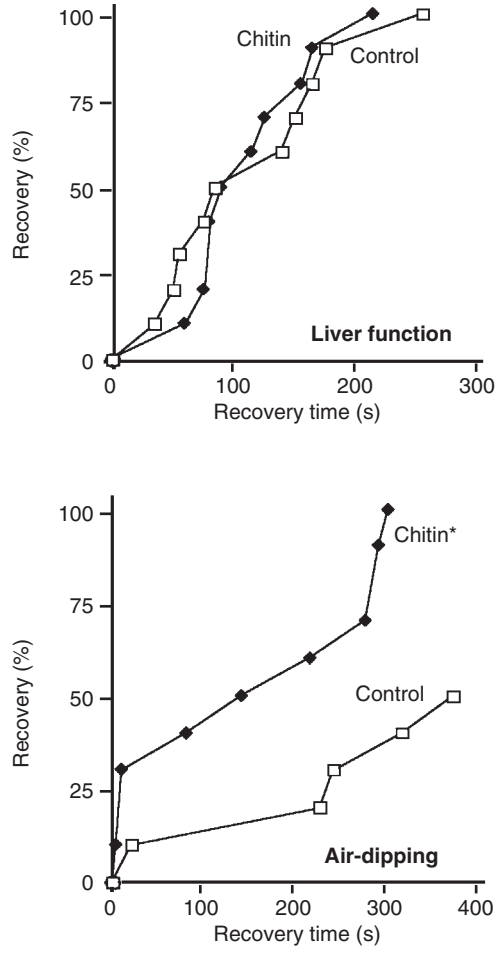

Fig. 10.6. Effect of dietary chitin on liver function and resistance to air-dipping in juvenile black sea bream (Source: Om *et al.*, 2003a). The recovery time from the succumbed condition following anaesthesia and air-dipping was recorded. *Significantly different from control ($P < 0.05$).

5 Gastric, Intestinal and Liver Chitinase Activity

Chitinolytic enzymes are widely distributed and of high activity in the gut and liver of various species of fishes (Okutani and Kimata, 1964; Yoshida and Sera, 1970; Micha *et al.*, 1973; Lindsay, 1984b; Matsumiya and Mochizuki, 1987; Kono *et al.*, 1987b, c). While the activity might be partly induced by the consumption of foods, the enzyme is already found in eggs and increases gradually in the course of embryogenesis of red sea bream (Kono *et al.*, 1987c). Some bacteria in the digestive tract of fish may also participate in chitin digestion. Chitin-decomposing bacteria are found in the digestive tract of cultured red sea bream and Japanese eel (Kono *et al.*,

1987b). Nevertheless, chitinase activity is endogenous in the rainbow trout gut, and independent of the bacterial flora (Lindsay *et al*., 1984). Improvement of physiological condition might be partly explained by the use of natural chitin by fish, although it is not clear whether chitinolytic activity is consistent with the dietary effects of chitin on physiological condition. Lindsay (1984b) showed no correlation between chitinase activity and the amount of chitin consumed by the fish species. Furthermore, the distribution of lysozyme activity was more discreet than that of chitinase.

6 Effect on Disease Resistance

It has been reported that intraperitoneal injection of chitin stimulates disease resistance such as phagocytic activity in the leukocytes of rainbow trout (Sakai *et al*., 1992). Dietary chitin stimulates the innate immune system of gilthead seabream *Sparus aurata* (Esteban *et al*., 2001). Madsen and Dalsgaad (1999) suggested that the stimulation of the immune response by dietary chitin resulted in better protection against bacterial infection and caused less deformities of the vertebral column in rainbow trout.

7 Negative Effects of Chitin

In contrast to positive effects, the growth of rainbow trout is depressed by increasing dietary chitin apparently due to digestibility factors (Lindsay *et al*., 1984). Both chitin and chitosan depress growth of tilapia *Oreochromis niloticus* × *Oreochromis aureus* regardless of the supplementation level (Shiau and Yu, 1999).

8 Function of Dietary Chitin

The positive effects of chitin on growth performance may be interpreted in terms of gut structure and function. Intestine length, which is an indicator of the diversified use of food and nutrients, is significantly elongated by dietary chitin (Om *et al*., 2003a). There appears to be a general relationship between intestinal length and dietary composition of feeding habits (Montgomery, 1977; Stroband, 1997; Cleveland and Montgomery, 2003). The finding that the intestine in the chitin-fed black sea bream is significantly longer than in the control group might be due to the physical state of chitin passing through the gut (Fig. 10.3). The long intestine presumably prolongs the evacuation time of food in the gut by increasing surface area. Consequently, growth and feed efficiency might be improved. Function of chitin as dietary fibre should also be considered, because fish fed a diet supplemented with cellulose, another form of polysaccharide fibre, at 10% had slightly higher growth (Kono *et al*., 1987a).

Suppression of lipogenesis and activation of lipolysis also occurs when diets are supplemented with algae (Mustafa and Nakagawa, 1995), catechin (Nakagawa *et al*., 2000), docosahexaenoic acid (Om *et al*., 2003b) and ascorbate (Ji *et al*., 2003). The presence of chitin in fish diets interferes with the bacteriolytic activity of lysozyme in trout stomach (Lindsay, 1984a). Chitin in the gut contents of wild fish and the observed effects of dietary chitin imply its importance in artificial diets for cultured fish. Juvenile fish might require, or at least benefit from, chitin as a food supplement in a quantity equivalent to that found in the gut of wild fish. The supplementation of nutritional substances that are widely distributed in natural food organisms, might normalize the metabolism and physiological condition of cultured fish. However, the contradictory effects of dietary chitin indicate that further studies are needed to clarify its functions.

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11 Plant Saponins

GEORGE FRANCIS AND KLAUS BECKER

Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim (480), D 70593 Stuttgart, Germany

1 Introduction

The saponins are naturally occurring surface-active glycosides mainly produced by plants, but also by lower marine animals and some bacteria (Riguera, 1997; Yoshiki *et al*., 1998). They consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroid in nature.

Saponins are known to inhibit mould and protect plants from insect attack (Hostettmann and Marston, 1995). When present in the diet in moderate to high quantities, they are believed to have several negative effects on animals (Cheeke, 1996). Dietary saponins derived from different plants have been implicated as the causative factor for depression of feed intake and reduction in weight gain in salmonid fish (Bureau *et al*., 1998), accentuation of ruminant bloat and causation of photosensitization (Cheeke, 1996), inhibition of active nutrient uptake (Johnson *et al*., 1986) including vitamins (Jenkins and Atwal, 1994) and minerals (Southon *et al*., 1988), anti-fertility action in animals (Tewary *et al*., 1973; Quin and Xu, 1998) and reduction in protein digestibility (Shimoyamada *et al*., 1998). On the other hand, saponins also have long been known to possess properties useful to humans as they are the active components in a large number of traditional 'herbal medicine' preparations. They are able to bind ammonia and are hence considered to reduce release of this gas into the atmosphere (Cheeke, 1996; Makkar *et al*., 1999), reduce the rumen protozoa number and thus increase the efficiency of rumen microbial protein production (Newbold *et al*., 1997; Makkar *et al*., 1998) and lower serum cholesterol which might reduce the risk of cardiovascular diseases (Sugano *et al*., 1990; Harwood *et al*., 1993). Antiviral activity of saponins from *Glycyrrhiza radix*, immunostimulant activity of saponins from *Quillaja saponaria* Molina, and

hypo-glycaemic and anti-diabetic activity of saponins from fenugreek (Petit *et al*., 1993; Kensil, 1996) have been reported.

Triterpenoid saponins have been detected in many legumes such as soybeans, other beans, peas, lucerne, etc. and also in alliums, tea, spinach, sugarbeet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. Steroid saponins are found in oats, capsicum peppers, aubergine, tomato seed, alliums, asparagus, yam, fenugreek, yucca and ginseng. One example of an extensively studied group of triterpenoid saponins is produced from *Q. saponaria*, a tree native to the Andes region. The bark was peeled off and extracted with water by the indigenous peoples as a shampooing agent, and by the Shamans as an overall curing agent. *Yucca schidigera* is the most common commercial source of steroid saponins. Partially purified *Q. saponaria* Molina saponin preparations such as Quil A, have found widespread use in veterinary vaccines (Kensil, 1996). Saponins are important in human nutrition because of their widespread occurrence in food constituents such as legumes.

Saponins are considered to be highly toxic to fish when present in water. Newinger (1994) identified saponins as the main active compounds present in the most effective fish poisoning plants in Africa. They also are considered to be the active components of many traditionally used fish poisons, like mahua oil cake (Francis *et al*., 2001a). Saponins are considered to be prominent antinutrients limiting the levels of inclusion of several alternate plant protein sources in the diets of cultured fish (for a collection of relevant references, see Francis *et al*., 2001a).

Taking a brief look at the aquafeed scenario in relation to feed additives, considerable potential is seen for plant-based compounds. Feeds account for more than 50% of the total production costs in intensive aquaculture. Increasing feed efficiency, especially by improving the metabolic assimilation of dietary nutrients is of high priority in contemporary animal production. Any reduction in feed costs would have a direct positive effect on profitability of aquaculture. Higher efficiency of supplementary feed utilization in high feed-input intensive aquaculture would also address concerns regarding excretion of nitrogen (N) and phosphorus (P) to the environment. The use of synthetic substances such as antibiotics and steroid hormones that have been conventionally used to increase the efficiency of feed assimilation by animals, either is or will soon be prohibited in several countries. In the EU for example, the use of antibiotics as growth promoters in animal feeds has been completely banned from the year 2006 onwards. There could be increased demand for natural substances for inclusion in aquafeeds as feed efficiency enhancers and growth promoters from the aquafeed industry. One factor that has generated scepticism regarding the use of natural substances as feed additives has been the difficulty to standardize their properties with regards to the presence of active components in the parent plant material, their extraction, and purification without loss of biological function. With the technical advancement of analytical tools and procedures, these limitations to the use of natural feed additives have been partially overcome, paving the way for their greater use in future.

This chapter presents a synthesis of results and conclusions obtained from our experiments with two commonly cultured fish, namely common carp and Nile tilapia, fed diets containing crude extracts and purified concentrates of triterpenoid saponins from *Q. saponaria* (effects of saponins from this plant in fish have been previously reviewed in Francis *et al*., 2005) and *Gypsophila paniculata*, and a concentrate of steroid saponins from *Y. schidigera*. The *Quillaja* saponins (QS) were obtained from Sigma (No. 2149; Sigma, St. Louis, USA) and the *G. paniculata* saponins (GS) from Serva Electrophoresis GmbH, D-69115 Heidelberg, Germany (special order). The *Yucca* saponins (YS) were concentrated from *Y. schidigera* powder (DK sarsaponin 30^{TM}, Desert King International, Chula Vista, CA 91911, USA) by separating saponins present in a water extract into butanol and then evaporating off the solvent. All known biologically active steroidal substances such as 5β-spirostan-β-ol, and sarsasapogenin and smilagenin monodesmoside saponins present in *Y. schidigera*, have been previously found to be completely butanol extractable (Killeen *et al*., 1998). The main aglycone (sapogenin) moiety in QS is quillaic acid, a triterpene of the oleanane type. The dominant sapogenins in the *Yucca* concentrate are sarsasapogenin and smilagenin.

2 Effects of Saponin-containing Diets on Cultured Fish

2.1 Feed intake and behaviour

We observed that common carp *Cyprinus carpio* and tilapia *Oreochromis niloticus* ate standard fishmeal-based diets mixed with up to 1000 mg/kg of all the saponin concentrates that were evaluated without any hesitation. The fish consumed the pellets containing saponins as soon as they dropped into the aquaria with no apparent differences in palatability between the control diet and saponin-containing diets. There was no mortality or abnormal behaviour of fish fed up to this concentration of saponins. On the other hand, standard diets containing 2000 mg/kg of the *Quillaja* saponin concentrate induced high mortality in first-feeding tilapia larvae (Steinbronn, 2002). Mortality started after 3 weeks of intensive feeding of the diet containing 2000 mg/kg of QS and 93 out of 460 fish larvae died over a period of about 5 days. The mortality stopped completely after the feeding intensity was reduced and the saponin-containing diet was gradually replaced with a standard diet (the control diet was a standard diet containing 40% protein, 10% lipid, 10% ash and had 20 kJ/g gross energy with the protein source being fishmeal).

2.2 Effects on growth and feed efficiency

Common carp and Nile tilapia juveniles fed diets containing QS (150 and 300 mg/kg in the diet) had a significantly higher rate of body mass gain

(Francis *et al*., 2001b, 2002a). The average final body mass of carp fed QS was about 18% higher and that of tilapia was more than 20% higher than that of fish that had similar average weights at the start of the respective experiments but which did not receive QS (Figs 11.1 and 11. 2).

The growth promoting effects of QS was most pronounced in the carp fed the 150 mg/kg diet; whereas, the dietary level of 300 mg/kg induced maximum effects in tilapia. The absolute increase in weight was higher compared to the control even at higher dietary levels of 700 mg/kg in Nile tilapia (Francis *et al*., 2002c). The growth-promoting effects of QS (Table 11.1) were most pronounced during the initial period of feeding (Francis *et al*., 2001b, 2002a). It was therefore hypothesized that there was probably an adaptation by the fish to continuous intake of QS. An experiment was therefore designed to provide discontinuous ingestion of saponins to counteract any adaptive responses of the fish (Francis *et al*., 2002b). Consumption of diets containing saponins during alternate weeks, however, did not result in retention of the more pronounced initial growth-promoting effects during the entire experimental period in carp. The haemolytic triterpenoid *Gypsophila* saponins concentrated using chromatography did not significantly increase growth rate at levels of between 5 and 250 mg/kg in diets of common carp after 8 weeks of feeding even though absolute growth was higher in all the saponin fed groups compared to the control (Table 11.2; Francis *et al*., unpublished data).

Concentrated steroidal YS at levels of 50 and 100 mg/kg (YS050 and YS100 groups) also did not affect growth of common carp significantly. Here the YS050 group seemed to perform better than the YS100 and control groups at the end of a 10-week feeding experiment (Fig. 11.3; Francis *et al*.,

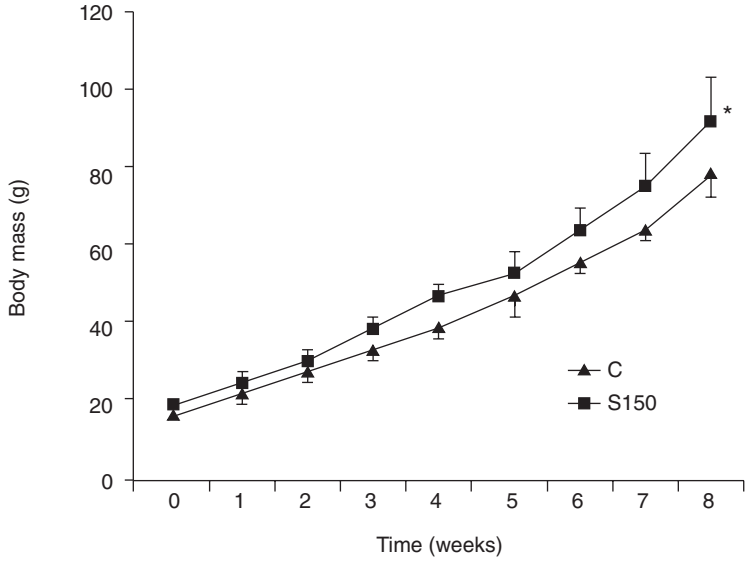

Fig. 11.1. Body mass increase of common carp fed a control diet (C) or a diet containing 150 mg/kg (S150) (Source: Francis *et al*., 2002a). *Significantly different from C ($P < 0.05$).

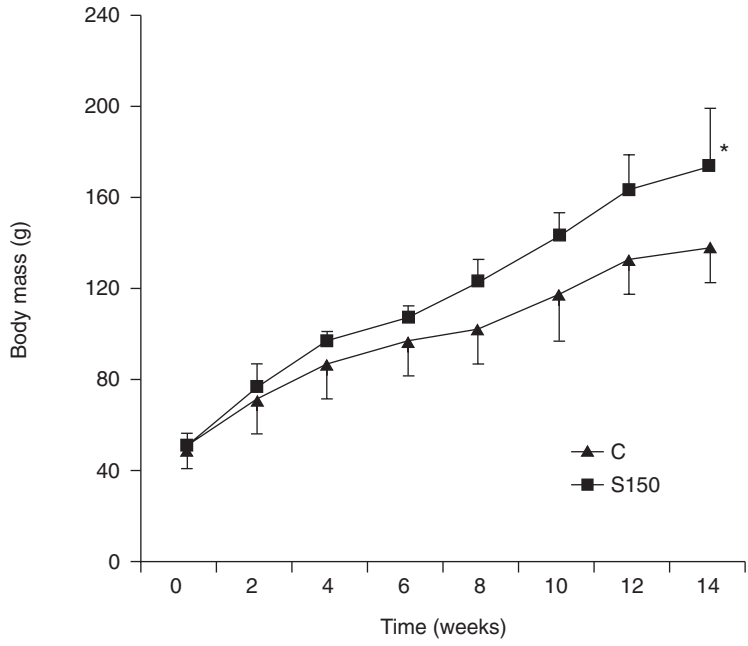

Fig. 11.2. Body mass increase of Nile tilapia fed a control diet (C) or a diet containing 300 mg/kg (S300) *Quillaja* saponin (Source: Francis *et al.*, 2001b). *Significantly different from C ($P < 0.05$).

Table 11.1. Efficiencies of protein accretion and energy retention of common carp and Nile tilapia fed diets containing 150 mg/kg (S150) or 300 mg/kg (S300) of *Quillaja* saponin (QS) (Source: Francis *et al.*, 2001b, 2002a).[a]

	Efficiency of:			
	Protein accretion (PPV)[b]		Energy retention (ER)[c]	
	Control	QS diet[d]	Control	QS diet[d]
Common carp	37.8 ± 1.5	40.4 ± 3.0	28.2 ± 2.0	32.2 ± 4.4
Nile tilapia	32.0 ± 5.4	37.2 ± 6.8	23.7 ± 4.4	32.7 ± 6.9

[a] Values mean ± SD. The differences between the averages presented for the different parameters for either fish were not statistically significant.
[b] PPV = (total protein gain/total protein fed) × 100.
[c] ER = (energy gain/total feed energy) × 100.
[d] The QS diets contained 150 mg/kg QS in the feed for common carp and 300 mg/kg QS in the feed for Nile tilapia.

unpublished data). The metabolic growth rate (MGR = live weight gain (g)/average metabolic live weight ($kg^{0.8}$)/day) of the YS100 group was higher than that of the control group until the seventh week of the experiment, the difference between the two widening up to this time. Thereafter the MGR of the control group started to increase compared to the

Table 11.2. Initial and final average body masses, metabolic growth rate (MGR) and feed conversion ratio (FCR) of carp fed experimental diets containing different levels of concentrated *Gypsophila* saponins.[a]

	Initial weight		Final weight		MGR[b]		
Fish group[d]	Average	SD	Average	SD	Average	SD	FCR[c]
Control	13.1	1.8	43.6	8.1	10.1	1.2	1.30
GS5	14.7	2.1	60.4	7.8	12.5	1.1	1.00
GS17	14.6	1.0	59.5	12.3	12.3	2.4	1.01
GS150	14.1	1.1	53.8	15.9	11.3	3.1	1.08
GS250	14.9	2.5	58.7	9.4	12.1	1.7	1.06
GSR150	13.5	1.5	52.3	10.6	11.6	2.4	1.08

[a] Values mean ± SD. The differences between the averages presented for the different parameters for either fish were not statistically significant ($P < 0.05$).
[b] MGR = live weight gain (g)/average metabolic live weight ($kg^{0.8}$)/day.
[c] FCR = feed consumed/live weight gain.
[d] GS5, GS17, GS150, GS250, carp fed purified haemolytic *Gypsophila* saponin at 5 mg/kg, 17 mg/kg, 150 mg/kg and 250 mg/kg, respectively; GSR150, carp fed 150 mg/kg raw *Gypsophila* saponin mixture.

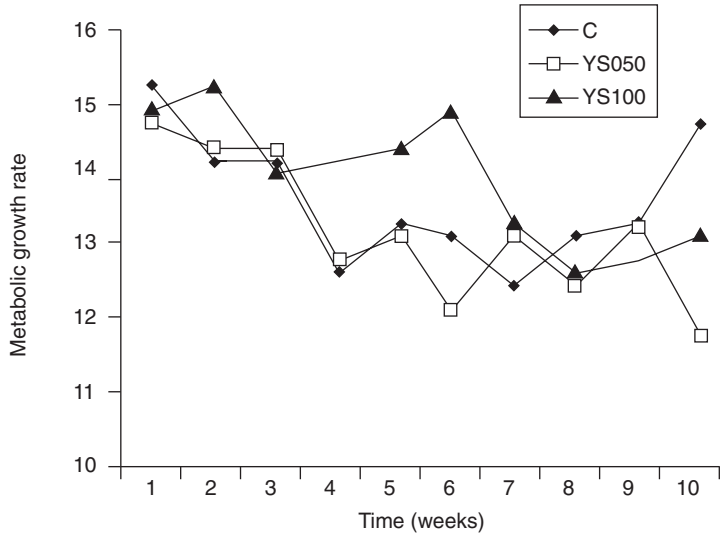

Fig. 11.3. Average weekly metabolic growth rate (live weight gain (g)/average metabolic live weight ($kg^{0.8}$)/day) of carp fed a control diet (C) or diets containing 50 mg/kg (YS050) or 100 mg/kg *Yucca* saponin (YS100). The difference between the C and YS050 groups was not statistically significant at any stage of the experiment.

YS100 and continued to do so until the end of the experiment. The YS050 group had the lowest growth rate throughout the experiment.

The addition of QS to the diet also reduced the amount of feed required for the synthesis of tissue protein. The food conversion ratio (FCR) was lower in carp fed a diet containing 150 mg/kg (0.82 ± 0.07) and tilapia fed

300 mg/kg (1.40 ± 0.19) of QS compared to the respective controls (0.89 ± 0.04 and 1.61 ± 0.34, respectively). Common carp fed diets containing GS and YS did not differ significantly from controls in regard to FCR (Francis *et al*., unpublished observation) with values of 0.91 for control, 0.96 for YS050 and 0.91 for YS100. In the case of the GS the absolute FCR value was lowest in the GS5 group and highest in the control group (Table 11.2; Francis *et al*., unpublished observations).

Differences in growth rate and feed efficiency of fish fed saponin-containing diets were not always significantly higher compared to controls because of the high variability among treatment groups. The lack of homogeneity in the growth-promoting effects of dietary QS was also evident during feeding experiments where the number of treated fish was relatively high, both in the laboratory (Francis *et al*., 2002c) and field trials (Steinbronn, 2002). The variability was despite the fact that fish used in the individual experiments were of the same lineage, age and body mass. The factors that may have caused variability in the growth response of carp and tilapia to dietary QS are not clear.

The mechanisms contributing to growth-promoting effects of saponins, especially QS which induced significant growth increases, are yet to be fully clarified. Diverse effects of dietary saponins include an increase in the permeability of intestinal membranes to dietary nutrients (Francis *et al*., 2002d) and/or a stimulation of the activity of digestive enzymes, which increases the efficiency of feed nutrient utilization. Dietary QS significantly increased the activity of carp gut enzymes, amylase and trypsin, and liver enzymes, lactate dehydrogenase (LDH) and cytochrome c-oxidase (CO) (Serrano *et al*., 1998). This shows that it could stimulate digestion of proteins and carbohydrates in the gut and promote both the respiratory chain and lactate fermentation. The ratios of LDH to CO decreased with QS supplementation indicating the promotion of aerobic metabolism.

It has also been observed that QS does not cause any obvious damage to the intestinal membranes in tilapia fry when present up to 700 mg/kg in the diet (Francis *et al*., unpublished observations). Neither was any abnormality observed in the liver or kidney tissue of tilapia fed QS up to 700 mg/kg over a period of 6 months. Initial investigations into the effects of saponins on membrane transport reveal an increase in paracellular transport of inert markers on application of QS to the mucosal side of isolated tilapia intestinal membrane (Francis *et al*., unpublished observations).

It also remains to be determined whether the saponins themselves or their breakdown products (e.g. sapogenins) in the intestines enter the blood of the fish and cause their effects systemically. From the extent of effects that saponins have on various physiological processes it is expected that either saponins or their breakdown products enter the body through the intestinal membranes. We have described the ability of saponins to influence serum hormone levels (Francis *et al*., 2002d). However, some dietary components may produce systemic effects even without actually entering the body. It has been reported that hormones such as ghrelin,

synthesized primarily in the stomach wall, could act as intermediaries between stomach, hypothalamus and pituitary and may be involved in energy balance (Tschöp *et al*., 2000). It remains to be seen whether saponins induce the synthesis and release of such hormonal intermediaries in the digestive system.

A factor that makes it more difficult to interpret the effect of dietary saponins is the presence of a number of individual compounds with different properties in the saponin mixtures used in our feeding trials. We conducted some experiments to see if there are functional differences between purified haemolytic saponin fractions and crude extracts for their growth-promoting effects. Initial results indicated no differences in growth or any of their nutrient assimilation parameters between carp fed raw *Gypsophila* saponin mixture or any of the levels of haemolytic saponins (5–250 mg/kg of the diet). The growth performance of all the saponin-fed groups was higher than that of the control fish in this experiment (Francis *et al*., unpublished observations). Further experimental results might clarify whether individual compounds possess higher potency as growth enhancers in fish.

Even though the results seem to indicate a stimulatory effect of saponins, particularly QS, on fish growth, gaps exist in our understanding of the mechanism of action of the saponins in fish. Future research in this area should concentrate on understanding the physiological mechanisms by which dietary saponins increase growth and feed conversion efficiency in carp and tilapia.

2.3 Effects on respiratory metabolism

A comparative study of the metabolic rate inferred from oxygen consumption among carp and tilapia fed saponins showed a shift in the metabolic activity in saponin-fed fish towards anabolic synthesis. The consumption of oxygen per unit body mass gain was lower in these fish (Fig. 11.4); the retention of feed energy was higher and the loss of feed energy was lower (Francis *et al*., 2001b, 2002a, b). Interestingly, there seems to be a lowering of oxygen demand for growth of fish in the presence of saponins in the diet. This would have positive implications in tropical aquaculture systems where dissolved oxygen is often a limiting factor. The higher efficiency of oxygen utilization may point to a higher synthesis efficiency of protein and lipid leading to a reduction in synthesis heat dissipation. The obvious decrease in oxygen consumption rate is, however, somewhat contradictory to the observed stimulation of CO by QS mentioned previously which indicates increased oxidative phosphorylation. Increased retention of feed energy for growth and lower nutrient excretion are also beneficial from both production and environmental points of view.

In common carp fed steroidal YS, the pattern of effects on metabolic rate and oxygen consumption was not uniform (Francis *et al*., unpublished observations). Here the oxygen consumed per unit body mass gain (OC)

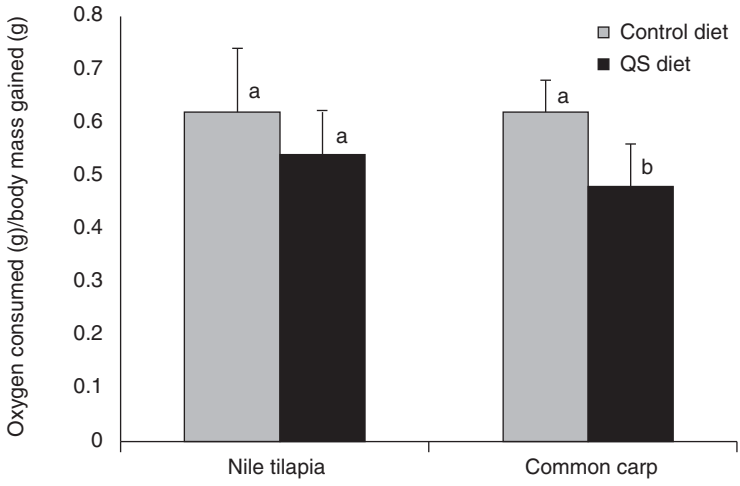

Fig. 11.4. Oxygen consumed (g) per unit body mass gained (g) in common carp and Nile tilapia fed diets containing 150 mg/kg or 300 mg/kg of *Quillaja* saponin (QS), respectively, compared to a control diet (C) (Source: Francis *et al*., 2001b, 2002b). Different letters on columns indicate a statistical difference ($P < 0.05$).

was highest in the YS050 group (13.7% higher than the control). The OC values of the control group (C) and the YS100 group were similar at the end of the experiment. The weekly average metabolic rate (MR, calculated as oxygen consumption in $mg/kg^{0.8}/h$; average of 168 hours) values for the whole experimental period also showed a similar pattern. The average MR values (± standard deviation) were 373 ± 19, 398 ± 32 and 366 ± 29 for the C, YS050 and YS100 groups, respectively. It needs to be mentioned that the metabolic rates of the YS100 group were considerably lower than those of the control group during the first 5 weeks of the experiment. The values then caught up with the control group values during the remaining weeks of the experiment (Fig. 11.5).

2.4 Effects on carcass composition, organic mass and nutrient assimilation

There were no obvious differences in carcass composition between control and saponin-fed fish. Generally there was increased carcass dry matter content in fish that consumed diets containing saponins; however, the differences were statistically significant only in one experiment (Francis *et al*., 2002b). The presence of QS in the diet did not uniformly affect the ratio of the viscera to muscle mass although the hepatosomatic and intestine-somatic indices tended to be lower in saponin-fed carp and tilapia (1.62 ± 0.21 and 2.94 ± 0.38 compared to 1.85 ± 0.28 and 3.19 ± 0.35 in carp and 2.00 ± 0.37 and 1.55 ± 0.31 compared to 2.7 ± 0.35 and 1.59 ± 0.19 in tilapia; Francis *et al*., 2001b, 2002b).

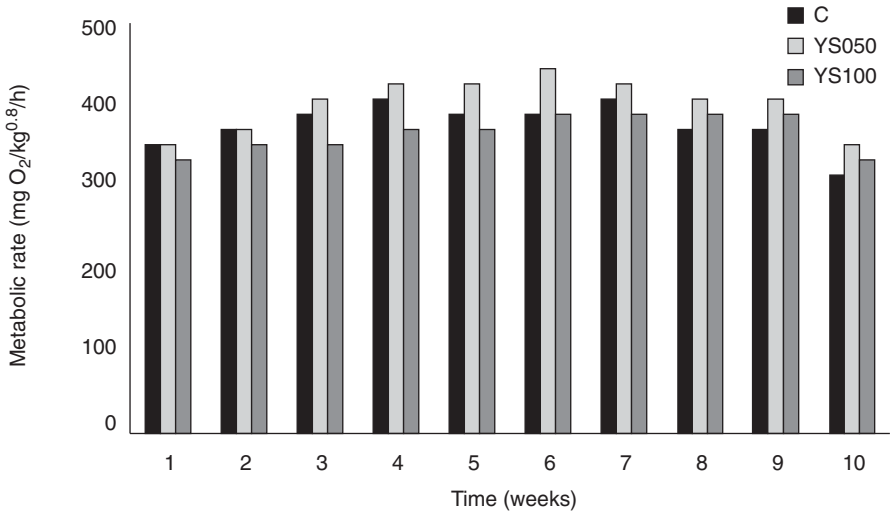

Fig. 11.5. Weekly average metabolic rate (MR; mg O_2/kg$^{0.8}$/h) of carp fed a control diet (C) or diets containing 50 mg/kg (YS050) or 100 mg/kg *Yucca* saponin (YS100). The difference between the C and YS050 groups was not statistically significant at any stage of the experiment.

Protein and energy accretion (Table 11.1) of fish fed saponins was higher in almost all the experiments including the ones where growth and body mass gain were not significantly influenced. Lipid assimilation also tended to be higher in fish fed both triterpenoid (Francis *et al.*, 2001b, 2002a, b) and steroid saponins. Common carp fed YS at the level of 100 mg/kg had an average carcass lipid content that was 16% higher than that of the control group. Average ash content was also lowest in the YS100 group. These effects could be explained by the increased digestion and absorption use of proteins and especially carbohydrates caused by stimulation of gut enzymes by saponins. However, the hypothesis that saponins might have increased absorption and hence availability of dietary nutrients leading to higher assimilation requires further confirmation.

2.5 Effects on tilapia reproduction

Sexually mature female tilapia consuming a diet containing 300 mg/kg of QS did not spawn over a period of more than 3 months, whereas fish fed the control diet and reared under similar conditions spawned regularly. This observation was followed up by conducting laboratory experiments and field studies to further explore the effects of dietary QS on tilapia reproduction. Regularly spawning adult tilapia when put on a diet containing 300 mg/kg of QS stopped egg laying from the next ovulation cycle onwards (Francis and Becker, unpublished observations). In another experiment the sex ratio of tilapia larvae fed a diet containing 700 mg/kg of

QS continuously over a 6-month experimental period deviated significantly from the normal 50:50 ratio in favour of males (Francis *et al.*, 2002c). This deviation from the normal sex ratio in favour of males was also evident (but not statistically significant) in the treatment groups receiving lower quantities of QS (150 mg/kg diet) in the diet (Fig. 11.6).

Pond experiments conducted with a higher number of fish (about 500 larvae in each treatment in duplicate) fed a QS-containing diet for the first 6 weeks of life, however, did not confirm the laboratory results obtained regarding the effects of dietary QS on sex ratio (Steinbronn, 2002). The sex ratios of tilapia larvae fed diets containing 150 and 500 mg/kg of QS did not differ significantly from 50:50 ratio for males and females, but the share of males was slightly higher in both treatments as compared to the control group. On the other hand at the highest level of 2000 mg/kg diet, more females than males were counted in tilapia larvae fed these diets. This treatment group experienced 20% mortality during the early rearing phase when the saponin containing diets were being fed. It remains to be confirmed whether this might have been caused by differential mortality rates among the sexes during the experimental period. Previous reports (Pandian and Sheela, 1995) state the likelihood of sex-dependent mortality occurring among fish that were sex-reversed through the application of synthetic hormones. The GIFT tilapia (developed from the 'Genetic Improvement of Farmed Tilapia' project of ICLARM, currently World Fish Center) larvae used in the field trial have also been reported to generally

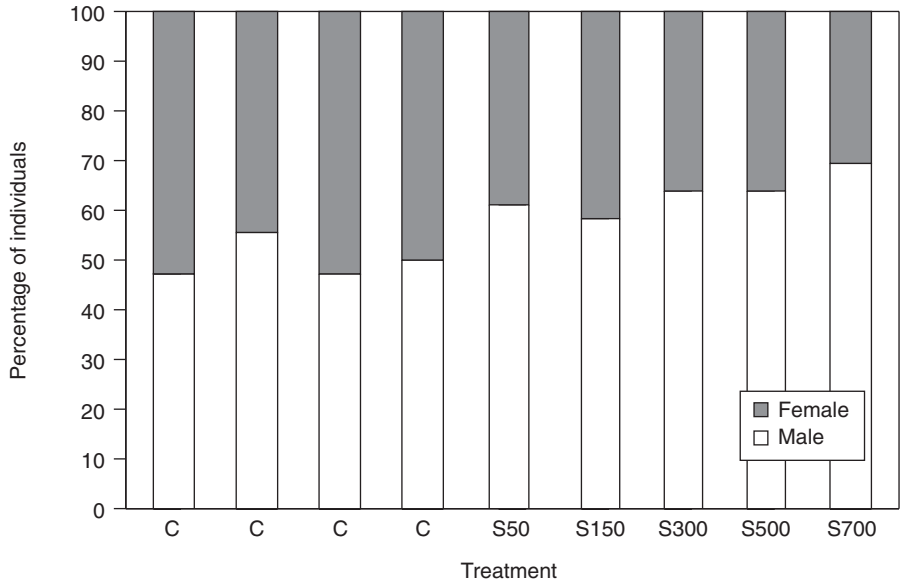

Fig. 11.6. Proportion of males and females in Nile tilapia larvae fed a control diet (C; saponin-free) and diets containing 50, 150, 300, 500 or 700 mg/kg of QS (S50, S150 … S700) (Source: adapted from Francis *et al.*, 2002c). The ratio of males and females in the S700 group differed significantly from the 50:50 ratio ($P < 0.05$).

have a higher number of female fish (Hussain *et al*., 2000). Continued observations revealed that production of fry was completely suppressed in ponds where fish from the 2000 mg/kg saponin group were stocked even after the removal of saponins from the diets (Steinbronn, 2002). This could point to a sterility of either males or females, which implies a potential for the control of reproduction in tilapia using QS. Normal fry production was observed in fish that previously received 150 and 500 mg/kg of QS.

Saponins have been previously reported to affect the release of hormones, such as luteinizing hormone (LH), from the pituitary (Benie *et al*., 1990) and this hormone is considered to regulate all aspects of teleost reproduction (Suzuki *et al*., 1988a), particularly final oocyte maturation and ovulation (Suzuki *et al*., 1988b). It was therefore hypothesized that induction of changes in the LH secretory pattern by QS or its degraded products absorbed from the intestine might be responsible for the observed effects on reproduction. QS was found to stimulate LH release from dispersed tilapia pituitary cells *in vitro* (Francis *et al*., 2002d). This effect was abolished in the presence of dilute calf serum. Serum LH values did not show any diet-dependent (QS containing or control diets) trend in either male or female tilapia *in vivo*. The retarding effects on egg production in adult females and the capacity for sex inversion in tilapia fry fed saponin-containing diets indicate effects at the hormonal level. Data from gonadosomatic index measurements also support this contention. Our efforts to identify any saponin-induced change in the level of one of the key hormones in reproductive functioning, the LH, did not reveal any dose dependent patterns. Once the optimum dietary level of saponins that produces complete sex inversion in tilapia fry or prevents egg production in female tilapia is determined, this effect of saponin will have considerable potential in tilapia aquaculture where one of the major problems is overproduction of fry that do not grow to marketable size. The effect of saponins on levels of reproductive hormones should be further studied by monitoring of hormones such as oestrogen, testosterone, 11-keto-testosterone and gonadotropic hormones *in vivo* and possibly by using cultured tilapia pituitary cells. *In vitro* studies have the advantage of requiring only small quantities of material for the identification of the individual compounds responsible.

3 Conclusions and Implications

Triterpenoid QS have a growth-promoting effect in common carp and Nile tilapia when given at low levels in the diet. The level of saponins in the diet need to be determined based on the fish species and the composition of the saponin mixture. More experiments are required before such conclusions can be drawn regarding the steroid YS. It is to be tested whether triterpenoid or steroid saponins from other plant sources with different aglycones and carbohydrate side-chains have similar growth-promoting effects in cultured fish. Both triterpenoid and steroid saponins generally

tended to reduce the metabolic rate of carp and tilapia and lower the oxygen demand for growth in both species. QS at 300 mg/kg inhibited egg production in tilapia. Dietary QS also has the potential to suppress reproductive capacity in tilapia when present in the diets during the first 6 weeks of larval life.

Plant saponins could thus be very valuable as an environmentally friendly feed additive for cultured fish. At least some of them have clear potential as growth promoters and seem to be capable of increasing the efficiency of feed utilization of cultured fish. The lowering of the demand of oxygen for growth is also important, especially in tropical aquaculture where the level of dissolved oxygen is often a limiting factor. In addition saponins could be very valuable as a replacement for environmentally undesirable synthetic steroid hormones in tilapia aquaculture with its potential for suppressing reproduction in culture ponds. What is needed is clarification of the mechanism of action of saponins so that a standard mixture with predictable effects can be recommended to aquaculture practitioners. There might also be other secondary plant compounds that may have similar positive effects on fish growth. A scan of the available literature shows that this area has remained almost untouched by fish nutrition research.

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12 Nucleotides

DELBERT M. GATLIN III AND PENG LI

Department of Wildlife and Fisheries Sciences and Faculty of Nutrition, Texas A&M University System, College Station, TX 77843-2258, USA

1 Introduction

Nucleotides comprise a group of biochemicals that have numerous essential physiological and biochemical functions. These functions include encoding and deciphering genetic information, mediating energy metabolism and cell signalling as well as serving as components of coenzymes, allosteric effectors and cellular agonists (Carver and Walker, 1995; Cosgrove, 1998). Although the roles of nucleotides administered exogenously have been debated for many years, their potential application in aquaculture has received heightened attention in recent years (Li and Gatlin, 2006). Nucleotides have traditionally been considered to be non-essential nutrients because neither overriding biochemical malfunctions nor classical signs of deficiency are developed in human or animal models. However, this opinion has been challenged by recent research which suggests that dietary nucleotide deficiency may impair liver, heart, intestine and immune functions (reviewed by Grimble and Westwood, 2000a). The modulatory effects of dietary nucleotides on lymphocyte maturation, activation and proliferation, as well as macrophage phagocytosis, immunoglobulin responses and genetic expression of certain cytokines also have been reported in humans and animals (reviewed by Gil, 2002). Nucleotide supplementation has been most frequently investigated in humans where exogenous nucleotides have been applied in clinical situations such as partial hepatectomy or during recovery from severe injuries such as burns (Grimble, 1996). In addition, nucleotide fortification of breast milk substitutes has been recommended to the US Food and Drug Administration for approval (Aggett *et al*., 2003).

Initial efforts to evaluate dietary supplementation of nucleotides for fish can be traced to the early 1970s, during which research mainly focused on the possible chemo-attractive effects of these compounds (Mackie, 1973; Kiyohara *et al*., 1975; Mackie and Adron, 1978). Much more recently,

 Dietary Supplements for the Health and Quality of Cultured Fish (eds H. Nakagawa, M. Sato and D.M. Gatlin III)

heightened attention associated with the potential benefits of dietary nucleotide supplementation to fish was aroused by the reports of Burrells *et al*. (2001a, b), which indicated enhanced resistance of salmonids to viral, bacterial and parasitic infections as well as improved efficacy of vaccination and osmoregulation capacity. Research pertaining to nucleotide nutrition in fish is rather limited to date but has shown rather consistent and encouraging beneficial results in fish health management although most of the suggested explanations remain hypothetical as systematic research on fish is far from complete. Because of increasing concerns of dependence on chemotherapeutants in aquaculture, research on immunonutrition for aquatic animals is becoming increasingly important (Gatlin, 2002). Research on nucleotide nutrition in fish is needed to provide insights concerning interactions between nutrition and physiological responses as well as provide practical solutions to reduce risks from infectious diseases in the aquaculture industry.

2 Nucleotide Biochemistry

The biochemical composition of nucleotides includes a purine or a pyrimidine base, a ribose or 2′-deoxyribose sugar and one or more phosphate groups. Major purine bases include adenine, guanine, hypoxanthine and xanthine and form nucleotides through a glycosidic bond between the N-8 nitrogen and C-1′ carbon of the pentose, while the C-5′ hydroxyl is esterified with the phosphoryl group(s) (Rudolph, 1994). Major pyrimidine bases include uracil, thymine and cytosine and form nucleotides in a similar way. Several common nucleotides containing ribose and deoxyribose sugars are depicted in Fig. 12.1.[1]

The purines and pyrimidines associated with nucleotides are synthesized from *de novo* pathways or obtained from salvage pathways as shown in Fig. 12.2 (reviewed by Rudolph, 1994; Carver and Walker, 1995; Grimble and Westwood, 2000a). Purine rings are synthesized in the cytosol of mammalian cells from glycine, aspartate, glutamine, tetrahydrofolate derivatives and CO_2 with considerable energy input, while pyrimidines are synthesized from aspartate, glutamine and CO_2 in the cytosol and mitochondria of mammalian cells. Presumably these pathways are also operative in fish. There is also a salvage pathway that conserves energy and maintains nucleotide homeostasis. Based on mammalian research, the salvage and *de novo* pathways vary markedly among various tissues and may be significantly influenced by metabolic needs or physiological functions. Although this information has not been confirmed in fish, nucleotide turnover in erythrocytes, lymphocytes, heart and brain of mammals primarily depends on supply from the salvage pathway. It also has been noted that dietary nucleotides modulate nucleotide metabolism of the liver (López-Navarro *et al*., 1995), which is the most important organ for nucleotide storage and inter-organ transport to meet physiological needs.

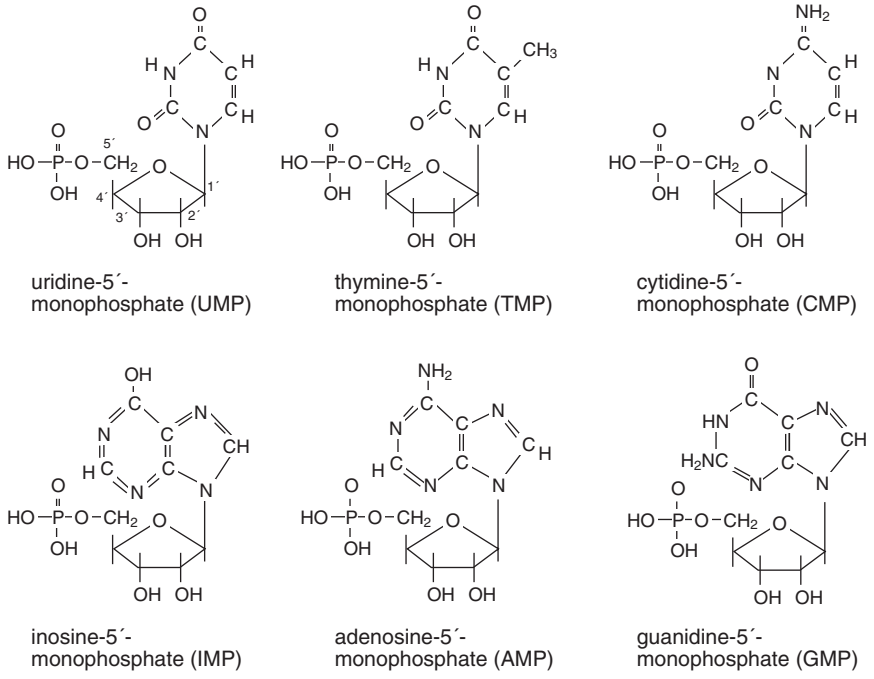

Fig. 12.1. Structures of common nucleotides.

3 Sources and Types of Nucleotides as Diet Supplements

Nucleotides are naturally present in all feedstuffs of animal and vegetable origin as free nucleotides and nucleic acids. Concentrations of RNA and DNA in feedstuffs depend mainly on their cell density (Gil, 2002). Clifford and Story (1976) reported the contents of purines and RNA in some feedstuffs including organ meats, seafoods and dried legumes. Devresse (2000) also reported the total contents (after complete hydrolysis) of purine and pyrimidine bases in common aquafeed ingredients such as fishmeal (1.4%), press cake fishmeal (0.4%), fish solubles (2.8%), yeast (0.9%), yeast extract (2.3%) and single-cell proteins (2.1%). Rumsey *et al*. (1992) reported that 12–20% of the total nitrogen in brewer's yeast *Saccharomyces cerevisiae* can be composed of RNA nitrogen, mainly in the purine and pyrimidine bases of the nucleoproteins.

Several different nucleotide supplements are now commercially available. These products consist of various mononucleotides and/or oligonucleotides derived from feedstuffs such as yeast. Most of the research to date on dietary supplementation of nucleotides for fish has employed the products from Chemoforma Co. (Basel, Switzerland) such as Optimûn (Burrells *et al*., 2001a, b; Leonardi *et al*., 2003; Low *et al*., 2003) and Ascogen S (Ramadan and Atef, 1991). Optimûn, contains AMP, CMP, UMP, IMP and GMP[1] as well as RNA, and is recommended to be supplemented at 0.2% of diet to provide approximately 0.03% exogenous nucleotides (Burrells *et al*.,

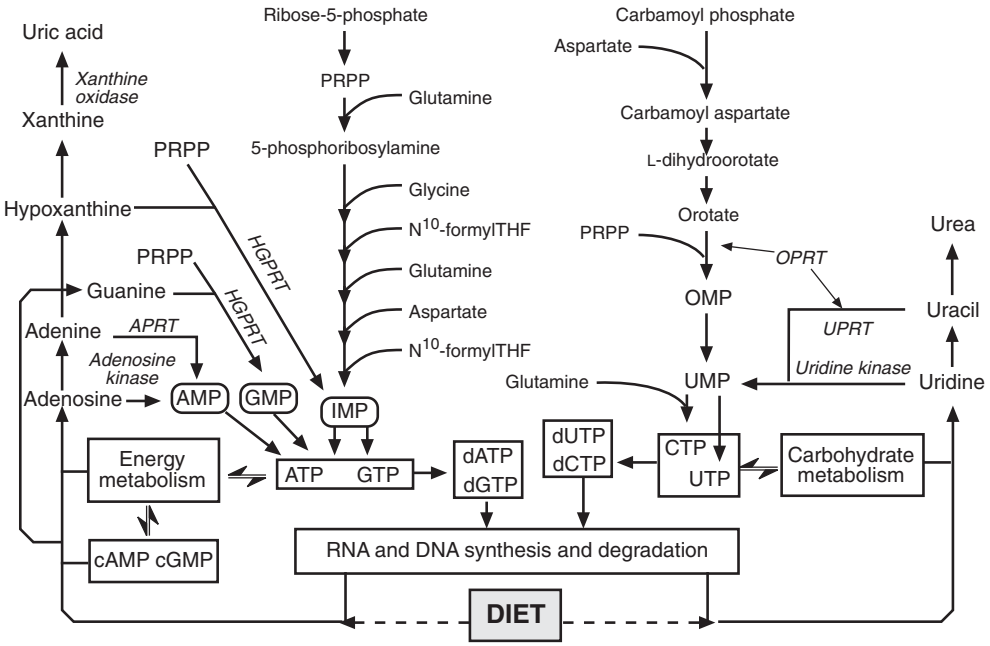

Fig. 12.2. Schematic overview of purine and pyrimidine salvage and *de novo* pathways based on mammalian research (Source: Grimble and Westwood, 2000a; courtesy of Humana Press, Totowa, New Jersey). Abbreviations include: PRPP, phosphoribosylpyrophosphate; N^{10}-formylTHF, N^{10}-formyl-tetrahydrofolic acid; *APRT*, adenine phosphoribosyltransferase; *OPRT*, orotidine phosphoribosyltransferase; *HGPRT*, hypoxanthine-guanine phosphoribosyltransferase.

2001a). Other companies such as Canadian Biosystem Inc. (Calgary, Canada) also have produced nucleotide products such as Ascogen P®, but specific concentrations of various constituents in that product are not disclosed.

4 Digestion and Absorption of Nucleotides and Related Metabolites

Research on digestion and absorption of nucleotides by fish is extremely limited to date. The presence and characterization of proteases and alkaline phosphatases in fish intestine have been well established. However, nuclease, the most important enzyme for nucleotide digestion in fish is poorly understood, although its presence has been reported for some fish such as rainbow trout *Oncorhynchus mykiss* (Roald, 1978). Therefore, the capacity of digesting exogenous nucleotides by various fish remains unknown at this time. It has been suggested by Borda *et al*. (2003) that nucleotides in their non-free form or in the form of nucleic acids tend to be tremendously stable and difficult to digest. Thus, those authors recommended a well-balanced cocktail

of free nucleotides. To the best of our knowledge, the digestibility and bioavailability of nucleic acids in natural feed ingredients, such as marine protein sources or brewer's yeast, for fish is currently unknown, although it appears that fish such as rainbow trout can utilize yeast nucleic acid extracts for growth, nitrogen retention and possibly non-essential amino acid synthesis (Rumsey *et al*., 1992). The nucleotide concentration in feed ingredients and bioavailability to fish, as well as information about the nucleotide pools in fish, need to be determined in order to obtain a greater understanding of nucleotide nutrition of fish.

5 Biological Effects of Nucleotides

5.1 Growth and feed utilization

The earliest studies of nucleotides in fish nutrition concerned their potential influences as palatability enhancers. Mackie (1973) first analysed the low-molecular weight fraction of squid and hypothesized nucleotide (AMP) and nucleoside (inosine) components as the main chemo-attractants. Kiyohara *et al*. (1975) also reported the presence of chemoreceptors on the lips of the puffer fish *Fugu pardalis* that responded to nucleotides (AMP, IMP, UMP and ADP) based on electrophysiological responses. These early experiments substantiated the chemo-attractive effect of dietary nucleotides on fish. Subsequently, Mackie and Adron (1978) tested 47 nucleosides and nucleotides and identified inosine and IMP as the most potent gustatory feeding stimulants for turbot *Scophthalmus maximus* based on feeding behaviour. Ishida and Hidaka (1987) tested gustatory sensitivity of various marine teleosts and found UMP was the most effective for most species, although ADP and IMP also were effective. Rumsey *et al*. (1992) subsequently observed that dietary supplementation with 2.5% and 4.1% yeast RNA extract or 1.85% guanine or 2.17% xanthine significantly increased cumulative feed intake of rainbow trout over a 12-week period. However, the behavioural or gustatory responses of fish to exogenous nucleotides have not always been consistent. For example, it was reported that aigo rabbitfish *Sigmanus fuscescens* did not respond to any nucleotides as did most other marine teleosts (Ishida and Hidaka, 1987). It also has been noted that the stimulatory effect of inosine or IMP on various fish have not been consistently observed (Métailler *et al*., 1983; Person-Le Ruyet *et al*., 1983). Ikeda *et al*. (1991), using jack mackerel *Trachurus japonicus* as an experimental model, found that IMP, GMP, UMP, UDP, UTP were effective feeding stimulants while nucleosides (including inosine, adenosine, guanosine, uridine) and other nucleotides (AMP, ADP, ATP, IDP, ITP, GDP, GTP, xanthosine 5′-monophosphate, 3′-IMP, 3′-UMP, 2-deoxy-IMP, allyltio-IMP) were not. Thus only IMP, but not inosine has been reported to have stimulatory effects on feeding of fish species including jack mackerel (Ikeda *et al*., 1991) and largemouth bass *Micropterus salmoides* **(**Kubitza *et al*., 1997). IMP may serve as a primary candidate for feed attractant research to further

explore complete replacement of fishmeal in aquafeeds for carnivorous species.

Borda *et al*. (2003) reviewed research concerning dietary nucleotide application to sea bream *Sparus aurata* larvae and hypothesized that an exogenous supply of nucleotides may promote growth of fish and crustaceans in the early stages to meet their high rate of cell replication. Person-Le Ruyet *et al*. (1983) also reported that turbot larvae (approximately 100 mg/fish) fed an inosine-supplemented diet (1.3% of diet for 6 days, 0.13% for 45 days) had significantly enhanced growth and survival after a 55-day period. Their subsequent study showed that 10 or 20 days of feeding a diet supplemented with 0.77% inosine also significantly increased weight gain of turbot larvae (approximate initial weight of 230 mg/fish). It was hypothesized that the growth-enhancing effect of inosine resulted from improved feed intake as live foods were discontinued, thereby promoting more rapid feed intake that reduced nutrient leaching into the water or possibly playing other roles in metabolism (Métailler *et al*., 1983). Beside inosine, growth-enhancing effects of nucleotide metabolite mixtures (such as Ascogen®, Chemoforma Co., Basel, Switzerland) occasionally have been observed in fish species such as tilapia *Oreochromis niloticus* × *Oreochromis aureus* (Ramadan and Atef, 1991) and rainbow trout (Adámek *et al*., 1996). A recent short-term feeding trial with juvenile red drum *Sciaenops ocellatus* conducted in our laboratory demonstrated that diets supplemented with 0.03 and 0.1% of a purified nucleotide mixture (equal amounts of AMP sodium, IMP sodium, CMP sodium, GMP sodium and UMP sodium coated with carboxymethyl cellulose, casein and gelatin) significantly enhanced weight gain and feed efficiency compared to that of fish fed an isonitrogenous basal diet (Li and Gatlin, unpublished results). However, the growth-enhancing effect of commercial nucleotide products on most juvenile or sub-adult fish appears to be rather marginal (Li *et al*., 2004a) based on current knowledge.

Information on the effects of dietary nucleotide supplementation on other production and quality traits of fish including body composition, fillet yield, taste and texture is extremely limited thus far. Juvenile red drum fed diets supplemented with Optimûn had significantly higher whole-body lipid content, but not intraperitoneal lipid deposition (Li *et al*., 2005). However, this phenomenon was not noticeable in hybrid striped bass *Morone chrysops* × *Morone saxatilis* (Li *et al*., 2004a, b). Although it is known that dietary nucleotides can influence levels of various lipids and/or fatty acids in certain tissues such as erythrocytes, plasma, liver and brain of mammals (Carver and Walker, 1995), such information in fish and potential physiological consequences are limited but deserve further investigation. Tacon and Cooke (1980) reported dietary inclusion of high levels of nucleic acids (10% of the diet) in the form of yeast extract increased the ash content of the rainbow trout carcass, although the possible influences of exogenous nucleotides on mineral absorption and metabolism are poorly studied at this time.

5.2 Reproduction and gastrointestinal function

Besides marginally enhanced growth performance of fish fed supplemental nucleotides, there are also a variety of other physiological responses that may be affected, including those related to reproduction and gastrointestinal function. One of the well-recognized uses of nucleotide supplementation in human nutrition is related to health of neonates and infants. Such a maternal strategy has been applied to a limited extent in fish. Gonzalez-Vecino *et al*. (2004) first studied nucleotide nutrition of broodstock haddock *Melanogrammus aeglefinus* and observed that first feeding success of larvae from nucleotide-fortified broodstock was significantly higher than that of larvae from broodstock fed a basal diet without supplemented nucleotides. In addition, the survival of larvae from broodstock fed the nucleotide-supplemented diet was over 30% greater than that of larvae from broodstock fed the basal diet. Also it was noted that gut development and size of larvae from broodstock fed the nucleotide-supplemented diet were significantly greater than those from broodstock fed the basal diet. A similar phenomenon also has been observed with sea urchins (A.L. Lawrence, Texas A&M University, 2005, personal communication).

Dietary nucleotides have been shown to have effects on the gastrointestinal (GI) tract in animal models, including physiological, morphological and microbiological influences. Dietary nucleotides or AMP alone can significantly increase growth and differentiation of the developing GI tract (Uauy *et al*., 1990). These compounds also have been reported to ameliorate intestinal injury and facilitate bifidobacteria predominance in the intestine (Carver and Walker, 1995). Morphological responses of human and terrestrial animal GI tracts to dietary nucleotides include increased villus height (Uauy *et al*., 1990), increased jejunum wall thickness and villus cell number (Bueno *et al*., 1994). At this time, however, research on gastrointestinal responses of fish to dietary nucleotides is limited except for several intestinal morphological studies. Burrells *et al*. (2001a) first detected morphological responses of Atlantic salmon *Salmo salar* intestine to dietary nucleotides by histological examination including increased mean fold height of proximal, mid- and distal intestine as well as total gut surface area in fish fed a nucleotide-supplemented diet. Borda *et al*. (2003) recently reported similar observations in juvenile sea bream. Thus, it is apparent that dietary nucleotides can beneficially influence intestinal health of fish. Because the intestine is a very important immune organ and a significant portion of dietary nucleotides are retained in the GI tract, the possible influences of dietary nucleotides on mucosa associated lymphoid tissue (MALT) should be regarded as a prioritized topic of nucleotide nutrition research, although current knowledge of MALT in fish is very limited. Because dietary nucleotides have been reported to promote intestinal microflora such as bifidobacteria in humans and various animals (Carver and Walker, 1995), the possible effect of dietary nucleotides on microbial ecology of the fish GI tract as a potential prebiotic is another interesting topic for further research.

5.3 Immune responses

5.3.1 Innate immunity

It is well established that dietary nucleotides can influence macrophage activity such as phagocytosis (Grimble and Westwood, 2000b; Gil, 2002) and activity of natural killer cells (Carver *et al*., 1990). Research with fish also has shown that exogenous nucleotides can influence both humoral and cellular components of the innate immune system. Sakai *et al*. (2001) reported that exogenous nucleotides could increase serum complement (alternative pathway) and lysozyme activity as well as phagocytosis and superoxide anion production of head kidney phagocytes of common carp (*Cyprinus carpio*). In addition, Li *et al*. (2004a) reported that hybrid striped bass fed an oligonucleotide-supplemented (Ascogen P®, Canadian Biosystem Inc., Calgary, Alberta, Canada) diet had higher blood neutrophil oxidative radical production than fish fed the basal diet. In contrast, an effect of dietary nucleotides on respiratory burst of head kidney cells of salmonids was not demonstrated (Burrells *et al*., 2001a). Devresse (2000) also suggested that exogenous nucleotides are key nutrients for the shrimp immune system, however, to the best of our knowledge, this hypothesis remains to be tested.

Low *et al*. (2003) recently reported changes in immune gene expression induced by dietary supplementation of nucleotides and discovered that non-specific immune components such as lysozyme expression were significantly decreased in the spleen and kidney of turbot fed a nucleotide-supplemented diet, but no effect was apparent in the gill. In contrast, interleukin-1β showed a significant increase in expression in the kidney of nucleotide-supplemented fish, whereas, expression of transferrin and transforming growth factor β was unaffected by nucleotide supplementation (Low *et al*., 2003).

5.3.2 Adaptive immunity

Nucleotides also have been shown to influence lymphocyte activity and immunoglobulin production. Jyonouchi *et al*. (1993, 1994) and Navarro *et al*. (1996) suggested nucleotides exert their greatest impact on the immune system by modulating immunoglobulin production. Ramadan *et al*. (1994) first reported that dietary supplementation of nucleotides (Ascogen, Chemoforma Ltd, Basel, Switzerland) had a marked immunopotentiating effect on both humoral and cell-mediated immune responses of tilapia after intramuscular injection or direct immersion with formalin-killed *Aeromonas hydrophila*. Antibody titres after vaccination as well as mitogenic responses of lymphocytes from fish fed the nucleotide-supplemented diet were much higher and significantly different than those of fish fed the basal diet. Similar phenomena also have been reported for other species such as rainbow trout (Burrells *et al*., 2001b; Leonardi *et al*., 2003) and hybrid striped bass (Li *et al*., 2004a). For example, Burrells *et al*. (2001b) observed that

Atlantic salmon fed a nucleotide-supplemented diet for 8 weeks had significantly enhanced specific antibody production compared to fish fed the basal diet. In addition, Leonardi *et al*. (2003) reported a significant enhancement of lymphocyte stimulation in rainbow trout fed a nucleotide-supplemented diet. The antibody titre of hybrid striped bass fed an oligonucleotide-supplemented diet after vaccination with formalin-killed *Streptococcus iniae* was threefold higher than that of fish fed a basal diet (Li *et al*., 2004a). Low *et al*. (2003) also reported that dietary nucleotides enhanced expression of immunoglobulin M and recombinase activating gene in gill and spleen of turbot but reduced their expression in kidney. Although the mechanisms of these various actions are practically unknown, nucleotides may be used as an 'oral adjuvant' and therefore enhance vaccination efficacy. Burrells *et al*. (2001b) explored this strategy in vaccination and reduced mortality of vaccinated Atlantic salmon from 6% to 2%. In summary, research on modulation of adaptive immunity by exogenous nucleotides has shown consistent results in various fish species; therefore, further research on this subject is warranted.

5.4 Stress responses

One of the more readily accepted hypotheses associated with the observed beneficial effects of dietary nucleotides in fish is that the typical stressors encountered by fish reared in aquaculture, such as poor water quality, excessive crowding and frequent handling, place additional demands on available nucleotides beyond those provided in typical aquafeeds, so that an exogenous supply may be beneficial (Burrells *et al*., 2001b; Low *et al*., 2003). One possible means by which dietary nucleotides beneficially influence the fish immune system is by partially offsetting the inhibitory effects of cortisol release associated with stress. Burrells *et al*. (2001b) first raised the hypothesis that dietary nucleotides could enhance stress tolerance and provided some evidence of this by comparing osmoregulatory capacity and growth performance of Atlantic salmon fed a nucleotide-supplemented diet and control diet after acute stress by seawater transfer. This hypothesis was not fully proven until Leonardi *et al*. (2003) observed that dietary nucleotides reduced serum cortisol levels of healthy rainbow trout after 90–120 days feeding as well as in fish infected with infectious pancreatic necrosis (IPN) virus. This stress reduction associated with dietary nucleotides also resulted in enhanced disease resistance of challenged fish in the study. Currently it remains unknown if exogenous nucleotides are involved in signalling pathways associated with stress responses or if various stressors have specific effects on nucleotide metabolism of fish.

5.5 Disease resistance

Survival after challenge with certain pathogens is generally assessed as a measure of disease resistance. It has been reported that dietary nucleotides

can enhance resistance of fish against various pathogenic organisms including viruses, bacteria and parasites, indicating a promising use of these biochemicals for health management in aquaculture.

Burrells *et al*. (2001a) observed that Atlantic salmon fed a nucleotide-supplemented diet for 2 weeks had a cumulative total mortality of 35.7%, compared to 48% for fish fed the basal diet, 53 days after initial contact with fish previously injected with infectious salmon anaemia (ISA) virus. The difference in mortality between the two treatments after 39 and 45 days of cohabitation was also statistically significant. Also Leonardi *et al*. (2003) reported that all rainbow trout fed a nucleotide-supplemented diet for 60 days survived injection of IPN virus, whereas all virus-injected fish fed the basal diet died. Enhanced resistance to various pathogenic bacteria also has been reported for several fish species including salmonids (Burrels *et al*., 2001a), common carp (Sakai *et al*., 2001) and hybrid striped bass (Li *et al*., 2004a). Burrels *et al*. (2001a) reported that after bath challenge with *Vibrio anguillarum*, rainbow trout fed a nucleotide-supplemented diet had cumulative mortality of 31%, while fish fed the basal diet and β-glucan-supplemented diets had mortality values of 49% and 43%, respectively. Burrels *et al*. (2001a) also reported cohabitation of coho salmon *Oncorhynchus kisutch* with fish infected with *Piscirickettsia salmonis*, a rickettsia-like intracellular γ-proteobacteria, resulted in 76.8% mortality in fish fed a basal diet; whereas, only 46.9% mortality was observed in fish fed the nucleotide-supplemented diet. However, the mechanism(s) contributing to this significant enhancement in survival after *P. salmonis* challenge have not been fully defined. In another study, Sakai *et al*. (2001) orally treated common carp with either nucleotide-suspended saline or an equal amount of dextrin (control group) and then injected the fish intraperitoneally with 0.1 ml of 3×10^7 cells/ml suspension of *A. hydrophila* and determined the bacterial number in blood, liver and kidney 2, 4, 8 and 12 h after injection. In all of the tissues from fish treated with nucleotides, no *A. hydrophila* cells were detected 12 h after challenge, however, the number of bacteria in the blood and liver of control fish reached 1×10^3 cells/ml. Li *et al*. (2004a) also reported that juvenile hybrid striped bass fed a nucleotide-supplemented diet for 7 or 8 weeks prior to exposure to *S. iniae* had reduced mortality (3% trial 1, 13% trial 2) compared to fish fed a basal diet (20% trial 1, 40% trial 2). The mortality of hybrid striped bass fed the nucleotide-supplemented diet after re-exposure to *S. iniae* was 52% and significantly lower than the 83.8% mortality experienced by fish fed the basal diet. Besides viral and bacterial pathogens, dietary nucleotides also significantly reduced the number of sea lice infecting Atlantic salmon (Burrells, 2001).

Because pathogenic bacteria may have various infection routes and induce numerous immune responses of the host (Ellis, 1999), the protective mechanism(s) of exogenous nucleotides should be further characterized. However, rather consistent results from various experiments have indicated that dietary nucleotides enhance resistance of fish to numerous different pathogens. This phenomenon may have important application for disease control in aquaculture.

6 Concluding Remarks and Research Needs

At this time existing information on the various applications of dietary nucleotides in aquaculture is rather limited but shows considerable promise (Table 12.1). Additional research in several different areas is needed to more fully characterize how these compounds function biochemically and how

Table 12.1. Research on dietary supplementation of nucleotides (NT) in fish (Source: Li and Gatlin, 2006; courtesy of Elsevier).

Authors	Nucleotide form	Dose	Length of administration	Species	Initial size	Effect[a]
Ramadan and Atef (1991)	Ascogen S (Chemoforma, Augst, Switzerland)	2 and 5 g/kg diet	16 weeks	Hybrid tilapia	21 days old	Growth ↑ Survival ↑
Ramadan *et al.* (1994)	Ascogen (Chemoforma, Augst, Switzerland)	5 g/kg diet	120 days	Hybrid tilapia	30 days old	Antibody titre after vaccination ↑ Mitogenic response of lymphocyte ↑
Adámek *et al.* (1996)	Ascogen (Chemoforma, Augst, Switzerland)	0.62, 2.5 and 5 g/kg diet at 1% bw/day	37 days	Rainbow trout	163.4–169.7 g/fish	Growth ↑
Burrells *et al.* (2001a)	Optimûn (Chemoforma, Augst, Switzerland)	2 g/kg diet, containing 0.03% NT, 2% bw/day	3 weeks	Rainbow trout	217 ± 62 g/fish	Survival after challenge with *V. anguillarum* ↑
		2 g/kg diet, containing 0.03% NT, 1% bw/day	2 weeks	Rainbow trout	53–55 g/fish	Survival after challenge with infectious salmon anaemia virus ↑
		2 g/kg diet, containing 0.03% NT, 2% bw/day	3 weeks	Coho salmon	100 g/fish	Survival after challenge with *P. salmonis* ↑
		2 g/kg diet, containing 0.03% NT, 2% bw/day	3 weeks	Atlantic salmon	60 g/fish	Sea lice infection ↓
Burrells *et al.* (2001b)	Optimûn (Chemoforma, Augst, Switzerland)	2 g/kg diet, containing 0.03% NT at 1.5% bw/day	3 weeks before vaccination and 5 weeks post-vaccination	Atlantic salmon	34.7 ± 9.6 g/fish	Antibody titre ↑ Mortality ↓
		2 g/kg diet, containing 0.03% NT at 1.5% bw/day	8 weeks	Atlantic salmon	43 ± 3.0 g/fish	Plasma chloride ↓ Growth ↑
		2 g/kg diet, containing 0.03% NT	10 weeks	Atlantic salmon	205 g/fish	Intestinal fold ↑

Continued

Table 12.1. *Continued.*

Authors	Nucleotide form	Dose	Length of administration	Species	Initial size	Effect[a]
Sakai *et al.* (2001)	Ribonuclease-digested yeast RNA (Amano Seiyaku Co-op, Tokyo, Japan)	15 mg/fish	3 days	Common carp	100 g/fish	Phagocytosis ↑ Respiratory burst ↑ Complement ↑ Lysozyme ↑ *A. hydrophila* infection ↓
Leonardi *et al.* (2003)	Optimûn (Chemoforma, Augst, Switzerland)	NA	120 days	'All-female' rainbow trout	80–100 g/fish	B lymphocytes ↑ Resistance to IPN virus ↑ Plasma cortisol ↓
Low *et al.* (2003)	Optimûn (Chemoforma, Augst, Switzerland)	2 g/kg diet, containing 0.03% NT to hand satiation daily	15 weeks	Turbot	120.9 ± 5.1 g/fish	Altered immunogene expression in various tissues
Li *et al.* (2004a)	Ascogen P (Canadian Biosystem Inc., Calgary, Canada)	5 g/kg diet, fixed ration approaching satiation daily	7 weeks	Hybrid striped bass	7.1 g/fish (trial 1), 9.1 g/fish (trial 2)	Neutrophil oxidative radical production ↑ Survival after challenge with *S. iniae* ↑

[a] Symbols represent an increase (↑) or decrease (↓) in the specified response.

they should be administered in the diet to obtain more consistent and favourable responses.

Dose and duration are primary considerations in administration of immunostimulants such as nucleotides (Sakai, 1999), although efforts to explore optimization of these administration protocols with nucleotides have been limited to date. Fish such as salmonids and sea bass *Dicentrarchus labrax* have been shown to tolerate high dietary levels of nucleic acids and/or yeast by virtue of their active liver uricase (Kinsella *et al*., 1985; Rumsey *et al*., 1992; Oliva-Teles and Goncalves, 2001). Thus, toxicity associated with nucleotide administration may not be as much of a concern with fish as compared to terrestrial animals. For example, Rumsey *et al*. (1992) reported that yeast RNA extract at levels up to 4.1% of diet did not depress growth of rainbow trout. However, an earlier study showed the growth-depressing effects of bacterial RNA extract (10% of diet) on rainbow trout associated with increased serum urea and carcass ash content, while RNA extract at 2.5% and 5% of diet did not reduce growth (Tacon and Cooke, 1980). However, Adámek *et al*. (1996) reported that 0.62 and 2.5 g Ascogen/kg diet increased growth and feed efficiency of rainbow trout, while 5 g Ascogen/kg diet led to growth depression of rainbow trout and goldfish after 37 days of feeding. Although the conclusiveness of this study was somewhat limited due to statistical considerations, it does indicate that attention to dose-optimization of nucleotide products for aquaculture is warranted. Future progress in identifying molecular mechanisms of

nucleotide signalling may provide useful information to address these issues including optimal dosage.

The administration regime for optimum responses of immunostimulants in aquaculture is another area requiring further research. In some instances, prolonged administration of immunostimulants such as peptidoglycan and levamisole may cause undesirable side effects on growth and disease resistance of cultured fish (e.g. Matsuo and Miyazano, 1993; Li *et al*., 2004b). Currently, there is no definitive evidence indicating the efficacy of dietary nucleotides is strictly associated with administration duration. However, Leonardi *et al*. (2003) observed that mitogenic response of B lymphocytes from rainbow trout was influenced by dietary nucleotide after 60 days of feeding, but not after 120 days. Also it has been observed that hybrid striped bass fed an Ascogen P®-supplemented diet for 16 weeks failed to show any enhancement of innate immune responses (including blood neutrophil oxidative radical production, serum lysozyme and extracellular and intracellular superoxide anion production of head kidney cells) which was not consistent with the results after 8 weeks of feeding the same diet (Li *et al*., 2004a). Although this evidence is rather circumstantial, such observations may suggest that administration length or regime should be taken into consideration in the use of dietary nucleotides. However, comprehensive research is needed to clarify the possible effects of prolonged feeding of nucleotides on immunity and disease resistance of fish.

Also additional research is needed to explore specific effects of various types of nucleotides to gain a better understanding of their potential applications. The selection of appropriate sources of nucleotide should be a primary consideration to study in the future. To date, most publications on nucleotide supplementation for fish have used patented or registered commercial products; therefore, information pertaining to concentrations and ratios of various types of nucleotides are generally unavailable. As such, it is very difficult to quantitatively estimate or compare the effects of supplemented nucleotides on the immune responses of various fish species. For example, yeast such as *S. cerevisiae* is an important source of nucleotides. However, lack of processing procedures or detailed information about this product makes it difficult to determine if the nucleotides are present in the form of mononucleotides or polynucleotides (oligonucleotides). Based on information from terrestrial animals, mononucleotides and polynucleotides may have different effects on the host. The immunostimulatory effect of microbial oligonucleotides or oligodeoxynucleotide primers also has been shown *in vitro* (Laing *et al*., 1999; Meng *et al*., 2003) as well as *in vivo* (Tassakka and Sakai, 2002) with fish, although these oligonucleotides were not from yeast. Although early attempts at exploring nucleotide supplementation for fish have been encouraging, improvements in methodology, especially the selection of nucleotide sources, are essential for future advancements in this area.

Although nucleotides have diverse physiological functions, health management is the most promising use of dietary nucleotides for the aquaculture industry. However, current information on dose,

administration regime, even nucleotide type is rather limited to ensure the efficacy of these biochemicals under various physiological and ecological situations. Research on possible age/size related responses of fish to dietary nucleotides is needed as well as additional information on the digestion and absorption processes of nucleotides. At least the form(s) of nucleotides most readily absorbed by fish should be screened for development of dietary supplements. Grimble (2001) addressed concerns about the sensitivity of various genotypes to immunonutrients. To the best of our knowledge, this type of information is not available for fish, although it is well established that genetic polymorphisms influence growth and some physiological responses such as lysozyme production of fish. Thus, potential genetic variation to nucleotide supplementation in various fish species is another area of research that should be explored.

Exogenous nucleotides may be involved in cell signalling pathways as well as serve as nutrients for biosynthesis. For example, the influences of nucleotide availability on gene transcription rate have been reported in murine intestinal epithelial cells (Walsh *et al*., 1990, 1992) and intestine (Valdés *et al*., 2000; Sánchez-Pozo and Gil, 2002). Development and application of novel tools in molecular biology will be necessary to more precisely and comprehensively detect various alterations in expression of specific genes in various tissues and organs of fish in response to nucleotide supplementation. Low *et al*. (2003) set an example for future research in this area. Further progress in deciphering genomes of fish is needed for the development of powerful analytical tools such as microarrays that may shed light on the various influences of nucleotides.

Note

[1] Abbreviations for nucleotides referred to in this chapter are as follows: AMP, adenosine-5′-monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CMP, cytidine-5′-monophosphate; GMP, guanidine-5′-monophosphate; GDP, guanidine diphosphate; GTP, guanidine triphosphate; IMP, inosine-5′-monophosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; TMP, thymine-5′-monophosphate; UMP, uridine-5′-monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate.

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13 Nutrigenomics

STÉPHANE PANSERAT,[1] SÉVERINE KIRCHNER[2] AND SADASIVAM KAUSHIK[1]

[1]*UMR NuAGe, INRA-IFREMER – University Bordeaux 1, 64310 Saint-Pée-sur-Nivelle, France;* [2]*Department of Pharmacology and Physiology, UMDNJ, New Jersey Medical School, Newark, NJ 07103, USA*

1 Introduction

There is a growing recognition that dietary micro- or macronutrients are potent signals that influence the metabolic programming of cells and have an important role in the control of homeostasis as well as growth and development.

The major reactions of the biochemical pathways leading to metabolism of essential nutrients are relatively well known. These have come mostly through studying the substrates and products of the reactions and the enzymes catalysing them. Regulations of the reactions have furthermore focused on the activity and specificity of the enzymes in terms of allosteric control and post-translational modifications. One way to study changes in flux through a particular metabolic pathway in response to altered diet would be to measure substrate and product concentrations of a particular reaction. This however is not feasible for multicellular organisms on a large scale. A complementary approach is through analysis of genes that encode these enzymes using recently developed genomic technologies. Although altering enzyme levels may not necessarily result in altering flux through that pathway, significant alterations in the expression of the enzyme-encoding genes may reflect responsive flux changes. In addition, this approach may identify molecules that regulate specific metabolic pathways, such as transcription factors or components of signal transduction cascades.

Classical molecular techniques to study gene expression are hybridization-based approaches such as Northern blot and *in situ* hybridization as well as PCR-based techniques such as the real-time reverse transcription-polymerase chain reaction (real time RT-PCR). These techniques are highly informative and give reliable data on gene expression, each one having its own utility, strengths and weaknesses

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(Reue, 1998; Bustin and Nolan, 2004). However all these techniques can study only a few identified genes at one time.

Nutritional genomics (nutrigenomics) refers to research that investigates the interaction between nutrition and the genome. This research is well developed in humans for evaluating foods and nutritionally bioactive compounds to promote health and prevent diseases (Gillies, 2003; Muller and Kersten, 2003; Kaput and Rodriguez, 2004) (see web sites focused on nutritional genomics such as http://nutrigenomics.ucdavis.edu/index.htm in the USA, http://www.nugo.org/everyone in Europe and http://www.nutrigenomics.org.nz/ in New Zealand). Nutrigenomics is a discovery science driven by the paradigms of molecular biology, enabled by microarrays technology, and integrated on an informatics platform. It is important to recognize that nutrients, in contrast to specific pharmacological molecules, can have a number of direct and indirect effects on gene expression. Indeed, organisms have to process a large number of different nutrients which can reach high intra-cellular concentrations. Each nutrient can also bind to numerous targets with different affinities and specificities. Nutrients interact with transcription factors or regulate transcription factors to control gene expression. Indeed, transcription factors such as peroxisome-proliferator-activated receptors (PPARs), sterol regulatory element-binding protein (SREBP), liver X receptor (LXR), carbohydrate response element-binding protein (ChREBP), etc. are the main agents through which nutrients influence gene expression. Detailed information on such regulation has been provided by Muller and Kersten (2003).

There are molecular biological techniques which can provide information on a number of genes at the same time and can be used in nutritional studies. Gene expression profiling may be performed by using microarray technology, which can monitor the expression of thousands of genes simultaneously. Moreover, these genes can have known or unknown functions. The technique relies only on simple hybridization to DNA segments affixed to a single nylon filter or glass side. More details on DNA microarray technologies can be seen in Hirshi *et al*. (2001). Other integrative approaches such as Differential Display RT-PCR (Harris, 2000) and Serial Analysis of Gene Expression (SAGE) (Velculescu *et al*., 1995) also may be applied but have some drawbacks which limit their usefulness. Both differential PCR and SAGE approaches are not stand-alone techniques. Differential display PCR can often express false leads when low-copy RNAs are being amplified.

2 Candidate Gene Approach in Fish Nutrition

In order to show the usefulness of the molecular approach in fish nutrition, we will outline a few examples of recent studies on nutritional regulation of candidate gene expression for proteins involved in digestion and intermediary metabolism in fish.

2.1 Nutritional regulation of digestive physiology at a molecular level

Context: suppression of live diets and replacement by inert-formulated diets in marine fish larvae

Production of marine fish larvae and juveniles (European sea bass *Dicentrarchus labrax*, gilthead seabream *Sparus aurata*, red sea bream *Chrysophrys major*) in commercial hatcheries still depends on the supply of live prey, such as rotifers and *Artemia* (Watanabe and Kiron, 1994; Cahu and Zambonino-Infante, 2001). As was already demonstrated in freshwater cyprinids or other small-egg larvae (Charlon and Bergot, 1984; Kaushik, 1988), compound diet substitution for live prey is crucial for lowering production costs and for sustaining production of high and constant quality juveniles.

Until now, compound diet substitution for live prey is generally performed after some weeks of life in the marine fish hatchery, while freshwater species can be fed compound diets as early as mouth opening. A number of studies have attempted to determine the timing of initiation of feeding and/or gastrointestinal functionality in fish larvae. Utilization of molecular techniques may help to answer to this question.

Digestive (pancreatic and intestinal) enzymes of sea bass larvae can be adapted to the composition and the quantity of diets and this adaptation may be due to a molecular regulation of their gene expression (Peres *et al*., 1998; Zambonino-Infante and Cahu, 1999). For example, Peres *et al*. (1998) suggested that the coordinated decrease between specific activity and mRNA levels of amylase enzyme is transcriptionally regulated during larval development. As such, amylase mRNA and activity are very high during young larval stages and decrease during the development of larvae. Overall, results show that digestive function of the different digestive organs (pancreas and intestine) are efficient before the onset of exogenous feeding and that fish larvae have, to a certain extent, the capacity to adapt their enzyme secretion and activity to diet composition. However, formulation of a larval diet must take into consideration the genetically programmed pattern of enzymes.

Another major aim of the studies conducted now in larval nutrition is to improve larval quality. It appeared that nutrients affect development of fish larvae and their quality (reviewed by Cahu *et al*., 2003). Several physical anomalies have been described in fish larvae and particularly skeletal malformation linked to nutrients. For example, high dietary retinoic acid levels result in higher incidence of bone deformities in Japanese flounder larvae. The teratogenic effect of retinoic acid observed in embryonic and postembryonic stages was explained by a depression of sonic hedgehog (shh), known to be a transcriptional factor (Suzuki *et al*., 1999). Understanding the action of the dietary components at the molecular level will allow improvements in the quality of hatchery-reared larvae.

2.2 Nutritional regulation of lipid metabolism

Context: suppression of fish oil/fishmeal by vegetable products without negative consequences on fish product quality

2.2.1 Fatty acid biosynthesis

Stagnation in feed grade fisheries, along with increased demand for fish oils, has dictated that alternatives to fish oils must be found if aquaculture is to continue to expand and supply more of the global demand for fish (Sargent *et al*., 2002). The only sustainable alternative to fish oils is plant (vegetable) oils, which can be rich in C18 but devoid of the essential fatty acids such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), compromising their nutritional value. Understanding the molecular basis of fatty acid biosynthesis and its regulation in fish is indeed necessary to best exploit and eventually manipulate the activity of the fatty acid bioconversion pathways to enable efficient and effective use of vegetable oils in aquaculture.

The pathway whereby C20 and C22 fatty acids are biosynthesized from their C18 precursors is particularly important in fish. Many vertebrates can convert the C18 n-3 fatty acid to long chain n-3 fatty acids such as EPA and DHA via a pathway involving a series of fatty acid desaturation and elongation reactions (reviewed by Sargent *et al*., 2002). Freshwater fish seem to express all the enzymes involved in long chain polyunsaturated fatty acid (LC-PUFA) biosynthesis whereas marine fish are unable to produce DHA at a significant rate due to apparent deficiencies in one or more steps in the pathway (Sargent *et al*., 2002). In recent years, significant progress has been made in characterizing fatty acid desaturates involved in LC-PUFA synthesis. Cloned cDNAs for Δ6-desaturases were isolated and functionally tested in freshwater fish (zebrafish *Danio rerio*, rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar*, common carp *Cyprinus carpio*) and marine fish (turbot, gilthead seabream) (Hastings *et al*., 2001; Seiliez *et al*., 2001, 2003; Zheng *et al*., 2004). Moreover, a strong nutritional regulation of Δ6-desaturase gene expression by lipids and carbohydrates was observed (Fig. 13.1). However, so far the Δ5-desaturase gene has not been characterized in marine fish in contrast to freshwater fish (Hastings *et al*., 2004), suggesting a genetic determinism in the dependence of marine fish to fish oils rich in n-3 LC-PUFA. To our knowledge, the presence of EPA and DHA in diets is still necessary to produce high quality marine fish (rich in n-3 PUFAs) for human food.

2.2.2 Link between dietary lipid quantity and quality and tissue lipid: importance of lipid storage

There is a strong relationship between the dietary lipid level intake and the lipid levels found in the whole body and especially in muscle of fish (Corraze *et al*., 1999; Sargent *et al*., 2002). Deposition of excess lipid in the

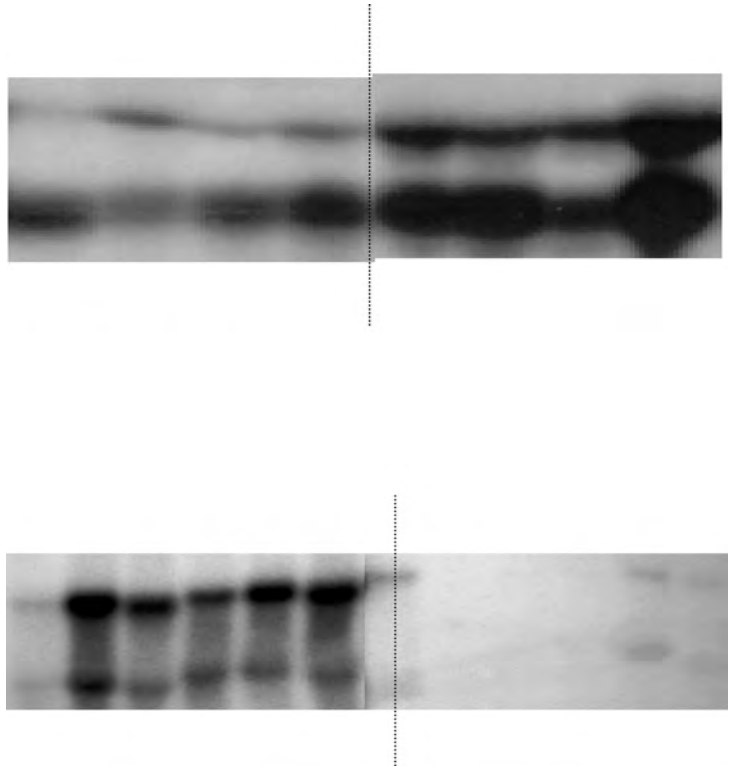

Fig. 13.1. Nutritional regulation of Δ6-desaturase gene expression in livers of rainbow trout (A) and gilthead seabream (B) (Source: adapted from Seiliez *et al.*, 2001, 2003). Rainbow trout were fed with fish oils (FO) or vegetable oils (VO) whereas gilthead seabream were fed with a highly unsaturated fatty acid (HUFA)-rich diet (+HUFA) or an HUFA-deprived diet (−HUFA). There is probably inhibition of desaturase gene expression by its enzymatic products (i.e. n-3 PUFAs). Molecular mechanisms are unknown.

body will clearly be a more serious problem in those species that tend to store lipid in the flesh. However, it is well known that the levels of lipids in the flesh and the tissue localization of the lipid storage (muscle, liver and perivisceral fat tissue) vary considerably among species (Sargent *et al.*, 2002), populations and sometimes between individuals in the same population (Quillet *et al.*, 2005). It is not clear to what extent genetic factors contribute to the observed variations (Sargent *et al.*, 2002). Mechanistic studies about dietary lipid storage are thus necessary. Preliminary studies have been performed about key enzymes such as the lipoprotein lipase (LPL) involved in lipid transport and storage. LPL plays a central role in lipoprotein metabolism, hydrolysing triglycerides and phospholipids in chylomicrons and very low density lipoproteins (VLDL). In contrast to what is observed in mammals, the LPL gene is expressed in livers of adult rainbow trout and red sea bream (Lindberg and Olivecrona, 2002; Liang *et al.*, 2002): thus LPL in fish liver functions similar to lipase in higher

vertebrates. Moreover, in red sea bream, the LPL gene is regulated in a tissue-specific fashion by the nutritional state (Liang *et al*., 2002) suggesting that regulation of LPL may affect lipid storage in fish differently depending upon the tissue.

2.3 Nutritional regulation of glucose metabolism

Context: incorporation of plant-based protein feedstuffs necessarily involves inclusion of carbohydrates

Teleosts in general do not utilize dietary carbohydrates very well and there appear to exist inter-species differences (reviewed by Wilson, 1994; Moon, 2001). In the context of replacement of fishmeal with plant feedstuffs naturally rich in carbohydrates in diets for carnivorous fish, it is important to analyse nutritional regulation of glucose metabolism in order to understand why these species have some difficulties utilizing high levels of digestible carbohydrates. Different hypotheses (Wilson, 1994; Moon, 2001) have been proposed in the context of understanding inter-species differences in dietary carbohydrate utilization. Recent studies in this area have tried to use molecular approaches to answer some of these questions.

2.3.1 Glucose transport: importance of glucose transporters (Gluts)

Glucose transport inside the cells is the first step for glucose utilization in any organism (Wood and Trayhurn, 2003). Studies at the molecular level have been useful to distinguish distinct types of glucose transporters and to detect their presence or absence in fish. Glucose transporter 1 (Glut1; a ubiquitous glucose transporter), glucose transporter 2 (Glut2; a transporter of high concentrations of glucose either into liver/pancreas or out of the intestine to blood) and glucose transporter type 4 (Glut4; an insulin-sensitive transporter involved in glucose transport of muscle and fat tissues) have all been found in rainbow trout through cloning of the corresponding genes and analysis of their expression (Teerijoki *et al*., 2000; Capilla *et al*., 2002). The existence of these transporters is now proven, however, more studies are needed to analyse the nutritional control of their expression and especially their function such as their capacity of translocation to the membrane (for Glut4).

2.3.2 Glucose metabolism: equilibrium between glucose utilization/storage/bioconversion (glycolysis, glycogenogenesis, lipogenesis) and glucose production (gluconeogenesis/glycogenolysis)

The liver plays a key role in coordinating body metabolism in response to nutritional status (Pilkis and Granner, 1992). Much of the regulatory effects occur initially in the liver, which then modulates activities of other organs regarding nutrient utilization and metabolism. Both metabolic pathways

leading to synthesis and degradation are active in the liver. One of the hypotheses to explain low dietary carbohydrate utilization is an atypical regulation of hepatic glucose metabolism in fish fed with high levels of carbohydrates.

The first metabolic pathway is involved in the storage of end products of glucose utilization (glycolysis, lipogenesis, glycogenogenesis). When mammals are fed with carbohydrates, there is induction of the enzymes in these metabolic pathways mainly linked to the increase of mRNA levels (Pilkis and Granner, 1992). This and other laboratories have found that the first enzyme of glucose phosphorylation, glucokinase, is indeed highly induced in rainbow trout and gilthead seabream after feeding carbohydrates (Caseras *et al*., 2000; Panserat *et al*., 2000a) and this induction is related to higher glucokinase gene expression as in mammals (Fig. 13.2). The cloning of glucokinase cDNA as well as its nutritional regulation were the first demonstrations of the possible adaptations of carnivorous fish to carbohydrates by mechanisms similar to those in mammals. The initial hypothesis for the absence of an inducible glucokinase in fish liver (Wilson, 1994) was clearly refuted by these studies (at least in these species) and

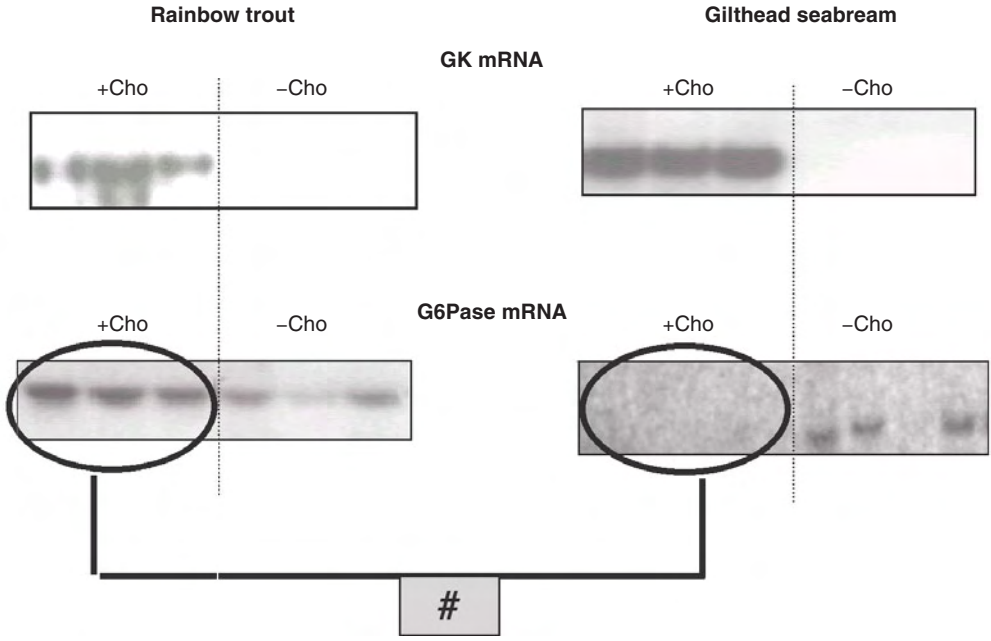

Fig. 13.2. Regulation of hepatic glucose metabolism by dietary carbohydrates in rainbow trout and gilthead seabream (Source: adapted from Panserat *et al*., 2000a, 2001b, 2002b). Two key enzymes involved in the glucose/glucose-6-phosphate cycle (glucokinase (GK) and glucose-6-phosphatase (G6Pase) (Pilkis and Granner, 1992)) have been studied at a molecular level by Northern blotting. +Cho, diet with digestible carbohydrates (20%); −Cho, diet without carbohydrate; #, there is always persistent G6Pase gene expression in rainbow trout fed with carbohydrates in contrast to gilthead seabream.

suggest that this step is not the limiting factor to explain the low dietary carbohydrate utilization by fish.

The second metabolic pathway is that corresponding to the production of endogenous glucose by the liver. Two metabolic pathways are involved: glycogenolysis and gluconeogenesis. In contrast to mammals (Pilkis and Granner, 1992; Van de Verve *et al*., 2000) and gilthead seabream (Caseras *et al*., 2002; Panserat *et al*., 2002b), there was no decrease in gluconeogenic gene expression, nor in the activities of glucose-6-phosphatase, fructose-1,6-biphosphatase (FBPase) and phosphoenolpyruvate carboxykinase, irrespective of the nutritional status of rainbow trout. Regardless if the trout were fasted or fed with or without dietary carbohydrates, the enzyme activities or the mRNA levels did not vary (Panserat *et al*., 2000b, 2001a, b) (Fig. 13.2). This suggests that, as in diabetic humans, there is a persistent high level of endogenous glucose production by liver in trout leading to a competition between exogenous (dietary) glucose and endogenous glucose as a source of energy. The reasons for the absence of the regulation of hepatic gluconeogenic enzymes by dietary carbohydrates are not yet clear but may be due to the high levels of dietary gluconeogenic amino acids (main substrates for glucose production) and fatty acids in the diets (Panserat *et al*., 2002a; Kirchner *et al*., 2003). Postprandial gluconeogenesis in extra-hepatic tissues such as intestine and kidney also may be very important (Kirchner *et al*., 2005) as shown by the molecular expression of the gluconeogenic FBPase enzyme in these tissues (Fig. 13.3). Use of potential inhibitors of gluconeogenic pathways such as metformin (an anti-diabetic drug) may be useful to test if there is improvement of dietary carbohydrate utilization in rainbow trout in these conditions.

2.4 Nutritional regulation of phosphorus absorption in fish intestine

Context: optimization of dietary phosphorus utilization

In rainbow trout, the absorption of dietary phosphorus from different sources appears to be only about 40–50% (reviewed by Lall, 2002), meaning that the majority of phosphorus ingested is released into the aqueous environment. Excessive discharge of phosphorus from aquaculture facilities, therefore, can cause excessive algal blooms, eventually leading to the destruction of the ecosystem. Feeds must contain adequate available phosphorus levels to meet the requirements of fish and the feeding practices should be adopted to minimise phosphorus loads to the environment. It is however clear that better understanding of the mechanisms underlying absorption of dietary phosphorus will help improve absorption of dietary phosphorus by the fish.

Little was known about dietary phosphorus-responsive genes in animals. In addition to genes known to be associated with phosphorus metabolism such as type II sodium phosphate co-transporter (NaPi-II) (Sugiura *et al*., 2003), Sugiura *et al*. (2004) have found in rainbow trout, dietary phosphorus-responsive genes such as intestinal meprin 1A (a

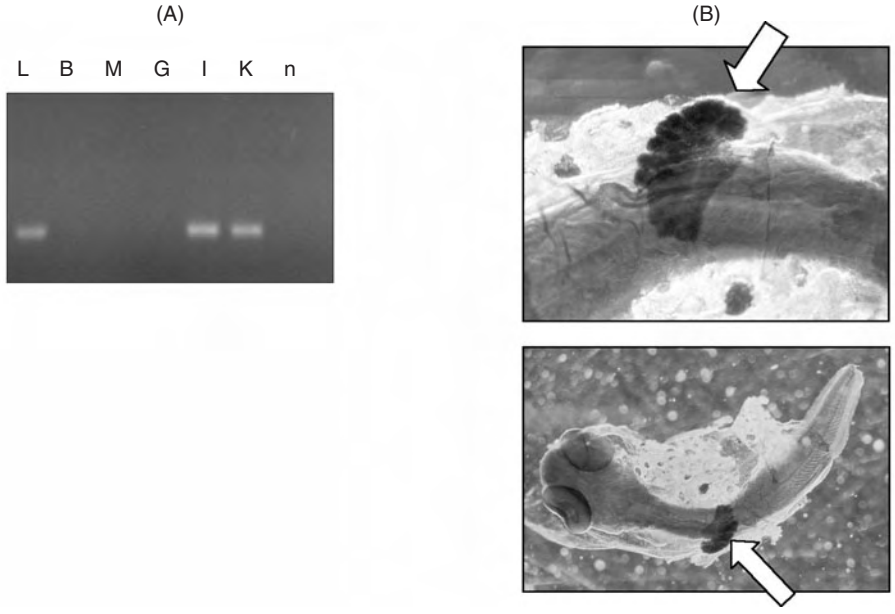

Fig. 13.3. Tissue specificity of the gluconeogenic enzyme, fructose-1,6-biphosphatase (FBPase) gene expression in rainbow trout. Analysis by (A) RT-PCR in juvenile rainbow trout (Panserat *et al.*, 2001b) and (B) *in situ* hybridization (*in toto*) in trout embryo (Source: Escaffre *et al.*, unpublished data). (A) FBPase gene expression (from left to right): L, liver; B, brain; M, muscle; G, gill; I, intestine; K, kidney; n, negative control. (B) FBPase mRNA in liver is shown by a blue coloration marked by an arrow.

peptidase) and cysteine sulphinic acid decarboxylase not previously known to be associated with phosphorus deficiency. These genes are among the earliest steady-response genes capable of predicting phosphorus deficiency. Traditionally the phosphorus status of fish has been estimated on whole body phosphorus, bone phosphorus and blood phosphorus levels, but the sensitivity of these indicators are insufficient in contrast to these new molecular bioindicators which can serve for optimization of dietary phosphorus levels in aquaculture diets.

3 Nutrigenomics in Farmed Fish: New Tools for Fish Nutrition Studies

The interface between the nutritional environment and cellular molecular or genetically determined processes is generally referred as 'nutrigenomics' including genomics, transcriptomics, proteomics and metabolomics (Muller and Kersten, 2003). Genomics cover molecular marker development, linkage mapping, quantitative trait loci (QTL) analysis and characterization of genomic resources and can be exploited for understanding all physiological functions. In this section, we will review current studies related to nutrigenomics in fish.

3.1 Genomics research initiatives in farmed fish

Teleosts are diverse groups of more than 23,000 species, by far the largest group of vertebrates. One characteristic feature of all orders of fish is that at one or other point of their evolution, they have undergone whole genome duplication (Taylor *et al*., 2003) and thus offer unique systems for comparative genomic studies and understanding vertebrate evolution. Initially, model fish species for genomics were zebrafish and medaka (*Oryzias latipes*), two freshwater species – models for developmental and genetic research – and the pufferfish (*Fugu rubripes* and *Tetraodon nigroviridis*) (Cossins and Crawford, 2005). However, genomic studies on a farmed fish species provide unique information concerning the genetic mechanisms governing performance traits in aquatic environments in relation to genome evolution, as well as having an economic significance in that they can inform sustainable breeding practices.

Over the past 5 years, genomics programmes[1] have been initiated for 'model' farmed fish species (Thorgaard *et al*., 2002; Liu, 2003; Rise *et al*., 2004): for carnivorous fish such as salmonids (rainbow trout and Atlantic salmon) and striped bass *Morone saxatilis*, for omnivorous channel catfish *Ictalurus punctatus* and for semicarnivorous tilapia *Oreochromis mossambicus* (see the following web sites in the USA: http://www.animalgenome.org/aquagenomics/ and http://www.genome.iastate.edu/; in Canada: http://web.uvic.ca/cbr/grasp/; and in France: http://www.inra.fr/agenae/). For all these fish species, quantitative nutritional requirements are known (National Research Council, 1993). Taken rainbow trout as an example, Thoorgard *et al*. (2002) have provided a comprehensive overview of opportunities for exploiting the tools of genomics in several research areas including fish nutrition.

3.1.1 Systematic characterization of genes and/or full length cDNAs – large-scale isolation of expressed sequence tags

Development of genomic resources (cDNA libraries) is the first step for genomic studies and the list of expressed sequence tag (EST) resources is rapidly increasing. The initial task consists of constructing cDNA libraries representing a maximum of expressed RNAs for a species. Under the French AGENAE (Analysis of Breeding Animals' Genome) programme, cDNA libraries were constructed for rainbow trout using different tissues (more than ten tissues from larvae to adults) (Aegarter *et al*., 2004). These cDNA libraries, after normalization, are used to undertake analysis of ESTs. EST analysis (sequencing) has proven to be one of the most efficient approaches for gene identification, gene expression profiling and cataloguing. Also it produces markers for genetic analysis and resources for the development of cDNA/oligonucleotide microarrays on glass or nylon. Even though half of the fish EST resource has been provided for zebrafish (Cossins and Crawford, 2005), gene indexes for farmed fish (Atlantic salmon, rainbow trout, channel catfish, etc.) have been developed and are

available at three major sites. These are: (i) The Institute of Genome Research (TIGR http://www.tigr.org/tdb/tgi/); (ii) National Center for Biotechnology Information (NCBI – UNIGENE http://www.ncbi.nlm.nih.gov/UniGene/; and (iii) National Institute of Agronomic Research (INRA – SIGENAE http://ensembl-sigenae.jouy.inra.fr/).

3.1.2 Genome sequencing

Three fish species for which almost complete genome sequences are available are the zebrafish (*D. rerio*) and medaka and two marine pufferfish species (*F. rubripes* and *T. nigroviridis*), with an unusually small genome size (Aparicio *et al*., 2002; Furutani-Seiki and Wittbrodt, 2004; Jaillon *et al*., 2004) (see ENSEMBL http://www.ensembl.org/ Nucleic Acids Res. 2005 33: D447–53). Sequence information for aquaculture fish species will be complementary data to the information of genome sequencing of zebrafish, medaka and pufferfish: knowledge of existence of genes involved in specific metabolic pathways in these fish species as well as the number of paralogs[2] can be helpful to improve the understanding of nutritional metabolism in farmed fish at a molecular level. However, these 'model' fish species (zebrafish, medaka, fugu) are reared in great numbers despite the lack of information on their nutritional requirements. Information about optimal nutrition of zebrafish is sparse, except some studies of the effects of dietary n-6 PUFA (Rowe and Eckhert, 1999) and dietary boron on zebrafish growth rate and embryogenesis (Meinelt *et al*., 2000).

3.2 Transcriptomics: application in fish nutrition

The challenge for the next decade is to identify nutrient-influenced molecular pathways and determine the down-stream effect of specific nutrients. Nutrigenomics assists in this identification because it allows the genome-wide characterization of genes, the expressions of which are influenced by nutrients. It is only with a complete understanding of the biochemical links between nutrition and the genome that we will be able to comprehend fully the influence of specific nutritional interventions on mechanisms affecting fish growth, health, physiological well-being and flesh quality.

To briefly illustrate the interest of nutrigenomics we hereby present two examples from rats: effects of dietary intake of proteins on hepatic transcriptome and of dietary n-3 PUFAs on brain gene expression.

The first example is drawn from Endo *et al*. (2002) who studied the effects of dietary protein intake on hepatic transcriptome. In the context of protein malnutrition it is of first importance to determine the comprehensive list of genes that are affected by protein deficiency. Compared to rats fed a 12% casein diet, when rats were fed a protein-free diet, 281 genes were increased or decreased by twofold, and interestingly the majority of them were identified for the first time as responders to

protein nutritional status. Indeed, one gene with a drastic change was Id (inhibitor of DNA binding) proteins involved in the regulation of multiple genes. Thus it seems that protein nutrition affects transcription of numerous genes through regulation of the expression of transcription regulators.

The second example is a study on the effects of dietary n-3 PUFAs on brain gene expression (Kitajka *et al.*, 2004). Since PUFAs are known to be essential structural components of the central nervous system, a nutrigenomics approach with high density microarrays was used to reveal brain-gene expression changes in response to different PUFA-enriched diets in rats. PUFA-enriched diets led to significant changes in expression of several genes in the central nervous system including transcriptional modulators. This result may give insights to understanding the beneficial effect of n-3 PUFAs on the nervous system. Moreover, PUFAs can alter gene transcription through different ways as they can act directly as transcription factors or modify gene expression through specific transcription factors. This type of analysis combined with the use of other 'omics' as well as functional gene studies may in the future help to discover new regulatory pathways linked to dietary PUFAs.

3.2.1 Hepatic gene expression profiles (transcriptomes) in fish under different nutritional statuses

To our knowledge, such an approach has not been fully exploited in fish. Some recent studies have dealt with relatively small numbers (1000) of genes to analyse variation of transcriptome expression linked to handling stress, chemical contaminants, temperature changes, etc. (Koskinen *et al.*, 2004; Krasnov *et al.*, 2004). Here, we will present our recent results in terms of nutrigenomics (Kirchner *et al.*, unpublished).

Given the high protein requirement of fish, we wanted to analyse the gene expression profile in the liver of rainbow trout under two protein intake levels (low and high) as well as in fasting fish. In an earlier study, we found that feeding rainbow trout low levels of protein in the background of similar intakes of carbohydrates, there was a postprandial glycemia, which reached very high values. This observation was not explained by analysing candidate proteins in liver and other tissues and necessitated further studies using high-through tools (Kirchner *et al.*, 2003, 2005). We thus analysed the hepatic transcriptome from fish with low and high levels of protein intake and compared them to fasted fish. We used trout cDNA nylon macroarrays (9452 cDNA clones) prepared from ESTs characterized by the French AGENAE programme. The statistical analysis using SAM (Statistical Analysis of Microarrays) methodology showed that only one gene was over-expressed in fish fed low protein compared to fasted fish and to fish fed with a standard high protein diet. This gene is a member of the inhibitor of growth family: it is involved in cell multiplication. We speculate that low protein intake may specifically induce a stress in fish liver leading to inhibition of growth/cell division which can be linked to the very high value of glycemia observed.

3.3 Other 'omics' approaches which can be combined with transcriptomics data: proteomics and metabolomics and their application in fish nutrition

Study of metabolic profile (metabolites) found in a cell, tissue or organism (metabolomics; Whitfield *et al*., 2004), and study of the full complement of proteins (proteomics) found in a cell, tissue or organism (Barnes and Kim, 2004) are also interesting complements to transcriptomics in nutritional research.

To our knowledge, still in its infancy, no metabolomics studies have yet been undertaken in fish nutrition. Some preliminary analyses have been performed using proteomics analysis. In proteomics, protein extraction followed by high resolution two-dimensional electrophoresis, coupled with gel image analysis allow identification and expression of hundreds of proteins. Martin *et al*. (2003) and Vilhelmsson *et al*. (2004) studied the effect of fasting, quality of proteins and total replacement of fishmeal by plant proteins on liver proteomes. Pathways shown to be affected by dietary plant substitution were those involved in primary energy generation, maintenance of reducing potential, bile acid synthesis and transport and cellular protein degradation (Vilhelmsson *et al*., 2004). This protein expression profile (for which the effects on the abundance of several stress response proteins were notably absent) differed from those observed for fish fed a soybean-based diet (Martin *et al*., 2003).

3.4 Post-genomic tools to study gene function in fish

To understand the relevance of genomic information, for any given organism, functional analysis of specific genes will be required, for instance, for genes differentially expressed between two nutritional statuses. Because the functions of many of these genes cannot be deduced only from their sequences or their gene expression profile, other techniques for studying gene functions are needed. Indeed, variation of gene expression, suppression of gene expression or gene over-expression, is essential to analyse the function of genes differentially expressed.

3.4.1 Gene over-expression using transgenic technologies

The first transgenic fish was reported in 1985 (Zhu *et al*., 1985). Currently, much research is underway with a number of teleosts: salmonids, cyprinids, catfish, tilapia, etc. (Sin, 1997). Germ-line transgenic fish have been mainly produced by microinjection of gene constructs into the fertilized egg shortly after fertilization (Sin, 1997). This technology is fast and easy due to the transparency and large size of most fish eggs (however this is not so for all fish species). The major inconvenience of this approach is the low efficiency of transgenesis.

Production of transgenic fish offers a valuable means of studying gene function because it allows the detection of phenotypes that have been

altered by a gain of function. Up to now, transgenesis has rarely been employed in order to study fish nutrition directly, except in two cases of improvement of dietary carbohydrate utilization by over-expression of glucose transporter 1 and hexokinase II in rainbow trout (Krasnov *et al*., 1999a; Pitkanen *et al*., 1999) and acquisition of the capacity to synthesize ascorbic acid (gluconolactone oxydase; Krasnov *et al*., 1999b). Results, however, are far from convincing due to technological issues, including high levels of mosaicism and absence of expression of the transgenic product. Other techniques, such as co-transfection of plasmids bearing meganuclease recognition sites with expression vectors encoding meganuclease, seem to hold promise for improving results provided by transgenesis in fish due to integration at the one-cell stage (Thermes *et al*., 2002). Meganuclease is an extremely rare cutting endonuclease which acts to solely digest the injected DNA, diminishing the mosaic expression of the transgene and improving transgenesis frequency in medaka.

3.4.2 Gene down-expression using specific inhibition of endogenous gene expression

An alternative technique to transgenesis, called gene knock-out, can be used to disrupt endogenous genes. Although there have been many gene knock-out studies in murine species, this technique has not yet been demonstrated to work well in fish due to the absence of characterized embryonic stem (ES)-like cell lines until now (Fan *et al*., 2004). Therefore, gene knock-down could be a novel tool to use for examining the functions of a gene whose sequence is known. The demand for knock-down technology in biology is rising exponentially as genes of interest emerge at an ever-increasing rate from genomic studies, and therefore the central task is one of relating gene(s) to function(s).

Gene knock-down technology via the use of morpholinos phosphorodimidate oligonucleotides (AMOs) has been demonstrated as a useful technique for understanding gene functions during embryonic development in zebrafish (Nasevicius and Ekker, 2000). One study developed a preliminary effective gene knock-down technique using AMOs in rainbow trout embryos (Boonanuntanasarn *et al*., 2002). However, this technique cannot be used for stable long-term gene knock-down because it consists of chemically modified oligonucleotides. Gene knock-down mediated by RNA interference (RNAi) is also a powerful tool in the down regulation of gene expression (Dykxhoorn *et al*., 2003). Recently, a study has shown the utility of small interfering RNAs (siRNAs) for gene knock-down in rainbow trout embryos (Boonanuntanasarn *et al*., 2003). All these preliminary studies have to be confirmed.

In conclusion, when it is combined with transgenic techniques, siRNAs *in vivo* may become tissue-specific and conditional, opening new challenges for studying gene function in farmed fish.

4 Future Directions

We described here the importance of the nutrigenomics approach to understand and to improve fish nutrition in the future. A different/parallel approach (not discussed in this review) is to study the genetic polymorphism of fish linked to nutritional parameters such as feed intake and lipid storage for example (Mambrini *et al*., 2004; Quillet *et al*., 2005). With the availability of resource families (Mambrini *et al*., 2004; Quillet *et al*., 2005) and DNA markers (from genomics sources) (for review see Liu and Cordes, 2004) it is expected that greater successes will be achieved in the near future for marker-assisted selection. Nutrigenetics refers to the retrospective analysis of genetic variations among individuals with regards to their response to specific nutrients (Muller and Kersten, 2003; Ordovas and Mooser, 2004). There is no reason to think that this is not possible with fish, opening new perspectives in selection programmes to improve fish nutrition.

Finally, another point of view is that of systems biology, as shown by studies by Ideker *et al*. (2001a, b) who produce an integrated approach to build, test and refine a model of a cellular pathway, in which the yeast galactose-utilization pathway components are analysed using DNA microarrays, quantitative proteomics, and databases of known physical interactions. Systems biology is the global analysis, ideally, of all of the elements in a biological system in response to hypothesis-driven perturbations (Ideker *et al*., 2001a; Auffray *et al*., 2003). It proposes to study the biological organism as a system, rather than study its elements one or a few at a time, as has been the approach in molecular and cellular biology in the past. These systems approaches are now being extended to multicellular organisms and more complex biological systems, and in the future such an approach could be used to study nutritional systems in fish as a useful tool for the development of new diets.

Notes

[1] Two examples of genomics programmes for salmonids are the French INRA AGENAE (Analysis of Breeding Animals' Genome) and the Canadian GRASP (Genomics Research on Atlantic Salmon Projects).

[2] Paralogs: Sequences that perform different biological functions in the same species that likely arose by duplication and divergence from a common ancestral sequence. A whole genome duplication occurred at the base of the teleost radiation (Taylor *et al*., 2003; Jaillon *et al*., 2004).

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14 Economics and Food Safety

FUMIO SAKAMOTO

Kagoshima Industrial Trading Co. Ltd, Kagoshima 892-0821, Japan

1 Feed Additives and Supplemented Food

1.1 Feed additives

For the purpose of ensuring production and a steady supply of safe livestock and to promote production and distribution of safe and high-quality feed, feed additives are regulated and official standards are stipulated, and examination based on the standards are conducted. In Japan the stipulation covers domestic animals and fish. Feed manufacturers are obliged to stipulate such items as the name of the feed, the type of feed, the year and month of manufacture, the manufacturer's name and address, the manufacturing facility and its address, the target livestock, net weight, amounts of crude protein/crude fat/crude fibre/crude ash/calcium/phosphate in the ingredients, raw material classification (such as grain, vegetable press cake, animal-derived feed, bran and others) and their formulation. Authorized feed additives are also subject to the regulation under the Japanese Feed Safety Act. This covers the usage of feed additives and their target livestock, and naming rules, determination of active ingredients and dilution material which are stipulated in the legislation in order to ensure the safe and effective usage of feed additives. In Japan, feed additives are defined as 'material which is used for the purpose of preventing feed quality deterioration designated by Ministry of Agriculture, Forestry and Fisheries'.

1.2 Supplemented food

A supplement refers to a food in which a specified nutrient such as a vitamin or mineral, which tends to be deficient in the diet, is supplied. In

 Dietary Supplements for the Health and Quality of Cultured Fish (eds H. Nakagawa, M. Sato and D.M. Gatlin III)

the USA, the Food Additive Amendments to the Federal Food, Drug and Cosmetic Act enacted in 1958 were greatly amended in 1994 when The Dietary Supplement Health and Education Act (DSHEA) was passed by Congress in October of that year. This combined with The Nutrition Labeling and Education Act (NLEA) enforced in 1990 and the amended version of NLEA 1992's Dietary Supplement Act (DSA) extended to 1993, led to the legal definition of a nutrient supplement food taking effect. As in other countries, the USA insists that nutrient supplements are considered as a specifically categorized 'food'. The DSHEA (USFDA, 1995) defines a dietary supplement as a product that contains at least one of the following dietary ingredients: a vitamin, a mineral, an herb, an amino acid, and by combined use of these ingredients in the form of extracts or concentrates, it will supply extra nutrients and help satisfy the nutritional requirements or metabolism. Nutrient supplement foods are:

- designed to be taken through the mouth in the shape of pills, capsules, tablets or liquid;
- not intended to substitute for the ordinary diet;
- labelled as 'dietary supplements'.

In the USA, food supplements are under the jurisdiction of the Center for Food Safety and Applied Nutrition (CFSAN) of the US Food and Drug Administration (USFDA).

In the European Union (EU), the EU directive (Directive 2002/46/EC; European Parliament, 2002a) came into force in 2002 and defined 'food supplement' as 'the food to supplement diet', 'the products which contain nutritious or physiologically effective agents or the concentrates of one or more substances' in the form of 'capsule, troche, tablet, pill, powder or ampoule'.

In the marketplace, food supplements include ingredients such as vitamins, minerals, amino acids, essential fatty acids, fibre and herbal extracts, but the directive currently limits supplemental food to vitamins and minerals. Scientific investigations on the effectiveness of other extracts or ingredients are expected by July 2007.

Research and work on the definition and directions for use of food supplements has just started. In the field of fish feed, there are still issues to be discussed. The physiological effectiveness of feed supplements and feed safety should be investigated and treated in the same way as food safety. The use of feed supplements requires a scientific basis and knowledge.

2 Practicality and Economic Efficiency of Supplements

2.1 Fish quality

There are many projects going on regarding quality management of cultured fish among aquaculturists reflecting the consumer's preference. Price competition due to excess production will only put business in difficult situations. The management started to seek differentiation and

have made an effort to establish regional brands since the 1980s. Regional quality differentiation occurs due to variation in specific production conditions, the quality of management and the consumer's preference. Increasing local processing of cultured fish is one of the factors promoting the establishment of regional brands. As the proportion of direct sales to the large-scale supermarkets rather than mass distribution through the marketplace increases, there are likely to be more requests for better regional brand image. To pursue establishment of the local brand, efforts must be made by members of the same production area or aquaculturists' co-op to produce products that are consistent with respect to quality, size and the culture methods used. Since it is also an issue to supply a uniform product throughout the year, fish for processed products must be cultured rather than depending on excess catch. Therefore, now the aquaculture industry is required to introduce the same quality management systems as most other production industries. In recent years, through the experience of price fluctuation and dynamic change of distribution, aquaculturists are eager to meet consumers' interests, shifting their attitude to apply the production methods which are safe and environmentally friendly.

2.2 Economic efficiency of supplements

Yellowtail and red sea bream fed algae (such as *Spirulina*, brown algae and *Chlorella*) powder or the extract as a feed supplement are reported to have improved texture caused by the change of carcass lipid and lipid composition due to a change of lipid metabolism. Algae that are currently used as feed supplements are confirmed to contain several active ingredients. These algae abound in micronutrients such as minerals, carotenoids and vitamins discussed in Chapter 9. But domestic production of algae is gradually decreasing, while imports of algae are slightly increasing. In Japan, algae powder used for cultured fish feed these days is mainly *Ascophyllum* from Norway. Other ingredients are kelp from South America, a kind of *Eisenia* (*Ecklonia*) from South Africa and a brown algae *Lessonia* from Chile. For livestock, the brown algae *Ascophyllum* from Norway is widely sold in the form of powder or paste for pig, cattle, horse, sheep, chicken and fish, and is recognized to be effective for growth promotion, appetite and improvement of flesh quality. Research reports on feed for cultured fish show that the fish fed with small amounts of algae show better growth rate, better feed efficiency, improved carcass quality and higher resistance to illness. Among foreign counties, Norway was successful at improving feed mixture quality to achieve improved feed efficiency and rate of muscle formation in Atlantic salmon and others. This enabled Norway to produce a high-quality product at a very competitive price in the global market. In Japan, producers are hoping for the retail fish price to go up but they are not making any effort to reduce costs and to be competitive. In this situation, it will be very difficult to try to establish a low cost production system including technical improvement of feed producers.

3 Evaluation of Safety

3.1 Food and safety

Since the public has become aware of food poisoning caused by contamination with *Escherichia coli*, *Salmonella* and other Enterobacteria, there has been a strong demand for safe food. The recent bovine spongiform encephalopathy (BSE) issue and concerns about the deception of food labelling have caused consumers to want higher food safety standards than we have ever experienced before. There are many reasons for the strong demand for food safety by consumers. One is that the internationalization of logistics of farmed products, livestock and seafood has made the distribution and origination of food visible to consumers. Another is that we have experienced a period of high economic growth and the status of food has become more to do with maintaining our health rather than just a tool to satisfy nutritious needs. In addition, food is the essential item to maintain life and growth for the young to the old. Consumers reasonably wish to have a reliable information source among the surfeit of misinformation that abounds in response to anxiety about food safety.

From the end of World War II to the present, the case for food safety has been changing. In the past 10 years, issues such as BSE, endocrine-disrupting chemicals, genetically engineered foods, and *E. coli* O157 are deeply connected with production processes. In risk management of food security, the risk management of the processing stage is emphasized. From now on, producers not only need to aim to achieve higher efficiency but also to allocate experts who have a good knowledge of food safety at the processing stages to reduce risks.

The outbreak of BSE had strong social impacts. It was this incident that triggered the issue of revising food safety security systems, but since the late 1990s there have been many other serious cases where food safety is at risk. Food security issues are social problems that need to be readdressed.

In Europe, many of these food safety problems such as BSE, dioxin and *Listeria* have arisen in the food industry. Agricultural producers, food industries and distributors are starting to focus on food safety issues each from their own standpoint. A review of regulations started within the EU and on 12 January 2000 the European committee published a White Paper regarding food safety (EFSA, 2000). This raised the general principles regarding the policy requirements for regulation of food, established an early warning system and founded a European food safety organization.

On 8 November 2000 the committee put a proposal to the European Parliament and Council of the EU. It was 'a draft to implement general food law, to organize a European Food Safety Authority and to implement regulations regarding the food safety issue'. Regulation (EC) No. 178/2002 of the European Parliament and of the Council came into being on 28 January 2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying

down procedures in matters of food safety (European Parliament, 2002b). In Europe, the concept of food safety covers a wide range of matters such as achievement of obstruction-free distribution, observance of international regulations, application of a risk analysis system and a risk prevention principle. In the future, conventional law will be revised according to general food law.

In the USA, food safety management systems focus on control of bacteria in the production process, for instance, the bacteria reduction programme and Hazard Analysis Critical Control Points (HACCP). They put up a slogan 'farm to table' which lays emphasis on the importance of the food safety security system within the whole food chain, but there is a lack of transparency in traceability or production and distribution information. In the USA, people pursue economic efficiency and the matter of cost is always the issue. The cost which consumers would be willing to pay for the assurance of food safety always becomes a point of discussion. It is said that establishment of a traceability system can only be realized if the cost which consumers are willing to bear exceeds the actual cost of the establishment of the system.

In Japan, the effort to establish an EU-type food safety management system has been proceeding. Further discussion on the outstanding issues needs to progress, such as who will pay for the cost of food safety, and how much we can afford to tolerate risks. It is time that we need to meet a consensus as a nation. Japan has a geographic feature that the land is surrounded by sea so that it is a matter of national character. However, we have been lacking the sense of urgency like 'what to do if the infectious disease spreads tomorrow' that led to the cases of BSE for instance. Since the self-sufficiency ratio of food is obviously low in Japan, we are dependent on massive imports of food. Therefore it is becoming more important to have risk management systems. The report of the BSE survey board points out every issue for securing food safety and shows the way that industry and the administration should proceed. The report consists of three parts. Part 1 refers to 'the investigation on the measures the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Health, Labor and Welfare employed on the event of outbreak of mad cow disease' which covers from 1986, when the first case of BSE was reported in the UK, until 2001, when the first BSE case was confirmed in Japan. The report pointed out that the Ministry of Agriculture, Forestry and Fisheries had been informed of the situation in foreign countries and also that Japan had been named by the EU as a country at high risk of BSE contamination. However, no preventative action was taken nor was the risk management system established. Moreover, mismanagement of information at the announcement of a BSE outbreak deepened distrust of the administration of food safety and resulted in social disturbance. The second part of the report summarizes seven items to be solved as administrative issues. These are:

1. Lack of a sense of crisis and risk management.
2. An administration which gives priority to producers and is not facing concerns of consumers.

3. Lack of transparency in the process of policy making by the administration.
4. Lack of communication between the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Health, Labor and Welfare.
5. The advice from experts is omitted.
6. Inconsistency in disclosure of information and insufficient understanding by consumers.
7. The need to identify the problems of relevant laws and the current system and reform them.

The survey board concluded that the action taken in 1996 was a total failure. There was mismanagement of information at the announcement of a BSE outbreak without any effective measures being put in place, and the reaction to rumours was too cautious and the damage due to unfounded rumours resulted in a large disturbance of food administration.

The Codex Alimentarius Commission proposes introducing a 'risk analysis' method in every country to maintain up-to-date food safety levels. It is essential to work on objective criteria for estimating risks and evaluating them from an economic standpoint.

Today's consumers recognize that there is no such thing as risk-free food. They are making a judgement between the level of safety and the cost to pay for it. Excessive demand for low price food means that necessary work is omitted and the use of high-risk materials and processes is encouraged. It is necessary that a minimum cost to reduce risk should be paid. Producers' and consumers' points of view on economic efficiency always contradict one another, but mutual understanding is desirable regarding the security of safety. Consumers emphasize the importance of reliability of the safety information and the information source. Professional competence free from financial interest, morality, and verification and decision making by a third party are the grounds for reliability. From the production stage to the consumers' table, producers need to complete a proper procedure at every stage of processing.

3.2 Cultured fish and safety

Concerns over cultured fish are spreading among consumers these days. Producers are making efforts to earn consumer confidence by attempting to culture fish without the use of chemicals and with consideration of environmental concerns. Internationally, the Codex Alimentarius Commission published *Proposals for International Standards of Cultured Marine Products*. At present, there is not a specific standard of hygiene code for safety management of the processing stage, so it is considered to be reasonable to apply eight items of the 'General Principle of Food Hygiene' for hygienic management in the production stage. The Ministry of Agriculture, Forestry and Fisheries in Japan has conducted an introductory project into hygienic production for the livestock industry for the purpose

of securing food safety of farm products and established an expert committee. The committee formulated 'Guidelines of hygienic management of livestock' which should act as a basis of safety management for each production stage of five types of livestock, laying hens, broiler chickens, pigs, beef cattle and dairy cattle. Production processes associated with farm products should be thoroughly analysed from the primary inputs, such as feed and young livestock, to the final product that will be consumed. To achieve security for food safety, every step of the processing stage must be thoroughly observed. There are many objections to the introduction of the HACCP method to the production process, but it has earned international consensus to manage the safety of the production process with compliance to the 'General Principle of Food Hygiene' that was adopted with HACCP.

As in the past, healthy livestock and poultry have been considered as safe and good quality food. People have recognized that healthy livestock and poultry were fed with good feed, which would not have a detrimental influence to human health. But a lot of feed additives and animal medicines are utilized and these remain in the livestock, and metabolic conversion occurs. It becomes obvious that these residues will have a detrimental influence on those who consume these products. In the case of Minamata disease, a minimal amount of organic mercury dissolved in seawater had been accumulated in fish and shellfish organs through bioaccumulation, and people who consumed too much of these fish were affected. So now an area of considerable concern is that a small amount of feed additive can become concentrated in livestock through bioaccumulation and then metabolically converted in livestock products. Feed for livestock also needs to be evaluated and countermeasures have to be taken for the safety of human consumers.

3.3 Evaluation of safety

As outlined above, feed materials may accumulate in livestock and may even be transformed into new noxious substances in meat, eggs and fish that when consumed are deleterious to human health. These risks of safety should be evaluated one by one and countermeasures should be taken for each issue. Evaluation of safety is considered to be an operation of decision-making regarding the correlation between risk and dose, bearing in mind the intensity of action and any metabolic transformation that may have taken place.

Substances in food and feed that need to be evaluated for safety are any of those substances which will detrimentally influence human health if they exist in food and feed in excessive amounts. Safety evaluation is a complex process because elements of food and feed are complex. Therefore, meticulous planning of analysis for each of the biological and non-biological adverse factors is required. Implementation of analysis, investigation on outcome and a final decision concerning safety is needed.

One of the problems regarding safety of feed and feed materials concerns the natural or artificial chemicals other than nutrient substances

that may be added. For example, these may include medicine supplied by a veterinarian, feed additives, pesticide residues or materials washed out from containers and packaging. Prompt and adequate responses or suspensions of artificially added additives are possible in accordance with the results of analysis of scientific and physiological characteristics and toxicity. For these issues, generally accepted methods of safety evaluation are to first set a tolerance threshold for the hazard and then to measure the maximum level of content. The permissible concentration of a particular chemical is set as a standard by the administration. Also, restrictions of use defined as 'usage standards' are usually set not to exceed the limit.

Acceptable Daily Intake (ADI) is the maximum permitted amount of chemical residue intake per day throughout the life of a human which supposedly does not cause any harmful influence according to the latest poison information. The ADI is indicated by the amount of chemical per day for a given weight. The permissible concentration is the chemical density in food and feed allowed for a chemical residue on any specific process of production or distribution and this is indicated in ppm.

At current standards, 'no residue' of a highly toxic substance is to be found in any food and feed. In general, 'no residue' means that the substance is not detected by a previously approved quantitative test. The definition of the term 'no residue' is vague at present, so that the residual permissive density of each chemical must be determined. Quantitative methods of food monitoring should be established which enable us to detect the permitted density with enough sensitivity. Also we need to have a surveillance organization to omit foods that contain chemical residues that exceed the permitted density.

From the point of view of toxicology, the appearance of a symptom resulting from the residue of a medicine or chemical in the animal is said to be very rare. However, marine and livestock foods make up a large percentage of total food intake these days so confirmation that these foods are safe to consume is highly desirable.

Medicine and chemicals dispensed to animals disappear exponentially so, theoretically, once dispensed a medicine lingers in the body for life. Therefore the permitted level of the residue of a medicine or chemical should be preauthorized. In cases where toxicological evaluation is not complete, very low residue density should be made the standard and lower densities judged negligible. By establishing quantitative tests for residue detection that are able to detect the standard, if no sign of residue is found then 'no residue' can be declared.

Plants and animals (fish, shellfish, poultry, livestock, etc.) which are used as foods or feed materials can take in hazardous or toxic substances during a lifetime and in some cases these may be accumulated and ultimately damage human health. However, contamination by unexpected hazardous substances is very rare, but nevertheless we need to predict the possibility of this occurring and devise countermeasures. The strategy of how to cope with these rare cases requires quite a different approach from that used for feed additives and animal medicines. An effective method is

to estimate potentially risky substances in advance, take samples regularly and monitor the samples against accumulated scientific data. However, today's monitoring programme has not yet defined the standard value, provisional standard value or regulation value for such risky substances, although domestically and internationally research is going on regarding hazardous toxic metals and artificial chemical compounds to accumulate data. It is necessary to keep a close check on all food and feed materials that are used daily, and it is desirable to establish a daily system of surveillance.

The safety of residual chemicals in food and feed is evaluated by every country and also internationally by the authority of the United Nations. The joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Codex Alimentarius Commission has established a Standards Committee regarding residues of veterinary medicines in food and it established a global rule on the Maximum Residue Limit (MRL) for each medicine. The Joint Expert Committee on Food Additives (JECFA) of FAO/WHO published the MRL original plan. JECFA's members are experts in toxicology and its related fields, government officials and non-governmental organizations (NGOs). They set the MRLs based on toxicological data. The MRLs set by the expert committee are adopted as global food standards and are recommended to each country. In the past, the decision whether to adopt global standards for domestic regulation or not was left to the discretion of each country. However, since the inauguration of the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) was ratified every country has a duty 'to have a domestic standard for sanitary plant quarantine measures that comply with the international standard, instruction or recommendation unless otherwise stipulated by law'.

Consumers' demand for safety management of food is expected to increase hereafter. In Japan, in accordance with the FAO/WHO principle, the introduction of a positive list system for setting MRLs as a residue standard of the Food Sanitation Law, article seven, will soon be adopted.

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