Barbara Richardson
Editor

Tilapia and Trout

Harvesting, Prevalence and Benefits

Fish, Fishing and Fisheries

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TILAPIA AND TROUT

HARVESTING, PREVALENCE AND BENEFITS

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TILAPIA AND TROUT

HARVESTING, PREVALENCE AND BENEFITS

BARBARA RICHARDSON
EDITOR

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New York

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PREFACE

This book discusses the harvesting, prevalence and benefits of tilapia and trout. Chapter One begins with a review of the risks and benefits of tilapia. Chapter Two provides a human health risk assessment of heavy metals in the consumption of the fish. Chapter Three studies the utilization of by-products and waste generated from the tilapia processing industry. Chapter Four reviews thermal ecology of brown trout and the climate change challenge. Chapter Five examines reparative neurogenesis in the adult trout brain and peculiarity of development in the trout's brain cells in primary culture. Chapter Six focuses on the effects of plant-based feeds on the immune responses of rainbow trout.

Chapter 1 – Tilapia is popular fish because of its low to moderate fat and high quality of protein content. Therefore, this fish has wide diversified health benefits in terms of reducing weight, boosting overall metabolism, building strong bones, decreasing the risk of various chronic diseases and preventing arthritis as well as cancer. However, environmental contaminants, originated both from the anthropogenic and natural sources, possess potential harm to living species. It usually refers to chemical, biological and physical contaminants and aquatic species like fishes are sensitive to these contaminants because of its bioaccumulation. So far, about 118 environmental contaminants related to fish are discovered which contains potential harm of human health. Therefore, human beings are also an important driver for assessing and controlling environmental contaminants. Tilapia species are one of the most widely cultured fresh water fish and the demand of tilapia is exponentially increasing in recent years especially in warm counties like Egypt, Middle East, Malaysia etc. Although Tilapia feed on a wide variety of dietary sources, but specific dietary requirements are still lacking and the

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interactions among nutrients and with cultured conditions are not completely understand. The contamination of water by chemical is a worldwide problem and it disturbs ecological balance of aquatic environment because of its usually non-degradable condition. In addition, fishes are the main inhabitants that directly contaminated by polluted water in many locations. For instance, in Egypt, metal concentrations in Nile Tilapia determined a specific bioaccumulation pattern. Therefore, human health hazard index indicated that the cumulative risk from cadmium exposure significantly increased due to higher consumption rate of Tilapia. Similarly, in Malaysia, the cancer risk calculations due to consumption of Tilapia exceeded the USEPA's acceptable risk level for cadmium (2.1×10^{-6}) and nickel (7.3×10^{-4}), along with the average carcinogenic risk (2.4×10^{-4}). Accordingly, in Nile Tilapia cadmium acts as a stressor and leads to metabolic alternations such as increase of glucose concentration in white muscle and sub-lethal concentrations increases phosphofructo kinase in red muscle. Cadmium can adversely affect organisms at relatively low level exposure and can damage liver, tests, nervous system, kidney, spleen and bone marrow of human being. Therefore, specific dietary of Tilapia species should be found out for cultured fish along with special focus on reducing the contamination of water body.

Chapter 2 – The concentrations of cadmium (Cd), copper (Cu), chromium (Cr), nickel (Ni), lead (Pb) and zinc (Zn) in fish tilapia (*Oreochromis* sp.) were cited from 9 publications and assessed for human health risks. The six cited metal data in the muscles and livers from 22 and 5 tilapia populations, respectively, were calculated for the estimated daily intake (EDI) and target hazard quotients (THQs) of metals in the fish consumption. These values showed that all investigated metals were not hazardous or not posing potential risk for the average and high level fish consumers. However, in some locations with high metal concentrations, were found to exceed the recommended maximum permissible safety limits, while THQ values also exceeded 1, suggesting that the water and fish of some locations or fish farms are not completely safe for human health. Therefore, consumption of tilapia harvested from the wild and fish farms which are potentially receiving anthropogenic wastes should be limited. Also, it is advisable to discard the livers of the tilapia fish populations before consumption because there were less potential human health risks of heavy metals to the consumers on the fish muscle.

Chapter 3 – Tilapia processing industry generates large amount of waste and by-products annually. Full utilization of these waste and by-products is of interest due to economic, environmental and sustainable concerns. By-products and waste such as skin, scale, bones, frames, heads, viscera, low

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grade meat and other waste are usually produced during tilapia processing. A comprehensive review on utilization of tilapia processing waste and by-products is focused in this chapter. Tilapia wastes and by-products in forms of solid and liquid contain high content of organic compounds causing environmental pollution and disposal problems. These waste and by-products can be converted into food and food ingredients with functional properties using physical and biological processes. For instant they can be used for production of value added and novel products such as proteins hydrolysates, collagen, gelatin and bioactive peptides. The peptides hydrolyzed from Tilapia protein have shown good nutritional value with high protein content and essential amino acids as well as bioactivities. Several activities of bioactive peptides such as antioxidant, Ca-binding peptides, angiotensin I-converting enzyme (ACE) inhibitory peptides and antimicrobial are reported. This paper also reviews current research on these functional and bioactive properties. In addition, promising technology for fully utilization of these waste and by-products is also introduced.

Chapter 4 – Climate change is one of the most important global processes that the Biosphere is experiencing, and fish as a part of the Biosphere are not indifferent to this process. Coldwater fish and, particularly the Brown trout (*Salmo trutta*), are very sensitive to the predicted changes in temperature of rivers and streams. Fish are poikilotherms and their body temperature and physiological rates are dependent on the environmental temperature, as this is a key factor of their ecological niche. Gill surface is directly related to respiratory capacity which in turn is related to the oxygen content in the water. At the same time, water capacity to contain oxygen depends on the water temperature and thereby growth rate is also linked to temperature. As a consequence, changes in environmental temperature will directly affect Brown trout survival in its thermal limits. Climate change predictions point to Brown trout natural distribution shifting to the North and East and losing southern territories, in its rear edge. Coldwater fish will not only suffer because of extreme and selective summer temperatures but also as a result of thermal changes in the different phases of the life cycle. In many places, Brown trout will be very close to their limit of physiological efficiency therefore compromising the production of somatic and reproductive biomass. Some ability for adapting to high temperatures is possible but physiological extremes may exist that cannot be forced. Genetic variance and heritable variation in phenotypic plasticity could compensate the effects of changing temperature regimes, but sudden changes might impede adaptation because plasticity is limited.

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Chapter 5 – Fishes have remarkable ability to effectively rebuild the structure of cells and nerve fibers after central nervous system injury. However, the underlying mechanism is poorly understood. In order to address this issue, the authors investigated the proliferation and apoptosis in contralateral and ipsilateral optic nerves, after stab wound injury to the eye of an adult trout *Oncorhynchus mykiss*. The qualitative and quantitative assessment of proliferation and apoptosis in the cells of the optic nerve of a trout has been made using antibodies against PCNA and the TUNEL method. The authors have found that proliferation and neurogenesis in proliferative brain regions, the cerebellum, and the optic tectum were significantly enhanced after the eye injury. PCNA labeling of optic nerve one week after injury revealed heterogenous population of proliferating cells.

TUNEL-labeling gave a qualitative and quantitative assessment of apoptosis in the cells of optic nerve of trout two days after injury. After damage to the optic nerve apoptotic response was registered, and mass patterns of cell migration were found. The maximal concentration of apoptotic bodies was detected in the areas of mass clumps of cells. It is probably indicative of massive cell death in the area of high phagocytic activity of macrophages/ microglia. One week post-trauma, the authors observed proliferation in the integrative centers of trout brain: cerebellum and optic tectum. In optic tectum, PCNA-positive radial glial like cells were identified. Proliferative activity was detected in the dorsal proliferative (matrix) area of the cerebellum and in parenchymal cells of the molecular and granular layers whereas local clusters of undifferentiated cells which formed neurogenic niches were observed in both the tectum and cerebellum, after injury.

The differentiation of neuronal cells detected by labeling cells with antibodies against the protein HuC/D occurred in the proliferative zones of the telencephalon, the optic *tectum*, cerebellum, and medulla of a trout within 2 days after the injury. The authors have shown that the HuC/D expression is higher in the proliferative brain regions than in the definitive neurons of a trout. *In vitro* analysis of brain cells of trout showed that the cells in suspension compare with monolayer retain high proliferative activity, as evidenced by PCNA-immunolabeling. Phase contrast observation showed mitosis in individual cells and the formation of neurospheres which gradually increased during 1-4 days of cultivation.

Chapter 6 – The rapid growth of the aquaculture during the last two decades has been accompanied by an increasing demand for aquafeeds. From several years, fish meal and oil were used as the main sources of proteins and lipids in the diets, respectively. However, with no expectations to increase the

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production of both ingredients to meet the demand, the industry now is using alternative sources of protein and lipids. Particularly, plant-origin meals and oils are being using in several species of carnivorous fish such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Most of the research has been focused in the growth performance and few attention have been given to the immune responses of the fish fed diets with plant meals and oils. In here, we present results of immune responses (non-specific mainly) of fingerlings and juveniles of rainbow trout fed different diets with high contents of different soybean products. In general, non-specific immune responses (lysozyme activity, macrophage burst activity) were not affected by the inclusion of high levels of soybean protein products during periods around 90 days. Besides these finding, a brief review of the effects of another immune responses are included for the rainbow trout when fed other plant-origin protein sources.

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Chapter 1

RISK AND BENEFITS OF TILAPIA

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ABSTRACT

Tilapia is popular fish because of its low to moderate fat and high quality of protein content. Therefore, this fish has wide diversified health benefits in terms of reducing weight, boosting overall metabolism, building strong bones, decreasing the risk of various chronic diseases and preventing arthritis as well as cancer. However, environmental contaminants, originated both from the anthropogenic and natural sources, possess potential harm to living species. It usually refers to chemical, biological and physical contaminants and aquatic species like fishes are sensitive to these contaminants because of its bioaccumulation. So far, about 118 environmental contaminants related to fish are discovered which contains potential harm of human health. Therefore, human beings are also an important driver for assessing and controlling environmental contaminants. Tilapia species are one of the most widely cultured fresh water fish and the demand of tilapia is exponentially increasing in recent years especially in warm counties like Egypt, Middle East, Malaysia etc. Although Tilapia feed on a wide variety of dietary sources, but specific dietary requirements are still lacking and the

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interactions among nutrients and with cultured conditions are not completely understand. The contamination of water by chemical is a worldwide problem and it disturbs ecological balance of aquatic environment because of its usually non-degradable condition. In addition, fishes are the main inhabitants that directly contaminated by polluted water in many locations. For instance, in Egypt, metal concentrations in Nile Tilapia determined a specific bioaccumulation pattern. Therefore, human health hazard index indicated that the cumulative risk from cadmium exposure significantly increased due to higher consumption rate of Tilapia. Similarly, in Malaysia, the cancer risk calculations due to consumption of Tilapia exceeded the USEPA's acceptable risk level for cadmium (2.1×10^{-6}) and nickel (7.3×10^{-4}), along with the average carcinogenic risk (2.4×10^{-4}). Accordingly, in Nile Tilapia cadmium acts as a stressor and leads to metabolic alternations such as increase of glucose concentration in white muscle and sub-lethal concentrations increases phosphofructo kinase in red muscle. Cadmium can adversely affect organisms at relatively low level exposure and can damage liver, tests, nervous system, kidney, spleen and bone marrow of human being. Therefore, specific dietary of Tilapia species should be found out for cultured fish along with special focus on reducing the contamination of water body.

Keywords: aquatic environment, cultured fish, contaminants, human health

INTRODUCTION

'Tilapia' is the most popular and most widely farmed freshwater fish in the world (Popma and Masser, 1999). It belongs to the family Cichlidae, order Perciformes and comprises of more than 70 species (Cnaani and Hulata, 2008). Tilapia is originated from Africa and the Middle East and occurred abundantly in tropical and subtropical regions (Cnaani and Hulata, 2008). Previously, tilapia can only be found in the South and Central America, the Indian subcontinent and the Middle East until the introduction of these fishes to all over Asia, southern Europe and southern USA for commercial purposes throughout the 20th century (Keenleyside, 1991). The introduction of tilapia in Asian countries occurred in the late 1940s when *Oreochromis mossambicus* spread widely to increase the food fish supplies in the Asian region (Eknath and hulata, 2009). However, O. *mossambicus* is substituted by O. *niloticus* that has a better growth performance in ponds (Ng and Romano, 2013).

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Of ten genera of tilapia, three genera are considered as commercially important which are *Oreochromis*, *Tilapia* and *Sarotherodon* (Macintosh and Little, 1995). Among all species of tilapia, members of the genus *Oreochromis* are favored for aquaculture, for example, the blue *tilapia*, O. *aureus*, the red *tilapia*, O. *niloticus*, O. *mossambicus* and the Nile *tilapia*, O. *niloticus* (Pullin, 1991). Based on overall statistic conducted by Food and Agriculture Organization of the United Nations (FAO, 2011a), the most commonly farmed tilapia is the Nile *tilapia* and O. *niloticus*, and the worldwide production of these tilapias reached to 2.5 million metric tons in 2009.

Most of tilapia production comes from Asia, accounting for up to 80% of the global production (Eknath and Hulata, 2009). Among Asian countries, China dominated the vast majority of aquaculture production of tilapia in 2009 which is over 1.2 million metric tons followed by Indonesia at over 250 000 metric tons and the least contribution of farmed tilapia production is the USA which is only 9979 metric tons or <0.5% of worldwide production (Ng and Romano, 2013). The USA is known as the main tilapia importer which accounts for 91.8% of global exports (Ng and Romano, 2013). The top countries in tilapia production since 1980 until 2007 are China (including Taiwan), Egypt, Philippines, Indonesia, Thailand, Brazil, Honduras, Columbia and Malaysia (Eknath and Hulata, 2009). It was recorded that the worldwide trade of tilapia increased from US$1.7 billion in 2000 to US$5.0 billion in 2010 and it is estimated to further increase in near future (FAO, 2011b).

SPECIALTY OF TILAPIA

There are three important factors for the remarkable success of tilapia as a farmed fish including their good taste, high nutritional content and very easy to culture. Tilapias are known as “aquatic chicken” because it has high growth rates, adaptability to a wide range of environmental conditions and ease in rearing (El-Sayed, 2006). It has high demand daily due to their delicate taste, mild flavor and tender flakes when cooked (Suresh and Bhujel, 2012). Several benefits of tilapia consumption such as weight reduction, increasing metabolism, strengthening the bones and reducing the possibility of various chronic diseases are contributed by high nutritional content of its flesh. The nutritional characteristics of tilapia that have been described by the United States Food and Drug Administration (FDA), for example, low fat content of about 1 g total fat (0.5 g saturated fat), 21 g protein, 40 mg sodium, 55 mg

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cholesterol, 90 mg omega-3 fatty acids and 93 calories (9 from fat) per 113 g (4 oz) fillet portion (Cnaani and Hulata, 2008).

Several characteristics that make tilapia ideal for aquaculture include their ability to survive in shallow and turbid waters, high marketability, variation in culture systems, high resistance to diseases and parasites and easy to hold and breed in a captive environment (Pullin, 1983; Gupta and Acosta, 2001a, Ng and Romano, 2013). Besides, tilapia can tolerate crowding and can survive in relatively stress and unfavorable conditions such as low dissolved oxygen (<2 mg/l) and high ammonia levels (~50 mg/l) for longer periods than most other cultured fish (Suresh and Bhujel, 2012). Another beneficial characteristics of tilapia is its relative ease of genetic manipulation to increase their productivity (Ng and Romano, 201). As a result of genetic manipulation, hybridization among *O. niloticus* and *O. aureus* yield male populations that have a better quality such as faster growing than non-hybrid counterparts (Ng and Romano, 201). Additionally, tilapia can ingest various types of food and can be fed low cost diets from terrestrial sources (Ng and Romano, 2013).

FEEDING AND NUTRITION

The profitability of tilapia production is determined by an appropriate feeding strategy (Bahnasawy et al., 2003). The most important feeding characteristic of tilapia, for example, is its feeding from a wide variety of food including plants or plant-derived components, animal protein and fat-based organisms as well as artificial pellets (Bowen, 1982; Jauncey and Ross, 1982). They can also be fed natural food organisms such as plankton, some aquatic macrophytes, planktonic and benthic aquatic invertebrates, larval fish, detritus and decomposing organic matter (Popma and Masser, 1999; Cnaani and Hulata, 2008). However, a good quality of food with an adequate amount of nutrient as well as an excellent feeding management should be provided to ensure a sustainability and productivity of tilapia (Ng and Romano, 2013).

Several studies on nutrition have been conducted to investigate the optimum nutrition requirement of tilapia, for example, El-Sayed (1999) identified some alternative protein sources for tilapia, Lim et al., (2011) conducted a study on the lipid and fatty acid requirements of tilapia while Jauncey (2000), Shiau (2002) and El-Sayed (2006) wrote the book chapter on nutritional reviews on tilapia. A more comprehensive publication on feeding and nutrition of tilapia were produced by Suresh and Bhujel (2012) and Ng and Romano (2013). These reviews focused on the nutritional requirements

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and comprehensive information about biology and culture of commercially important tilapia species.

Tilapia has a good ability to feed on unpelleted feeds more efficiently than other cultured species (Popma and Masser, 1999). This is due to the two pharyngeal plates of fine teeth in tilapia that help in physical grinding of plant tissues and a stomach pH < 2 which helps to break the cell walls of bacteria and algae. Tilapia is most commonly given pelletized food to avoid any nutrient loss. According to Popma and Masser (1999), the digestible energy requirements for economically optimum growth of tilapia including 8.2 to 9.4 kcal per gram of dietary protein and a similar amount of vitamin and mineral as other warm water fish species required. Together with other warm water fish, tilapia requires the same ten essential amino acids such as lysine (Lys), methionine (Met), tryptophan (Trp), arginine (Arg), phenylalanine (Phe), histidine (His), isoleucine (Ile), leucine (Leu), threonine (Thr) and valine (Val) (Ng and Romano, 2013).

Protein

Among the costliest ingredients in the fish diets is the protein (El-Sayed, 1999). It composed of amino acids (AA) and the amount of protein requirement is determined by the species' digestibility, life stage of a fish and amino acid profile of the protein (Ng and Romano, 2013). For optimal performance, a higher protein (30-40%) is required by tilapia fry and spawning females as compared to the grow-out stages (20-30%) (Siddiqui et al., 1998; Sweilum et al., 2005 and Abdel-Tawwab et al., 2010). The main live protein diets of tilapia fry are detritus and neuston while juveniles feed on detritus and periphyton (Tran et al., 2011). At the initial larval stages, the use of live food is important to enhance the growth performance and survival rate. There are several factors for the availability of natural foods in ponds such as soil fertility, type and amount of fertilizers added, and the number and weight of fish stocked (Tran et al., 2011).

The best pelletable protein source for tilapia is fishmeal because it includes a complete amino acid profile, high digestibility and involves a residual lipid content that contains beneficial fatty acids (Ng and Romano, 2013). Although fishmeal is chosen as the important food source for tilapia but there is limitation for continuous supply of fishmeal due to a rising cost and unpredictable reliability (Ng and Romano, 2013). Thus, another alternative animal sources were made to replace fishmeal such as the introduction of

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feather meal, meat bone meal and poultry by-products. Among the animal sources, blood meal showed the best growth and feeding efficiency followed by poultry by-product (El-Sayed, 1998). The use of 66% of feather meal to replace a mixture of fishmeal and meat bone meal in the diets of Nile tilapia fry is reported by Bishop et al., (1995). However, Guimaraes et al., (2008) claimed that the protein digestibility of feather meal and meat bone meal significantly lower to Nile tilapia as compared to poultry by-products and fishmeal.

Another low-cost protein diet for tilapia is the terrestrial plants (Ng and Romano, 2013). Soybean meal is the most common plant protein used in tilapia diets. However, it is also substituted by a lower price, a higher protein content (40-44%) and relatively good amino acid profile ingredient such as cottonseed meal and rapeseed or canola meal (El-Sayed, 1999). Because tilapias are opportunistic omnivores, their diet pattern is considered as interesting. Young tilapias are carnivorous and preferred zooplankton as their diets and when they enter a juvenile stage, their diets shift to plant material or detritus origin (Bahnasawy et al., 2003). Based on observation, *Oreochromis* species feed primarily on microscopic plant materials while *Tilapia* species prefer large plants (Bahnasawy et al., 2003).

Lipid and Fatty Acid

Lipid and fatty acid are important in fish diets as it improves digestibility and concentrated energy source as well as one of the important components to cellular membranes, precursors to hormones and helps in the absorption of lipid-soluble vitamins (Ng and Romano, 2013). In addition, it also improves diet quality when oil coating to extruded pellets enhances the palatability and appearance to fish (National Research Council (NRC), 2011). It was reported that fatty acid requirement of Nile tilapia was reported to be 0.5% for lonoleic (Takeuchi et al., 1983). Comparatively, the redbelly tilapia recorded approximately 1% of both linoleic and arachidonic acid for fatty acid requirement (Kanazawa et al., 1980).

A balance amount of fatty acid should be provided for the essential fatty acid requirement of tilapia as excess amount of fatty acid will affect the growth of tilapia. For example, Kanazawa et al., (1980) and Huang et al., (1998) identified that high dietary n-3 PUFA reduced the growth of tilapia while an increase of 1% of dietary a-linolenic lowered the growth of blue tilapia (Stickney and McGeachin, 1983). It was suggested that a maximum

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lipid levels for tilapia diets should range from 5 to 12% and excessive amount of lipids may cause substantial carcass and visceral deposition of fats (Bahnasawy et al., 2003).

Carbohydrate

Carbohydrate is an inexpensive nutrient and energy source for tilapia (Shiau and Peng, 1993). It provides stability for pelleted feeds and important for growth improvement of fish (Anderson et al., 1984). Fish has low ability to digest and utilize starches in which the performance of fish is determined by the complexity of starch and the presence of intestinal bacteria (Leenhouwers et al., 2007). The example of indigestible carbohydrates sources is a-starch, cellulose, sodium alginate, chitin and kaolin (Kihara and Sakata, 1997). In tilapia, a slower metabolizing complex sugars, such as starch, dextrin and disaccharides is effectively utilized as compared to glucose based in term of growth performance, feeding efficiencies and energy storage (Anderson et al., 1984; Shiau and Suen, 1992; Shiau and Peng, 1993; Tung and Shiau, 1993; Shiau and Chuang, 1995; Hsieh and Shiau, 2000).

Excessive starch content might cause detrimental growth in fish (El-Sayed and Garling, 1988). High starch diets resulted in a condition which is referred as ‘diabetic’ due to an extended duration of hyperglycaemia or inadequate insulin levels (Wilson, 1994; Shiau, 1997). Excessive blood glucose levels in tilapia is excreted through urine (Tung and Shiau, 1993; Lin et al., 2000). One of the beneficial characteristic of Nile tilapia is their intestinal bacteria can easily ferment a-starch effectively than others and producing short chain volatile fatty acids (VFA) (Ng and Romano, 2013). Furthermore, the gut of tilapia is capable of fermenting all tested carbohydrate sources such as glucose, wheat starch, arabinoxylan and whole wheat (Leenhouwers et al., 2008).

Vitamins and Minerals

Vitamins and minerals are added to the diets of tilapia in order to improve their diet quality. Although tilapias mostly feed on natural food that already contains a lot of vitamins, however the adding of extra vitamin in the diet is questionable in many cases (Bahnasawy et al., 2003). The amount of vitamin and minerals added in tilapia diets is not fixed and depending on the species

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and their culture conditions or dietary composition. The function of minerals includes the function of enzyme for a better digestion, gas exchange, acid-base balance and osmoregulation (Ng and Romano, 2013). These nutrients are added to tilapia diets because it can be obtained from brackish or marine environment while tilapia are mostly farmed in freshwater or low salinity waters. Similar with other nutrients, excessive or deficient amount of mineral can reduce the performance of the fish.

CULTURE AND PRODUCTION OF TILAPIA

Tilapias are regarded as the most significant food fishes cultured in tropical and subtropical countries (Beveridge and McAndrew, 2000). With high global production which is 2.3 million tons in 2006 ($2.4 billion), tilapia have become one of the world's most important farmed fish (Fitzsimmons, 2008). Additionally, tilapias are categorized in the top ten of the major cultured species list by the Food and Agriculture Organization of the United Nation's (FAO) in 2004. Tilapias are cultured in at least 85 countries and the main producers are China (~ 900,000 tons), Egypt (~ 200,000 tons), the Philippines (~ 145,000 tons), Indonesia (~ 140,000 tons), Thailand (~ 100,000 tons), Taiwan (~ 90,000 tons) and Brazil (~ 70,000 tons) (Cnaani and Hulata, 2008). However, Fitzsimmons (2016) reported that tilapia production in China is 43% (~1800000 metric tonnes) (Figure 1), whereas the production in Malaysia is 1% (~38768 metric tonnes) (MAMPU, 2016).

Among the most commonly cultured tilapia outside of Africa are Nile tilapia (*O. niloticus*) while Blue tilapia (*O. aureus*), Mozambique tilapia (*O. mossambicus*) and the Zanzibar tilapia (*O. urolepis hornorum*) are less commonly farmed species (Popma and Masser, 1999). These species can be recognized by different banding pattern on the caudal fin and color patterns on the body and fins. For example, Nile tilapia have strong vertical bands on its caudal fin with gray or pink pigmentation in the throat region while Mozambique tilapia have weak or no bands on the caudal fin with a more yellow coloration (Popma and Masser, 1999).

Tilapia can be grown in a wide range of cultured systems from extensive to semi-extensive systems as well as monoculture to polyculture with other fish (Cnaani and Hulata, 2008). Among all tilapia species, Nile tilapia (*Oreochromis niloticus*) is the most important which constitutes 90% of all tilapia cultured outside Africa (FAO, 2004). Tilapia can also be cultivated in pond with the addition of inexpensive organic and inorganic fertilizers in order

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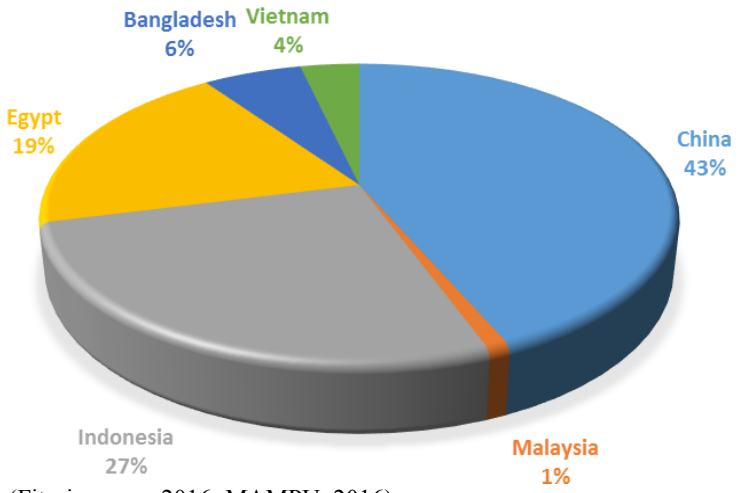
to increase pond productivity such as plankton production to have a greater fish production (Diana and Lin, 1998; Liti et al., 2002).



Source: (Fitzsimmons, 2016; MAMPU, 2016).

Figure 1. Tilapia production (%) in several countries of the world (2015).

ENVIRONMENTAL CONTAMINATES

Environmental contaminants, originated both from the anthropogenic and natural sources, are substances that, when accidentally or deliberately introduced into the environment, may have the potential to harm living species (GOC, 2016). Environmental contamination usually refers to chemical, biological and physical contaminants and aquatic species like fishes are sensitive to these contaminants because of bioaccumulation. Environmental contaminants both from the natural and anthropogenic sources are responsible to contaminated the aquatic environment. Specifically, chemical pollution is very dangerous in terms of its carcinogenic characteristics. In addition, the concentration and chemical fractionation of Cr, Ni, Cu, As, Cd and Pb have been globally reported as alarming toxic heavy metals because of its abundance and persistence in the aquatic environment (Islam et al., 2015; Mohiuddin et al., 2011). Moreover, human being is the principal driver for assessing and controlling environmental contaminants (Vallero, 2010) along with ecosystem that is a good receptor of contaminants. The Environmental Contaminants Encyclopedia emphasized about 118

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environmental contaminants related to fish which is also link to human health risk (Irwin et al., 1998).

Tilapia species are one of the most widely cultured fresh water fish and this cultured tilapia represents more than 70% in the world of the tilapia production (USDA, 2010). Moreover, the demand of tilapia is exponentially increasing in the recent years especially in the warm counties (El-Sherif and El-Feky, 2008) such as Egypt, Middle East, Malaysia etc. Although Tilapia feeds on a wide variety of dietary sources, but specific dietary requirements are still lacking and the interactions among nutrients and with cultured conditions are not completely understand for the various Tilapia species (USDA, 2010).

The contamination of water by metal compound is a worldwide environmental problem (Almeida et al., 2001). Heavy metal contamination may have detrimental effects on the ecological balance of the aquatic environment because of its non-degradable condition as well as fishes are the main inhabitants that directly suffer from the contamination and it has been reported in many location of the world (Omer et al., 2012). For instance, in Egypt, the metal concentrations in Nile Tilapia showed a specific bioaccumulation pattern. Therefore, the human health hazard index indicated that the cumulative risk from cadmium (Cd) exposure greatly increases with the increasing fish consumption rate (Omar et al., 2013; Omer et al., 2012). Moreover, ammonia, which is very common environmental contaminants, is available from manmade as well as natural sources and it is highly toxic to Tilapia if accumulated in the body (Kucuik, 2014). Accordingly, in Nile Tilapia cadmium acts as a stressor and leads to metabolic alternations such as increase of glucose concentration in white muscle and sub-lethal concentrations of cadmium increases phosphofructo kinase and LDH in red muscle. Cadmium can adversely affect organisms at relatively low exposure concentrations and leads to pathological conditions in liver, tests, brain, nervous system, kidney, spleen and bone marrow of human being (Almeida et al., 2001). Therefore, a strict worldwide guideline for fresh and sea water is required in terms of reducing water pollution instead of separate guidelines produced by USEPA, EU, WHO along with country wise guideline.

BIOACCUMULATION IN TILAPIA

Several literatures reported high levels of toxic metals in many ecosystems in the world (Silva et al., 2004). A rapid increase in domestic and industrial

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wastewater are caused by a continuous increase of urbanization and industrialization (Molina et al., 2011). The heavy metals that enter the aquatic environment accumulate in tissues and organs of aquatic organisms and has become one of the important worldwide problem (Malik et al., 2010). The problem arises because of inability of heavy metals to be degraded. It is either deposited, assimilated or incorporated in water, sediment and aquatic animals (Linnik and Zubenko, 2000). As a result, the heavy metals that accumulate in the body of aquatic organisms enter the food chain and also affects the consumers from the highest level (Akan et al., 2009).

The bioaccumulation process of heavy metals occurred via the food chain and indirectly causing health risks to human consumers through food assimilation (Agah et al., 2009). For example, cancer risk assessment conducted by Environmental Protection Agency (USEPA) identified the guideline for the consumption of tilapia. Based on their observation, a weekly consumption of 540 g or more for 70 years may resulted in increase of upper bound cancer risk by 1 in 100,000 consumers. An observation by Jent et al., (1998) and Rashed (2001) indicated that fish collected from the water near to agricultural area has higher concentration of cadmium (Cd) and copper (Cu) in the liver of fish. Comparatively, different observation was obtained by Philips and Rasso (1978) and Malik et al., (2010) in which high accumulation of lead (Pb) and chromium (Cr) was found in the kidney of tilapia.

Fish is commonly used to examine the contamination of aquatic ecosystem based on its important role of a food source (Blasco et al., 1998; Agah et al., 2009). A study conducted by Molina et al., (2011) on bioaccumulation in Nile tilapia (*Oreochromis niloticus*) from Laguna de Bay, Philippines found that Nile tilapia consumption can resulted in an excess of 38 cancer cases per 100,000 populations during the dry season and 9 cancer cases per 100,000 populations in wet season due to chronic oral exposure to arsenic. The other heavy metal such as chromium is also considered as carcinogenic for human through inhalation, while the level of carcinogen in cadmium, lead and mercury are not clearly obtained.

HUMAN HEALTH RISK

Continuous exposure to heavy metals' bioaccumulation through fish consumption can increase the risks to human health. A high concentration of heavy metals is contributed by waste disposal, vehicular exhaust, atmospheric deposition, fertilization, use of pesticides, and application of sewage sludge in

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arable land (Cui et al., 2005; Zheng et al., 2007; Khan et al., 2013; Zhao et al., 2014). The toxicity, persistence and bioaccumulation of heavy metals is considered dangerous because of their high accumulation inside living bodies may lead to harmful diseases over time (Khan et al., 2010). The harmful heavy metals are mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), copper (Cu), zinc (Zn), and nickel (Ni). A study conducted by Molina et al., (2011) on the assessment of the human risks to human health associated with the exposure to heavy metals bioaccumulation in Nile tilapia classified arsenic and chromium as a carcinogenic while cadmium, lead and mercury are either possible and probable carcinogens. Another assessment focusing on arsenic bioaccumulation in *O. Mossambicus* has been conducted by Liao and Ling (2003) provide some information on fish consumption guideline and water quality regulations which is beneficial for maintaining fish and human health.

Direct exposure to heavy metals is caused by three main factors such as food consumption, inhalation and dermal contact in which food consumption accounts for more than 90% of exposure (Loutfy et al., 2006; Cao et al., 2014). For example, arsenic, cadmium and lead are exposed through oral route. Some diseases that are related to arsenic exposure include various types of internal cancers such as liver, bladder, respiratory and gastrointestinal tracts (Molina et al., 2011). Cadmium contaminated food is also carcinogenic in which it resulted to both chronic and acute health problems such as renal tubular damage, osteoporosis and cancer (Turkdogan et al., 2003; Liu et al., 2013). In different case, prolonged period of Pb exposure is harmful for adults by initiating kidney issues and high blood pressure and affected children by retarding their physical and mental development (Rahman et al., 2013).

Surprisingly, excessive amount of zinc and copper are also toxic to human and animals. Although these elements are essential for a normal body function and insufficient amount of these elements may cause diseases such as anorexia, immune dysfunction and poor wound healing (Lei et al., 2015) but excessive concentration can increase toxicity of the body. Excessively high copper concentration can cause headache and irritation of the nose, mouth and eyes (Dieter et al., 2005; Rahman et al., 2013) while zinc toxicity resulted in sideroblastic anemia (Frisbie et al., 2009; Muhammad et al., 2011).

Alam et al., (2015) reported the average carcinogenic risk (2.4×10^{-4}) from heavy metal ingestion through tilapia consumption largely exceeded the recommended level (i.e. 10^{-6}) proposed by USEPA (Table 1). Specifically, the carcinogenic risk from Ni (7.3×10^{-4}) in Malaysia through consuming tilapia is higher than the value Ni (3.0×10^{-4}) reported in the tropical wetlands of India

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(Table 1). In India bioaccumulation of Ni in tilapia is mainly from the natural sources and it might be associated with lung and nasal cancer. However, in Taiwan Blackfoot diseases were reported might be due to the As contaminated tilapia consumption and the carcinogenic risk of As (Arsenic) was calculated in the range of 2.49×10^{-6} to 6.24×10^{-6}. Similarly, Liao et al., (2008) also reported that Blackfoot disease might be associated with the consumption of As (3.4×10^{-5} to 9.3×10^{-5}) contaminated tilapia in Taiwan. However, in 2007 the calm consumption of tilapia in Taiwan reported the carcinogenic risk of As in the range of 0.28×10^{-6} to 4.52×10^{-6}. In addition, the oyster samples of Taiwan were reported very high ranger for As (1.26×10^{-6} to 3.82×10^{-6}). Similarly, Blackfoot disease might be also associated with the consumption of As (3.4×10^{-5} to 9.3×10^{-5}) contaminated tilapia in Taiwan.

SOCIO-ECONOMIC IMPACTS

Fish and fisheries contribute a lot of benefits for nutritional supply and livelihood security in most of the countries mainly for the developing countries. Fish dominates the amount of protein intake for human consumption. Depending on capture fisheries by millions of people across the world resulted in insufficient fish supply due to continuous increase in demand and over exploitation of fish from coastal fisheries. Thus, another alternative based on aquaculture activities provide additional production of food fish to meet the global demand. The aquaculture production reached an annual growth of about 9% in 2000, while in 1970 it was only 1.3% for captured fisheries (Tacon, 2003). Thus, aquaculture is considered as the fastest growing food production sector in the last three decades (FAO, 2006).

Table 1. Carcinogenic risk associated with tilapia consumption in various countries

Country	As	Ni	Cd	Reference
(2015) Malaysia	-	7.3×10^{-4}	2.1×10^{-6}	(Alam et al., 2015)
(2015) Malaysia	6.76×10^{-5}	-	-	(Low et al., 2015)
(2011) India	-	3.0×10^{-4}	-	(Bhupander and Mukherjee, 2011)
(2008) Taiwan	9.3×10^{-5}	-	-	(Liao et al., 2008)
(2007) Taiwan	4.52×10^{-6}	-	-	(Liu et al., 2007)
(2006) Taiwan	6.24×10^{-6}	-	-	(Jang et al., 2006)
(2006) Taiwan	3.82×10^{-5}	-	-	(Liu et al., 2006)
(2003) Taiwan	5.25×10^{-4}	-	-	(Liao and Ling, 2003)

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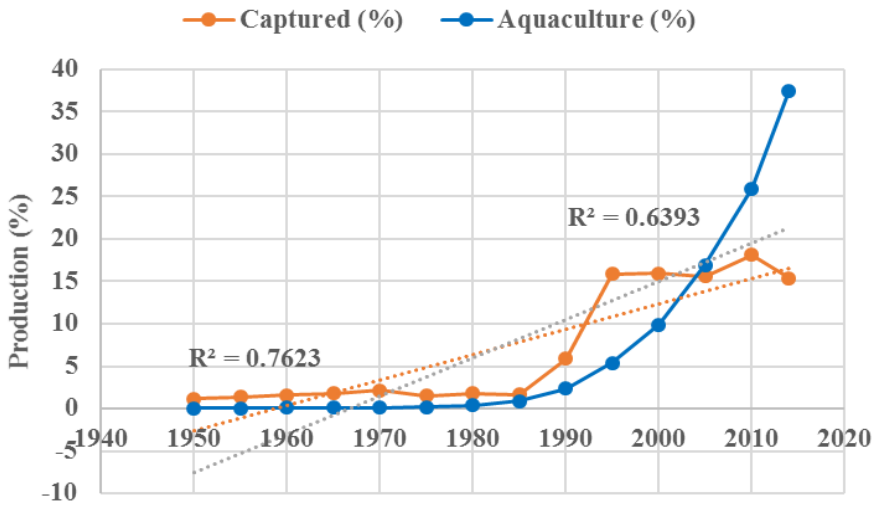

Source: (FAO, 2016).

Figure 2. Global trend of *Oreochromis niloticus* production (1950 to 2014).

The captured and aquaculture production of *Oreochromis niloticus* show a significant (R^2 = 8) and (R^2 = 6) increasing trend respectively during 1950-2014 (Figure 2). The aquaculture of *Oreochromis niloticus* in 1950 was 1590 tonnes, whereas in 2014 the production increased to 3670259 tonnes (FAO, 2016). Similarly, the captured of *Oreochromis niloticus* in 1950 was 18000 tonnes, whereas in 2014 the production became 233811 tonnes. However, the captured production of *Oreochromis mossambicus* (Figure 3) does not show a significant increase trend (R^2 = 3), but the aquaculture of *Oreochromis mossambicus* shows a significant increasing trend (R^2 = 8) during 1950-2014 (FAO, 2016a). The aquaculture of *Oreochromis mossambicus* in 2014 was 42363 tonnes, but in 1950 there was no practise of aquaculture of *Oreochromis mossambicus.* Moreover, the contribution of aquaculture to total global fisheries was ranging from 3.2% in 1950 (638,577 metric tonnes) to 5.2% in 1970. The production further increased in 1980 (9.6%) and 1990 (16.3%). An outstanding rate of aquaculture production was achieved in the early 2000s e.g., an annual rate of 32.1% in 2000, 34% in 2001 and 35.2% in 2002 (Al-Sayed, 2006). Among the most preferred fish for aquaculture is tilapia and it is chosen as the second most cultured fish based on its marketability, stable market price and easy to culture (Wang and Lu, 2016).

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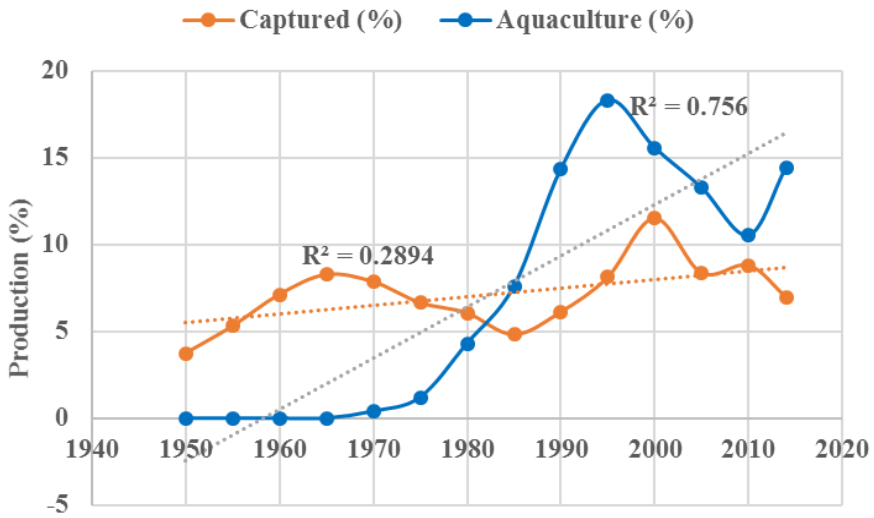

Source: (FAO, 2016a).

Figure 3. Global trend of *Oreochromis mossambicus* production (1950 to 2014).

Tilapia is cultured in more than one hundred countries and is considered as the most productive and internationally traded freshwater food fish (FAO, 2013). It was also found that the increase in global sales of aquaculture tilapia was statistically highly significantly ($R^2 = 0.97$) during 2000-2013 (Figure 4). The global sales in 2013 was USD 9000 000 000 whereas in 2000 it was USD 1615 321 000. The culture of tilapia is affected by environmental condition where the fish is cultured. Several factors such as disease and environmental pollution can reduce the quality and productivity of tilapia. In some cases, disease attack resulted in severe economic losses in various countries including USA, Israel, Brazil, China and Thailand (Eldar et al., 1994; Shoemaker et al., 2001; Suanyuk et al., 2008; Evans et al., 2009; Mian et al., 2009). For example, in China, a serious *Streptococcus* infection with 20-50% infection rate causes high fish mortality (50-70%) in the main production area (Ye et al., 2011). In other case, a continuous increase of the amount of heavy metals in the environment and aquatic ecosystem occurs due to anthropogenic activities, urbanization, industrialization and agriculture practices (Abdel-Baki et al., 2011). The environmental pollution might cause a great implication for human health due to a consumption of infected shellfish. The examples of outbreak diseases are typhoid fever, infectious hepatitis and several viral diseases such as cholera, phycotoxins and depuration (Al-Sayed, 2006).

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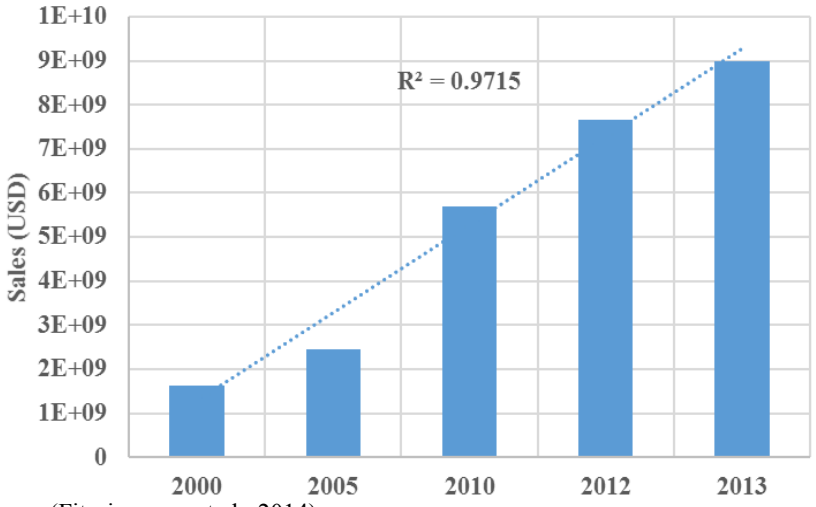

Source: (Fitzsimmons et al., 2014).

Figure 4. Global sales of aquaculture tilapia (2000 to 2013).

CONCLUSION

Natural food supply is the best nutrient source for the well development of tilapia. However, a good growth rate of tilapia completely not only depend on natural food but also on the supplementary feeds. Although dietary alternatives- based on protein ingredient such as fishmeal, fish oil and soybean meal- are considered as the most preferred alternative diets for tilapia but it requires higher cost for a long period. To recommend a more economical diets, plant based ingredients or a mixture of plant and animal protein ingredients should be beneficial. Additionally, comprehensive research on feed formulation is also important to provide economically and commercially alternative diets for tilapia. Moreover, one worldwide guideline for the aquatic environment should be produced to implement instead of country wise guidelines in order to reduce the water contamination.

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Chapter 2

HUMAN HEALTH RISK ASSESSMENT OF HEAVY METALS IN THE CONSUMPTION OF TILAPIA: AN ASSESSMENT BASED ON REPORTED DATA

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ABSTRACT

The concentrations of cadmium (Cd), copper (Cu), chromium (Cr), nickel (Ni), lead (Pb) and zinc (Zn) in fish tilapia (*Oreochromis* sp.) were cited from 9 publications and assessed for human health risks. The six cited metal data in the muscles and livers from 22 and 5 tilapia populations, respectively, were calculated for the estimated daily intake (EDI) and target hazard quotients (THQs) of metals in the fish consumption. These values showed that all investigated metals were not hazardous or not posing potential risk for the average and high level fish consumers. However, in some locations with high metal concentrations, were found to exceed the recommended maximum permissible safety limits, while THQ values also exceeded 1, suggesting that the water and

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fish of some locations or fish farms are not completely safe for human health. Therefore, consumption of tilapia harvested from the wild and fish farms which are potentially receiving anthropogenic wastes should be limited. Also, it is advisable to discard the livers of the tilapia fish populations before consumption because there were less potential human health risks of heavy metals to the consumers on the fish muscle.

Keywords: fish, heavy metals, estimated daily intakes, target hazard quotients

INTRODUCTION

The consumption of food, via ingestion, is often regarded as one of the most important pathways of human exposure to heavy metal toxicities. The concentrations of heavy metals in the edible fish, whether marine or freshwater, have been widely reported in the literature (Otachi et al. 2014; Štrbac et al. 2014; Yap et al. 2015; Mbewe et al. 2016; Saha et al. 2016). From biomonitoring point of view, these data are important for present and future references. Once the maximum permissible levels for food safety in the edible muscle parts have been exceeded, this should raise public concern to urge the government in power to implement further check or to penalize the person or party responsible for the occurrence.

The development of cancerous cells in various organs of human body is viewed as quite omnipresent nowadays as a result of metal consumption in their food (Zweig et al. 1999), through daily newspapers in both local and international. Owing to the toxicity, long persistence, bioaccumulation and biomagnifications of heavy metals (Eisler 1988), contamination of heavy metals through the food chain can trigger human health risks (Zweig et al. 1999; Taweel et al. 2013; Iqbal and Shah 2014). The transfer of bioaccumulated metals in the fish tilapia to human beings via the food chain have been also proven and confirmed based on the many reported reports found in the literature (Leung et al. 2014).

Unlike gills which is the first organ in contact with the environmental water, the metals bioaccumulated in the muscles and livers of fish are believed to have been fully assimilated considered and therefore, demonstrating evidence of long-term (past) exposure to contamination events (Taweel et al. 2013). In view of that, metal levels in both organs of tilapia fish are plausibly helpful for evaluating contaminant impact and assessing past contamination events in the aquatic ecosystem.

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The use of tilapia fish as a good bioindicator of heavy metal pollution in the aquatic ecosystem is attributable to several advantages (Barak and Mason 1990). Firstly, they can be widely found in all aquatic ecosystems, ranging to freshwater to marine coastal environment. For examples, the tilapia *Oreochromis mossambicus* have been focused in the heavy metal study for human health risk implications in Bangladesh (Kawser Ahmed et al. 2015); Secondly, they have relatively stable population size, providing a long-term record of environmental stresses; Thirdly, there is abundant literature for their extensive life-history and environmental response for tilapia fish; Fourthly, the metals accumulated in the their muscles can reflect the influence of anthropogenic origins (George et al. 2012) since their food sources are inclusive of both aquatic and terrestrial origins that are influenced by anthropogenic impacts. In particular, tilapia's food includes an array of natural foods such as benthic organisms, planktons, detritus and decomposing organic matter (Nakayama et al. 2010). Consequently, elevated metal levels found in the muscles of tilapia could be a result of their ingestion of a plethora of contaminated sediments, accompanied by other food items.

In this study, estimated daily intake (EDI) and target hazard quotient (THQ) were employed to determine the human health risk assessments (HHRA). The THQ is firstly proposed by USEPA (1989, 2000) and is an integrated risk index for the risk assessment of metals in contaminated foods. The THQ value has been recognized as one of the reasonable parameters for the risk assessment of metals associated with the consumption of contaminated fish (Rahman et al. 2012; Kawser Ahmed et al. 2015; Taweel et al. 2013). According to USEPA (1989, 2000), a THQ value below 1 means the exposed population is unlikely to experience obvious adverse effects, whereas a THQ above 1 means that there is a chance of noncarcinogenic effects, with an increasing probability as the value increases.

The objective of this paper was to assess the potential human health risk of six heavy metals (Cd, Cr, Cu, Ni, Pb and Zn) exposure via consumption of the fish tilapia populations from nine publications in the literature.

MATERIALS AND METHODS

Sources of Data Cited

In this study, the keywords 'metals in Tilapia or fish' were searched in the well-known databases including SpringerLink and ScienceDirect. A total of 85

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publications were found. However, only eight papers with clear mean data presentation of Cd, Cu, Cr, Ni, Pb and Zn in the *Oreochromis mossambicus* or *O. niloticus* were selected. Another good paper is also selected from African Journal of Biotechnology by Abdulali et al. (2011) for Malaysian *O. niloticus*. Therefore, a lot of nine publications with the six metals were cited and included in the present study. The selection of those papers is based on three major criteria: 1) all the published papers must have published at least six metals in the fish tilapia whether *O. mossambicus* or *O. niloticus*, 2) the HHRA based on the selected six metals (Cd, Cu, Cr, Ni, Pb and Zn) in the muscles and livers of tilapia have not been published before. Therefore, present study based on the six metals by using similar adult body weights and consumption rate will give a better and meaningful comparison of HHRA, and 3) all the data cited have been verified through quality control and quality assurance which have been clearly demonstrated or reported. All these data have been checked with standard/certified reference materials with acceptable metal recoveries.

Data Treatment for Human Health Risk Assessment

For human health risk assessment (HHRA), all data that were originally reported in mg/kg wet weights, except for those by Abdulali et al. (2011) for Malaysian *O. niloticus* and Mahboob et al. (2014) for Saudi Arabian *O. niloticus*, were all presented in dry weight basis. Hence, those data were converted to wet weight from dry weight basis by using a conversion factor of 0.208 (George et al. 2012; Rahman et al. 2012).

i) Estimated daily intake (EDI)

The EDI of heavy metals was calculated using the following formula (USEPA 1989):

$$\mathrm{EDI} = \frac{\mathrm{Mc} \times \text{consumption rate}}{\text{body weight}}$$

where Mc is the metal concentration in the soft tissue of fish in wet weight basis, average body weights were 64.0 adults (Lim et al. 2010) while the consumption rate as 160 and 320 g/ day/person for average level (AL) (FAO, 2005, 2009; Agusa et al., 2007) and high level (HL) consumers, respectively

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The oral reference dose (RfD) was used in this study to evaluate the EDIs of metals in fish. The RfD values (µg/kg/day) used in this study were: Cd: 1.00; Cu: 40.0; Ni: 20.0; Cr: 3.00; and Zn: 300, provided by the USEPA's regional screening level (USEPA 2015) while PbRfD (4.00) followed that by (Storelli, 2008).

ii) Target hazard quotient (THQ)

In this study, a non-cancer risk assessment method was based on the use of THQ, a ratio between the estimated dose of contaminant and the reference dose below which there will not be any appreciable risk. The THQ determined with the formula described by USEPA (2000):

$$\mathrm{THQ} = \frac{\mathrm{EF} \times \mathrm{ED} \times \mathrm{CR} \times \mathrm{Mc}}{\mathrm{RfD} \times \mathrm{ABW} \times \mathrm{AET}} \times 10^{-3}$$

where EF is exposure frequency (365 d/year); ED is the exposure duration (70 years), equivalent to the average lifetime; CR is the consumption rate as cited above; Mc is the metal concentration in wet weight basis; RfD is the oral reference dose as cited above; ABW is the average adult body weight (64.0 kg); AET is the averaging exposure time for non-carcinogens (365 d/year ×ED); and 10^{-3} is the unit conversion factor.

iii) Total hazard index (HI)

For the risk assessment of multiple heavy metals contained in fish, a total hazard index (HI) was employed by summing all the calculated THQ values for the determined metals (Jian et al. 2013). For the risk assessment of multiple metals contained in fish, a total THQ or total hazard index (HI) was employed by summing all the calculated THQi values of heavy metals (Jian et al. 2013).

$$\mathrm{HI} = \sum_{i=1}^{n} \mathrm{THQ}i$$

where, THQi is the targeted hazard quotient of an individual metal and n in the present study is 6 (Cd, Cu, Cr, Ni, Pb and Zn).

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RESULTS AND DISCUSSION

Metal Levels in the Muscles and Livers of Fish

The concentrations (mg/kg wet weight) of Cd, Cu, Cr, Pb, Ni and Zn, in the tilapia populations, are presented in Table 1. Based on muscles of 22 tilapia populations (Table 1), the ranges of concentrations (mg/kg wet weight) of Cd, Cu, Ni, Pb, Zn and Cr are 0.001-0.391, 0.19-5.22, 0.01-4.35, 0.001-11.3, 1.90-65.3, and 0.07-3.45, respectively. The metals in the muscles varied in the following sequence: Zn>Pb> Ni> Cu> Cr> Cd. Based on livers of 5 tilapia populations (Table 1), the ranges of concentrations (mg/kg wet weight) of Cd, Cu, Ni, Pb, Zn and Cr are 0.012-0.180, 0.97-219, 0.15-4.35, 0.02-1.00, 13.5-29.7, and 0.16-4.28, respectively. The metals in the livers varied in the following sequence: Cu> Zn> Cr > Ni>Pb> Cd.

In comparison of metal concentrations between the muscles and livers of tilapia, it is found the overall levels of Cd, Cu, Ni, and Cr in the livers (based on 5 populations) are significantly ($P< 0.05$) higher than those in the muscles (based on 22 populations). Specifically, based on the paper by Abdulali et al. (2011) on three sampling sites, the results showed that the levels of all six metals were significantly higher in the livers ($P< 0.05$) than in the muscles. In addition, Dhanakumar et al. (2015) reported higher levels (mg/kg dry weight) of Cu in livers (0.99) than muscles (0.05), Cr in livers (6.18) than muscles (1.10), Zn in livers (43.7) than muscles (12.5), Pb in livers (6.50) than muscles (0.42), and Ni in livers (0.93) than muscles (0.24), in the tilapia *O. mossambicus* collected from reservoirs of Cauvery delta region in India.

The general high accumulation of the six metals in the tilapia in the livers than in the muscles was supported by many reports (Jirsa et al. 2008; Otachi et al. 2014). For example, Otachi et al. (2014) reported that metal concentrations were higher in the liver than in the muscle of tilapia *O. leucostictus* while George et al. (2012) reported that the metal levels in the liver of the fishes *Mugil cephalus*, *Etroplus suratensis*, *Sillago sihama*, and *Arius arius* collected from the Indian Cochin backwaters were higher in the muscles. This could be attributable to different routes of fish organ exposure to polluted aquatic ecosystem and each organ specialised in the uptake, absorption, storage, regulation, and excretion by their respective ability (Roesijadi 1992; Roach et al. 2008). The high metal accumulation in the fish livers may be related to the metàllothionein binding to the metals as a means to reduce metal toxicity (Hadson 1988; Višnjic et al. 2010), reflecting the importance of the tilapia livers's role in storing and detoxifying metals.

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Table 1. Mean concentrations (mg/kg) of heavy metals in the muscles and livers of Tilapia cited from nine different references in the literature

No.	PN	Part	sp	Location	Cd	Cr	Cu	Ni	Pb	Zn	Reference
1	1	M	OM	(A: Zhongshan;	0.090	0.60	1.64	3.76	11.10	38.2	Leung et al. (2014)
1	2	M	OM	B: Shunde;	0.040	0.57	1.72	4.35	11.30	37.0	Leung et al. (2014)
1	3	M	OM	C: Tung Lung Chau;	0.030	0.28	0.92	2.53	5.60	26.8	Leung et al. (2014)
1	4	M	OM	D: Ma Wan;	0.020	1.05	1.40	3.78	9.18	29.0	Leung et al. (2014)
1	5	M	OM	E: Cheung Chau;	0.030	0.44	1.55	3.72	9.04	32.4	Leung et al. (2014)
1	6	M	OM	F: Kat O;	0.020	0.34	1.11	2.83	6.68	22.8	Leung et al. (2014)
1	7	M	OM	G: Mirs Bay;	0.020	0.33	1.07	2.94	6.19	21.3	Leung et al. (2014)
1	8	M	OM	H: Dachan Bay, Shenzhen	0.040	0.48	1.59	4.18	9.64	28.7	Leung et al. (2014)
2	10	DMWS	OM	Fangyuan Township, Changhua County (Taiwan; 2009)	0.010	2.46	3.17	2.27	0.14	61.4	Ling et al. (2013)
2	11	DMOS	OM	Fangyuan Township, Changhua County (Taiwan; 2009)	0.010	2.27	2.37	2.32	0.49	39.7	Ling et al. (2013)
2	12	VMWS	OM	Fangyuan Township, Changhua County (Taiwan; 2009)	0.090	3.45	3.87	2.88	0.43	62.5	Ling et al. (2013)
2	13	VMOS	OM	Fangyuan Township, Changhua County (Taiwan; 2009)	0.030	3.17	3.48	3.12	0.53	40.7	Ling et al. (2013)
3	14	M	OM	Lake Awassa, Ethiopia	0.001	0.07	0.54	0.01	0.001	3.7	Yohannes et al. (2013)
4	16	MH	OM	agro-ecological zones in Bangladesh	0.003	1.27	1.14	0.01	0.09	1.9	Kawser Ahmad et al. (2015)

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Table 1. (Continued)

No.	PN	Part	sp	Location	Cd	Cr	Cu	Ni	Pb	Zn	Reference
5	17	M	OM	A fishery farm in Pentung County, Southern Taiwan; 2003)	0.011	0.60	1.34	0.14	0.18	10.8	Lin et al. (2005)
6	18	M	OM	Two local markets in Kowloon (HK; 2004)	0.090	0.15	0.19	0.13	0.29	8.6	Cheung et al. (2008)
7	20	M	OM	Salton Sea, California/ MASGC (2000)	0.180	0.21	0.64	0.95	0.03	12.2	Moreau et al. (2007)
8	21	M	ON	Langat River	0.004	1.23	1.14	0.62	0.04	6.9	Abdulali et al. (2011)
8	23	M	ON	Cempaka River	0.006	1.25	0.71	0.67	0.03	7.7	Abdulali et al. (2011)
8	25	M	ON	UKM Lake	0.006	1.19	0.49	0.62	0.03	9.4	Abdulali et al. (2011)
9	27	M	ON	WadiHaneffah, Riyadh, Saudi Arabia	0.391	0.21	5.22	0.91	0.72	60.8	Mahboob et al. (2014)
9	28	M	ON	WadiHaneffah, Riyadh, Saudi Arabia	0.333	0.29	4.91	0.98	0.64	65.3	Mahboob et al. (2014)
				Overall (muscle= 22)							
				Minimum	0.001	0.070	0.19	0.01	0.001	1.90	
				Maximum	0.391	3.450	5.22	4.35	11.3	65.3	
				Mean	0.066	0.996	1.83	1.98	3.29	28.5	
				Std Deviation	0.105	0.988	1.42	1.50	4.29	20.4	
		L									
3	15	L	OM	Lake Awassa, Ethiopia	0.180	0.25	219	0.48	0.08	13.5	Yohannes et al. (2013)
7	19	L	OM	Salton Sea, California/ TSMP (1997-2000)	0.012	0.16	0.97	0.15	0.02	17.3	Moreau et al. (2007)
8	22	L	ON	Langat River	0.044	4.22	68.4	2.91	1.00	29.7	Abdulali et al. (2011)
8	24	L	ON	Cempaka River	0.096	4.16	93.4	4.35	0.52	23.3	Abdulali et al. (2011)

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No.	PN	Part	sp	Location	Cd	Cr	Cu	Ni	Pb	Zn	Reference
8	26	L	ON	UKM Lake	0.146	4.28	69.1	3.33	0.68	25.6	Abdulali et al. (2011)
				Overall (Liver= 5)							
				Minimum	0.012	0.16	0.97	0.15	0.02	13.5	
				Maximum	0.180	4.28	219	4.35	1.00	29.7	
				Mean	0.096	2.61	90.3	2.24	0.46	21.9	
				SD	0.069	2.20	80.1	1.84	0.41	6.48	

Note:

Populations of no. 15, 19, 22, 24 and 26, focused on the fish livers.

All mean concentrations were originally cited in mg/kg dry weight except for populations no. 21 to 28 in which the concentrations were converted to wet weight from dry weight basis by using a conversion factor of 0.208 (George et al. 2012; Rahman et al. 2012).

No.= Number of citations; Pop no.= Population number; sp= species;

M = muscle; L= liver; MH = muscle plus head;

OM= *Oreochromis mossambicus*; ON= *Oreochromis niloticus*.

DMWS = Dorsal muscle with skin;

DMOS = Dorsal muscle without skin;

VMWS = Ventral muscle with skin;

VMOS = Ventral muscle without skin.

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Previously, positive and strong correlation between Zn and metallothionein in liver tissues of Tilapia had been reported (Liu et al. 1996). The lower metal accumulation in the muscles than in the livers reflected the fact that a low levels of binding metallothionein in the muscle. This could help us to understand the tilapia muscle acted as a transitory tissue in the pathway of metal uptake and storage while the liver acted as a main organ for metabolism and respiration. Therefore, the tilapia liver can be considered as a target site for metal accumulation (Otachi et al. 2014). Comparison with Food Safety Guidelines.

The muscles were concentrated in the present study because it is the major contribution of human diet as reported by many researchers (Leung et al. 2014; Saha et al. 2016). For comparison purpose, the metal levels in the livers were also included.

Cadmium

The present concentrations (mg/kg ww) of Cd ranges (muscles, 0.001-0.391 and livers, 0.012-0.180) did not exceed the legal limits or food safety guidelines (FSG) (1.00 mg/kg ww) set by WHO (1989), Malaysian Food Regulations (MFR) (MFR 1985) and the European Union (EC 2006).

Cd is a ubiquitous trace metal which is extremely toxic to fish and is capable of producing chronic toxicity even when it is present at the concentration of 1.00 mg/kg (Rahman et al. 2012). According to Uluozlu et al. (2007), Cd can accumulate in the human body and may cause kidney dysfunction, skeletal damage, and reproductive deficiencies.

Copper

The present Cu ranges in the muscles (0.19-5.22) were also well below the FSGs suggested by WHO (1996) (30 mg/kg ww), and MFR (30 mg/kg ww; MFR 1985). However, the Cu levels (0.97-219) in the livers of all fish populations, except for that reported by Moreau et al. (2007), exceeded the above two safety guidelines.

Cu is an essential part of several enzymes and is necessary for metabolic function and synthesis of hemoglobin. However, high intake of Cu has been recognized to cause adverse health problem (Rahman et al. 2012) such as liver and kidney damage (Ikem and Egiebor 2005; Flemming and Trevors 1989).

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Furthermore, Cu may cause an additive toxic effect in association with Zn and Hg (Schmitt and Brumbaugh 1990).

Chromium

The present Cr ranges (muscles: 0.07-3.45 and livers: 0.16-4.28) have been to exceed the Chinese national standards set the limit of Cr at 2.0 mg/kg wet weight (GB 2762–2012) (Gu et al. 2015). However, according to Zhang et al. (2014), no limit has been established for Cr in aquatic foods by the Codex Alimentarius Commission of Australia, New Zealand, Japan, the United States, and Taiwan. The presence of Cr in the diet is essential due to its active involvement in the glucose metabolism, insulin function and lipid metabolism (Ahmed et al. 2015; Anderson 2000; Mertz 1993; Zhang et al. 2014). In addition, increased risk factors for diabetes mellitus and cardiovascular disease have been found to be related to suboptimal dietary intake of Cr (Anderson 2000; Mertz 1993). According to Gad (1989), Cr in hexavalent form is regarded as toxic and carcinogenic, ending in the target organ of the kidney.

Nickel

Ranges for Ni levels in the muscles (0.01-4.35) and livers (0.15-4.35) were also lower than those set by the US Food and Drug Administration (USFDA 2007) (Ni: 80 mg/kg ww), which is the only available and valid MPL in the literature (Yap et al. 2015).

Exposure to compounds of Ni has been shown in epidemiological studies to correlate with increased incidences of cancer in humans (Nordberg et al. 2007). International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence in humans for the carcinogenicity of Ni (IARC 2014). According to Forti et al. (2011), Ni normally occurs at very low levels in the environment and it can cause variety of pulmonary adverse health effects, such as lung inflammation, fibrosis, emphysema, and tumors.

Lead

The present Pb ranges (muscles: 0.001-11.3 and livers: 0.02-1.00) in some of the muscle and livers of tilapia could reach levels exceeding the limits set by the FSGs set by WHO (2.00 mg/kg ww; WHO 1996) and European Union

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(1.50 mg/kg ww; EC 2006) and MFR (1.00 mg/kg ww; MFR 1985). Therefore, it is suggested that the consumption of the fish collected from the contaminated sites could plausibly pose toxicological risks to the consumers.

According to a review by Lustberg and Silbergeld (2002), Pb exposure in the general population is associated with cancer risk. Pb is a non-essential element and it is well documented that Pb can cause neurotoxicity, nephrotoxicity, and many other adverse health effects and health disorders (Garcia-Lestonetal. 2010; Rahman et al. 2012).

Zinc

The present Zn ranges (muscles, 1.90-65.3 and livers, 13.5-29.7) were below the FSGs suggested by FAO (1983) (30 mg/kg ww) and MFR (100 mg/kg ww; MFR 1985) except for nine populations which exceeded both FSGs established by MFR (1985) which included three populations (Zhongshan, Shunde and Cheung Chau) by Leung et al. (2014), four populations (whether with or without skins in the dorsal or ventral muscles) by Ling et al. (2013) and two populations (WadiHaneffah, Riyadh, Saudi Arabia) by Mahboob et al. (2014).

Some authors reported that chronic exposure to Cu and Zn is associated with Parkinson's disease (Gorell et al. 1997) and these elements might act alone or together over time to induce the disease (Prasad 1983). Zn is involved in most metabolic pathways in humans, and deficiency can result in loss of appetite, inhibition of growth, skin changes, and immunological abnormalities (Tuzen 2009).

ESTIMATED DIETARY INTAKE (EDI) AND TARGET HAZARD QUOTIENT (THQ)

Average Level (AL) Consumers

Table 2 shows the values EDI, THQ and HI of heavy metals in the adult (64 kg) AL consumers (160 g/day) of tilapia of the cited data. The EDI values for the 22 populations based on muscles are 0.003-0.978 for Cd, 0.47-13.06 for Cu, 0.03-10.88 for Ni, 0.01-28.25 for Pb, 4.63-163 for Zn, and 0.18-8.63 for Cr. The THQ values of the 22 populations in the muscles are 0.003-0.978 for Cd,0.01-0.33 for Cu, 0.002-0.54 for Ni, 0.003-7.06 for Pb, 0.02-0.54 for Zn, and0.06-2.88 for Cr. The HI ranges from 0.13-8.60.

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Table 2. Estimated daily intakes (EDI), Target hazard quotient (THQ) and total hazard index (HI) of heavy metals in the adult (64 kg) average consumers (160 g/day) of Tilapia of all cited data in the literature

PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
1	EDI	0.225	1.500	4.100	9.400	27.750	95.500	-
	THQ	0.225	0.500	0.103	0.470	6.938	0.318	8.553
2	EDI	0.100	1.425	4.300	10.875	28.250	92.500	-
	THQ	0.100	0.475	0.108	0.544	7.063	0.308	8.597
3	EDI	0.075	0.700	2.300	6.325	14.000	67.000	-
	THQ	0.075	0.233	0.058	0.316	3.500	0.223	4.405
4	EDI	0.050	2.625	3.500	9.450	22.950	72.500	-
	THQ	0.050	0.875	0.088	0.473	5.738	0.242	7.464
5	EDI	0.075	1.100	3.875	9.300	22.600	81.000	-
	THQ	0.075	0.367	0.097	0.465	5.650	0.270	6.924
6	EDI	0.050	0.850	2.775	7.075	16.700	57.000	-
	THQ	0.050	0.283	0.069	0.354	4.175	0.190	5.121
7	EDI	0.050	0.825	2.675	7.350	15.475	53.250	-
	THQ	0.050	0.275	0.067	0.368	3.869	0.178	4.806
8	EDI	0.100	1.200	3.975	10.450	24.1	71.750	-
	THQ	0.100	0.400	0.099	0.523	6.025	0.239	7.386
10	EDI	0.025	6.150	7.925	5.675	0.350	153.500	-
	THQ	0.025	2.050	0.198	0.284	0.088	0.512	3.156
11	EDI	0.025	5.675	5.925	5.800	1.225	99.250	-
	THQ	0.025	1.892	0.148	0.290	0.306	0.331	2.992
12	EDI	0.225	8.625	9.675	7.200	1.075	156.250	-
	THQ	0.225	2.875	0.242	0.360	0.269	0.521	4.491
13	EDI	0.075	7.925	8.700	7.800	1.325	101.750	-
	THQ	0.075	2.642	0.218	0.390	0.331	0.339	3.995
14	EDI	0.003	0.175	1.350	0.025	0.010	9.200	-
	THQ	0.003	0.058	0.034	0.001	0.003	0.031	0.129

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Table 2. (Continued)

PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
16	EDI	0.008	3.185	2.845	0.030	0.225	4.625	-
	THQ	0.008	1.062	0.071	0.002	0.056	0.015	1.213
17	EDI	0.028	1.493	3.360	0.353	0.458	26.975	-
	THQ	0.028	0.498	0.084	0.018	0.114	0.090	0.831
18	EDI	0.225	0.375	0.475	0.325	0.725	21.525	-
	THQ	0.225	0.125	0.012	0.016	0.181	0.072	0.631
20	EDI	0.450	0.525	1.600	2.375	0.075	30.500	-
	THQ	0.450	0.175	0.040	0.119	0.019	0.102	0.904
21	EDI	0.010	3.078	2.844	1.560	0.094	17.160	-
	THQ	0.010	1.026	0.071	0.078	0.023	0.057	1.266
23	EDI	0.016	3.120	1.768	1.664	0.078	19.240	-
	THQ	0.016	1.040	0.044	0.083	0.020	0.064	1.267
25	EDI	0.016	2.964	1.227	1.560	0.073	23.400	-
	THQ	0.016	0.988	0.031	0.078	0.018	0.078	1.208
27	EDI	0.978	0.525	13.057	2.272	1.810	151.944	-
	THQ	0.978	0.175	0.326	0.114	0.452	0.506	2.552
28	EDI	0.832	0.718	12.282	2.439	1.607	163.228	-
	THQ	0.832	0.239	0.307	0.122	0.402	0.544	2.446
Overall (22)	EDI	Cd	Cr	Cu	Ni	Pb	Zn	
	Minimum	0.003	0.18	0.47	0.03	0.01	4.63	-
	Maximum	0.978	8.63	13.06	10.88	28.25	163.23	-
	Mean	0.17	2.49	4.57	4.97	8.23	71.32	-
	SD	0.26	2.47	3.55	3.75	10.74	51.02	-
Overall (22)	THQ	Cd	Cr	Cu	Ni	Pb	Zn	HI
	Minimum	0.003	0.06	0.01	0.002	0.003	0.02	0.13
	Maximum	0.978	2.88	0.33	0.54	7.06	0.54	8.60
	Mean	0.17	0.83	0.11	0.25	2.06	0.24	3.65

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PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
	SD	0.26	0.82	0.09	0.19	2.69	0.17	2.73
	Liver							
15	EDI	0.450	0.625	549.200	1.200	0.200	33.775	-
	THQ	0.450	0.208	13.730	0.060	0.050	0.113	14.611
19	EDI	0.030	0.395	2.435	0.370	0.038	43.250	-
	THQ	0.030	0.132	0.061	0.019	0.009	0.144	0.395
22	EDI	0.109	10.556	171.080	7.280	2.496	74.360	-
	THQ	0.109	3.519	4.277	0.364	0.624	0.248	9.141
24	EDI	0.239	10.400	233.480	10.868	1.300	58.240	-
	THQ	0.239	3.467	5.837	0.543	0.325	0.194	10.605
26	EDI	0.364	10.712	172.640	8.320	1.706	63.960	-
	THQ	0.364	3.571	4.316	0.416	0.426	0.213	9.306
Overall (5)	EDI	Cd	Cr	Cu	Ni	Pb	Zn	
	Minimum	0.03	0.40	2.43	0.37	0.04	33.78	-
	Maximum	0.45	10.71	549.20	10.87	2.50	74.36	-
	Mean	0.24	6.54	225.77	5.61	1.15	54.72	-
	SD	0.17	5.50	200.22	4.60	1.03	16.23	-
Overall (5)	THQ	Cd	Cr	Cu	Ni	Pb	Zn	HI
	Minimum	0.030	0.132	0.061	0.019	0.009	0.113	0.395
	Maximum	0.450	3.571	13.730	0.543	0.624	0.248	14.611
	Mean	0.238	2.179	5.644	0.280	0.287	0.182	8.812
	SD	0.174	1.835	5.006	0.230	0.259	0.054	5.197

Note: PN= Populations number follows those in Table 1.
Populations of no. 15, 19, 22, 24 and 26, focused on the fish livers.

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Table 3. Estimated daily intakes (EDI), Target hazard quotient (THQ) and total hazard index (HI) of heavy metals in the adult (64 kg) high level consumers (320 g/day) of Tilapia of all cited data in the literature

PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
1	EDI	0.450	3.000	8.200	18.800	55.500	191.000	-
	THQ	0.450	1.000	0.205	0.940	13.875	0.637	17.107
2	EDI	0.200	2.850	8.600	21.750	56.500	185.000	-
	THQ	0.200	0.950	0.215	1.088	14.125	0.617	17.194
3	EDI	0.150	1.400	4.600	12.650	28.000	134.000	-
	THQ	0.150	0.467	0.115	0.633	7.000	0.447	8.811
4	EDI	0.100	5.250	7.000	18.900	45.900	145.000	-
	THQ	0.100	1.750	0.175	0.945	11.475	0.483	14.928
5	EDI	0.150	2.200	7.750	18.600	45.200	162.000	-
	THQ	0.150	0.733	0.194	0.930	11.300	0.540	13.847
6	EDI	0.100	1.700	5.550	14.150	33.400	114.000	-
	THQ	0.100	0.567	0.139	0.708	8.350	0.380	10.243
7	EDI	0.100	1.650	5.350	14.700	30.950	106.500	-
	THQ	0.100	0.550	0.134	0.735	7.738	0.355	9.611
8	EDI	0.200	2.400	7.950	20.900	48.200	143.500	-
	THQ	0.200	0.800	0.199	1.045	12.050	0.478	14.772
10	EDI	0.050	12.300	15.850	11.350	0.700	307.000	-
	THQ	0.050	4.100	0.396	0.568	0.175	1.023	6.312
11	EDI	0.050	11.350	11.850	11.600	2.450	198.500	-
	THQ	0.050	3.783	0.296	0.580	0.613	0.662	5.984
12	EDI	0.450	17.250	19.350	14.400	2.150	312.500	-
	THQ	0.450	5.750	0.484	0.720	0.538	1.042	8.983

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PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
13	EDI	0.150	15.850	17.400	15.600	2.650	203.500	-
	THQ	0.150	5.283	0.435	0.780	0.663	0.678	7.989
14	EDI	0.005	0.350	2.700	0.050	0.020	18.400	-
	THQ	0.005	0.117	0.068	0.003	0.005	0.061	0.258
16	EDI	0.015	6.370	5.690	0.060	0.450	9.250	-
	THQ	0.015	2.123	0.142	0.003	0.113	0.031	2.427
17	EDI	0.055	2.985	6.720	0.705	0.915	53.950	-
	THQ	0.055	0.995	0.168	0.035	0.229	0.180	1.662
18	EDI	0.450	0.750	0.950	0.650	1.450	43.050	-
	THQ	0.450	0.250	0.024	0.033	0.363	0.144	1.262
20	EDI	0.900	1.050	3.200	4.750	0.150	61.000	-
	THQ	0.900	0.350	0.080	0.238	0.038	0.203	1.808
21	EDI	0.021	6.157	5.689	3.120	0.187	34.320	-
	THQ	0.021	2.052	0.142	0.156	0.047	0.114	2.532
23	EDI	0.031	6.240	3.536	3.328	0.156	38.480	-
	THQ	0.031	2.080	0.088	0.166	0.039	0.128	2.533
25	EDI	0.031	5.928	2.454	3.120	0.146	46.800	-
	THQ	0.031	1.976	0.061	0.156	0.036	0.156	2.417
27	EDI	1.955	1.050	26.114	4.545	3.619	303.888	-
	THQ	1.955	0.350	0.653	0.227	0.905	1.013	5.103
28	EDI	1.664	1.435	24.565	4.878	3.214	326.456	-
	THQ	1.664	0.478	0.614	0.244	0.803	1.088	4.892

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Table 3. (Continued)

PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
Overall (22)	EDI	Cd	Cr	Cu	Ni	Pb	Zn	
	Minimum	0.005	0.350	0.950	0.050	0.020	9.250	-
	Maximum	1.955	17.250	26.114	21.750	56.500	326.456	-
	Mean	0.331	4.978	9.139	9.937	16.450	142.641	-
	SD	0.525	4.943	7.096	7.506	21.486	102.037	-
Overall (22)	THQ	Cd	Cr	Cu	Ni	Pb	Zn	HI
	Minimum	0.005	0.117	0.024	0.003	0.005	0.031	0.258
	Maximum	1.955	5.750	0.653	1.088	14.125	1.088	17.194
	Mean	0.331	1.659	0.229	0.497	4.113	0.475	7.303
	SD	0.525	1.647	0.177	0.375	5.371	0.340	5.455
	Liver							
15	EDI	0.900	1.250	1098.400	2.400	0.400	67.550	-
	THQ	0.900	0.417	27.460	0.120	0.100	0.225	29.222
19	EDI	0.060	0.790	4.870	0.740	0.075	86.500	-
	THQ	0.060	0.263	0.122	0.037	0.019	0.288	0.789
22	EDI	0.218	21.112	342.160	14.560	4.992	148.720	-
	THQ	0.218	7.037	8.554	0.728	1.248	0.496	18.281
24	EDI	0.478	20.800	466.960	21.736	2.600	116.480	-
	THQ	0.478	6.933	11.674	1.087	0.650	0.388	21.211
26	EDI	0.728	21.424	345.280	16.640	3.411	127.920	-
	THQ	0.728	7.141	8.632	0.832	0.853	0.426	18.613

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PN	Muscle	Cd	Cr	Cu	Ni	Pb	Zn	HI
Overall (5)	EDI	Cd	Cr	Cu	Ni	Pb	Zn	
	Minimum	0.060	0.790	4.870	0.740	0.075	67.550	-
	Maximum	0.900	21.424	1098.400	21.736	4.992	148.720	-
	Mean	0.477	13.075	451.534	11.215	2.296	109.434	-
	SD	0.347	11.008	400.448	9.202	2.070	32.462	-
Overall (5)	THQ	Cd	Cr	Cu	Ni	Pb	Zn	HI
	Minimum	0.060	0.263	0.122	0.037	0.019	0.225	0.789
	Maximum	0.900	7.141	27.460	1.087	1.248	0.496	29.222
	Mean	0.477	4.358	11.288	0.561	0.574	0.365	17.623
	SD	0.347	3.669	10.011	0.460	0.517	0.108	10.395

Note: PN= Population number follows those in Table 1.
Populations of no. 15, 19, 22, 24 and 26, focused on the fish livers.

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Therefore, the THQ values of Cd, Cu, Ni and Zn in the muscles of 22 populations were below the oral reference doses (µg/kg wet weight per day) for AL consumers, indicating that normal consumption of fishes from the 22 populations would not result in health risk from Cd, Cu, Ni and Zn. Likewise, THQ values for Pb and Cr in the muscles some populations exceeded 1, indicating potential health risk to the AL consumers.

The EDI values for the 5 populations based on livers are 0.03-0.45 for Cd, 2.43-549 for Cu, 0.37-10.87 for Ni, 0.04-2.50 for Pb, 33.78-74.36 for Zn, and 0.40-10.71 for Cr (Table 2). The THQ values of the 5 populations in the livers are 0.003-0.45 for Cd, 0.061-13.7 for Cu, 0.019-0.543 for Ni, 0.009-0.624 for Pb, 0.113-0.248 for Zn, and 0.132-3.571 for Cr. The HI ranges from 0.395-14.6.

Therefore, the THQ values of Cd, Ni, Pb and Zn in the livers of 22 populations were below the oral reference doses (µg/kg wet weight per day) for AL consumers, indicating that normal consumption of fishes from the 22 populations would not result in health risk from Cd, Ni, Pb and Zn. Likewise, THQ values for Cu and Cr in the livers some populations exceeded 1, indicating potential health risk to the AL consumers.

High Level (HL) Consumers

Table 3 shows the values EDI, THQ and HI of heavy metals in the adult (64 kg) HL consumers (320 g/day) of tilapia of the cited data. The EDI values for the 22 populations based on muscles are 0.005-1.955 for Cd, 0.95-26.11 for Cu, 0.050-21.75 for Ni, 0.02-56.5 for Pb, 9.25-326 for Zn, and 0.35-17.3 for Cr. The THQ values of the 22 populations in the muscles are 0.005-1.955 for Cd, 0.024-0.653 for Cu, 0.003-1.088 for Ni, 0.005-14.1 for Pb, 0.031-1.09 for Zn, and 0.117-5.75 for Cr. The HI ranges from 0.26-17.2.

Therefore, the THQ values of Cu in the muscles of 22 populations were below the oral reference doses (µg/kg wet weight per day) for HL consumers, indicating that even the above normal consumption of fishes from the 22 populations would not result in health risk from Cu. On the other hand, THQ values for Cd, Ni, Pb, Cr and Zn in the muscles some populations exceeded 1, indicating potential health risk to the HL consumers.

The EDI values for the 5 populations based on livers are 0.06-0.90 for Cd, 4.87-1098 for Cu, 0.74-21.7 for Ni, 0.08-4.99 for Pb, 67.6-149 for Zn, and 0.79-21.4 for Cr (Table 3). The THQ values of the 5 populations in the livers

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are 0.06-0.90 for Cd, 0.12-27.5 for Cu, 0.04-1.09 for Ni, 0.019-1.25 for Pb, 0.225-0.496 for Zn, and 0.263-7.141 for Cr. The HI ranges from 0.79-29.2.

Therefore, the THQ values of Cd and Zn in the livers of 5 populations were below the oral reference doses (µg/kg wet weight per day) for HL consumers, indicating that even above normal consumption of fishes from the 22 populations would not result in health risk from Cd and Zn. However, THQ values for Cu, Ni, Cr and Pb in the livers some populations exceeded 1, indicating potential health risk to the HL consumers. This is due to the fact that high concentration of Cu was accumulated and stored in the fish liver. But, this is not a major problem since the livers are always discarded during consumption. Otherwise, the consumption of tilapia's livers of tilapia could create detrimental effects caused by Cu accumulation.

The THQ values below 1 in most tilapia populations especially for AL consumers in the fish muscles indicated no health-threatening concern due to the consumption of edible muscle of tilapia populations. Still, there is a need for regular monitoring of toxic metal pollution so as to obtain data for environmental protection and conservation measures. Yap et al. (2015) reported that almost all THQ value were below 1 except for Cu in the fish's liver from a polluted pond for both children and adults. They also reported the THQ values for Cd, Cu, Fe, Ni and Zn in the widely consumer part (muscles) from the two ponds were below 1, this indicated no health risk was present. Therefore, that implied that there was no risk to developing chronic systemic effects due to the intake of the above named metals (Copat et al. 2013) if the fish's muscles are consumed.

HI was used in the present HHRA because humans are always exposed to more than one metal pollutant and thus they could suffer combined or interactive effects (Li et al. 2013). The present HI values based on six metals were generally higher in HL than AL consumers, indicating that consumption of large amount of tilapia (muscles or livers) may suffer higher health risk.

CONCLUSION

Based on the assessment of human health risks (EDI and THQ) in the six metal concentrations of fish tilapia (*Oreochromis* sp.) cited from 9 publications, it is found that the metal levels in the muscles were not hazardous or not posing potential risk for the AL and HL consumers. However, in some locations with high metal concentrations, were found to exceed the recommended MPL, while THQ values also exceeded 1. This

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suggested that the environmental habitats (water, sediments and food sources) for some sampling sites or fish farms are not completely safe for human health. Therefore, consumption of tilapia harvested from the wild and fish farms which were potentially receiving anthropogenic wastes should be limited. Also, it is advisable to discard the livers of the tilapia fish populations before consumption because there were less potential human health risks of heavy metals to the consumers on the fish muscle.

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Chapter 3

UTILIZATION OF BY-PRODUCTS AND WASTE GENERATED FROM THE TILAPIA PROCESSING INDUSTRY

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ABSTRACT

The Tilapia processing industry generates large amount of waste and by-products annually. Full utilization of these waste and by-products is of interest due to economic, environmental and sustainable concerns. By-products and waste such as skin, scale, bones, frames, heads, viscera, low grade meat and other waste are usually produced during tilapia processing. A comprehensive review on utilization of tilapia processing waste and by-products is focused in this chapter. Tilapia wastes and by-products in forms of solid and liquid contain high content of organic compounds causing environmental pollution and disposal problems. These waste and by-products can be converted into food and food

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ingredients with functional properties using physical and biological processes. For instant they can be used for production of value added and novel products such as proteins hydrolysates, collagen, gelatin and bioactive peptides. The peptides hydrolyzed from Tilapia protein have shown good nutritional value with high protein content and essential amino acids as well as bioactivities. Several activities of bioactive peptides such as antioxidant, Ca-binding peptides, angiotensin I-converting enzyme (ACE) inhibitory peptides and antimicrobial are reported. This paper also reviews current research on these functional and bioactive properties. In addition, promising technology for fully utilization of these waste and by-products is also introduced.

Keywords: by-products, waste, industry, tilapia, processing

INTRODUCTION

Tilapia is an important food source containing high level of protein, low content of fat and carbohydrate. It is also excellent source of minerals and vitamins. It is the third most widely cultured fish, after carp and salmons [1]. Over the past years, the production of tilapia has been dramatically increased and has become one of China's leading exported fish. The production of farmed tilapia has shown a tremendous increase jumping from 465,953 metric tons in 2009 to 633,759 metric tons of live weight in 2014. The value of farmed tilapia has also observed a bully increase, going from US$843 million in 2010 to US$1,115 million in 2014 [2]. In general, about 16 tilapia species have been used for aquaculture production out of which ten species are commercially farmed. Global tilapia production was dominated by three species: the Nile tilapia *Oreochromis niloticus* (L.), the Mozambique tilapia *Oreochromis mossambicus* (Peters) and the blue tilapia *Oreochromis aureus* (Steindachner). Apparently, Nile tilapia was the most important farmed tilapia specie, representing more than 80% of total tilapia production [1]. It is also worthy to note that tilapia processing industry generates huge amounts of wastes with a wide variety of by-products such as skins, scale, heads, bones, viscera, frames, low grade meat and other (Figure 1 and Table 1).

Basically, Tilapia by-products are generally used for production of feeds and fertilizers that have low economic value. In view of utilizing tilapia industry, by-product utilization has been studying by several researchers all over the World. The amounts of wastes and utilization of by-products and waste generated from tilapia processing industry were showed in Table 1.

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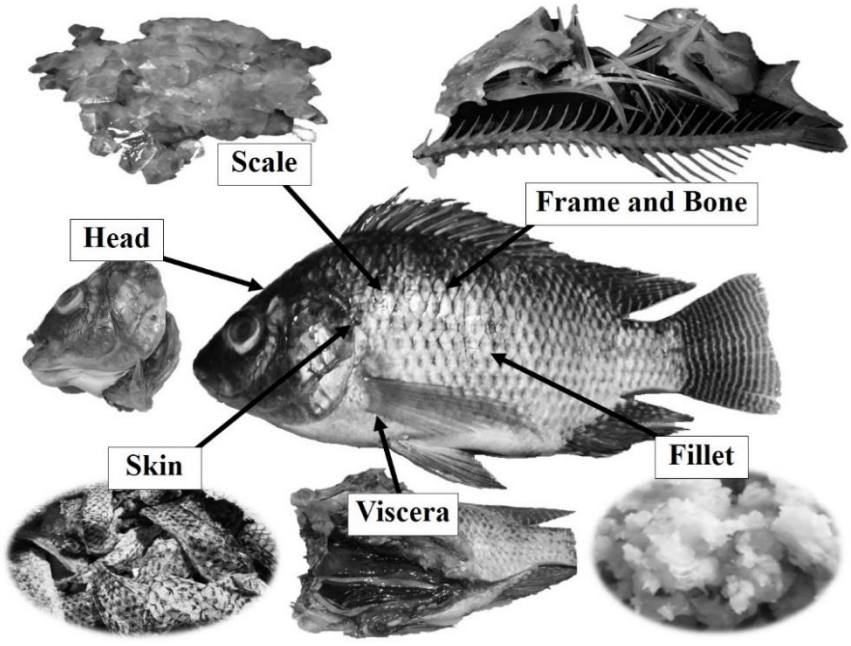

Figure 1. Tilapia by-products.

Table 1. Utilization of by-products and waste generated from tilapia processing industry

by-products	Amounts of wastes (%)	Utilization
Skin	4.9	Collagen, Gelatin, Bioactive peptides
Scale	2.0	Collagen, Gelatin, Bioactive peptides
Bone	3.9	Mineral, Nutrients
Frame	20.6	Bioactive peptides
Head	28.4	Fatty acid, Nutrients
Viscera	6.5	Enzymes
Low grade meat and other waste	3.7	Nutrients, Bioactive peptides

Several by-products, such as skin, bones, frames, heads and tails are usually generated during the processing of tilapia into fillet [3]. These by-products can be converted into tilapia by-product hydrolysate powder with beneficial functional properties [4]. Tilapia by-product hydrolysate has shown

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a good nutritional value with high protein content and essential amino acids. High value of ACE inhibition activity in tilapia by-product hydrolysate might be due to the presence of low molecular weight peptides [5]. Thus, this chapter focuses on the evaluation of all possible nutrients from tilapia by-products.

TILAPIA SKIN

Tilapia skin is an inherent protein source with average protein content (wet basis) of 21.30% [6]. Tilapia skin has been used as raw material for gelatin and collagen extraction [6-8] as well as for production of bioactive peptides such as antioxidant and ACE inhibitory [9-12]. Collagen and gelatin used in commercial production processes are usually derived from vertebrate organisms, such as cows, pigs, and chickens. However, porcine collagen is prohibited in Muslims and Jews religious. Therefore, researchers have focused on searching for new sources of collagen and gelatin and tilapia skin is good choice for production collagen and gelatin. Collagen is the crucial insoluble fibrous protein in the extracellular matrix and in connective tissue. Collagen hydrolysate is not precisely the same thing as gelatin. In the hydrolyzed form, the collagen is executed more rigorously, which certainly breaks up the proteins into smaller pieces. They both have the same amino acids, but different chemical properties. Generally, the production of collagen protein from fish skin includes: selection of the materials, preprocess, extraction, separation, and purification. Extraction is a core process collagen and there are many factors affecting processing efficacy and quality of final product such as pH, temperature, time, and protein concentration [6]. Acid-solubilized collagen and protease-solubilized collagen are found to be efficient methods for tilapia skin [13]. The yields of collagen from tilapia skin is approximately 27.2 g per 100 g dry weight [14]. The presence of hydrogen peroxide the strength and puncture resistance of tilapia skin collagen fibers decrease exponentially with respect to heating time [7]. There are three stages of degradation of fish skin collagen fibers: (1) dissolution of the outer membrane layer of the fiber bundle, (2) loosening and rupture of the fiber bundle, and (3) untwisting and exposure of microfibers. The effect of extraction temperature on the extraction capability and characteristics of acid-soluble collagen, particularly its triple helical structure have been investigated [15]. Fish collagen is usually extracted at low temperature (below 10°C) to maintain triple helix structure. However, extraction temperature of tilapia collagen can be increased upto 25°C to increase extraction efficacy. In addition, the

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extraction at 25°C for 5 h with 0.5 M acetic acid gave higher extraction efficiency than that in 0.3 M acetic acid [15]. All acid-soluble collagen obtained were identified as type I collagen. The amount of amino acids, proline and hydroxyproline, in acid-solubilised collagen is 210 residues per 1000 residues [16]. The collagen was used in many industries such as food supplement, cosmetics and personal care products. Furthermore, physical and chemical properties of collagen and gelatin extracted from tilapia skin have been reported [17].

Gelatin is a mixture of peptides and proteins produced by partial hydrolysis of collagen. Nowadays, gelatin is vastly used in pharmaceutical, food, medical and photography industries because of its superior gelling, thickening and film-forming properties compared with plant based hydrocolloids [11]. Specifically, gelatin features gel strength, viscosity and melting. Various factors effecting of gelation are composed of the average molecular weight, distribution of molecular weight, concentration of gelatin solution, time to gel gelation, temperature, pH and salt content [8]. The functional properties of gelatin are intimately related to amino acid composition and molecular weight distribution. In addition, proline and hydroxyproline are accepted to play a key role in the refolding of gelatin during gelling. Obviously, tilapia skin is a good resource of gelatin and bioactive peptides. Production process of gelatin has various steps. First of all, the fat and minerals are disposed of the raw materials. Afterwards different pre-treatment methods are used (enzymatic hydrolysis, alkaline or acid procedure), depending on the raw material and on the final application of the gelatin. Seconded, the pre-treated raw materials are now treated with hot water and extracted in several stages. Third, the extracted solutions are free of traces of fat and fine fiber in high-performance separators. Even the finest impurities are removed by filtration. In a last purification stage the gelatin is released of calcium, sodium, residual acid and other salts. Fourth, the gelatin solution is now concentrated in vacuum evaporator. After all, the highly concentrated gelatin solutions are sterilized, cooled, set and dried [14]. At present, enzymatic membrane reactor (EMR) a coupling of a membrane separation process with an enzymatic reaction which is used for production of tilapia skin gelatin. There are many parameters affecting bioactive activities of tilapia protein hydrolysate including degree of hydrolysis, peptide structure, amino acid composition, molecular weight, and type of protease used [5]**.** The tilapia skin gelatin hydrolysate obtained by progressive hydrolysis using properase E showed the highest degree of hydrolysis (18 %) and hydroxyl radical scavenging activity when compared with multifect neutral enzyme [10]. Two

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antioxidant peptides were identified and the amino acid sequences were showed in Table 2.

Table 2. Bioactive peptides derived from tilapia processing by-products

by-products	Bioactive peptides	Activity	Amino acid sequence	References
Skin	antioxidant	IC_{50} = 4.61 μg/mL and 6.45 μg/ mL	EGL (317.33 Da) and DEY (645.21 Da)	[10]
Skin	antioxidant	8.16 μg trolox/mg peptide	GETGPAGPAGAAGPAGPR (MW 1490.61 Da)	[12]
Skin	ACE inhibitory	59.32 % inhibition	GPEGPAGAR (MW 810.87 Da)	[12]
Scale	antioxidant	IC_{50} = 8.82 μM	DPALATEPDPMPF (1382.57 Da)	[19]
Frame	antioxidant	IC_{50} = 27.6 μg/mL	DCGY (456.12 Da)	[20]
Frame	antioxidant	IC_{50} = 38.4 μg/mL	NYDEY (702.26 Da)	[20]

Likewise, antioxidant and anti-hypertensive activities were prepared by trypsin hydrolysis of tilapia skin gelatin [12]. Trypsin A fraction showed the greatest reducing power among all hydrolysate fractions, while trypsin B fraction from gel filtration column was found to exhibit the best radical scavenging and ACE inhibitory activities. Moreover, alpha-chain subunits were separated from tilapia skin gelatin using ultrafiltration, and the physicochemical properties of obtained subunits have been investigated [8]. They found that α_1-subunit and α_2-subunit could be successfully separated by 100 kDa and 150 kDa MWCO membranes. Glycine was the most dominant amino acid in both α_1-subunit and α_2-subunit. Nowadays, preparation of edible gelatin films from tilapia skins not only can efficiently extend their potential value, but also provide a source for preparing edible films, which can be applied in the food package to improve product quality and reduce waste problems. In 2016, Chen et al. reported that effects of α_1/α_2 ratios and drying temperatures on the properties of tilapia skin gelatin films [14]. The results showed that films were prepared from α-subunits at α_1/α_2 ratio of 2/1, both tensile strength and elongation at break of films were the highest. The color of α-subunit based composite films was similar to that of α_2-subunit based films.

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On the other hand, the tensile strength and elongation at break of α-subunit based composite films prepared at 10^0C or 15^0C were much higher than those of films prepared at 20^0C or 25^0C. In addition, the extraction of black and red tilapia gelatin were carried out by a series of steps involving washings with 0.2% (w/v) sodium hydroxide and sulfuric acid, and 1.0% (w/v) of citric acid [18]. The gelatins obtained from the skins of red and black tilapias displayed different physicochemical properties. The gelatins from both the black and the red tilapias were snowy white, shiny and light-textured in appearance. The gelatin of black tilapia skin had a strong fishy odour while that of the red tilapia skin had a hardly detectable odour. Their pH values were in the vicinity of 3. The black tilapia skin gelatin was also significantly more viscous, had a higher melting point, and had higher total amino acid content. In short, tilapia skins are potential sources of gelatin and collagen. Moreover, it is new candidates towards the development of the ACE inhibitory and antioxidant agent isolated from the natural sources.

TILAPIA SCALE

During the process of tilapia, a great amount of tilapia scales are usually dumped. Tilapia scale can be a good source of raw material for production of collagen and gelatin [19, 21]. Fish scales are bio-composites of extremely ordered type I collagen fibres and hydroxyapatite $Ca_{10}(OH)_2(PO_4)_6$ [22]. Collagen is highly valued both as a food additive and a functional food ingredient. Specifically, collagen extracted from fish scale has less malodorous smell than that from fish skin and bone [23]. Tilapia scale type I atelocollagen hydrogels with aligned fibril structures is manufactured under a strong magnetic field [24]. The magnetic treatment had no effect on the nanostructure of collagen fibrils. Hydroxyapatite classified as bioceramic materials is the major mineral constituent of vertebrate bones and teeth. The effect of temperature on isolation and characterization of hydroxyapatile from tilapia fish scales have been studied [25]. Scales were subjected to heat treatment at different temperatures and microstructure of scales were observed. Tilapia scale was a good natural source of hydroxyapatite with 800 C as the optimum calcination temperature in hydroxyapatite production.

Gelatin extracted from tilapia scale is one best alternative to replace the porcine and bovine gelatin which can lead to the religious concerns and several diseases. Warm-blooded fish gelatin has similar properties to mammalian sources. Gelatin was extracted from the scale of black tilapia [26].

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The extraction was done by heating the scales in water for 1.5 h at 70°C. The scales gelatin gave high yield and foaming properties. Forthermore, pepsin-solubilized collagen was prosperously prepared from tilapia scale waste. Lyophilized collagen was dissolved in dilute hydrochloric acid to form acidic collagen solutions which was neutralized to form gel. Tilapia scales can be an effective source of collagen extraction that could be used as a potential biomaterial in biomedical applications. A novel extrusion–hydro-extraction process for extraction of collagen from tilapia fish scale was investigated [23]. Extruded scale samples had a 2–3 times higher protein extraction yield than that of non-extruded scale samples. All extracts contained hydroxyproline (61–73 residues/1000 residues) and hydroxylysine (5–6 residues/1000 residues) and were identified as type-I collagens.

Obviously, peptides with antioxidant properties were isolated from tilapia scale gelatin [19]. Gelatin was hydrolyzed using alcalase, pronase E, trypsin and pepsin. Among hydrolysates, alcalase-derived hydrolysate exhibited the highest antioxidant activity compared to other enzymatic hydrolysates. The effect of type I collagen derived from tilapia fish scale on odontoblast-like cells was reported [27]. Type I collagen purified from tilapia fish scale essentially enhanced the cell attachment to nearly two fold as compared to control. Type I collagen derived from tilapia scale, an underutilized resource, holds promise as scaffolding material in the application of tissue engineering in dental field. The calcium-binding activity of tilapia scale protein hydrolysates sequentially hydrolyzed by trypsin, flavor enzyme and pepsin were observed [28]. They found that the tilapia scale protein hydrolysates had higher calcium-binding activity. Tilapia scale protein hydrolysates have potential as functional foods for calcium supplementation. In summary, the peptide derived from tilapia scale gelatin acts as a candidate against oxidative stress and could be used as a potential functional food ingredient. The physicochemical studies revealed that extracted collagens could have promising applications in the food, medical, and cosmetic industries.

TILAPIA BONE

The tilapia bone is discarded or processed into bone meal for animal feed, which was not cautious [29] .Tilapia is generally processed into a frozen fillet, which could also originated by-products accounting for up to%15 of the fish weight, with fish bone being the major by-product [30] .Interestingly, bones were mainly considered as rich in minerals such as calcium and phosphorus

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and collagen but some special carbohydrate and lipids were also found [31]. Bone tissue was a considerable reservoir for storage of calcium and phosphates and was necessary in the regulation of plasma concentrations of these minerals. Due to the physiological significance of calcium and phosphorous in the soft tissues, calcium and phosphate present in the bones may be relocated to other tissues when the dietary supply do not meet the requirement. The proximate composition, fatty acid, amino acids and nutritional composition of flour from tilapia bone were analyzed [32, 33]. The results in tilapia bone flour were: moisture, protein, total lipids, and ash. There were many minerals in tilapia bone such as calcium, iron, potassium, magnesium, copper, sodium, zinc, and phosphorus. Moreover, a total of 22 fatty acids were detected in tilapia bone flour total lipids including linolenic acid, eicopentaenoic acid, stearic acid, oleic acid, alpha linolenic acid, palmitic acid, margaric acid and moroctic acid. The major amino acids were found in tilapia bone as glutamic acid, aspartic acid, lysine and leucine.

Gaining more product value from the tilapia bone would be one procedure to more effectively utilize the fish resource and contribute to sustainability. The potential of fish bone as a source of mineral supplement was reported [34] and tilapia bone powder had been developed into a supplementary form as a calcium capsule [35]. Calcium is considered as the rich mineral in the bone [36]. However, the direct application of fish bone powder to generate the functionality was limited by its low solubility because calcium in the bone was mainly found in the hydroxylappatite form as calcium phosphate, which was hard to solubilize. Development of calcium supplement from the bone of tilapia need to be investigated [35]. The optimal process to develop a calcium supplement from tilapia bones was soaking in 0.8% sodium hydroxide for 90 minutes, heating at 121°C for 90 minutes and drying at 90°C for 60 minutes. In addition, low solubility of calcium limits its bioavailability. Thus, extraction of calcium from fish bone powder was necessary for generating functionality as well as fulfilling the nutritional value of food products. Tilapia bone powder was extracted using acetic acid. The calcium content in the extract was 2,376 mg/L [36]. Moreover, the calcium extract was added to fish sausage by replacing the ice content in the recipe with the frozen calcium extract [32]. The sausage quality was compared to the control (without adding calcium). Proximate analysis showed that the addition of calcium resulted in moisture reduction while the ash contents increased by 1.42%. The lightness, assessed by colorimeter, increased slightly. Texture profile analysis revealed that the hardness and gumminess of the fish sausage increased upon adding the calcium extract. The sensory evaluation also indicated that the textural

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attribute of the fish sausage with calcium extract exhibited higher overall acceptance than the control. Tilapia bones was used to fortify flax seed and cinnamon cookies [37]. The panelists gave the best score to the12% tilapia bones cookies. The consumption of 100g of the12% enriched cookies provided 39%, 34%, 62%, and 57% protein, Ca, P, and Fe, respectively, along with 238% of the omega-3 fatty acids recommended by American Heart Association for those having cardiovascular disease. At last, tilapia bones represented a significant part of the cut offs from the filleting industry and a better utilization of this raw material for various applications was a matter of great scientific interest. Bones from whole tilapia and cut offs could was used as a raw material for health products.

TILAPIA FRAME

Tilapia fame is by-product form tilapia processing industry. There are bioactive peptides in tilapia fame. Nowadays, extraction of tilapia frame protein was hydrolyzed by pH-shift and protease hydrolysis. In term of pH-shift, the conditions of isolating protein from tilapia frame by-products by a pH-shift process were investigated along with their physicochemical and gelling properties [38]. The least solubility of tilapia frame protein was observed at pH 5.5 and gradually increased at both acid and alkaline sides. The highest solubility was observed at pH 2 and 12. Protein solubility of tilapia frame increased as the ratio of alkaline extraction medium and extraction time increased. An alkaline pH-shift process productively removed fat as much as 95%. Breaking force and deformity of recovered protein gel from an alkaline pH-shift were higher than those from the acid counterpart. Furthermore, protease hydrolysis is interesting method recently. Tilapia frame protein was hydrolyzed by different proteases and was separated by ultrafiltration to obtain antioxidant peptides [20]. The tilapia frame protein hydrolysate obtained by trypsin exhibited the highest degree of hydrolysis and antioxidant activity. The IC_{50} values of two peptides on hydroxyl radical scavenging activity were 27.6 and 38.4 μg/mL, respectively. In addition, the effect of varying the aminopeptidase concentration and hydrolysis time in the production of a protein hydrolysate from tilapia frame was evaluated in terms of the obtained antioxidant and ACE inhibitory activities [39]. The use of 2% (w/w) flavourzyme 1000 L for 1 h yielded the highest levels of 2,2-diphenyl-1-picrylhydrazil free radical scavenging (90.4%), metal chelating (91.8%), thiobarbituric acid activity ratio (81.9%), and ACE inhibition (83.8%). In

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addition, tilapia frame protein hydrolysate contained a higher net amount of amino acids and a larger peptide molecular weight distribution compared to the unhydrolysed tilapia frame supernatant. In total, tilapia frame protein hydrolysate may be a suitable supplement to improve the functionality of food products and has the potential to be developed into new health foods.

TILAPIA HEAD

In many countries of the world, the enormous quantities of tilapia heads produced are often discarded into the environment and become a source of pollution. The fatty acid composition and nutrient inherent of tilapia heads, in natural and after processing in the form of flour, with the ultimate goal of its consumption by humans. There were practically studies on tilapia head products and they are very seldom utilized for food. Apparently, tilapia heads were normally discarded during the filleting operation. Table 3 showed that the fatty acid composition and nutrient potential of flour made from tilapia heads were investigated [40].

In 2012, Vignesh and Srinivasan reported nutritional composition of flour from tilapia head [33]. The major amino acids such as glutamic acid (0.9967%), aspartic acid (1.837%), lysine (1.048%) and leucine (0.807%) were found in tilapia head.

Table 3. Proximate composition of natural tilapia heads and tilapia head flour [40]

Constituents	Natural tilapia heads	Tilapia head flour
Moisture	67.24 %	6.01 %
Crude protein	16.48 %	38.41 %
Ash	5.72 %	19.38 %
Total lipids	9.56 %	35.46 %
Omega-3	206 mg/ 100 g	731 mg /100 g
Palmitic acid	1999 mg/ 100 g	7699 mg/ 100 g
Oleic acid	3,128 mg/ 100	11,447 mg/ 100 g
Linoleic acid	1,018 mg/ 100 g	3,784 mg/ 100 g
Polyunsaturated Fatty Acids	1,414 mg/ 100 g	5,226 mg/ 100 g
Monounsaturated Fatty Acids	4,274 mg/ 100 g	15,743 mg/ 100 g
Saturated Fatty Acids	2,982 mg/ 100 g	11,191 mg/ 100 g

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The tilapia head flour possessed seven essential minerals in milli grams per gram (mg/gm) such as calcium (56.7 mg), iron (3.098 mg), potassium (16.78 mg), magnesium (15.67 mg), copper (1.414 mg), sodium (34.67 mg) and zinc (0.343 mg). Thus, tilapia heads can be used as a low-cost raw material for food fit for human consumption. The flours made from the tilapia head may result as significant alternative food in the human diet.

TILAPIA VISCERA

In the part, tilapia viscera were habitually feeds and fertilizers that had a low value. There was growing interest in obtaining higher value biochemical and pharmaceuticals from tilapia viscera, notably enzymes. Nowadays, enzymes have several applications in the food industry. Enzymes are mainly derived from plant, animal and microbial sources, whereas their complement, derived from marine and other aquatic sources, have not been vastly used. Very wide ranges of sources are used for commercial enzyme production. It may be extracted from any living organism. There are various organs in tilapia viscera such as spleen, liver, stomach, intestine etc. In 2001, Taniguchi et al. reported that tilapia viscera was a good source of digestive enzyme [41]. Tilapia processing originates large amounts of solid and liquid wastes. Normally, more than half of the raw material weight is untouched. There have been relatively many attempts to use digestive enzymes as industrial processing aids. Tilapia is cold-blooded animal and vary plentifully in their feeding habits and temperature preferences, and so it is anticipated that their digestive enzymes will also exhibit diversity. In recent years, additional applications of proteases in the tilapia industry have appeared. The utilization of tilapia viscera for producing enzymes and also to the reduction of waste disposal problems were intrigued for food industry. There are four main steps for digestive enzyme production from viscera tilapia including extraction, separation, purification and concentration (Table 4).

Alternatively, lipase of the intestine of tilapia was purified by ammonium surface precipitation, followed by ion-exchange chromatography, chromatofocusing, and gel filtration [41]. The specific activity of the purified enzyme was 177 times higher than that of the crude extract. In term of protease enzyme, an acidic protease from tilapia viscera was precipitated by ammonium sulfate [42]. The enzyme showed the highest activity and purification-fold when precipitated at 40–60% ammonium sulfate. Purification fold and activity were increased after purification by dialysis and gel filtration.

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Table 4. Step for production of digestive enzymes from tilapia viscera

Production	Process
Extraction	Homogenization
	Hydrolysis
Separation	Ammonium sulfate precipitation
	Ultrafiltration
	Centrifugation
Purification	Gel filtration
	Diafiltration
Concentration	Ultrafiltration
	Freeze dried

The K_m and V_{max} values of enzyme were 0.77mM and 2.22mM/min, respectively, while the catalysis efficiency (V_{max}/K_m) was 2.88. Moreover, an alkaline protease was extracted from the intestine of tilapia [43]. The enzyme was purified in three steps: heat treatment, ammonium sulphate fractionation and gel filtration, presenting yield and purification of 30% and 22-fold, respectively. This enzyme showed K_m for the hydrolysis of benzoyl-DL-argininep-nitroanilide equal to 0.755 mM.

In 2006, Hinsui et al. reported that the trypsin and chymotrypsin activities of extraction of spleen, liver, stomach, intestine and mixed viscera of tilapia were compared [44]. Noticeably, intestine was the best sources for trypsin and chymotrypsin. Trypsin and chymotrypsin fractions were extracted from intestine of tilapia by 30-70% saturated ammonium sulfate precipitation, dialyzed, acetone precipitation and separated by affinity chromatography column. Specific activities of trypsin and chymotrypsin were 0.529 and 0.380 unit/mg protein, respectively. The purities of trypsin and chymotrypsin fraction were increased by 5.56 and 3.62 folds, respectively. Similarly, trypsin from the intestine of tilapia was purified by the following techniques: acetone precipitation, ammonium sulfate fractionation, gel filtration, and ion exchange chromatography [45]. The purified enzyme was determined to be homogeneous by polyacrylamide gel electrophoresis and sodium dodecyl sulfate (SDS)-PAGE. The behavior of the enzyme for the hydrolysis of casein followed Michaelis-Menten kinetics with Km of 0.46 mg/mL. In term of amylase, tilapia viscera extracts were prepared using water, NaCl solution and acetone homogenization [46]. The saline solution (5% w/v) yielded the extract with the highest amylase specific activities of 1,800 unit/mg protein, respectively. At this condition, amylase activity had risen 5 times before

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declining to about 3 times of its initial activity after 90 min. Precipitation of other protein by using 30 or 35% ammonium sulphate effectively improved specific activity of both enzymes of the extract. Specifically, optimum pH and temperature of enzyme from tilapia viscera is showed in Table 5. In addition, the utilization of the enzymatic hydrolysate of tilapia viscera were separately added to tilapia surimi to explore their inhibitory effects on lipid and protein oxidation during refrigeration [47]. The enzymes were separated by ultrafiltration fraction which its fraction improved the color of tilapia surimi. The hydrolysate demonstrated strong antioxidant capacity against both lipid and protein oxidation in surimi during storage, which, however, was inferior to that of its ultrafiltration fraction. Accordingly, the active digestive enzymes could be prepared from tilapia viscera that can several applications in the food industry.

LOW GRADE MEAT AND OTHER WASTE

In addition, low grade meat, and other waste such as blood, fin and tail are interesting because it has nutritional value. In 2013, Chareonphun et al. reported bioactive compound in tilapia meat including antioxidant, ACE inhibitory, and calcium-binding peptides [48, 49].

Apparently, the degree of hydrolysis and substrate concentration significantly affected the production of mass of peptides and their bioactive properties. This is in accord with the report of Kangsanant et al. [50] and Al-Souti [51].

Table 5. Enzyme extracted from tilapia viscera

Tilapia viscera	Enzymes	Optimum pH	Optimum temperature	References
Intestine	Lipase	7.5	35 ^{0}C	[41]
Viscera	Acidic protease	2.5	35 ^{0}C	[42]
Intestine	Alkaline protease	8.0	50 ^{0}C	[43]
Viscera	Trypsin	9.0	80 ^{0}C	[44]
Viscera	Chymotrypsin	9.0	60 ^{0}C	[44]
Intestine	Trypsin	9.0	60 ^{0}C	[45]
Viscera	Protease and amylase	8.0	60 ^{0}C	[46]

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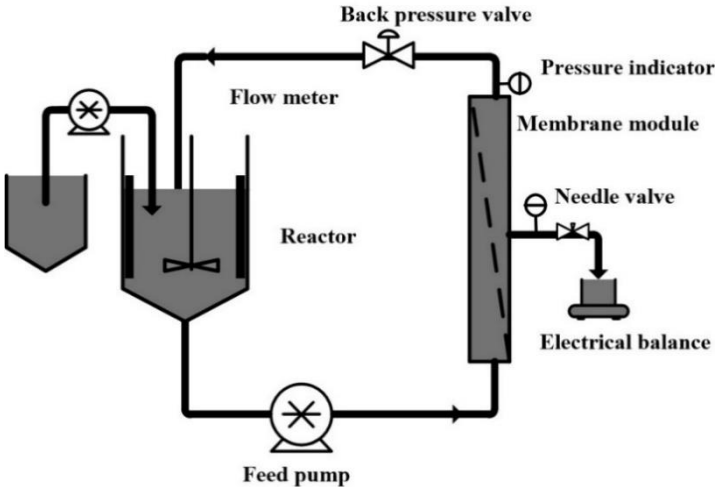

Figure 2. Schematic diagram of continuous enzymatic membrane reactor system.

The tilapia muscle is rich in antioxidants and acted as a potent free radical scavenger and provided protection against oxidative stress. Recently, continuous enzymatic membrane reactor (CEMR) system was used for production of bioactive peptides from tilapia muscle (Figure 2).

A conventional method is performed as a various step procedure. An initial step is an extracting protein from raw material with a suitable solvent. Protein hydrolysis that involves the hydrolytic degradation of this peptide bond using alkali, acid or enzyme and separation by membrane filtration is the following step. Obviously, enzymatic hydrolysis can be carried out in batch reactor, where the enzyme is mixed with protein allows to breakdown peptide bond. The main disadvantage of this system is not only a higher cost of protease that have to be inactivated after used but also low yield of desirable molecular weight peptides [53]. The development and application of CEMR for the hydrolysis of proteins have been applied to overcome many problems. The CEMR is the system of alternative for hydrolysis process involving proteins which has higher productivities and more uniform products than traditional method. In CEMR, the substrate is continuously fed to the reactor in order to compensate for permeate and maintain constant volume in the reactor. The CEMR has been successfully used in the production of bioactive peptides from tilapia protein. Interestingly, production of antioxidant and Ca-binding peptides from tilapia muscle protein using membrane bioreactor were investigated [53]. The investigation was focused on the effect of process parameters on antioxidant and Ca-binding peptides conversion and

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productivity during continuous enzymatic membrane reactor. It was found that proteins have to be constant feeding rate continuously with Alcalase 2.4L. Furthermore, it gave high antioxidant and Ca-binding peptides conversion and productivity. In addition, tilapia blood, tilapia fin and tilapia tail are regularly animal feeds and fertilizers, but there are rarely studied until now. All in all, low grad meat of tilapia, tilapia blood, tilapia fin and tilapia tail are rich in nutritional and bioactive content which might represents a dietary source to enrich bioactive compounds intake among human subjects in the future.

CONCLUSION

Utilization of by-products and waste generated from tilapia processing industry (skin, scale, bones, frames, heads, viscera, low grad meat and other) are very important currently. In general, the tilapia waste has potential applications in aquaculture/animal feed production. In recently, it could serve as a good source of various composition including collagen, gelatin, mineral, nutrients, fatty acid, bioactive peptides, and enzymes. Alternatively, tilapia by-products and waste can be as a low-cost raw material and a valuable alternative ingredients in pharmaceutical, food, medical and other industries.

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Chapter 4

THERMAL ECOLOGY OF BROWN TROUT AND THE CLIMATE CHANGE CHALLENGE

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ABSTRACT

Climate change is one of the most important global processes that the Biosphere is experiencing, and fish as a part of the Biosphere are not indifferent to this process. Coldwater fish and, particularly the Brown trout (*Salmo trutta*), are very sensitive to the predicted changes in temperature of rivers and streams. Fish are poikilotherms and their body temperature and physiological rates are dependent on the environmental temperature, as this is a key factor of their ecological niche. Gill surface is directly related to respiratory capacity which in turn is related to the oxygen content in the water. At the same time, water capacity to contain oxygen depends on the water temperature and thereby growth rate is also linked to temperature. As a consequence, changes in environmental temperature will directly affect Brown trout survival in its thermal limits. Climate change predictions point to Brown trout natural distribution shifting to the North and East and losing southern territories, in its rear edge. Coldwater fish will not only suffer because of extreme and selective summer temperatures but also as a result of thermal changes in the different phases of the life cycle. In many places, Brown trout will be

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very close to their limit of physiological efficiency therefore compromising the production of somatic and reproductive biomass. Some ability for adapting to high temperatures is possible but physiological extremes may exist that cannot be forced. Genetic variance and heritable variation in phenotypic plasticity could compensate the effects of changing temperature regimes, but sudden changes might impede adaptation because plasticity is limited.

Keywords: adaptation, allometry, climate change, distribution, life history, oxygen use, physiological efficiency, preferred temperature, thermal niche, thermal tolerance

INTRODUCTION

Climate change is a hot spot in the conservation of coldwater fish and thermal ecology is the necessary way to face the issue. The increase in the water temperature of rivers and streams which is predicted for this current century could compromise Brown trout (*Salmo trutta*) populations in their natural range. The definition of the Brown trout thermal niche in its different life stages, the understanding of the habitat and community constraints interacting to limit the response to the stream thermal regime, are fundamental factors for evaluating the effects of climate change on populations. Thus, from an ecological point of view, thermal physiology is seen as a means of evaluating the probable future of Brown trout populations.

This chapter is aimed to show how temperature influences the physiology and ecology of Brown trout and the consequences of climate change on its life history and distribution.

THE THERMAL REGIME OF RIVERS AND STREAMS

Water temperature is a key factor in defining and determining the state of an aquatic ecosystem (Caissie 2006) with a large influence on fish biologic success (Wootton 1990, Jobling 1995). It is a well-known fact that temperature is a major factor in fish energy balance as it affects the food intake rate, the growth efficiency and the metabolic rate (Forseth & Jonsson 1994).

The energy flux in the interphase air/water is one of the most important factors determining the temperature of a surface water body (Edinger et al.

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1968). This flux is the result of the energy exchange primarily produced through net short wave radiation (solar radiation), net long wave radiation, evaporative heat and convective heat transfer. Thus, the stream temperature can be mainly explained because radiation heats the water and its immediate surrounding (riverbed and air) establishing a close relationship between the air and the stream temperature (Mohseni & Stefan, 1999). Empirically, the air temperature can accurately explain the variability of the stream temperature (86-96%) (Crisp & Howson 1982). Other important drivers can grouped in topography, streamflow and the riverbed features (Caissie 2006). Likewise, when headwaters receive groundwater discharge, the stream temperature is closely related to the aquifer temperature that feeds it (O'Driscoll & DeWalle 2006).

Water temperature is not constant and experiences daily and annual variations (Figures 1 and 2). Daily fluctuations often have the minimum around the sunrise and the maximum at mid-afternoon. In cold headwater reaches, daily fluctuations are usually slight and increasing as the river becomes larger and less dominated by groundwater discharges and more affected by meteorologic conditions (Caissie 2006). Both, water and air temperatures run parallel, although water temperature fluctuations lag behind air temperature fluctuations (Stefan & Preud'Homme 1993, Matuszek & Shuter 1996, Stoneman & Jones 1996, Mohseni et al. 1998, Santiago et al. 2016c). The mass of water also influences the thermal stability itself, since it is a function of the thermal capacity which is proportional to the specific heat and the involved mass (Stelczer 1987). This means that a greater mass of water (larger river or lake) is more thermostable than another of a smaller size. Below 30°C (approximate temperature at which the specific heat is minimum), lower temperature, higher specific heat and, consequently, the thermal capacity increased at equal mass. Logically, these effects are in turn under the influence of the increase ratio of surface exposed to the atmosphere in relation to increasing mass (Stefan & Preud'Homme 1993).

Moreover and in a general way, rivers are also cooled in summer and warmed in winter through heat fluxes of the riverbed (Caissie & Giberson 2003). These heat fluxes may be especially important in autumn due to a significant thermal gradient as a consequence of the accumulated residual summer heat in the ground (Alexander et al. 2003). The heat flux through the riverbed is mainly a function of the geothermal heating occurring through conduction and advective heat transfer from the contribution of groundwater and hyporheic exchange (Lapham 1989). Its importance is relative against the effect of exchange with the air (Evans et al. 1998), but it can be very

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significant to explain the reduction of the temperature range variation of a river against what is expected by the mere action of the afore mentioned principle main factors (mainly radiation).

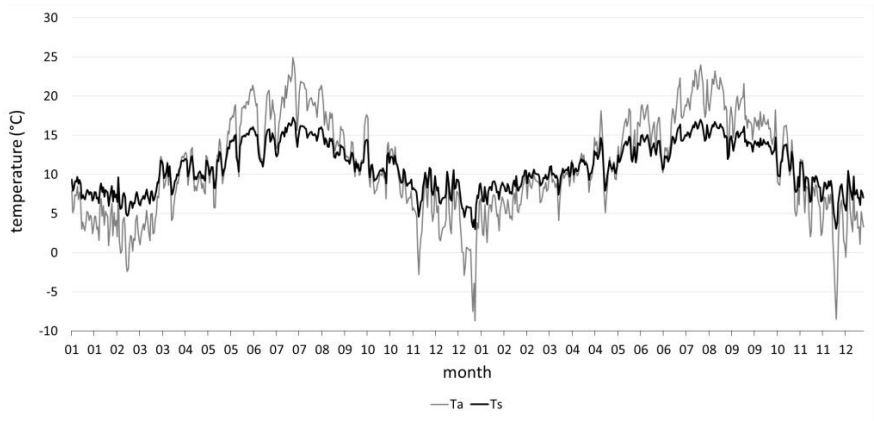

Figure 1. Daily mean stream temperature (*Ts*) and air temperature (*Ta*) at an associate meteorological station in a mountain stream at Central Spain (latitude: 41ºN).

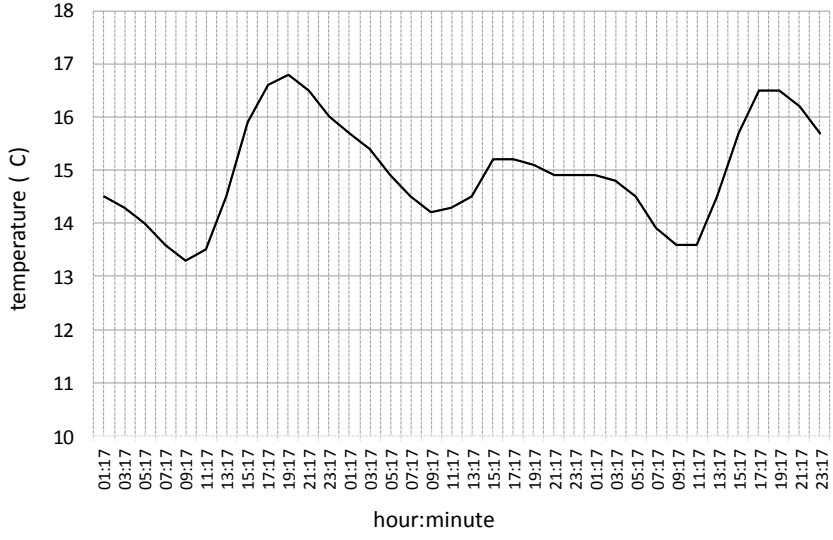

Figure 2. Daily oscillations of the stream temperature. Data recorded every 2 hours.

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All this explains that, under natural conditions, the temperature of the running water in the temperate region varies usually between 0ºC and 25ºC, reaching 35°C in tropical rivers (Lewis 2008), and can touch the 40ºC in rivers of warm desert areas (Matthews & Zimmerman, 1990). Temperatures above these values only occur naturally in volcanic waters and hot springs (Meybeck et al. 1996).

TEMPERATURE EFFECTS ON PHYSIOLOGY: GROWTH

Physiological efficiency

In the lifetime of an animal, the energy obtained from food that is not used to sustain life is invested in biomass, firstly somatic and then reproductive (Sibly & Calow 1986): thus, the amount of ingested food is a prime determinant in somatic growth rate. Growth is the net result of two continuous processes with opposite trends, anabolism and catabolism. What is the surface that governs anabolism? It seems logical that since the respiratory rate is defining the anabolism, the surface in question is that which limits breathing. Thereby we see that the respiratory rate is a consequence of the reaction between the fuel provided by the fish through the digestion of food (sufficient condition) and the available oxygen in the blood (the necessary condition) (Pauly 1981).

The reasons for gills becoming strong candidates for being the physiologically effective surface in fish can be summarized in (Pauly 1981):

i. Gills regulate the intake of oxygen in the body and oxygen is essential for the synthesis of body substance.
ii. Even in the most aerated water, there is very little oxygen in comparison to the atmosphere. Besides, water is 840 times denser and 55 times more viscose than air, containing 1/30 times oxygen, and wherein the diffusion through the membranes takes 300,000 times longer than in the air.
iii. The gills of the fish grow in proportion to a power of weight lesser than unit. The value of this exponent is the power that links fish energy metabolism and weight.
iv. The total oxygen amount which can diffuse into a body as function of time follows the law of Fick's diffusion (Jobling 1995),

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$$M = (P_W - P_B) \cdot GA \cdot K \cdot d^{-1}$$

M being the diffusion rate (ml·h^{-1}), (P_W-P_B) the partial pressure difference between water and blood (atm), *K* the Krogh diffusion constant, that describes the tissue diffusion features (oxygen milliliters diffused across 1 mm^2 of tissue in 1 minute and 1 atm of oxygen pressure), *GA* is the gill area in mm^2 and *d* is the length of the diffusion route (lamellar wall thickness separating blood and water, in µm). Of these parameters, only *GA* can vary as body size increases, thereby making the gill size the key regulator of oxygen uptake in the growing fish.

v. Large gills expose fish to several problems, including;
   a. Swimming: Very large gills increase resistance to water flux, and a large amount of energy is needed to overcome it.
   b. Ecomorphology: The large gills needed by large fish require modification of the entire head and front of the body, and even the feeding mode.
   c. Functionality: A large gill area implies the existence of very small spaces between the secondary gill lamellae, which are in permanent risk of obstruction.
   d. Chemical Susceptibility: The gills of fish should be relatively 'open' to the external environment, which also means greater exposure to the dissolved toxic substances in water. Thereby, gills are a very weak first line of defense against osmotic stress.
   e. Parasites: The gill tissue of fish is an ideal environment for parasites (a lot of blood which is also well oxygenated).

Potential problems associated with the development of extremely large gills suggest that for any species of fish, a given gill size should imply that it allows a good supply of oxygen for potential and rapid body growth but only up to a limit related to the optimum for the busy ecological niche.

As a rule, the total gill area of a fish of any size can be expressed by the equation

$$GA = GAI \cdot W^{d_g}$$

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where *GA* is the gill area, *W* is the weight of fish in freshwater fish, and d_g is an exponent whose value for freshwater fish is 0.76 (between 0.36 and 1.13, Palzenberger & Pohla 1992) or 0.88 (according to White et al. 2006), and *GAI* it is a constant characteristic of each species called gill area index. G.M. Hughes (en Palzenberger & Pohla 1992) gave a *dg* value of 0.93 for the Rainbow trout (*Oncorhynchus mykiss*), and Niimi & Morgan (1980) of 1.13. Palzenberger & Pohla (1992) found that these values in the high range of variation of the exponent were related to active species. If the weight is given in grams and the area in square centimeters, *GAI* is expressed as the area (cm^2) of a fish gill weighing 1 g. As to relative surfaces, in an adult fish gills represent 60 to 75% of the total body surface area (Jobling 1995).

It has been said that the body weight tends to increase faster than the gill area, and relative gill area (= gill area / weight of the fish) decreases with increasing body size (Pauly 1981). Consequently, the oxygen supply per body weight unit decreases as the size (and weight) increases, resulting in a relatively lower energy metabolism and a consequent lower rate of synthesis (Peters 1983). On the other hand, the amount of body substance degraded per time unit increases in direct proportion to body weight, and the growing fish gradually reaches a point where the synthesis of body substances is just sufficient to replace the degraded substances, and where net growth is zero (the asymptotic size).

As for the relationship between oxygen consumption and weight (Pauly 1981, Saint-Paul 1983):

$$Q = d \cdot W^{d_q}$$

being *Q* the consumption of O_2, *W* the weight of the fish, d_q an exponent value between 0.5 and 0.95, and *a'* a constant characteristic of each species.

De Jager & Dekkers (1975) suggested that d_g (the exponent of the weight in the formula of gill area) and d_q should be equal, getting in their own work values of 0.811 and 0.826 respectively for both exponents (assigning a unique value *d* equal to 0.82). Winberg (1960) gives a value of *d* = 0.81 for salmonids.

Again, Pauly (1981) demonstrated in *Micropterus salmoides* and *Cyprinus carpio* that the oxygen supply determines growth performance when other potentially limiting factors remain constant (e.g., food, temperature).

Saint-Paul (1983) described the relation between the differences in the metabolic rates at different temperatures by mean of the following expression:

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$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{(T_2 - T_1)\cdot 10^{-2}}$$

where Q_{10} represents the fitting of the metabolic rate at a change of 10ºC, and K_i is the metabolic rate ($mgO_2/100g/h$) at T_i ºC.

Reducing the amount of available O_2 seems to have an effect on the growth rate by means of both types of factors, direct (by leading a reduction in the rate of synthesis) and indirect (by reducing food intake by reducing appetite). It could be argued, therefore, that it is the reduced intake which reduces the growth of fish kept at low O_2 concentration, not the low concentration of O_2 itself. On the other hand, the reduced 'appetite' that fish show at low oxygen levels appear to be a regulatory factor to prevent an excess of aminoacids which could produce an additional cost of excretion. Under low oxygen availability conditions aminoacids cannot be used for new body synthesis nor as combustible substances, since in both cases oxygen is needed (Pauly 1981).

The efficiency of feed conversion (α = weight increase/food intake) in fish is usually expressed as

$$\alpha = cte \cdot W^b$$

being b negative and its absolute value $|b|$ should be close to (1-d), or it can be said that the relative gill size reduction that occurs with increased weight of the fish explains most loss of conversion efficiency.

Arriving at this point, physiological efficiency connects with the temperature in two ways: by the solubility of oxygen in water and the rate of metabolic reactions, both mediated by temperature. At the same time, salinity and atmospheric pressure also influence the oxygen solubility, and the oxygen saturation level for each temperature is related to the availability of the dissolved oxygen. The Law of Henry connects the concentration (C) and the partial pressure (P) of a given gas in a given liquid by means of the constant of Henry (k_H) at a constant temperature (T), it is possible to write it as a function of temperature (Figure 3):

$$P(T) = k_H(T) \cdot C$$

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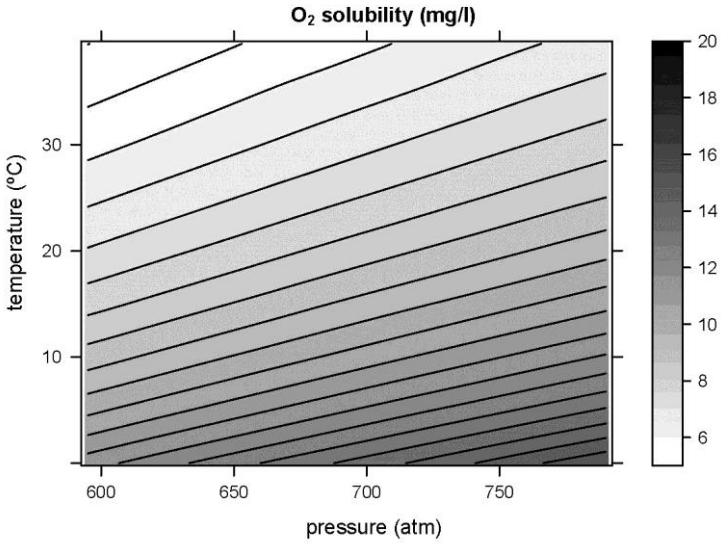

Figure 3. Solubility of the oxygen (O_2) in water at various temperatures (ºC) and pressures (atm) (from Lewis 2006 data).

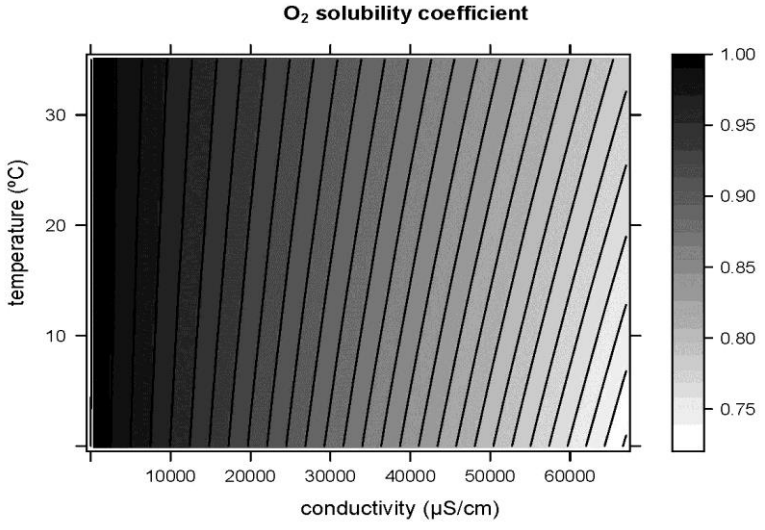

Figure 4. Correction coefficient of the oxygen (O_2) solubility as a function of temperature (ºC) and salinity, the last expressed as electric conductivity (µS/cm at the normalized temperature of 25ºC) (from data of Lewis 2006).

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In chemical thermodynamics, the integrated form of the van't Hoff's equation shows the solubility of a gas at constant salinity as a function of temperature. Thus, solubility β is obtained:

$$\ln \beta = a_1 + a_2 \cdot T^{-1} + a_3 \cdot \ln T + a_4 \cdot T + a_5 \cdot T^2 + \cdots$$

where a_i is constant for each gas and T the absolute temperature. If salinity is also variable

$$\ln \beta = A_1 + A_2 \cdot \left(\frac{100}{T}\right) + A_3 \cdot \ln\left(\frac{T}{100}\right) + S‰ \cdot \left[B_1 + B_2 \cdot \left(\frac{T}{100}\right) + B_3 \cdot \left(\frac{T}{100}\right)^2\right]$$

where A_i and B_i are constants and S‰ is salinity in per mil (Weiss 1970). In this way, a correction coefficient of the solubility can be obtained (Figure 4).

Respiration: Influence of Activity and Temperature

In poikilotherms, the metabolic rate is strongly linked to the body temperature that is the temperature at which chemical reactions occur in the organisms and, therefore, it has a direct effect on the performance of the metabolic processes and the growth expectation (Atkinson 1994, Sibly & Atkinson 1994). In fish, the external and internal temperatures are closely related due to the high specific heat of water. Elliott (1981) points out that the greatest heat transfer within the fish environment occurs through the body wall, even when an organ that is in contact with the external environment (the gills) is also an effective heat exchanger.

It is really difficult to distinguish between the direct and indirect effects of temperature on organisms. Water temperature is critical for aquatic organisms through its effects on metabolic rates and, consequently, on the growth and development period (Ricker 1979). The relationship between oxygen consumption at rest in fish and the ambient temperature is curvilinear (Johnston et al. 1991), increasing with the temperature so that a tropical fish at a temperature of 30°C requires about six times the amount of oxygen that a polar fish at 0ºC needs for resting metabolic rate (Clarke & Johnston 1999). Thus, these aquatic organisms breathe and eat more in warmer water than in colder water, although other constraints to high metabolism, such as

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availability of oxygen are found. It must not be forgotten that the metabolic reactions need to be performed within a temperature range that determines their efficiency, or even the inability to take place. Each living organism will have maximum and minimum temperatures between which it can live, and these limits can change during the life history. In fish, unusually high temperatures can cause growth inhibition, lead to pathological crisis, and cause changes in the migratory cycles (Dietrich et al. 2014, Jeffries et al. 2012, Hari et al. 2006, Todd et al. 2012). In general, changes in water temperature affect plant communities, communities of aquatic invertebrates and fish, as well as productivity at each trophic level (Ward & Stanford 1979, Carpenter et al. 1992, Petts & Amoros 1996, Huryn & Wallace 2000, Lessard & Hayes 2003, Velasco et al. 2003, Butterwick et al. 2005, Cid et al. 2008, Passerini et al. 2016).

It has been said that water temperature affects solubility. The same occurs to oxygen demand by fish, and it can also affect the growth rate due to its effect on concentration thresholds and minimum oxygen saturation (Forseth & Jonsson 1994). Elliott et al. (1995) verified how at high oxygen concentrations, fluctuations of between 85% and 100% saturation had no significant biological effect on growth rates. As it follows from Fick's equation, the greater the difference in oxygen partial pressure between water and fish blood, the higher the rate of diffusion of oxygen to the gills; thus, the rate of diffusion will be greater when there are a higher concentration of oxygen in the water and a higher oxygen demand by the fish. This means that, in conditions of oxygen saturation and sufficient food, fish find their optimal lower respiratory temperature at which they are able to maintain a more active metabolism by maximizing the difference of partial pressure between water and fish blood. The gills of the fish are the gateway communicating with the air containing oxygen, and the body weight tends to increase faster than the gill area (Pauly 1981). Consequently, the oxygen supply per body weight unit decreases as the size (and weight) increases, and results in a relatively lower energy metabolism and a lower rate of synthesis (Peters 1983). The oxygen supply is so important that thermal tolerance of organisms is probably explained by the oxygen limitation (Pörtner et al. 2000, Pörtner 2001, 2002).

On the other hand, the temperature influences the water viscosity (Gordon et al. 2004), implying an indirect influence on the fish energy performance because the cost of sustaining life at certain temperatures must be added to the required activity to find enough food, whereas at low temperatures, the water viscosity increases and thereby its resistance to be penetrated by the fish. So, viscosity makes swimming and breathing energetically more expensive.

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To make gas exchange between water and gills more efficient, blood flow circulates in the opposite direction to the flow of the water through the gills. This increased efficiency is due to the ability to maintain a constant pressure gradient of oxygen throughout the water path. The extraction efficiency of oxygen from water in the gills can reach 75-80% (Jobling 1995).

When a fish enters in activity it requires more oxygen but also decreases the rate of water flux, that is, the contact time between the water and the gill tissue. This time is reduced from 250ms in resting fish to 30ms swimming at high speed but attenuates its reduction through various mechanisms among which the lamellar 'recruitment' stands out: only 60% of secondary lamellae are irrigated with blood in a resting fish, but as the fish begins to swim more lamellae are flooded with blood. Thus the lamellar 'recruitment' serves to increase the effective gill area for oxygen uptake and gas exchange (Jobling 1995).

Pumping a dense and viscous liquid on the gills, such as water, consumes energy and as the activity of the fish increases muscles progressively uses more energy, including those responsible for respiratory pump (it is estimated that 10% of fish oxygen taking is consumed in pumping water through the gills). Thus, the energy cost of ventilation increases with increasing activity and some species adopt an alternative ventilatory strategy by swimming at high speeds. For example, several species of salmonids, when they rest or swim at low speed, use ventilatory cyclical movements, involving the gill muscles. At high speeds they use the swimming muscles to force water over the gills by keeping their mouths open. This alternative ventilatory system allows to the fish to obtain an opercular pumping supply at the same time they swim at high speeds, and it yields energy savings. These savings are, however, less than the required energy to activate the respiratory pump. This strategy of swimming with the open mouth means that there is a disruption of the hydrodynamic body profile presented to the water, and this leads to an increase in turbulence. Consequently, there is an increase in cost of swiming for fish adopting this kind of forced ventilation, although there are energy savings compared to ventilation by pumping at any speed (Jobling 1995).

All this means that, while remaining outside the lethality, the temperature has a direct influence on the fish respiration rate either by its control over the maximum load of available oxygen (the higher the temperature, the lesser oxygen availability) or by converting the medium denser and less penetrable - more expensively respirable - (the lower the temperature, the higher the viscosity).

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On the other hand there is thermal stability: regardless of specific adaptations, temperature variations have a more pronounced effect on small fish than on those that are larger, depending on their mass (the higher the mass, the greater the thermal inertia) (Elliott 1994).

The Brown Trout Thermal niche

Far from being simply a physiological constraint, water temperature is a main component of the ecological niche of poikilotherm animals (e.g., Magnuson & Destasio 1996, Wehrly et al. 2003, Angilletta 2009, Finstad et al. 2011, Santiago et al. 2016a) and it affects a wide spectra of functions such as the energetic metabolism (Elliott & Hurley 1999, 2001), protein metabolism (McCarthy & Houlihan 1997), osmoregulation (McCormick et al. 1997), blood and reproductive maturation (Jeffries et al. 2012), reproductive timing (Warren et al. 2012), gametogenesis (Lahnsteiner & Leitner 2013), early development (Pörtner et al. 2006, Réalis-Doyelle et al. 2016), cardiac function (Farrell 1997, Vornanen 2016), gene expression (Meshcheryakova et al. 2016, White et al. 2011), pollutant susceptibility (Reid et al. 1997) ecological relationships (Fey & Herren 2014, Hein et al. 2013), fish behaviour (Colchen et al. 2016) and even "personality" (Frost et al. 2013).

Two levels of ecological niche can be distinguished: fundamental niche and realized niche. The fundamental niche is the multidimensional space of ideal environmental conditions under which a species can persist (Spotila et al. 1989), and the realized niche is the narrower space that is limited by diverse factors such as interspecific competition (Raven et al. 2010).

Magnuson & Destasio (1996) defined the thermal niche as the preferred temperature and the interval in which fish spend all their time. This concept seems to approach the realized niche more than the wider fundamental thermal niche. The latter is better adjusted to the concept of a physiological thermal niche that is the zone of physiological thermal efficiency at which trout have a positive somatic growth (3.6°C-19.5°C in Elliott et al. 1995, c. 5°C-23.0°C in Forseth et al. 2009). The space of physiological thermal efficiency is a property of each species which can be restricted by the action of other constraints. For example, for a given thermal efficiency space, depending on the scarcity of food, fish could lose efficiency for growing or surviving at extreme temperatures. These concepts are shown in the Figure 5.

All the foregoing implies that the temperature range at which trout will be found might be equal or narrower than what would be expected if fish used the

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entire temperature range in which it is able to persist. This allows us to deduce that other concomitant environmental constraints to temperature are working together with the temperature to constrict/limit the ability of trout to take up the stream habitat (Armstrong et al. 2003, Ayllón et al. 2013). Sometimes, other chemical (water quality), physical (substrate) and biological (community) traits can be found as determinants of the limits of Brown trout distribution even though the temperature is quite suitable (Power 1997, Tokeshi 1993).

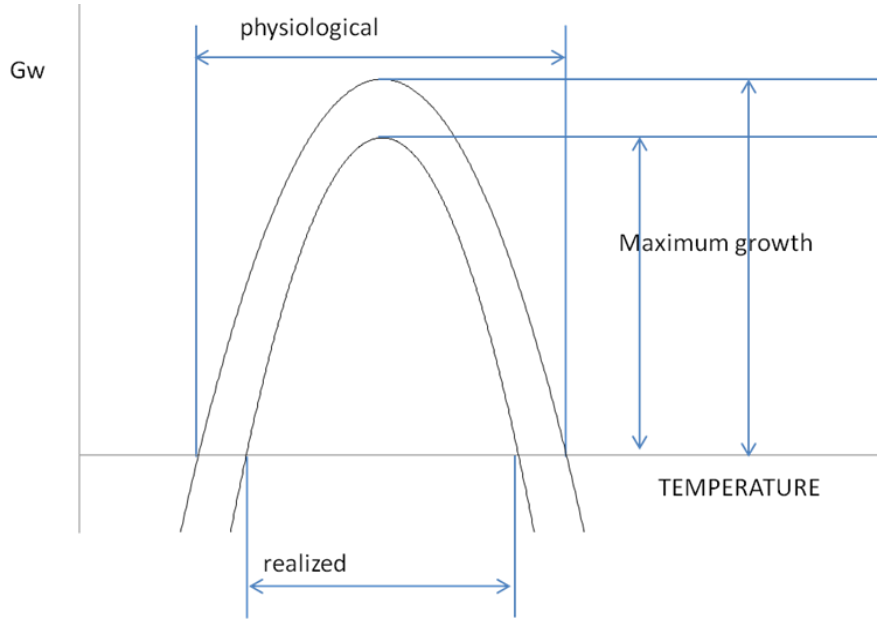

Figure 5. Growth rate (G_w) as a function of environment (water) temperature. Physiological niche is associated to the space of physiological thermal efficiency in laboratory (ideal) conditions. Realized niche is the observed response of trout in the wild when exposed to other constraints.

The range of temperature is usually wider for the physiological niche than for the realized niche. This is because, between the limits of the former, the physiological performance is not enough to counteract the competition with other sympatric species, or the limit reaches are not adequate due to the combination with other physical factors (Santiago et al. 2016a). If trout are not competitive, there will be a buffer-zone in that the species may well live but only if out-competing species are absent. It is possible to detect the maximum temperature at which trout performance makes viable populations and the trout

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are not out-competed by other species in the stream community. Santiago et al. (2016a) found the realized thermal niche of the Brown trout to have the upper limit between 18.1ºC-18.7ºC at Central Spain. Finstad et al. (2011) found that Brown trout and Arctic char (*Salvelinus alpinus*) sharing spatial and trophic niches coexist or exclude each one to other in function of productivity or temperature. Temperature and competition are also known to act together in other fish (McMahon et al. 2007) and ectothermic groups (DeBach & Sisojević 1960, Barata et al. 1996, Buckley & Roughgarden, 2006) determining animal distribution.

It has been said that physiology is mainly controlled by temperature as occurs in gametogenesis. Lahnsteiner (2012) found that Brown trout's spermatic motility was low between 9-15ºC and maxima between 3-7ºC. Maturation of genetic material in males and females points to be optimum around 7ºC, independently of the thermostability (Lahnsteiner & Leitner 2013).

Different developmental stages show different thermal tolerance in Brown trout. Eggs can survive in the range of 0-13ºC (Elliott & Elliott 2010) but between 0-1ºC the development is arrested (Maddock 1974) while other authors set the lowest limit for embryo development at 4ºC, with a low survival at 2ºC (Stonecypher et al. 1994). Réalis-Doyelle et al. (2016) found the maximum embryo and larvae survival and minimum malformations in the range 4-6ºC, this trend hardly changing above 10ºC. At 12ºC survival is scarcely 1%. Other authors (Jungwirt & Winkler 1984, Humpesch 1985, Ojanguren & Braña 2003) describe maximum embryo survival in the range 7-10ºC. Anyway, Lahnsteiner (2012) found that late developmental stages were less temperature sensitive than early stages.

Alevin thermal tolerance is wider than in previous stages. Thus, ultimate lethal temperatures (UL) are 0º and 22-24ºC, and incipient lethality (IL) appears at 0ºC and 20-22ºC (Elliott & Elliott 2010). Parr/smolt UL are -0.8ºC and 26-30CºC, IL 0-0.7 and 22-25ºC (Ojaguren et al. 2001), and feeding occurs between 0.4-4ºC and 19-26ºC (Elliott et al. 1995, Ojanguren et al. 2001, Forseth et al. 2009) but physiological efficiency (growth limits) can be narrower. For Brown trout fed with invertebrates, growth is between 2.9-3.6°C and 18.2-19.5ºC (optimum 13.1-14.1ºC) (Elliott et al. 1995, Elliott & Hurley 1999, Elliott & Hurley 2000a), fed with fish from 2.0 to 19.5ºC (optimum 16.6-17.4ºC) (Forseth & Jonsson 1994, Elliott & Hurley 2000a, b), and with pellets from 1.2-6.1 to 19.4-26.8ºC (optimum 116-19.1ºC) (Grande & Andersen 1991, Forseth et al. 2009).

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The concept of degree-days is used in fish biology to represent the total thermal energy needed to complete a process. The duration of a biological process (e.g., incubation) can be expressed as the sum of the daily mean temperature (ºC) of the necessary time (days) to finish that process. The embryonic development in Brown trout needs between 195-220 degree-days to the first eye pigmentation (Gjerdem & Gunnes 1978, Grande & Andersen 1990). From fecundation to hatching 444 degree-days are needed, and 220 additional degree-days to the emergence (the completed absorption yolk-sack). Nonetheless, the degree-days value in the embryonic development is lower in coldwater than in warmer water (Grande & Andersen, 1990), and the interval in which temperature can vary is determined by the thermal tolerance of the organism, as shown. Elliott & Hurley (1998) modelled the hatching time as a function of the temperature:

$$H_{50} = f_H(T) = C_{H_{50}} \cdot \left(\frac{T_1 - T}{T - T_0}\right)$$

H_{50} being the necessary time from fecundation to hatch the 50% of eggs, T_0 and T_1 the lowest and highest temperature limits for hatching, respectively, and $C_{H_{50}}$ is a coefficient representing H_{50} at a constant temperature $(T_1 + T_0)/2$.

In the same way, for the interval from hatching to the emergence of the 50% of alevins (A_i) can be written:

$$A_{50} = C_{A_{50}} \cdot \left(\frac{T_1 - T}{T - T_0}\right)$$

$C_{A_{50}}$ to $C_{H_{50}}$ being analogue for the time between the hatching and the emergence.

Temperature and Growth in the Brown Trout

Various models of somatic growth were successively built for Brown trout with the aim of improving their predictive power (Elliott 1975, Elliott et al. 1995, Elliott & Hurley 1995, 1999, 2000a, 2001). In them it has emphasized the study of the effect of temperature on growth at maximum feeding rate. Thus the temperature at which the metabolic efficiency for trout is maximum was identified, as various studies located it around 13.1ºC for 0+ and 1+ trout

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age-classes fed with invertebrates (Elliott et al. 1995, Elliott & Elliott 2010), 13.5ºC when fed with pellets (Forseth et al. 2009) and 16°C in larger trout between 2+ and 4+ fed with fish (Forseth & Jonsson 1994). The temperature range for growth observed by Elliott et al. (1995) was between 3.6-19.5ºC, and between 5-23ºC by Forseth & Jonsson (1994). Thus, one question may arise: why the maximum metabolic efficiency is obtained at a low temperature in comparison with a warm-blooded animal? This must due to a combination of several connected factors. Trout are ectotherm animals and this entails metabolic adaptations that make the organism work within a range close to ambient temperature (homeotherms or endotherms maintain temperatures above external ones most of the time), besides there is the complex fact that trout have evolved and adapted to live in cold oligotrophic water. In this scenario, trout have 'chosen' to develop a metabolism that takes full advantage of the high oxygen availability due to those low temperatures and oligotrophy. Thus, it is feasible to think that trout evolution has tended to 'find' the maximum metabolic efficiency at a equilibrium point in which the theoretical ascendant curve of metabolic activity (as oxygen demand) and descendent oxygen availability intersect (Forseth & Jonsson 1994).

As a result of all this rationale and according to Elliott (1994), it can be concluded that the water temperature (and related oxygen availability), fish size and level of energy intake (ration size) are the three most important variables to explain the trout growth.

Jobling (1983) proposed that relations between the specific growth rate (G_W) and weight (W) could be described by a power function of the form:

$$G_W = \frac{a}{W^b}$$

Elliott & Hurley (1995) calculate values of a and b in relation to the temperature T, so the formula could be rewritten in the form:

$$G_W(T) = \frac{a(T)}{W^b}$$

Now, we have a theoretical model of the growth rate of trout on condition of the nonexistence of limiting factors other than the temperature.

Knowing the average temperatures and homogeneous short periods during the growth time, one can infer the theoretical maximum growth of trout at a given period, by applying the formula (Elliott et al., 1995):

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$$E\left(\bar{W}_t^b\right) = \left(\bar{W}_0^b\right) + \frac{b \cdot c}{100} \cdot \left[\frac{(T_1 - T_{LIM}) \cdot t_1}{T_M - T_{LIM}} + \frac{(T_2 - T_{LIM}) \cdot t_2}{T_M - T_{LIM}} + \cdots \frac{(T_k - T_{LIM}) \cdot t_k}{T_M - T_{LIM}}\right]$$

where $T_{LIM} = T_L = 3.56$ºC if $T \leq TM$ or $T_{LIM} = T_U = 19.48$ºC if $T > T_M$, T_L and T_U being the lowest and highest temperatures at which the growth rate is zero. The exponent b is the potential transformation of the weight that produces a linear growth over time (0.308), and c is the growth rate of one gram of trout at the optimum temperature (2.803). The five parameters of the equation have biological significance and remain constant for the temperature range 3.56-19.48ºC.

$$\left(\bar{W}_0^b\right)$$

and

$$E\left(\bar{W}_t^b\right)$$

are the observed and expected average weight on the potential transformation scale, respectively, and T_1, T_2, ... T_k are average temperatures of t_1, t_2, ... t_k days.

As was mentioned previously, this refers to a model with water temperature as the only limiting factor and it is adjusted for sexually immature fish, but there are other factors that are noted sooner or later.

As the model was formulated by Elliott et al. (1995):

$$\lim_{G_W \to 0} W_t = \infty$$

and consequently

$$\lim_{t \to \infty} W_t = \infty$$

This means that, with no other limiting factor, trout will indefinitely continue growing even with a very small G_W, but it would mean a very substantial increase of net biomass due to the initial weight of the individual

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that grows with this rate. In the hypothetical case of an immortal individual, it would never stop growing, its weight tending to infinity.

This does not happen and, in addition to other environmental factors such as food availability, there are physiological conditions constricting the growth. In general, increased body size reduces the physiological efficiency of its parts (Sibly & Calow 1986). It has been observed that there is an allometric relationship between the gill surface and body size (Pauly 1981, Hughes 1984) which influences the performance of breathing. In the same way, an allometry exists between the stomach surface and body size, which influences the digestibility (Wootton 1990). Probably, this last relationship is linked to the fact that the maximum food intake increases at a slower rate than the maintenance ration and, consequently, the growth prospect is reduced with increasing size. This would lead to reducing the expected growth if only the limiting effect of temperature is taken into account, an inflection point appearing in the growth curve and an asymptotic value such that

$$\lim_{t \to \infty} W_t = W_\infty$$

with $W_\infty \neq \infty$.

This leads to the growth model of von Bertalanffy (1938), in which the parameters representing the growth limits appear:

$$L_t = L_\infty \cdot \left(1 - e^{-K \cdot (t - t_0)}\right)$$

when the length L is substituted for the potential relation to the weight ($W = q \cdot L^b$), we have:

$$W_t = W_\infty \cdot \left(1 - e^{-K \cdot (t - t_0)}\right)^b$$

The inflection point in which the first effect of asymptotic growth would occur would be reflected by:

$$\frac{d^2 W}{dt} = 0$$

or does not exist. Jensen et al. (2000) applied the model of Elliott et al. (1995) to 42 trout populations where water temperature explained 74.9% of growth.

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By including other environmental parameters these authors improved the explanatory power of the model by up to 81.3%.

Moreover, if the model of Elliott et al. (1995) were to be applied continuously over time, it would be ignoring the fact that there is a period during which reproductive biomass, not somatic biomass, is produced. Reproductive biomass does not contribute to the animal body growth but it has a high evolutionary significance in terms of fitness (Sibly & Calow 1986). In this period of reproductive growth, even in conditions of maximum performance metabolism, somatic growth would be very different to other periods.

In short, in the absence of other ecological considerations, growth can be maximized to its physiological limits, -with their constraints- or, under the assumption that high growth rates entail survival costs, to be an optimal compromise between fitness costs and benefits of different growth rates. Optimization models can also make predictions about when the animals would stop growing and begin to reproduce. The optimal size depends on the relationship between the size and development time, and size and fecundity. By modeling, it can be clearly illustrated that for the evolution of a larger size there must be a substantial increase in fertility by means of a small increase in development time (Sibly & Calow 1986).

Finally, having noted the main conditions governing fish growth, the following considerations should be made regarding the effect of demography: Does the population density affect the growth rate because there are other fish in competition? If this were true, would the density effect be noticed while enough food or behavioral interference between animals (territoriality) exists diverting time and energy to the territorial competition?

The existence of territorial and/or feeding competition is a symptom of density dependence. If there were no density dependence regulation there would be no difference in growth rates between dense or sparse populations. The density dependence is linked to a single main factor which is intraspecific competition (Sinclair 1989), although this competition is often linked to the availability of resources, especially those involving energy constraints. The Energetic Equivalence Rule (Damuth 1981, 1991, Nee et al. 1991) argues that the amount of energy that each species uses per unit of area of habitat is independent of body mass, although several studies suggest that this is not necessarily so (Marquet et al. 1995). It seems logical that the metabolism of the population depends on the size of its individuals, however the question is whether this is due to some characteristic of the species, its feeding habits, or, linking it to the concept of density dependence, if it is due to some emanated

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conditioning from the population structure. Thus, the structure of the population and its density (Deverill et al. 1999, Jenkins et al. 1999, Nordwall et al. 2001, Lorenzen & Enberg 2002, Lobón-Cerviá 2005, Kaspersson & Höjesjö 2009) and the limitations imposed by the environment through the temperature (Elliott & Elliott 2010) are considered major factors affecting growth and, consequently, the size and weight of fish and, particularly, of Brown trout.

CLIMATE CHANGE AND STREAM TEMPERATURE

Air temperature trended to ascend during the last century throughout the world, showing a more pronounced rise beginning in the seventies (IPCC 2013). As a consequence, aquatic ecosystems underwent alterations in water temperature regimes also at the planetary level (e.g., Chessman 2009, Kaushal et al. 2010, Orr et al. 2015, Chen et al. 2016). The 5th Assessment Report (AR) of the International Panel of Climate change (IPCC 2013) predicts that the generalized significant increases of the air temperature will continue during the 21st century including the western Palearctic which is the natural area of Brown trout distribution. Predictions show annual increases in air temperature of 4.5ºC (up to 6.0ºC in summer) in the worst climate change scenario (RCP 8.5 [IPCC 2013]) and mean precipitation reductions around 19% (24% in summer) for the Mediterranean region and the greater part of Europe at the end of the century. From Poland to the east and the north, the annual mean increase of temperature will be around 6.0ºC, and 2.5ºC in the European Atlantic facade. During summer, mean increases will reach 4.5ºC in Central and Northern Europe and 3.5ºC in the Atlantic area. Annual precipitation variation will rise progressively from Mediterranean area to the north, without significant changes in medium latitudes in Central Europe and showing significant increases of 15% in the Baltic area and around 25% in the Scandinavian Arctic. Using the SRES A2 scenario of the 4th AR of the IPCC (Nakicenovic et al. 2000, IPCC 2007), Van Vliet et al. (2013) predict that this increase will affect river and stream temperatures that could suffer a rise of daily mean temperature >2.5ºC in Southern Europe, between 1.0-2.0ºC in the British Isles and most of the Scandinavian Peninsula, and in the range 2.0-2.5ºC in Central Europe and southeastern Scandinavian Peninsula. More precise studies predict a higher increase in air and stream temperatures than did the IPCC (2013) and Van Vliet et al. (2013) (e.g., up to 8ºC for air temperature and 4ºC for stream temperature in Central Spain [Santiago et al.

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2016b]) which could produce more relevant ecological consequences (Chapman 1928, Magnuson et al. 1979, Angilletta 2009). In addition, the warming up of the water is exacerbated by the streamflow reduction as several authors have verified (Moatar & Gailhard 2006, Pekarova et al. 2008, Santiago et al. 2016c). Streams showing a low baseflow are more sensitive to warming (Carlson et al. 2015, Santiago et al. 2016c). While droughts are not expected to increase in the near future in the Mediterranean area (IPCC 2013), air temperature increases could cause subsequent increasing evapotranspiration and less water availability in rivers and streams. Consequently, climate change may affect the aquatic ecosystems (Wade 2006, Woodward et al. 2010, Arismendi et al. 2012) and the habitat availability for many fish species because of increases in water temperature and decreases in flow rate (Hughes 2000, Parmesan 2006, Santiago et al. 2016a,b,c). Van Vliet et al. (2013) predict reductions of mean streamflow in the range of 25-50% in southern and central latitudes in Europe (in the upper part of the range for southern latitudes and in the lower part for central latitudes, including the British Isles) and increases between 0-25% in the Scandinavian Peninsula which could increase up to 50% in the mainland. However, it must not be forgotten that climate scenarios in Van Vliet et al. (2013) (taken from IPCC 2007) are more benign than in the more recent 5th AR IPCC report (IPCC 2013).

CLIMATE CHANGE EFFECTS ON THE DISTRIBUTION AND SURVIVAL OF THE BROWN TROUT

Changes in the Native Distribution

Distribution and biological efficiency of coldwater fish will experience significant changes because of climate change being especially sensitive at the rear edge (*sensu* Hampe & Petit 2005) of their distribution. Williams et al. (2015) reviewed a long list of papers and identified a significant amount of climate change impacts and effects on salmonid species and their habitat. Temperature stands out among the main impacted factors from climate change. DeWeber and Wagner (2015), for example, found temperature to be the most important determinant of the probability of Brook trout (*Salvelinus fontinalis*) occurrence. Santiago et al. (2016a) also found temperature as the main predictor of presence/absence of Brown trout in two studied streams in Central Spain ahead of other aspects of physical habitat and fish community.

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Based on this, studies dealing with predicting the effect of climate change on salmonids distribution are increasing. *Inter alia*, Meisner (1990) simulated the climate warming in two streams in Ontario and predicted reductions of the thermal habitat of the Brook trout by 30% and 42% for an increase of 4.1ºC in summer air temperature. Ruesch et al. (2012) estimated the thermal habitat loss for *Oncorhynchus tshawytscha*, *O. mykiss* and *Salvelinus confluentus* will be between 39–95%, 51–87% and 86–100% respectively at the John Day basin (Oregon) by the year 2100. Stewart et al. (2015) also predict significant loss of thermal habitat for coldwater fish in Wisconsin where stream temperatures will increase by 1 to 2ºC by mid-century in 80% of stream lengths and up to 3ºC in 99% of stream lengths by late century.

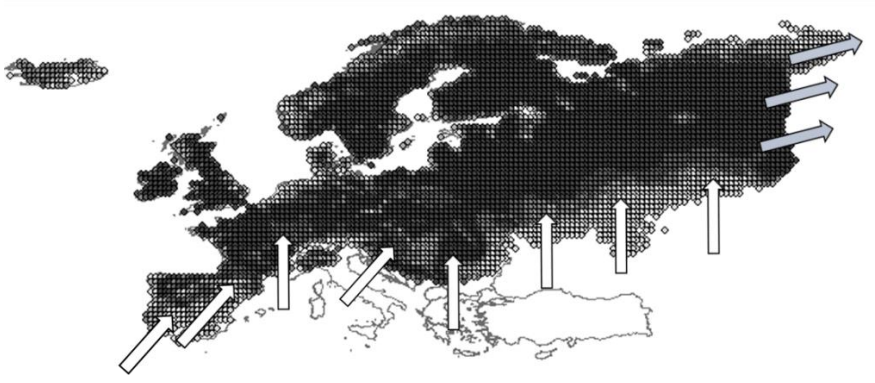

Figure 6. Current distribution of the native Brown trout (the darker the dots, the higher the probability of occurrence). The arrows show the direction of the predicted distribution shift (white arrows: at the rear edge; grey arrows: at the leading edge). The most likely way to colonize new waterbodies would be by means of marine migration (probability of occurrence data: AquaMaps).
[http://www.aquamaps.org/search.php#tools]

There are several projections about the effect of climate warming predictions on Brown trout distribution (Figure 6 shows a synthesis of predictions). Lassalle & Rochard (2009) made an analysis at a coarse scale of the relationships between fish distribution and physical features of the great European basins and predicted Brown trout "to lose all its suitable basins in the southern part of its distribution area (Black Sea, the Mediterranean, the Iberian Peninsula and the South of France), but likely to continue being abundant in northern basins." Environmental conditions may become suitable for salmonids and able to sustain new populations eastwards, in the Arctic rivers along the Russian continental coast (Jonsson & Jonsson 2009). Graham

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& Harrod (2009), in a more spatially refined study of the distribution in the British Isles detected that the response to climate warming is likely to be variable depending on the location, so that the loss of habitat is only predicted to occur at some southern or lowland populations. Almodóvar et al. (2011) predict the loss of almost the entire stream length inhabited by Brown trout in Northeastern Spain, and Filipe et al. (2013) do it for the 57% of the studied reaches in West and Central Europe. Santiago et al. (2016a) predict reductions of thermal habitat as large as the 11% and 56% of the length of two streams in Central Spain. Methodological and scale differences can be behind the lack of similarity between these studies, but the relevance of the impact of climate change in all cases is a common conclusion. Studies on the South rear edge of the Brown trout range are attracting special attention of researchers because of their biogeographical relevance. In this area, Santiago et al. (2016c) show a wide range of situations which can mark the shift of the distribution area of Brown trout in Southwestern Europe. Temperatures will raise less in the mountains than in the Iberian central peneplain and less in karstic mountains than in granitic ones. At the same time, the Mediterranean facade will be more sensitive to warming and waterflow reductions than the Atlantic facade. Thus, Brown trout populations in karstic mountains in Northern Spain (Cantabrian Mountains and calcareous parts of Pyrenees) are better able to resist the climate warming than eastern ones in granitic parts of Pyrenees. The same thing could occur in other parts of Europe. Probably, the less pronounced thermal response of rivers and streams in the karstic areas will allow a better persistence of the Brown trout population, although it would be also linked to changes in waterflow regimes.

Decreases in summer waterflow will reduce the size of usable habitat and this involves reducing the total carrying capacity of a given river (Muñoz-Mas et al. 2016). This results in smaller and less resilient populations.

Life History

Jonsson & Jonsson (2009) predicted that changes in water temperatures and flow will affect migration, ontogeny, growth and life history traits of Brown trout.

Coldwater fish will not only suffer because of extreme and selective summer temperatures but also as a result of thermal changes in the different phases of the life cycle. Increases in temperatures in winter and early spring, being benign for juvenile and adult fish, can significantly reduce the survival

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of eggs and larvae. Simulations for Central Spain predict temperature increases that jeopardize the survival of the fertilized reproductive material and the consequent recruitment.

Brown trout will be very close to its limit of physiological efficiency compromising the production of somatic and reproductive biomass. The increase in the temperature of rivers and lakes can affect growth by changing the length of the growing season. The growing season can be extended by increasing winter temperatures or shortened by increasing summer temperatures. Regarding growth temperatures, increased mean temperature could increase or decrease growth efficiency depending on whether the average temperature is higher or lower than the optimum temperature. Thus, in the same river, two situations may occur: increased growth in the coldest reaches and a reduction in the warmest ones. Nonetheless, Reist et al. (2006) suggest that trout in the coldest rivers are specifically adapted to low temperatures and short growing seasons, and increased temperatures will negatively affect growth rates, age/size structure, and abundances of northern populations. Several authors (Beer & Anderson 2013, Cianfrani et al. 2015) showed how juvenile salmonids' weight will be lower affecting fish survival and reducing Brown trout biomass production as a consequence.

Moreover, regarding the production of reproductive material, it is known that the larger females produce more and larger eggs with higher survival probability (McFadden et al. 1965, Bagenal 1969, Lobón-Cerviá et al. 1997).

Being located in temperatures of physiological stress can increase the susceptibility of fish to disease. Thus, susceptibility coupled to better conditions for the development of pathogens and parasites make global warming a factor of increased risk of certain infectious and parasitic diseases of fish (Karvonen et al. 2010) but morbidity is according to the biology of each disease and environmental and demographic local conditions. As an example, Hari et al. (2006) studied the warming in Alpine streams during the last 25 years and detected a relation between stream temperature increase and the incidence of temperature dependent Proliferative Kidney Disease at lower reaches of the Brown trout distribution, affecting the actual boundary.

The fragmentation of populations is another handicap for the persistence of the trout. Coldwater fish may find climate refugia in mountain streams (Isaak et al. 2016) as a response to climate warming but a large retraction of their populations may thermally isolate them: headwater reaches located in areas of thermal sensitivity produce the disconnection of the tributaries network previously connected by the main channel, now uninhabited by these fish. At the limits of climate refugia (reaches able to sustain populations) small

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summer thermal refugia (temporally and spatially limited) could be found located in places such as the cooler pools, places where tributaries meet, and header reaches (Crozier et al. 2008) so this would be the only alternative for fish survival. The existence of thermal or climate refugia in a sensitive region is highly geologically dependent, being related to the existence of both, aquifers more resistant to warming and cool, deep aquifers.

Thermoregulatory behaviour is part of the strategy of use of the thermal refugia (Reynolds et al. 1979, Goyer et al. 2014). This strategy consists of performing short excursions (e.g., <60 min in experiments with Brook trout) to sustain the body temperature below a critical temperature threshold, enabling fishes to exploit resources in an unfavourable thermal environment (Pepino et al. 2015). Brown trout were observed using pool bottoms during daylight hours to avoid the warmer and less oxygenated surface waters in thermal refugia (Elliott 2000). Nevertheless, if the warm events became too long, the thermal refugia could become completely insufficient, thus compromising fish survival (Brewitt & Danner 2014, Daigle et al. 2015).

Adaptation Capacity

Increasing evidence makes it necessary to consider the evolutionary responses to global warming (Angilletta 2009). A key question is whether understanding the physiology and genetic underpinnings of thermal tolerance can improve predictions of the ability of fish to adapt to higher temperatures (McCullough et al. 2009). Genetic models suggest that the current and predicted warming rhythm will lead to the extinction of certain species because organisms will not be able to adapt. The maladaptation level during warming is due various factors including the stochasticity of temperature, the size of the population, the additive genetic variance, the rate of gene flow, and the interactions between species (Angilletta 2009). Theoretically, directional selection would be adequate to enhance the stability of genetic variances and covariances (Jones et al. 2004) but not much is known about the genetic structure of thermal reaction norms. However, these limitations will be crucial to determine if adaptation rhythm is sufficient to compensate the effect of the predicted environmental changes (Angilletta 2009). Forseth et al. (2009) did not find local adaptation to prevailing temperatures in their analysis of several populations but Jensen et al. (2008) observed a relative thermal plasticity at early stages of development of brown trout. This effect varied among populations due to locally adapted reaction norms according to the temperature

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regimes in their living environments. These authors suggest that additive genetic variance and heritable variation in phenotypic plasticity could compensate the effects of changing temperature regimes, but sudden changes might impede adaptation because plasticity is limited. Ayllón et al. (2016) suggest that the necessary time for adaptation is too long in relation to the climate change rythm. Jonsson & Jonsson (2009) mantains that salmonid populations do not show thermal growth adaptations probably because they have evolved and they live in environments with seasonal, annual and spatially variable temperatures where phenotypic plasticity is more advantageous than genetic fixation. Elliott & Elliott (2010) reviewed a large number of studies and reached the general conclusion that salmonids show little intraspecific variation to support the hypotheses for thermal adaptation, except in very cold rivers. Beacham & Withler (1991) observed heritability for tolerance to high water temperatures in a study with Chinook salmon (*Oncorhynchus tshawytscha*) even though it only occurred in northern coldwater experimental individuals and it did not occur in southern warmwater individuals.

Some ability for adapting to high temperatures is possible but physiological extremes may exist that cannot be forced. It must not be forgotten that physiological efficiency is species specific and dependent on the relationship between temperature and oxygen capacity to transfuse. It may be possible to "stretch" the realized niche but the physiological niche cannot be easily influenced. The frequencies of the fittest genotypes are likely to shift to the range of displaced temperatures, although high temperature stochasticity may dilute the quantitative adaptation.

There are many fronts in the research of coldwater fish and further investigation is necessary to develop all the questions discussed in this chapter. More accurate predictions will permit a more successful response to the challenge of avoiding the loss of Brown trout native populations.

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- Santiago, J.M.; Solana, J.; Alonso, C.; Garcia de Jalón, D.; Muñoz-Mas, R.; Martínez-Capel, F.; Pórtoles, J.; Monjo, R. & Ribalaygua, J.. Stream flow regime, temperature and climate change: the loss of fish habitat. In: *Proceedings of the 11th International Symposium on Ecohydraulics. Melbourne, Australia*; (2016). p. 26791.
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Chapter 5

REPARATIVE NEUROGENESIS IN THE ADULT TROUT BRAIN AND PECULIARITY OF DEVELOPMENT IN TROUT'S BRAIN CELLS IN PRIMARY CULTURE

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ABSTRACT

Fishes have remarkable ability to effectively rebuild the structure of cells and nerve fibers after central nervous system injury. However, the underlying mechanism is poorly understood. In order to address this issue, we investigated the proliferation and apoptosis in contralateral and ipsilateral optic nerves, after stab wound injury to the eye of an adult trout *Oncorhynchus mykiss*. The qualitative and quantitative assessment of proliferation and apoptosis in the cells of the optic nerve of a trout has been made using antibodies against PCNA and the TUNEL method. We

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have found that proliferation and neurogenesis in proliferative brain regions, the cerebellum, and the optic tectum were significantly enhanced after the eye injury. PCNA labeling of optic nerve one week after injury revealed heterogenous population of proliferating cells.

TUNEL-labeling gave a qualitative and quantitative assessment of apoptosis in the cells of optic nerve of trout two days after injury. After damage to the optic nerve apoptotic response was registered, and mass patterns of cell migration were found. The maximal concentration of apoptotic bodies was detected in the areas of mass clumps of cells. It is probably indicative of massive cell death in the area of high phagocytic activity of macrophages/microglia. One week post-trauma, we observed proliferation in the integrative centers of trout brain: cerebellum and optic tectum. In optic tectum, PCNA-positive radial glial like cells were identified. Proliferative activity was detected in the dorsal proliferative (matrix) area of the cerebellum and in parenchymal cells of the molecular and granular layers whereas local clusters of undifferentiated cells which formed neurogenic niches were observed in both the tectum and cerebellum, after injury.

The differentiation of neuronal cells detected by labeling cells with antibodies against the protein HuC/D occurred in the proliferative zones of the telencephalon, the optic *tectum*, cerebellum, and medulla of a trout within 2 days after the injury. We have shown that the HuC/D expression is higher in the proliferative brain regions than in the definitive neurons of a trout. *In vitro* analysis of brain cells of trout showed that the cells in suspension compare with monolayer retain high proliferative activity, as evidenced by PCNA-immunolabeling. Phase contrast observation showed mitosis in individual cells and the formation of neurospheres which gradually increased during 1-4 days of cultivation.

Keywords: optic nerve injury, neurogenic niche, radial glia, reparative neurogenesis, primary culture of brain cells, neurospheres, proliferation, PCNA, HuC/D, trout, matrix areas of brain, TUNEL-labeling, apoptosis

1. INTRODUCTION

Among vertebrates, fish are known to be able to effectively restore the structure of cells and fibers after damage of the central nervous system. They have the ability to restore the number of damaged cells by production of new cells in the matrix areas of the brain and neurogenic niches, and the ability to restore the structure of damaged axons of neurons in the spinal cord pathways [1]. However, it is currently unknown how this process is related to the

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neurogenesis in the adult brain and what elements of the matrix areas of the brain are involved in the reparative neurogenesis in fish. The evolutionarily ancient animal groups are often used as a convenient model for neurogenic studies in adults. The brain of such animals has a large number of periventricular proliferative zones and active zones of secondary neurogenesis [2, 3]. In contrast to the mammalian brain, numerous proliferative regions have been found in adult fish. The presence of such regions was described in *Apteronotus leptorhynchus* [4], *Sparus aurata* [5], three-spined stickleback *Gasterosteus aculeatus* [6], *Danio rerio* [7, 8], and three species of the genus *Austrolebias* [9].

We investigated the change in the cellular composition of proliferative brain regions in response to eye damage in an adult trout. Optic nerve injury often induces massive nerve cell death and irreversible visual functional impairment in mammals, such as cat [10], rabbit [11], and mouse [12]. Lower vertebrates, like zebrafish [13], *Rana pipiens* [14] and *Litoria moorei* [15], however, can recover visual function due to survival of retinal ganglion cells (RGCs). In goldfish, about 90% of RGCs survive and rapidly regrow axons to the optic tectum about 2 weeks after axotomy [16]. Fish has excellent potential to regenerate RGC axon to the optic tectum within 5 days after optic nerve crush [17]. It can restore visual function, compared with 16 weeks for sunfish [18], 30–50 days for goldfish [19] and 40 days for cichlid [20]. However, whether RGC survival or neurogenesis is required for visual functional recovery is still a matter of controversy [21, 22]. The regenerative ability of the adult brain requires a series of coordinated cellular processes: neuronal progenitor cell proliferation and migration to injury sites, neuronal differentiation, cell survival, and the integration of the new neurons into existing neural circuits. However, the regeneration efficiency of neurons in the injured mammalian brain is extremely low [23]. In contrast to mammals, the adult central nervous system (CNS) of teleost fish exhibits a high capacity for neuronal regeneration after injury [1]. Thus, comparative studies in zebrafish and mammals should reveal both general and divergent properties of adult neurogenesis.

The regenerative processes in the brain of fish after the damaging impact are determined by a number of factors, which distinguish the dynamics of this process from that in other vertebrates, particularly mammals and humans [24, 25]. It is known that the brain injury in the mammalian brain results in a number of pathological changes associated with the development of an

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inflammatory response to the toxic effects of glutamate and other inflammatory mediators, and further pathological changes associated with processes of secondary inflammation and involving massive cell death [26, 27]. As a result of CNS trauma, the mammalian cells are exposed to severe necrosis, and only a small part of them is eliminated via apoptosis [28]. In the fish brain, the cell response to the trauma develops in a different scenario. Apoptosis is observed 5 minutes after the injury, which progresses in the next few days [7]. The elimination of damaged cells is carried out by phagocytes (microglia/macrophages), which remove damaged cells very effectively and provide a "clean" cell death without the remaining damaged cellular material and the development of secondary inflammation [29]. The replacement of the large amounts of dead cells resulted from the damage in the fish brain appears from various sources: the radial glia, centers of primary and secondary proliferation, and neurogenic niches.

Here, to investigate the cellular aspects underlying the strong ability of fish to undergo neuronal regeneration, we developed a trout model of adult stab wound injury of eye and optic nerve. Using this model, we tried to reveal a series of regenerative processes in the injured optic nerve and some integration centers of the brain: the optic tectum and the cerebellum. We studied the proliferation of endogenous neuronal progenitor cells in the tectal and cerebellar proliferative zones, the migration of neuronal progenitor cells from the cerebellar matrix proliferative zones towards the injury site, and the proliferative activity of different types of cells both in terms of adult neurogenesis and neurogenic niches. We examined apoptosis in the optic nerve of adult trout 2 days after injury followed by the proliferative response of cells in the optic nerve and brain integration centers (the optic tectum and the cerebellum) after stab wound injury to the eye. Using morphological analysis and quantification of proportion of proliferating cell nuclear antigen (PCNA)-immunopositive cells in control and damaged fish, dorsal matrix zone of trout cerebellum and optic tectum was characterized at 1 week after injury of eye.

Primary culture of nerve cells from adult trout's brain was established to study the properties of cells in the CNS of adult trout and their proliferative potential *in vitro*. After 4 days of culture, immunohistochemical staining was performed to analyze the proliferative potential of cells in the brain of adult trout. This study was designed to investigate whether adult trout can be used as a novel model for studying neuronal regeneration.

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2. Cellular Response in the Trout's Optic Nerve After Injury

After the mechanical impact on the trout eye, the morphology of optic nerve at the side of the damage and on the contralateral side was studied. The morphological structure of the trout optic nerve on the contralateral side is shown in Figure 1A. In this region, four morphological cell types were identified among the optic nerve fibers (see Table 1).

Solitary TUNEL-labeled bodies in the form of small granules of different sizes and dense apoptotic bodies were observed on the contralateral side (Figure 1B). In our studies, TUNEL-positive cells with DNA fragmentation are shown in Figure 1B–E. After mechanical damage to the eye, distribution of TUNEL-positive elements in the optic nerve was uneven. TUNEL-positive cells were distributed on the damaged side and the number of TUNEL-positive cells was highest in the area of injury (Figure 1D). Intense peroxidase labeling of the nuclei of apoptotic cells shows signs of DNA fragmentation. Dark peroxidase labeled granules (apoptotic bodies) sometimes formed homogeneous conglomerates (Figure 1D, E). These structures were located at the site of the nucleus, evenly distributed in the cytoplasm, either shifted to the cytoplasmic membrane or grouped with one of the poles of the cell soma. For TUNEL-positive cells characterized by the presence of intact cell membrane around them with no foci of inflammatory infiltration, they sometimes exhibit an intense labeling of fibers in the optic nerve (Figure 1F). This effect arises from the rupture of karyolemma of the major damaged retinal cells, TUNEL-labeled fragmentation of chromatin in the cytoplasm, followed by its spread to some distance as a result of anterograde transport.

At 2 days after injury, we found that there were a large number of type 1 and type 2 cells that migrated on the ipsilateral side (Figure 1C). These cells formed longitudinal migratory flows (Figure 1C), surrounded by type 3 cells, and the number of which was also significantly increased. The average number of cells on the ipsilateral side was 2.3 fold greater than that on the contralateral one. Along with the patterns of cell migration on the ipsilateral side, we observed massive accumulation of apoptotic bodies and numerous TUNEL-labeled cells (Figure 1D).

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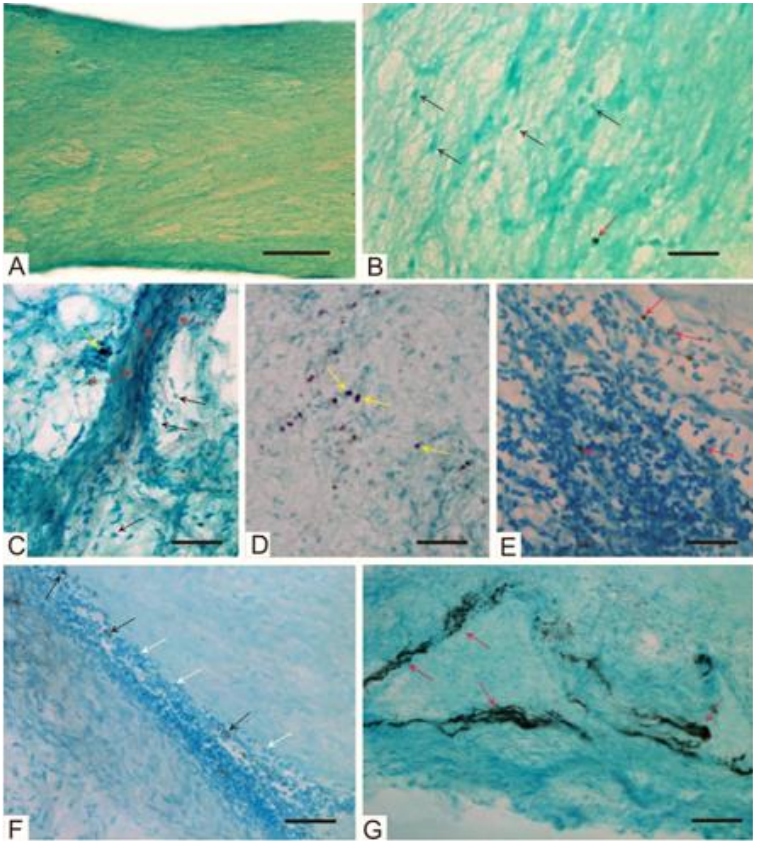

Figure 1. Morphological structure and apoptosis in the optic nerve of trout *Oncorhynchus mykiss*. A - general view of the contralateral optic nerve. B - cells (black arrows) and TUNEL-labeled granules (red arrow) in the contralateral nerve. C - patterns of cell migration (type 1 cells shown by black arrows, type 2 cells – red arrows, apoptotic bodies - yellow arrows) in the ipsilateral optic nerve. D - accumulation of apoptotic bodies (yellow arrows) in the proximal ipsilateral optic nerve. E - a large cluster of types 3 and 4 cells (red arrows) in mesaxons of ipsilateral optic nerve, red arrows indicate the different types of TUNEL-labeled elements. F - general view of the proximal portion of the ipsilateral optic nerve, white arrows show type 1 migrating cells, and black arrows indicate TUNEL-labeled apoptotic bodies. G - TUNEL-labeled fibers in the proximal ipsilateral optic nerve (red arrows). Immunoperoxidase TUNEL labeling in combination with methyl green staining. Scale bars: 200 μm (A) and 50 μm (B–G).

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Table 1. Morphological parameters (mean ± SEM) and optical density of proliferating cell nuclear antigen-immunopositive (PCNA+) optic nerve cells after stab wound injury of trout eyes

Type of cells	Large diameter (μm)	Small diameter (μm)	The optical density of PCNA-immuno labeling (UOD)	
			Min.	Max.
1 (large)	12,1 ± 0,8	5 ± 0,4	-	-
2 (elongated)	8,4 ± 0,2	4,2 ± 0,5	108	124
3 (round)	8,6 ± 0,3	6,1 ± 0,3	107	118
4 (small)	6,9 ± 0,06	5 ± 0,02	97	120

Patterns of cell degranulation representing the earlier stages of the apoptotic process and TUNEL-labeled fragments of degranulated chromatin were detected along with apoptotic crescents-like bodies (Figure 1E). A significantly increased number of cells were observed in the regions separating the individual fiber optic bundles – mesaxones (epineurium) (Figure 1F). In these regions, we also observed TUNEL-labeled apoptotic cells (Figure 1F). Along with the TUNEL-labeled cells on the ipsilateral side, portions of TUNEL-labeled fibers (Figure 1G) were observed.

Considering the complex nature of the mechanical damage affecting different membranes and structures of the eye, the optic nerve, and the adjacent oculomotor muscles together with the connective tissue, the adjacent muscle fibers of the oculomotor muscles were investigated along with the optic nerve (Figure 2A). The postinjury study of the morphological structure of TUNEL-labeled and stained with Brachet's methyl green muscle bundles revealed a large number of cells different types in the connective tissue surrounding the muscle bundles, previously detected in contra- and ipsilateral optic nerves (Figure 2A). These cells were also detected along radial fibers in the area between the optic fibers and adjacent oculomotor fiber bundles (Figure 2B). We consider that these cells appearing in the region of the optic nerve damage represent the regional group of connective tissue macrophages migrating to the site of injury to remove the fragments of the damaged optic nerve and adjacent muscle cells.

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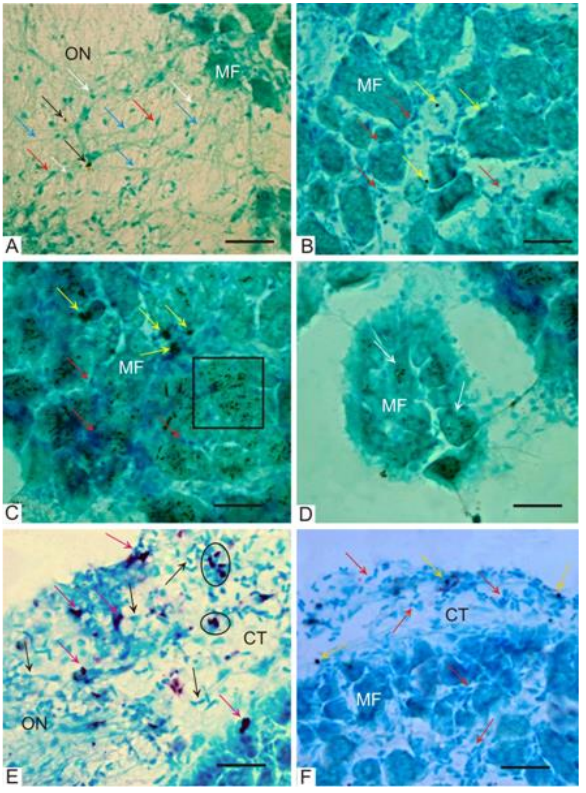

Figure 2. Morphological structure and TUNEL-labeling of oculomotor muscle adjacent to the damage nerve of trout 2 days after injury. A - reconstruction of horizontal sections containing a fragment of damaged optic nerve and adjacent muscle tissue; there are 1-4 cells types (cells of 3rd type shown by white arrows, 1st type - red arrow), apoptotic bodies (black arrows) and radially oriented fibers (blue arrows); B - cross-section of oculomotor muscle fibers (MF), yellow arrows indicate of TUNEL-labeled apoptotic bodies, red arrows - cells infiltrating the space between the muscle bundles; C - cross-section of the oculomotor muscles, square delineated fragment containing diffuse TUNEL-labeled material, dense apoptotic bodies indicated by yellow arrows, cells intermuscular spaces are shown by red arrows; D - an isolated muscle fragment comprising diffuse TUNEL-labeled structures (white arrows); E - the area containing fragment of damaged optic nerve (ON) in combination with a peripheral portion of oculomotor muscles (MF), surrounded by connective tissue (CT), conglomerates apoptotic bodies contoured by ovals, single apoptotic bodies show by pink arrows, numerous cells of 1-4 types shown by black arrows; F - cross-section of oculomotor muscle fibers surrounded by a shell containing cells of 1-4 types (red arrows) and TUNEL-labeled bodies (yellow arrows). Immunoperoxidase TUNEL-labeling in combination with methyl green staining. Scale bar 50 µm.

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The study of the morphological structure of individual muscle bundles showed the initial stages of the apoptotic process, the fragmentation of chromatin appearing as numerous diffuse TUNEL-labeled fragments in muscle fibers after the injury (Figures 2C, D). However, we do not exclude the presence of necrotic and inflammatory process in these areas, since disordered chromatin condensation without its binding to the nuclear membrane and karyolysis were sometimes observed in the muscle fibers, which is not typical for apoptosis (Figure 2D). We observed the sites of inflammatory infiltration around the muscle bundles (Figure 2B).

The maximum accumulation of TUNEL-positive apoptotic cells was detected in regions where ipsilateral optic fibers adjoined to the muscle fibers (Figure 2E). The connective tissue surrounding muscle fiber bundles contained apoptotic bodies and 1–4 types of cells (Figure 2F).

To evaluate the proliferative activity in the injured optic nerve, IHC of PCNA-labeling was carried out 1 week after the injury. The results of PCNA-labeling showed a high level of proliferative activity of cells in the ipsilateral optic nerve (Figure 3A). Among immunopositive cells, the cells of 2, 3 and 4 types were found, but the activity of PCNA was also identified in migrating cells type 1 (Figures 3A, B). The distribution of PCNA+ cells was uneven; the cells of types 3 and 4 often formed local clusters (Figure 3A). The results of the densitometric analyzes indicated that there were differences in the optical density in cells of types 2, 3, and 4 (see Table 1). The densitometric analysis showed that the difference between the minimum and maximum optical density in the cells was 22–30%.

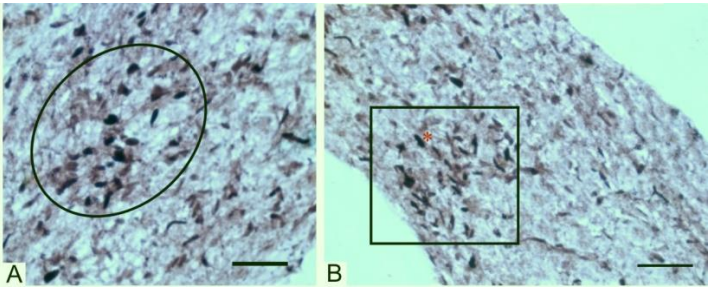

Figure 3. Localization of proliferative cell nuclear antigen (PCNA) in damaged optic nerve of trout 1 week after optic nerve injury. A - clusters of intensely labeled type 2 and 3cells (contoured by oval) in the deep layers of the damaged optic nerve. B - accumulation of immunopositive type 1 cells (contoured by square) in the surface layers of the damaged nerve, a red asterisk shows cells where mitosis was finished. Scale bars: 50 μm.

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3. Comparative Characteristics of Proliferation and Apoptosis in Damaged Optic Nerve of Trout and Goldfish

Our results showed that both apoptosis and proliferative activity took place in the optic nerve after the mechanical damage of the trout eye. These processes prevail on the side of injury, since the number of TUNEL-labeled cells was 6.5 times higher and the number of proliferating cells was 2.8 times higher than those on the contralateral side. The structure of the optic nerve in a trout, as well as in other fish, is formed by axons of retinal ganglion cells and glia cells (Schwann cells, oligodendrocytes, and astrocytes) and has some connective tissue containing macrophages/microglia, which activate in response to a damaging impact. Presently, the overall picture of the interactions between astrocytes/ganglion cells during regeneration of ganglion cells in fish is poorly understood. For example, in studies on goldfish, the network of GFAP-immunopositive reactive astrocytes was discovered 7 days after the crush of the optic nerve [30]. These cells appeared simultaneously with the growth of axons in the damaged optic nerve. However, the damaged area remained GFAP-negative, and astrocytes were excluded from the area of the damage during regeneration. Nona et al. suggested that the fibers of the optic nerve of goldfish regenerated so quickly that they were not myelinated when they reached the brain [31]. It is assumed that the myelin formation in regenerating axons of the optic nerve is determined not only by glial cells but also by additional factors of the microenvironment of the optic nerve, whose origin is not clear yet.

The analysis of morphological parameters, the type of PCNA-immunopositivity, the optical density of PCNA labeling of the optic nerve cells in a trout allowed to identify four types of cells (see Table 1). These data indicate the heterogeneity of the population of proliferating cells in the optic nerve. We suppose that the proliferating cells may include microglia and Schwann cells. We suggest that heterogeneous cell types were present among the apoptotic bodies, including optic nerve macroglia (astrocytes and Schwann cells) and the resident fibroblast-like cells appearing as a result of the damaging impact. We found no data in the available literature discussing the process of apoptosis of non-neuronal cells in the optic nerve of fish. Presently, in the studies of regeneration of the optic nerve in the lower vertebrates, the

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participation of retinal ganglion cells in the regeneration can be considered the most studied [32-34]. Some analogues can be found between the studies of the optic nerve regeneration in mammals and fish, mainly because there are pronounced differences in gene expression found in these vertebrate groups.

The data on the goldfish demonstrate that Schwann cells of unknown origin appear in the area of the optic nerve damage, which are absent in the damaged area prior to the myelination of axons. According Nona et al. [31] such Schwann cells begin to proliferate after a few axons reach the brain during the regeneration; and then only a small number of cells exits the cell cycle and joins the regenerating axons by participating in the formation of myelin. Remyelination is completed 3 months after the damage of the optic nerve in a goldfish. Regenerating axons become myelinated with the participation of both oligodendrocytes and Schwann cells distally (near the brain) of the damaged area [35].

In studies on goldfish, in the optic nerve of adult fish Pax2-positive and Pax2-negative cells are present [36]. Pax2 is a well-known transcription factor which participates in optic nerve development. It assures the correct arrival and package of the newly formed retinal axons and the adequate differentiation of the newly formed glial cells. Pax2 protein expression is continuous throughout adult life in the goldfish optic nerve. It has been proposed that optic nerve head astrocytes, including Pax2 positive cells, come from migrating astrocyte precursors of the retina during development [37, 38]. However, Macdonald et al. [39] suggested that Pax2 positive cells in the zebrafish optic nerve originate from the optic stalk cells expressing this transcription factor during the development.

Double labeling of Pax2 and PCNA in the optic nerve of goldfish showed that few proliferating Pax2-positive/PCNA-positive cells were observed in the optic nerve head. In the lateral intraorbital zone, these cells were located in the area that forms a boundary between the photoreceptor segments and the optic nerve [36].

Astrocytes have been shown to play an important role during the process of regeneration which allows the fish visual system to completely regenerate, but this does not occur in mammals [40]. In teleosts, the Pax2+ astrocyte population and pax2a gene expression are modified in the optic nerve head after a peripheral growth zone cryolesion when the regenerating RGC axons reach the optical nerve head [41].

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4. Cellular Response in the Trout Brain After Optic Nerve Injury

Proliferative activity in the cerebellum of trout. PCNA immunoreactivity was found in the dorsal and ventral area of cerebellar body. In the dorsomedial region of the cerebellum, we observed large clusters of PCNA+ cells forming *dorsal matrix zone* (Figure 4A). In the DMZ, a lot of cells migrated in radial and tangential directions (Figure 4B). At the dorsal part of molecular layer, radially migrating cells were dominated; at the superficial level of molecular layer, we identified tangentially migrating cells (Figure 4B). High-density distribution of PCNA-negative cells was observed near the PCNA-immunopositive cells in the DMZ (Figure 4B). Such accumulation of PCNA+ cells with irregular shape was also observed directly under the DMZ (Figure 4C). We believe that this cell formation is a *regional neurogenic niche*, formed as a result of traumatic exposure. In the molecular layer of the dorsomedial part of cerebellar body, we also observed round PCNA- cells. The density of their distribution increased in the dorsal direction (Figure 4B). PCNA+ cells in the DMZ included highly labeled oval cells and elongated, fusiform and/or rod-shaped cells (Figure 4C). In the cerebellar ventral region, the concentration of PCNA+ cells was much lower (Figure 4D). Separate PCNA+ parenchymal cells were identified in the molecular layer (Figure 4D). In the superficial area of the granular layer we observed single small accumulation of PCNA+ parenchymal cells. PCNA+ cells were often found among granular cells. From dorsal side of granular layer, the number of PCNA+ cells was much higher (Figure 4D). In the infraganglionic plexus, small parenchymal PCNA+ cells were found (Figure 4E). Sometimes, small clusters of both PCNA+ and PCNA- cells were also identified in the territory of infraganglionic plexus (Figure 4F). We believe that these clusters represent regional neurogenic niches, proliferative activity in which was induced by mechanical trauma.

Proliferative activity in the tectum of trout. After mechanical trauma of the eye, numerous immunopositive cells were identified in the inner tectum layers (Figure 5A, B): the surface fibrous and cellular layer (SFCL), the central gray layer (CGL), the central white layer (CWL), and the periventricular layer (PVL). In the marginal layer, PCNA+ cells of radial glia were identified (Figure 5C, D). The distribution density of PCNA+ radial glia was relatively low and amounted to 33 ± 7 cells per field. The ratio of PCNA-immunolabeled radial glial cells in the marginal layer and the inner cell layers in the tectum is shown in Figure 5E. The optical density of PCNA immunolabeling of radial

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glia cells varied: highly immunogenic cell (114.5 OD units) and less intensely labeled (104.4 OD units). In the inner layers of the tectum, the density level of PCNA+ was higher than in the cells of radial glia and was 123 OD units on average (Figure 5F). The maximum density level of PCNA in the cells of the tectum was 127.7 OD units and minimum was 116.2 OD units.

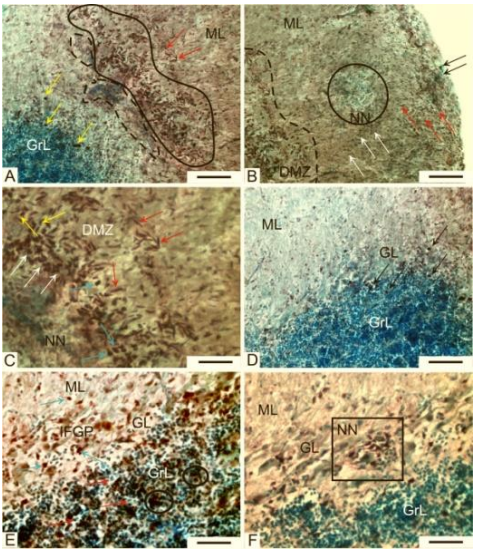

Figure 4. Localization of proliferative cells nuclear antigen (PCNA) in trout cerebellum 1 week after optic nerve injury. A - PCNA+ cells in the cerebellar dorsal matrix zone (DMZ, delineated by a solid line), the accumulation of PCNA– cells under the DMZ delineated by the dotted line, yellow arrows show intensively labeled oval cells in the granular layer (GrL), red arrows show rod-shaped migrating cells. B - dorsal part of the molecular layer (ML) of the cerebellum. Accumulation of PCNA– cells is delineated by circle, black arrows indicate the PCNA– tangentially migrating cells, red arrows point to PCNA– small round cells, white arrows indicate weakly labeled radially migrating cells, and NN represents neurogenic niche. PCNA+ cells in the cerebellar DMZ are delineated by dotted line. C - cellular composition of DMZ. Type 1 cells are shown by red arrows, type 2 cells by yellow arrows, type 3 cells by blue arrows, and type 4 cells by white arrows. D - ventral part of cerebellar body. Black arrows show PCNA+ cells in molecular (ML), ganglionic (GL) and granular layers. E - infraganglionic plexus (IFGP) in dorsal part of the cerebellum. Blue arrows show the PCNA+ cells in IFGS, red arrows point to PCNA– cells in GrL, ovals delineate a cluster of PCNA+ cells in the granular layer. F - fragment of ganglionic layer containing neurogenic niche (contoured by square). Peroxidase PCNA immunolabeling on transversal brain sections *in situ*. Scale bars: 100 µm (A, B, D) and 50 µm (C, E, F).

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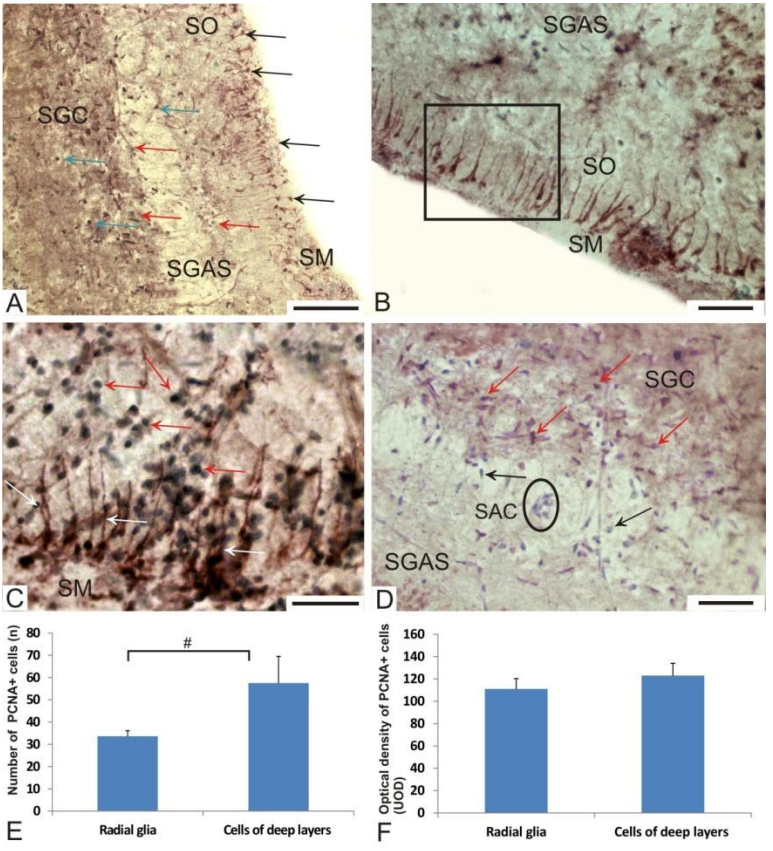

Figure 5. Localization of proliferative cell nuclear antigen (PCNA) in the optic tectum of trout 1 week after optic nerve injury. A - general view of optic tectum. Black arrows show PCNA+ cells of radial glia, blue arrows show PCNA+ cells in deep layers, red arrows point to PCNA– cells. B - radial glia (contoured by square) in *stratum marginale* (SM) of optic tectum. SO: *Stratum opticum*. C - PCNA+ radial glia (white arrows) and PCNA– cells (red arrows) at high magnification. D - PCNA+ cells (red arrows) in *stratum griseum et album superficiale* (SGAS), *stratum griseum centrale* (SGC), *stratum album centrale* (SAC), PCNA– cells (black arrows); cell cluster forming the neurogenic niche is contoured by oval. Scale bars: 100 µm (A, B, D) and 50 µm (C). E - number of PCNA+ elements: radial glial cells and immunopositive cells in the deep layers of optic tectum. Tukey's post-hoc test was used to determine significant differences in radial glia cells and immunopositive cells in deep layers. Error bars represent SEM (n = 5; #P < 0.05). F - optical density of PCNA-labeling in radial glia and cells in deep layers of optic tectum (mean ± SEM). UOD: Units of optical density.

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5. Proliferative Response in the Fish's Brain After Injury

After stab-wound injury, proliferative activity in trout brain was investigated in the cerebellum and optic tectum. The optic tectum is known to be the target of primary retinal projection. After mechanical eye injury, proliferative activity was found in the cerebellar DMZ, as well as in single parenchymal cells located in basal part of molecular layer, granular layer and infraganglionic plexus. In this study, we identified PCNA- clusters of activated cells in regional neurogenic niches. Proliferative activity was found in the dorsal matrix zone of cerebellum, which was described in the cerebellum of zebrafish [8] and *Apteronotus leptorhynchus* [7]. In the brain of zebrafish, during a 30 minute period, about 6,000 cells formed, which correspond to 0.2% of total cells in the brain [42]. Quantitative studies on the cerebellum *Apteronotus leptorhynchus* showed that during a 2 hour period, about 100,000 cells formed, which correspond to 0.06% of total brain cells [43]. In this study, several populations of intensively PCNA-labeled cells in different stages of mitosis were found in the DMZ, and less intensely labeled cells were considered post-mitotic elongated elements, migrating in radial and tangential directions outside from the DMZ towards damaged region. Another population of cells involved in the proliferative response of the CNS after mechanical damage represented PCNA- cell clusters with high cell density. These cell clusters were observed above and below the DMZ and in the infraganglionic plexus. We assume that these clusters of PCNA- cells are neurogenic niches activated after optic nerve injury. These observations are consistent with the data of Zupanc et al. [7, 43]. According to [7, 43], neural stem cells (NSC) are in specific proliferative zones "neurogenic niches" located in the molecular layer. Descendants of proliferated cells migrate along specific routes in the granular layer, where they are distributed evenly [7, 44]. The third population is PCNA+ cells arranged singly or in small clusters in the lower one third part of the molecular layer and granular layer.

The optic tectum is another sensory integration center in the trout brain. Earlier PCNA labeling for identification of proliferative activity of mesencephalic matrix zones was revealed in *Carassius carassius* [45]. It was found that PCNA labeling allows the identification of patterns of distribution of mitotically active cells in the brain that form morphogenetic fields - the matrix zones. Matrix zone labeled by PCNA in the mesencephalon, has also been identified in the adult sturgeon *Acipenser shrenkii* [46].

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After mechanical eye injury in trout, PCNA immunopositivity was detected in radial glial cells, single parenchymal cells of inner layers and in the periventricular tectal region. It is known that the populations of radial glia in the brain of fish are not homogenous and include rapidly and slowly proliferating cells [47]. We assume that after stab wound injury of trout optic tectum, proliferative activity was enhanced in slowly proliferating cells in radial glia. This assumption is based on data from densitometric analysis of PCNA activity in tectal radial glia cells. Compared with the density distribution of single parenchymal PCNA+ cells in the deeper layers of the optic tectum, the density distribution of PCNA+ cells in the radial glia was not high. We believe that after eye injury, only a part of radial glia population in the optic tectum proliferated. The optical density of radial glia immunolabeling allows allocation of a few cells with high PCNA activity (114 OD units). We interpreted these cells as neuronal precursors [48] in the state of asymmetric mitosis that was previously set for radial glia cells [49]. Another more numerous population of radial glia in the trout optic tectum had 104 OD units; such cells we considered as post-mitotic cells. A large cluster of PCNA+ cells were detected in the periventricular zone of caudal optic tectum, also found in other fish species [6, 7]. PCNA immunopositivity in the trout optic tectum was mainly found in small cells. At 1 week after optic nerve injury, the proliferative activity was mainly found in the dorsal matrix zone of the cerebellum, radial glia and primary periventricular zone of the optic tectum, and some parenchymal cells. We identified the radial and tangential patterns of cell migration in the cerebellum, among which we detected PCNA+ and PCNA- elements. We identified PCNA- neurogenic niches especially in the dorsal part of the cerebellum and in the optic tectum.

Our results showed that: (1) destroying the integrity of the eyes and injury to optic nerve head lead to intense proliferation and migration of cells in the optic nerve head region. We assume that optic nerve cells with proliferating reactive astrocytes arise in response to optic nerve injury. (2) Adjacent muscle fibers damaged by trauma are a source of regional macrophages involved in the neuroimmune interactions during the post-traumatic period. (3) As a result of combined mechanical eye injury and optic nerve crush in the sensory integration centers cerebellum and optic tectum, proliferative response occurred. In the dorsal part of the cerebellum in response to mechanical eye injury and optic nerve crush, we identified neurogenic niches and radial and tangential cell migration. The number of PCNA+ radial glia cells increased in

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the parenchyma and neurogenic niches of the optic tectum. These data indicate that injury of the optic nerve head can cause the proliferative response of cells in the matrix areas and activation of neurogenic niches.

6. Marker Neuronal Expression HuC/D in the Proliferative Zones of the Trout Brain after the Mechanical Injury of the Optic Nerve

Our study showed that the neuronal protein HuC/D is expressed in the definitive centers and proliferative regions of the adult trout brain. In the telencephalon, regions of neurogenesis adjoined to the external borders of the dorsal and ventral regions were found (Figure 6A, B, C, E, F). Since the telencephalon of teleosts has the everting structure of hemispheres, the proliferative zone (PZ) is located at the outer wall of the cerebral hemispheres. It was found that the PZ in the medial part of the dorsal region of hemispheres has a laminar structure; HuC/D expression was detected in the surface layers of the region indicating a very early neuronal differentiation of cells formed after the traumatic injury (Figure 6A). On the border of the dorsal and medial areas, the maximum number of HuC/D+ cells and the highest level of HuC/D activity was found among the cells formed in the posttraumatic period (Figure 6B). In the lateral zone, the local accumulations of HuC/D+ cells were present (Figure 6C). However, the total thickness of the proliferative layer and the proliferative HuC/D expression in cells in the proliferative region was minimal in this region and maximal in the dorsal region. The general scheme of the dorsal and ventral regions of the trout telencephalon and the distribution of proliferative zones are shown in Figure 6D.

In the *ventral* region of the trout *telencephalon* corresponding to the striatal formation of other vertebrates, the HuC/D expression was prominent in the definitive nuclei: dorsal and ventral (Figures 6E, F). Investigation of the expression of neuronal proteins HuC/D showed that the HuC/D expression level was significantly different in the definitive areas of the forebrain (medial, dorsal and lateral areas of the dorsal region and the dorsal and ventral regions of the ventral area), as well as in the PZ of the ventral region [3]. Typically, the newly formed cells in the ventral part of the proliferative region had a very high level of HuC/D activity [3], while the neurons of definitive regions the intensity of HuC/D immunolabeling could be low.

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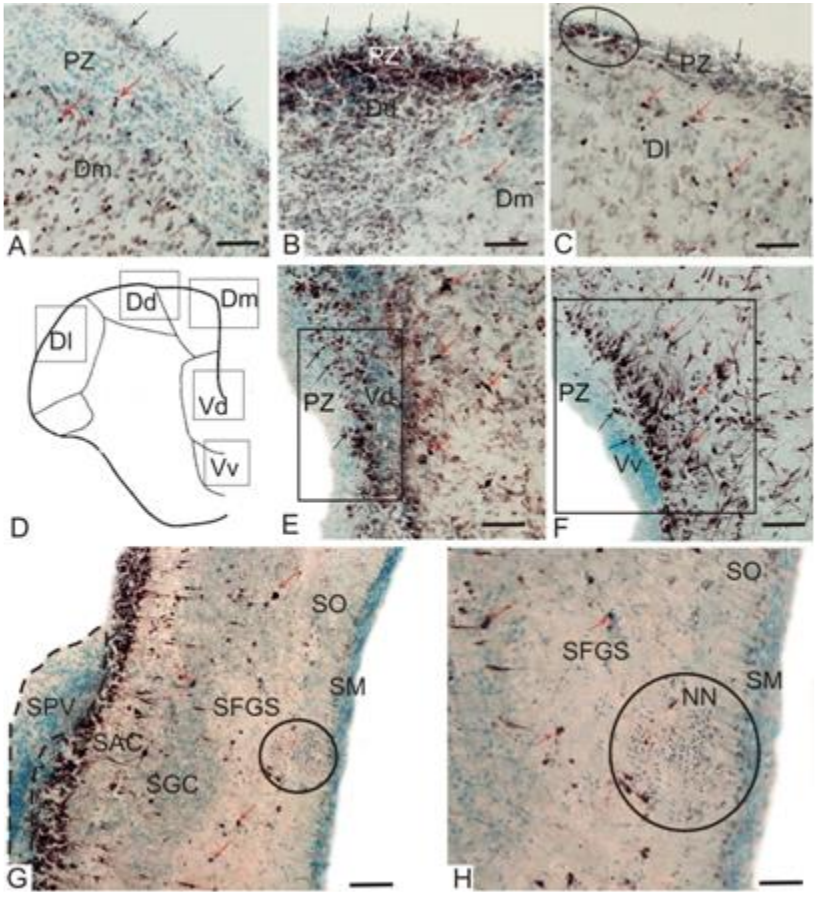

Figure 6. Localization of neuronal protein HuC/D in the proliferative regions of the telencephalon and optic tectum 2 days after the mechanical injury. A - HuC/D+ cells (black arrows) in the proliferative zone (PZ) and deep layers (red arrows) of the medial region of the telencephalon (Dm); B - at the border between the dorsal (Dd) and medial (Dm) regions; C - in the lateral region (Dl), an oval marks the conglomerate of HuC/D+ cells; D - dorsal and ventral regions of the trout telencephalon containing proliferative zones from figures A–C and E–F; E - HuC/D+ cells in PZ (marked with a square) of the dorsal nucleus of the ventral part of the telencephalon (Vd); F - ventral nucleus of the ventral region (Vv) (highlighted with a square); G - HuC/D-immunolabeling in the optic tectum, (designations are the same as at 5B–D), an oval contoures the neurogenic niche, the dotted line highlightes the caudal proliferative region of the tectum; H - neurogenic niche in the tectum (NN) at larger magnification. Immunoperoxidase labeling of HuC/D cells on sections *in situ*. Scale bar: 100 μm (A–C, E, F), 200 μm (G), and 50 μm (H).

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In the *optic tectum*, the HuC/D activity was found in mature neuronal populations of virtually all layers except the marginal layer (ML) and the optic fiber layer (OL) (Figure 6G). HuC/D+ cells were found in the different layers of the tectum (Figure 6G). In the surface fibrous and cellular and the central gray layers, cells of various types were found. In the caudal tectum, neurogenic areas of activity were identified: the periventricular layer with significantly higher thickness than that of the central and rostral area, and the appearance of neurogenic niches in the surface layers of the tectum (marginal and optical) (Figures 6G, H). In these areas, the HuC/D activity in a centrally located cells was not found, but single HuC/D+ cells or small groups of these cells were located on the periphery (Figure 6H), which indicated the undifferentiated pluripotent nature of the cells of these regions in the midbrain. In the neurogenic niches, HuC/D- small cells were found.

In the trout *cerebellum*, regions containing HuC/D+ cells were also identified in the definitive cell molecular, granular layers, and infraganglionic plexus, as well as in areas with neurogenic activity: DMZ, neurogenic niches, molecular and granular layers, and so called granular elevations 2 days after the mechanical injury (Figures 7A–F). DMZ was located in the dorsal-medial part of the molecular layer, just above the granular layer (Figure 7B). PCNA+/HuC/D- elongated cells were found in the DMZ; and highly HuC/D+ cells surrounded the DMZ and formed localized areas of increased density distribution (Figure 7B).

The local neurogenic niches morphologically similar to those in the tectum were found in the body of the cerebellum after the optic damage (Figures 7C, D). The most part of the neurogenic niche was HuC/D-, but HuC/D+ cells were located around and within the niche, which indicated neuronal differentiation of such cells early in the posttraumatic period (Figure 7D). The tangential and radial cell migration was found on the dorsal-lateral surface of the body of the cerebellum in the molecular layer (Figures 7E, F). HuC/D- elongated cells stained with methyl green formed multilayered series of migrating cells in the surface and inside the molecular layer (Figure 7E). In these tangential flows, the distribution density of HuC/D+ cells was smaller than in other parts of the molecular layer. Radial migration patterns were found in the lateral areas of the body of the cerebellum (Figure 7F). On the lateral and dorsal surface of the cerebellar body, the intensive HuC/D+ cells were often found in the surface layers (Figures 7C, D).

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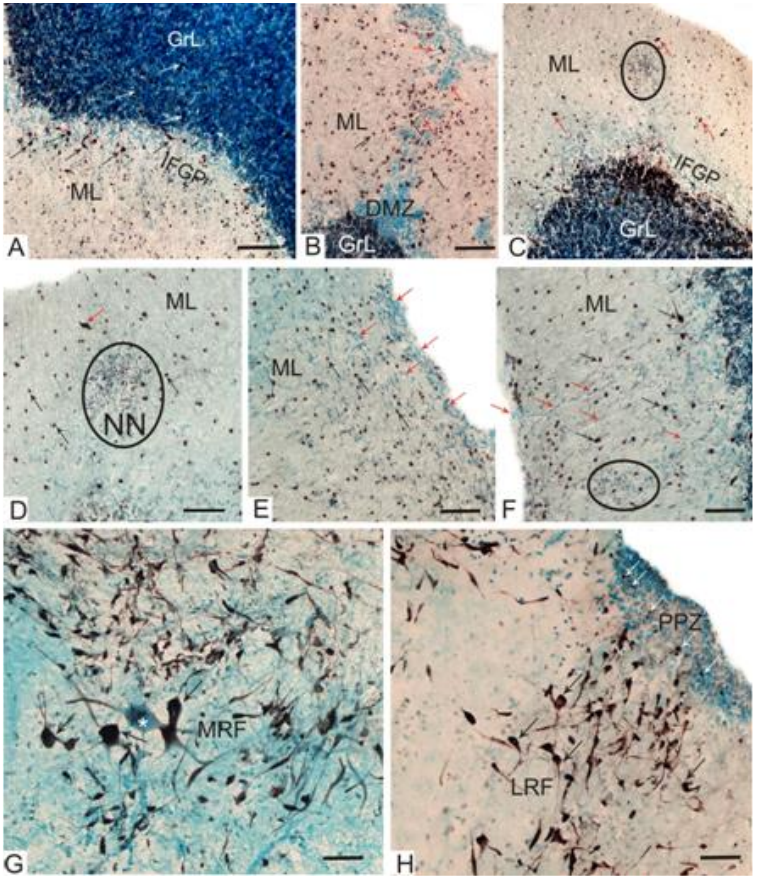

Figure 7. Localization of neuronal protein HuC/D in the proliferative zones of the cerebellum and medulla of a trout 2 days after the mechanical injury. A - general view of the cerebellar body, the black arrows show immunopositive differentiated IFGP cells, white is neurons of the granular layer; B - immunolocalization in DMZ, red arrows show HuC/D– migrating cells; C - in the dorsal part of the cerebellar body, an oval contours the neurogenic niche; D - a fragment containing neurogenic niche in the higher magnification; E - patterns of the tangential migration of HuC/D– cells (red arrows) in the surface layers of the molecular layer; F - radial migration of cells in the lateral part of the body of the cerebellum, an oval contours the neurogenic niche; G – in the medial reticular formation (MRF) of the medulla oblongata, white star marks a large neuron with low HuC/D activity, red arrows show highly differentiated neurons; H - in the periventricular proliferative zone (PPZ) and lateral reticular formation (LRF), white arrows indicate HuC/D+ cells in the PPZ. Immunoperoxidase HuC/D labeling in sections *in situ*. Scale bar: 200 μm (A, C) and 100 μm (B, D-H).

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In the trout *medulla oblongata*, the definitive cells were found in the reticular formation, and cells with HuC/D activity were found in the periventricular proliferative region (Figures 7G, H). Definitive neurons of the reticular formation had different levels of the HuC/D activity in large, medium, and small cells (Figure 7G). In the periventricular region, the high HuC/D expression was detected in cells of the medial, lateral, and dorsal proliferative zones (Figure 7H). Thus, all periventricular proliferative regions of the medulla oblongata were characterized by the presence of morphologically heterogeneous but intensively HuC/D labeled cells in contrast to the definitive areas of the reticular formation containing the largest brain cells but having heterogeneous (moderate or low) HuC/D activity.

7. Neurogenesis in Proliferative Brain Regions of a Trout Induced by Stab Wound Injury of Eye

After mechanical injury of the optic nerve, a significant proliferative activity was found in the brain proliferative regions of a trout. The newly formed cells in these areas are differentiated into various cell types, including neurons. To estimate the number of neurons formed as a result of the mechanical injury of the optic nerve and the retina, the proliferative brain regions, including secondary proliferative and periventricular zone of the forebrain, tectum, cerebellum, and brainstem of a trout, were investigated [3].

The results showed the intensive formation of new neurons in various centers of the brain. Neurogenesis has been reported in the dorsal and ventral regions of the matrix everted regions of the trout telencephalon. The studies in zebrafish showed that the newly formed cells began to express neuronal protein HuC/D 3–4 days after injury in the dorsal telencephalon [22, 50, 51]. These new neurons appeared near the injury site and in remote areas of the damaged regions. In the following days, the number of HuC/D+ cells gradually increased, especially in the area of the damage. Spatial and temporal distribution pattern of these cells showed that newly formed cells acquired phenotypic features typical of neurons during the migration to the site of injury [1]. According to our observations in a trout, the most intensive formation of new cells occurring 2 days after the injury was typical of the dorsal proliferative zone. The maximum distribution density of small, undifferentiated HuC/D+ cells located under the HuC/D- layer of proliferating cells has been identified in this area. Similar processes were found in other

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areas of the dorsal region: medial and lateral with specific structural organization, in particular, the medial PZ was larger in comparison with the lateral PZ. In the ventral region, a thick HuC/D- cell layer was detected both in the dorsal and ventral nuclei. In these structures, the layered organization separating PZ from definitive HuC/D+ differentiated cells was clearly visible. In the ventral nucleus, undifferentiated HuC/D+ cells located in the PZ were found, which indicated early neuronal differentiation of cells formed in the posttraumatic period. However, in the telencephalon of a trout, we did not find the typical neurogenic niches after the damage, unlike in other parts of the nervous system — the tectum and cerebellum. Nevertheless, HuC/D- undifferentiated cells were found in the dorsal region of the dorsal area along with HuC/D+ undifferentiated cells in the deep zone. We consider that the appearance of these elements in the Dd region is a response to the injury. The density of distribution of HuC/D- cells in the Dd was quite high. This could indicate the presence of specific types of neurogenic niches in the telencephalon of a trout, which was not previously found in fish.

In the tectum and cerebellum of a trout, the activation of large regional neurogenic niches occurred in the dorsal part of the body of the cerebellum (in the molecular layer) and the optical layer of the tectum, while smaller niches activated in the infraganglionar plexus and lateral areas of the cerebellar body. Another feature of the reparative neurogenesis in the tectum of a trout is the emergence of PCNA+ radial glial cells in the surface layers of the tectum. We consider this feature to be associated with adaptive properties of the brain center, which is a direct retinal projection and has a close functional relationship with the injured region. An increase in proliferative activity in the caudal tectum of a trout is also a sign of increased proliferative potential of the brain, and we consider it as an additional repairing reserve in the trout brain. An increase in the speed of neurogenesis in proliferative zones of the trout brain, particularly in the periventricular region of the medulla oblongata, in combination with a high HuC/D activity in the cells in the periventricular area, in our opinion, is also a complex response of the CNS to the reparative stimuli.

The mentioned studies suggest that an increase in the production of new neurons in the proliferative zone of the forebrain, the optic tectum, cerebellum and brain stem in adult trout occurred 2 days after the eye and optic nerve injury, in contrast to *D. rerio*, in which a similar response occurs 3–4 days after the injury [50]. In the telencephalon of adult trout, the most intense neurogenesis is observed in the dorsal area of the Dd proliferative region. In the optic tectum, neurogenesis is significantly enhanced in the caudal periventricular proliferative region among parenchymal cells in the layers of

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the tectum. In the surface layers (marginal and optical) of the tectum, neurogenic niches containing proliferating PCNA+/HuCD– cells and HuCD+ cells were discovered. In the cerebellum, there was a significant increase in cell proliferation in the dorsal proliferative region among isolated parenchymal cells of the molecular and granular layers. In the lateral part of the cerebellar body, the neurogenic niches with the structure similar to those in the optic tectum. In the medulla oblongata, the high proliferative activity of periventricular region and adjacent areas of the dorsal, lateral, and medial reticular formation with high levels of HuC/D activity was identified. Thus, our results suggest that neurons formed as a result of the reparative process are characterized by a high level of expression of HuC/D neuronal marker in comparison with the cells of definitive centers of the brain. Based on the data, we assume that this peptide plays an active role in the adult and reparative neurogenesis.

8. Evaluation of Proliferative Potential of Trout Brain Cells *In Vitro*

Primary culture of cells. During culture, small fractions of cells adhered on the surface of culture dish and formed a monolayer fraction. Some of these cells began to form outgrowths (Figure 8A). The greater proportion of the cells remained in suspension (Figure 8B–D). Investigation of the cells in suspension revealed that pretreatment of brain cells with trypsin or collagenase in different modes and conglomerates tended to form (Figure 8C, D). Analysis of the cellular composition of conglomerates revealed that some of them were composed of isometric elements, which may be the descendants of a single cell and therefore represent typical neurospheres (Figure 8E). Other types of conglomerates were composed of heteromorphic elements and were apparently formed for the second time after changes in the surface properties and the adhesiveness of cells during preparation of the primary cultures (Figure 8F). After analyzing the cellular composition of conglomerates, we found that the predominant cell types are small type 5 cells (Table 2). Mitosis was observed after 1 day of culture, in type 5 isometric cells, with a diameter of about 8 μm. On the second day of culture, the ratio between the fraction of cells in suspension and the cells in the conglomerate differed from that on the first day. All cells in the neurospheres were of round shape with the same diameter of the transparent light cytoplasm (Figure 8E).

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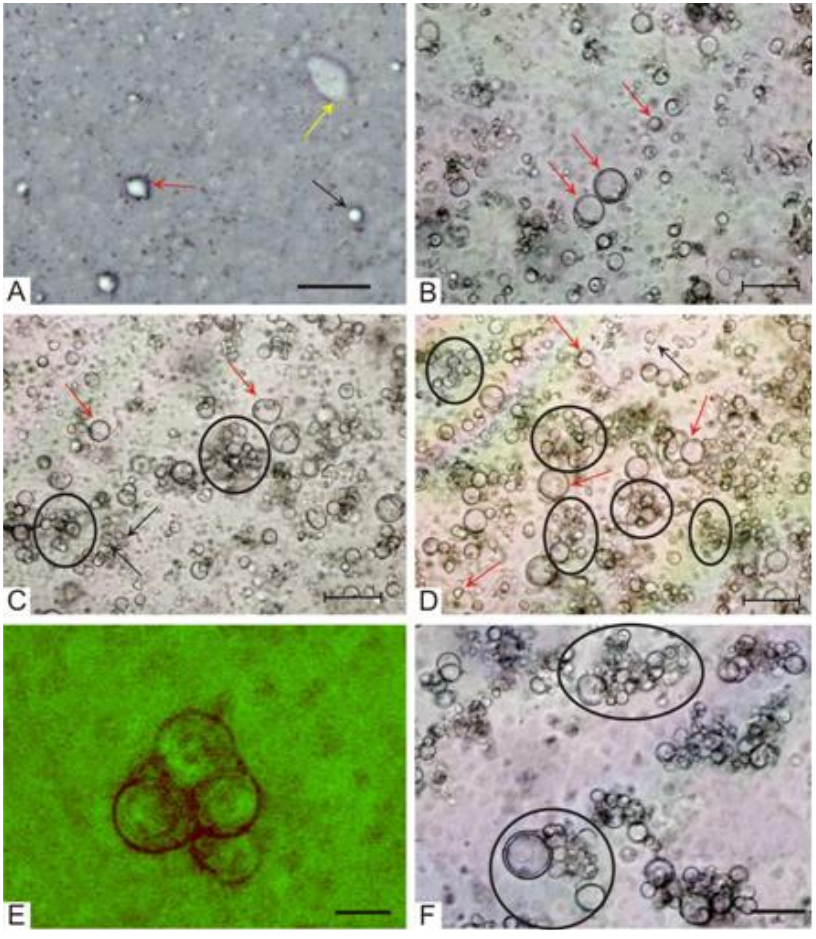

Figure 8. Phase contrast monitoring in primary culture of trout brain cells. A - cells in monolayer; colored arrows indicate the different types of cells: big cell (yellow arrow), cells with outgrowth (red arrow) and cell without outgrowth (black arrow). B - suspension fraction of brain cells after 1 day of culture. Red arrow shows single cells. C – suspension fraction of cells on the 2nd day of culture. Ovals contour cell conglomerates, and red arrows indicate single cells. D - on the 4th day of culture. E - general view of neurospheres. F - heterogeneous conglomerates of cells in suspension on the 4th day of culture. Scale bars: 50 µm (A–D, F), 10 µm (E).

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Table 2. Parameters of trout brain cells (mean ± SEM) in suspension and conglomerates after 1 and 2 days of culture (according to classification of Arevalo et al. (1995))

Type of cells	1 day suspension		1 day conglomerates		2 day suspension		2 day conglomerates	
	Size of cells* (µm)	%	Size of cells (µm)	%	Size of cells (µm)	%	Size of cells (µm)	%
2	25.6 ± 4.6/24.4 ± 4.7	12.3	21.5 ± 2.6/16.7 ± 1.3	4.7	21.7 ± 0.8/20.5 ± 1.7	5.4	22.3 ± 2.9/21.6 ± 2.6	5.7
3	17.3 ± 1.7/16 ± 2.3	16.4	16.3 ± 1.3/15.2 ± 2.5	19	16.8 ± 1.2/15.7 ± 1.3	17.8	16.2 ± 1.3/14.5 ± 0.8	27.6
4	12.3 ± 1.3/11.3 ± 1.5	58.7	12.3 ± 1.4/11 ± 0.9	23.8	12.5 ± 1.5/11.3 ± 1.6	52	11.1 ± 1.3/10 ± 1.3	27.8
5	8.9 ± 0.9/8.1 ± 0.8	12.3	7.6 ± 1/6.9 ± 1	52.3	8.4 ± 1.2/7.8 ± 1.4	24.6	8.3 ± 1.1/7.6 ± 1.3	38.8

***** - big and small sizes of cells are separated by a slash

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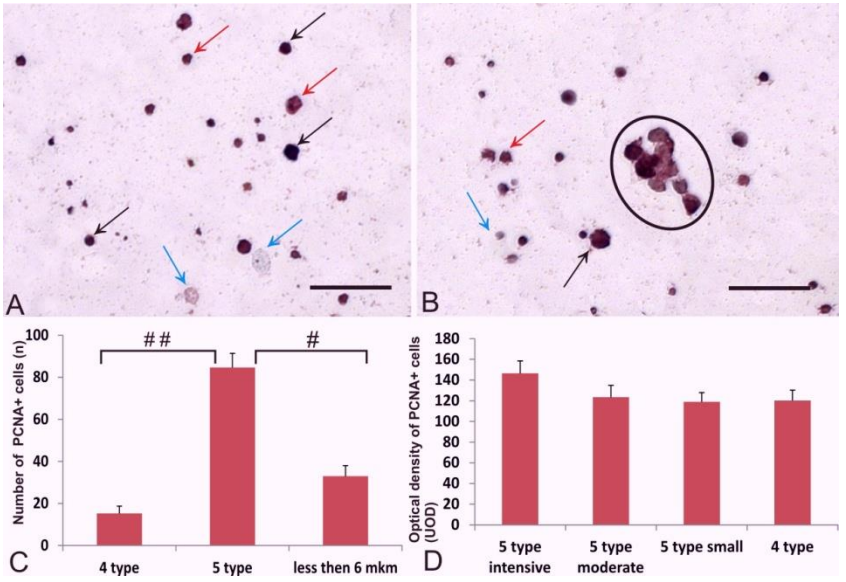

Figure 9. Proliferative cell nuclear antigen (PCNA) labeling of trout brain cells after 4 days in primary culture. A - highly active PCNA+ cells (black arrows), moderately active PCNA+ cells (red arrows) and PCNA– cells (blue arrows); B - conglomerate of highly and moderately labeled PCNA+ cells (contoured by oval); arrows represent the same cells as in A. Scale bars: 50 µm (A, B). C - Tukey's post-hoc test was used to determine significant differences in number of PCNA+ cells. Error bars represent (mean ± SEM, #P < 0.05, ##P < 0.001). D - optical density of PCNA+ cells (mean ± SEM).

Evaluation of proliferative activity in suspension fraction of trout brain cells. After 4 days of culture, cells in the brain of trout were analyzed for immunoperoxidase labeling of PCNA. The results of immunohistochemical labeling showed that PCNA-immunopositivity was present in small cells (Figure 9A, B). Type 5 PCNA+ cells, accounting for 84.7%, had a diameter of 6.9 ± 1.5/5.8 ± 1.3 µm and type 5 cells, accounting for 15.3%, had a diameter of 11.6 ± 1.1/9.2 ± 1.7 µm. Among type 5 cells, we identified a small subpopulation of cells with a diameter less than 6 µm. These cells accounted for one third of all type 5 cells (Figure 9C). Most of small type 4 and 5 cells had two levels of PCNA immunoreactivity: the high level with the OD of immunolabeled precipitates in cells being 146,6 ± 5,7 OD units and the low level with the OD of immunolabeled precipitates in cells being 123.4 ± 8.2 OD units (Figure 9D). Strong PCNA activity was detected in cells with the diameter of 8.3/7.8 µm; frequency of densely labeled cells per visual field was

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low and accounted for no more than 5–6 elements (Figure 9A). Occasionally, we encountered PCNA+ small cell clusters, which consisted of 2–3 cells and were densely labeled, and the remaining cells were less intensely labeled (Figure 9B). In small type 5 cells, the level of optical density was 118.8 ± 3.7 OD units.

9. Primary Culture of Trout Brain Cells

Results from this study showed that the linear volume of cell conglomerates increased with culture time. We also found that the sizes of conglomerates range from 33/19.9 to 99.6/62.7 μm on day 1 of culture and from 23/20.4 to 105/62.3 μm on day 4 of culture. This clearly illustrates the gradual linear increase in the size of conglomerates and gives the reason to conclude that the *in vitro* proliferation processes of cells of the CNS of adult trout can be recorded by cell culture. Findings regarding patterns of mitosis and of cells entering into differentiation processes and forming a monolayer suggest the utility of such primary culture systems for observing the process of proliferation and differentiation *in vitro*, which are otherwise difficult to be observed *in vivo*. Interestingly, after 4 days of culture, most of the cells were in suspension while others remained in the form of adherence [53].

We further confirmed that the majority of cells in suspension were proliferating but not dead and some of them conglomerates. We also observed higher proliferative activity in the suspension compared to in the form of monolayer. In this study, we did not pay special attention to creating special conditions for the subsequent differentiation of cells in the monolayer, and therefore, we did not add specific growth factors to the growth medium. Significant proliferative activity was observed in cells in suspension and as part of conglomerates. Immunohistochemistry analysis of suspension cells showed that type 4 small cells were in the proliferative state.

Analysis of the cellular composition of conglomerates revealed that some conglomerates are formed by isometric elements, which may be the descendants of a single cell and, therefore, represent typical neurospheres in suspension. Detection of neurospheres, which we interpreted as descendants of stem cells, was confirmed by immunohistochemical labeling of PCNA [53]. PCNA immunopositivity was found predominantly in single cells and small cells forming conglomerates. Unequal intensity of PCNA labeling of cells in the conglomerates in our opinion indicates heterochronic proliferation of these cell clusters. Thus, we consider the most intensely labeled cells as being able

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to direct the proliferation while cells with comparatively lesser intensity as the cells in which mitosis is complete. In trout brain cell suspension, we identified very small cells (less than 5 μm in size) with the highest activity of PCNA labeling. We believe that this type of cells are the most actively proliferated population of cells of the adult trout brain and maybe this type of cells may be located in the proliferative zones of adult trout brain.

In this study, we did not specifically isolate cells of the matrix areas of the brain, but culture of cells from the whole trout brain showed that cells retain much high capacity to proliferate. Perhaps one explanation for a significant proliferative potential in cultured trout brain cells is the lack of growth factors in the culture medium. The formation of two types of conglomerates, i.e., heteromorphic and homogeneous, indicates the high proliferative potential of brain cells to successfully proliferate in the culture.

Thus, we isolated cells from the brain of trout and cultured them *in vitro* under the standardized conditions as described above. We observed formation of heteromorphic and isomorphic (neurospheres) conglomerates with high proliferation activity along with the cells in suspension. As shown earlier, neuronal regeneration is intimately linked to adult neurogenesis. Different species of fish examined thus far also generate new neurons constitutively, both in large numbers and in many regions of the adult CNS. Comparative analysis has suggested that adult neurogenesis is a primitive vertebrate trait [54]. It is likely that the availability of all the cellular and molecular regulatory mechanisms necessary for the generation of new neurons in the intact CNS has greatly facilitated the repurposing of the cellular machinery for neuronal regeneration with only slight adaptive changes.

A broad understanding of the biology of adult neurogenesis and neuronal regeneration will also facilitate the analysis of the selective pressures that have caused the loss of the regenerative potential during the evolution of mammals. We tend to further study the process of differentiation in these cultured cells and propose them to be used as a model system for further study of mechanism underlying adult neurogenesis in the trout model.

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Chapter 6

EFFECTS OF PLANT-BASED FEEDS ON THE IMMUNE RESPONSES OF RAINBOW TROUT *ONCORHYNCHUS MYKISS* (WALBAUM)

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ABSTRACT

The rapid growth of the aquaculture during the last two decades has been accompanied by an increasing demand for aquafeeds. From several years, fish meal and oil were used as the main sources of proteins and lipids in the diets, respectively. However, with no expectations to increase the production of both ingredients to meet the demand, the industry now is using alternative sources of protein and lipids. Particularly, plant-origin meals and oils are being using in several species of carnivorous fish such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Most of the research has been focused in the growth performance and few attention have been given to the immune

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responses of the fish fed diets with plant meals and oils. In here, we present results of immune responses (non-specific mainly) of fingerlings and juveniles of rainbow trout fed different diets with high contents of different soybean products. In general, non-specific immune responses (lysozyme activity, macrophage burst activity) were not affected by the inclusion of high levels of soybean protein products during periods around 90 days. Besides these finding, a brief review of the effects of another immune responses are included for the rainbow trout when fed other plant-origin protein sources.

INTRODUCTION

The growth and intensification of salmonid culture during the last few years had caused an increased demand for feeds (Hardy 2010), and accordingly, the ingredients for their production. Fish meal and oil are still widely using as main protein and lipids sources (respectively), and even their production will be stable for the next decades, the demand will surpass the offer in a few years (Gatlin et al. 2007; Kaushik & Seiliez, 2010), so the aquaculture industry now is looking at new protein and lipid sources, such as the plant-origin meals and oils. Research in the use of plant-origin ingredients has been exhaustive in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), but mainly focused in growth performance and recently on the effect of these ingredients on gene expression. However, few information is available regarding the effect of such ingredients in the immune response of the salmonids (Burrells et al. 1999; Jalili et al. 2013; Krogdahl et al. 2000). Based on this, we have performed several feeding trials to determine the effects of plant-origin protein sources, mainly soybean products in the growth performance and some immune responses of rainbow trout fingerlings and juveniles, so we present the findings and we give a brief of how research must move on regarding the use plant protein sources in rainbow trout culture.

FEEDING TRIALS

Feed Formulation

Diets were formulated based on the nutritional requirements reported for the species by NRC (2011) and are show in Table 1. With the exception of

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Diet 2, the other three are based exclusively in soybean meal or soy protein concentrate.

Table 1. Diet formulations of the different feeding trials for rainbow trout

Ingredients (g/kg)	Treatments			
	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	0	200	0	0
Soybean meal	450	400	0	0
Soy protein concentrate	0	0	483.8	645.1
Spirulina powder	150	0	166.7	0
Yeast	15	15	15	15
Other[1]	290	290	290	290
α-cellulose	95	95	45	44.9
Proximate composition (% dry weight basis)				
Protein	38.0 ± 2	42.1 ±1.0	43 ± 1.0	43.1 ± 2.0
Lipids	10.2 ± 2	11.2 ± 0.7	10.9 ± 0.3	10 ± 1.1
Ash	6.0 ± 0.2	12 ± 0.5	7.0 ± 1.0	6.6 ± 1.3

[1]Other ingredients (g/kg): fish oil (50), soybean lecithin (50), dextrin (100), mixture of vitamins and minerals (40), wheat gluten (50).

Feeding Trials

The diets 1 and 2 were used under practical conditions in a trout farm "Rancho Los Alevines," located in the municipality of Amanalco de Becerra (State of México, México), by using 500 L fiber glass tanks. The other two trials were performed under laboratory conditions at the Laboratorio de Produccion Acuícola of the FES Iztacala, UNAM, by using a recirculating system with 100 L polypropylene tanks. Each diet was fed to triplicated groups at 7% of the total biomass per tank. Daily ration was divided into equal feedings, one in the morning and other during noon. In all cases, the trials were follow from a minimum period of 60 days. At the end of each trial, the fish were weighted to obtain the growth performance and then sacrificed to obtain the sample for the nonspecific immune responses.

Nonspecific Responses

Basically we determined two nonspecific responses: lysozyme activity in the blood serum and macrophage burst activity. Lysozyme activity in plasma was determined by the technique reported by Caruso et al. (2002). For the macrophage burst activity, kidneys were dissected and the macrophage were

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isolated and cultivated in Leibovitz L-15 medium supplemented with fetal bovine serum (Burrells et al. 1999). Macrophage burst activity was determined with the reduction of NBT (Chung & Secombes, 1988).

Statistical Analysis

Data were tested with the Shapiro and Wilk W test and Barlett´s test for normality and homoscedasticity, respectively. Data expressed as percentage were arcsine transformed and then tested. Since all data showed normality and homoscedasticity, they were compared by one-way ANOVA (package Prism 6.0 for Mac, GraphPad Sofware, Inc.). When found the statistical differences between treatments were evaluated by a Tukey multiple comparison test, considering an error of 5% ($P < 0.05$).

FINDINGS

Tables 2 to 5 show the growth performance of rainbow trout fed the different experimental formulations. Diets based on soybean meal showed lower values on the growth performance (Tables 2 and 3) when compared with a commercial diet, while fish fed the diets based on soy protein concentrate (Tables 4 and 5) showed higher growth than the observed on those fed the commercial. Previously, Castro et al. (2011) reported that soybean meal might substitute up to a 75% of fish meal in diets for rainbow trout fingerlings with our affecting the growth. Still, soybean meal contains several anti-nutrient compounds (Krogdahl et al. 2010) that might be affecting growth in the long term. With the use of soy protein concentrate, a refined product of soybean meal, some of the anti-nutrients are washed away and might be helping in protein utilization.

Table 2. Growth performance of rainbow trout juveniles fed a diet with soybean meal, Spirulina powder and the inclusion of Baker´s yeast. Data are the mean of three replicated groups ± standard error. No significant differences were observed at this level (P < 0.05)

Treatments	IW (g)	WG (%)	SGR (%/day)	Survival rate (%)
Diet 1	1.8 ± 0.1	350 ± 52	2.0 ± 0.2	67
Control	1.8 ± 0.2	390 ± 35	2.1 ± 0.1	78

[1]Initial weight. [2]Weight gain = ((final weight – initial weight)/initial weight) x 100.
[3]Specific growth rate = ((ln final weight – ln initial weight)/days) x 100.

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Table 3. Growth performance of rainbow trout juveniles fed a diet with soy meal and inclusion of Baker´s yeast. Data are the mean of three replicated groups ± standard error. Means with different letter in the same column differ significantly (P < 0.05)

Treatments	IW (g)	WG (%)	SGR (%/day)	Survival rate (%)
Diet 2	12.8 ± 1.1	702 ± 69a	2.3 ± 0.09a	82a
Control	12.7 ± 1.6	1039 ± 115b	4.8 ± 0.1b	50b

Table 4. Growth performance of rainbow trout fingerlings fed a diet with soy protein concentrate and *Spirulina* powder. Data are the mean of three replicated groups ± standard error. No significant differences were observed at this level (P < 0.05)

Treatments	IW (g)	FW (g)	WG (%)	SGR (%/day)	Survival rate (%)
Diet 3	1.3 ± 0.02	10.1 ± 0.8	660 ± 74	3.3 ± 0.1	80
Control	1.3 ± 0.02	9.2 ± 0.3	598 ± 31	3.2 ± 0.07	73

Table 5. Growth performance of rainbow trout fingerlings fed a diet with soy protein concentrate and yeast. Data are the mean of three replicated groups ± standard error. No significant differences were observed at this level (P < 0.05)

Treatments	IW (g)	WG (%)	SGR (%/day)	Survival rate (%)
Diet 4	1.3 ± 0.09	758 ± 50	3.5 ± 0.1	91
Control	1.3 ± 0.1	671 ± 66	3.2 ± 0.1	86

Nonspecific immune responses are the first line of defense of fish and are more important in them than has been observed for mammals. Particularly, one of the important molecule is the lysozyme, an enzyme that shows high potential for bactericidal and bacteriolytic activities against Gram-positive and negative bacteria (Saurabh & Sahoo 2008). So, it is widely used as a marker for the immune status in fish. The activities of lysozyme recorded in the four trials are lower than the reported previously by Verlhac et al. (1996) as normal levels. Still, our results (Figure 1) show that lysozyme activity in the blood serum of rainbow trout fed several diets with soy products showed similar levels to those found in the organism fed a commercial diet (control group). The particular importance is the fact that even in practical conditions, fingerlings and juveniles of rainbow trout showed higher o similar values than

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the fish fed the control diet. Stress during the production, specially stock density, might decrease the levels of lysozyme (Caruso & Lazard 1999). Other important aspect is the nutrition status of the organisms: according to Saurabh & Sahoo (2008) nutritional support plays an important role in the maintenance of health condition and the plant-origin proteins are able in maintaining the well-being of the rainbow trout.

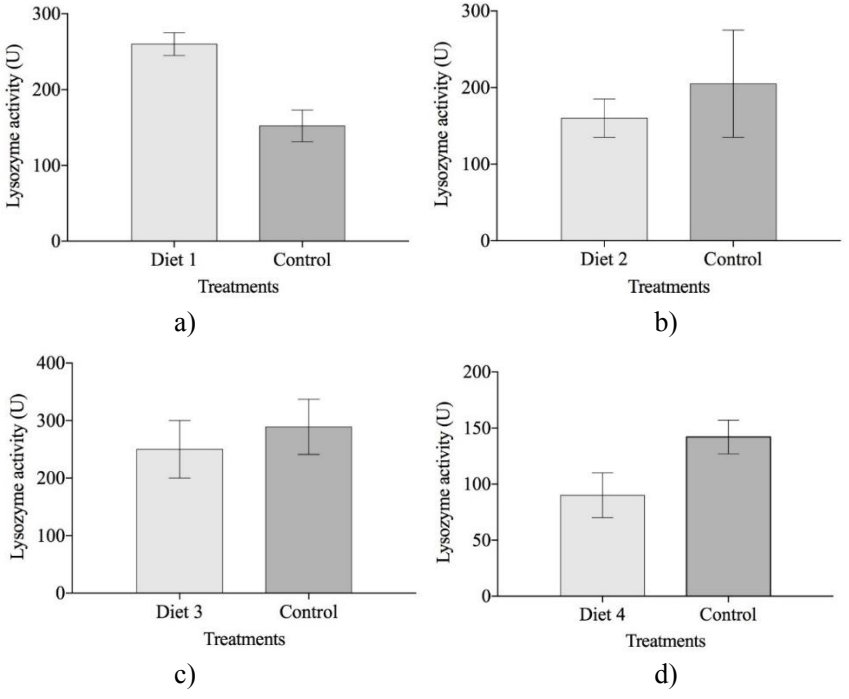

Figure 1. Lysozyme activity in the blood serum of rainbow trout fed different diet formulations based on soybean products. Each graph (letters A, B, C and D) represents a single feeding trial with the experimental diet and fish fed a commercial diet. Each column represents the mean of five replicate observations ± standard error. No significant differences were observed at this level ($P < 0.05$).

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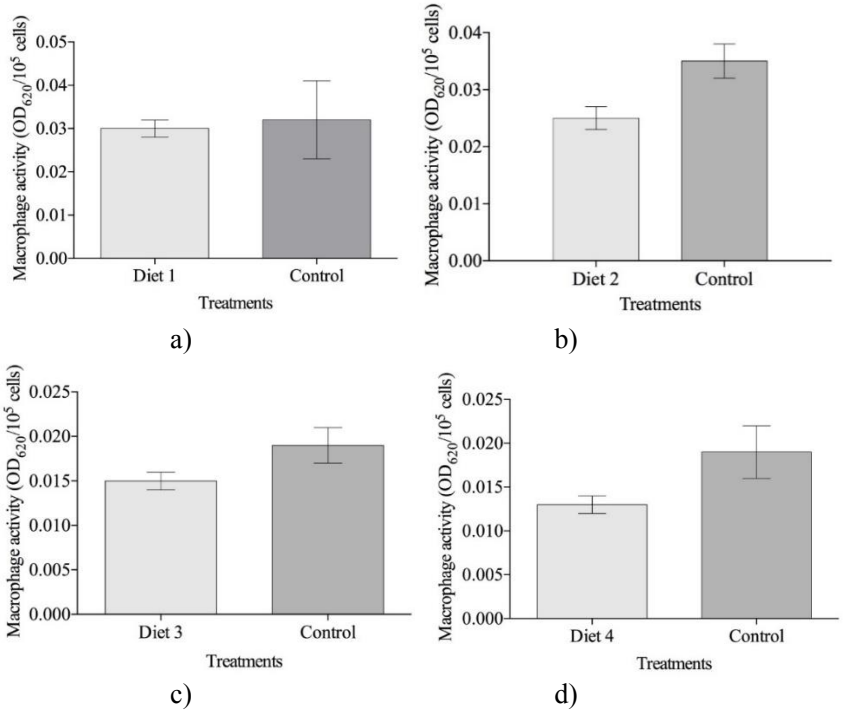

Figure 2. Kidney macrophage burst activity of rainbow trout fed different diet formulations based on soybean products. Each graph (letters A, B, C and D) represents a single feeding trial with the fish fed experimental diet and those fed a commercial diet. Each column represents the mean of five replicate observations ± standard error. No significant differences were observed at this level ($P < 0.05$).

As the lysozyme activity, the macrophage burst activity is an important nonspecific response in fish. Based in the production of reactive oxygen moieties such O_2- and H_2O_2, the macrophage burst is a potent microbicidal during episodes of infections (Chung & Secombes 1988). Previously, it was reported that soybean meal affects negatively the burst activity in rainbow trout (Burrells et al. 1999). However, the data obtained in the different trials with rainbow trout fed with high inclusion either soybean meal or soy protein concentrate, showed similar values when compared with the fish fed a commercial diet (Figure 2). This might indicate that during the fingerling stage, rainbow trout is able to use soybean meal as protein source without affecting the growth and nonspecific responses. During the feeding trial with Diet 2, the organisms began with a mean weight of 12.8 g and during this trial, rainbow trout fed the diets with soybean showed the biggest difference

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between the values of burst activity when compared with the control diet. It has been suggested that older organism became intolerant to soy products, but this affirmation need to be corroborate. Use of soy in high levels seems to be safe during the fingerling stage of rainbow trout, as not negative effects are detectable in growth and nonspecific immune response.

CONCLUSION

The use of plant-origin ingredients represents an opportunity for the Aquaculture industry to continue with the growth of the production. Soybean products, especially the soy protein concentrate, are able to be use as main protein sources in rainbow trout diets, as they do not have a negative effect on the growth and nonspecific immune responses, particularly during the fingerling2 stage of this species. Still, it is necessary to continue the research regarding the effects of such ingredients in the specific immune responses of rainbow trout. Other important thing is to use genomics tools to select organisms that show better growth and increased immune responses, both non- and specific, to develop specific genetic lines. This is particular important for Mexico, as rainbow trout production represents an annual production of 6,700 tons (CONAPESCA 2013), so selective programs are necessary to make culture a more profitable and sustainability activity.

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