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New Advances and Contributions to Fish Biology

Edited by Hakan Türker



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Contributors

Jacinto Elías Sedeño-Díaz, Eugenia López López, Claudia Turra Pimpão, Enio Moura, Rita Maria Mangrich Rocha, Ana Carolina Fredianelli, Luciana Do Amaral Gurgel Galeb, Francisco Pizzolato Montanha, Sebastian Reyes-Cerpa, Kevin Maisey, Felipe Esteban Reyes-López, Daniela Toro-Ascuy, Ana Sandino, Mónica Imarai, Miodrag Belosevic, Carlos Freitas, Alexandre Rivas, Caroline Campos, Igor Rechetnicow, James Kahn, Maria Correa, Michel Catarino, Javier Sánchez-Hernández, María J. Servia, Rufino Vieira-Lanero, Fernando Cobo, Ivan Viegas, John Jones, Miguel Pardal, Rui Carvalho, Glen Findlay Tibbitts

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Meet the editor



Hakan Türker is currently working as an Associate Professor in the Department of Biology at Abant İzzet Baysal University, Bolu, Turkey. He received his M.Sc. and Ph.D. from the Department of Aquaculture, Fisheries and Wildlife Biology, Clemson University, South Carolina, USA and did postdoctoral research at Tropical Biosphere Research Center, Ryukyus University, Okinawa, Japan. His current research interests include (a) integrated multitrophic aquaculture (b) fish biology (c) aquatic toxicology. He has published 14 papers in reputed international journals.

Contents

Preface XI

Section 1 New Advances and Contributions to Fish Biology 1

- Chapter 1 **Fish Cytokines and Immune Response 3**
Sebastián Reyes-Cerpa, Kevin Maisey, Felipe Reyes-López, Daniela Toro-Ascuy, Ana María Sandino and Mónica Imarai
- Chapter 2 **Cytokine Regulation of Teleost Inflammatory Responses 59**
Leon Grayfer and Miodrag Belosevic
- Chapter 3 **Regulation of Teleost Macrophage and Neutrophil Cell Development by Growth Factors and Transcription Factors 97**
Barbara A. Katzenback, Fumihiko Katakura and Miodrag Belosevic
- Chapter 4 **Freshwater Fish as Sentinel Organisms: From the Molecular to the Population Level, a Review 151**
Jacinto Elías Sedeño-Díaz and Eugenia López-López
- Chapter 5 **The Potential Impacts of Global Climatic Changes and Dams on Amazonian Fish and Their Fisheries 175**
Carlos Edwar de Carvalho Freitas, Alexandre A. F. Rivas, Caroline Pereira Campos, Igor Sant'Ana, James Randall Kahn, Maria Angélica de Almeida Correa and Michel Fabiano Catarino
- Chapter 6 **Evaluation of Toxicity in Silver Catfish 197**
Cláudia Turra Pimpão, Ênio Moura, Ana Carolina Fredianelli, Luciana G. Galeb, Rita Maria V. Mangrich Rocha and Francisco P. Montanha
- Chapter 7 **Functional and Structural Differences in Atria Versus Ventricles in Teleost Hearts 221**
Christine Genge, Leif Hove-Madsen and Glen F. Tibbitts

- Chapter 8 **Advances and Applications of Tracer Measurements of Carbohydrate Metabolism in Fish 247**
Ivan Viegas, Rui de Albuquerque Carvalho, Miguel Ângelo Pardal and John Griffith Jones

- Chapter 9 **Ontogenetic Dietary Shifts in a Predatory Freshwater Fish Species: The Brown Trout as an Example of a Dynamic Fish Species 271**
Javier Sánchez-Hernández, María J. Servia, Rufino Vieira-Lanero and Fernando Cobo

Preface

Fish is an important component of aquaculture with up to 80% of animal protein contribution especially in developing countries. Therefore, aquaculture is a bright spot with great potential in many countries worldwide. This potential raises the issue of achieving a sustainable and environmentally friendly aquaculture. Many areas need to be explored and developed. Updated information about some of the main issues that currently affects aquaculture was presented in this book for the scientific researchers in the field of aquaculture and fish biology. The book is sub-divided into nine distinct chapters. The importance of each of these contributions was briefly summarized here.

The understanding of the mechanisms that control inflammation in teleosts may allow for the development of strategies to prevent infectious diseases. Therefore, the main concepts of innate immune mechanism are reviewed in Chapter 1 by Sebastián Reyes-Cerpa, Kevin Maisey, Felipe Reyes-López, Daniela Toro-Ascuy, Ana M. Sandino, Mónica Imarai and Chapter 2 by Leon Grayfer and Miodrag Belosevic. Their reviews focus on the recent advancements in the understanding of the biology of hallmark fish pro-inflammatory cytokines. In the following Chapter 3 by Barbara A. Katzenback, Fumihiko Katakura and Miodrag Belosevic, the author are provided an overview of the current knowledge of the fish model systems on the sentinel cells (macrophages and neutrophils) of the innate immune response.

Jacinto E. S. Díaz, Eugenia L. in Chapter 4 discuss a short framework of effects of xenobiotics on the responses of freshwater fish across molecular to population level when have been exposed to environmental stressors. Their review considers the use of fish as sentinel organisms to assess the anthropogenic impacts over the freshwater ecosystems. Climate changes and dams are likely to represent the most important threats to freshwater fish around the world. The effects of climate change and dams on the ecosystem are reviewed by Carlos E. C. Freitas, Alexandre A. F. Rivas, Caroline P. Campos, Igor Sant'Ana, James R. Kahn, Maria A. A. Correa and Michel Catarino in Chapter 5. The silver catfish (jundiá) have been used for bioindicator of environmental contamination for many researches and can be used to aquatic biological systems. In Chapter 6 by Cláudia T. Pimpão, Ênio Moura, Ana C. Fredianelli, Luciana G. Galeb, Rita M. V. M. Rocha, Francisco P. Montanha aimed to review some aspects of the toxicology silver catfish.

Christine Genge, Leif Hove-Madsen and Glen F. Tibbits in Chapter 7 reviewed the roles of the atrium and ventricle in achieving variability with myocardial contractility among the teleost species. In chapter 8 Ivan Viega, Rui A. Carvalho, Miguel Â. Pardal, John G. Jones focus on the metabolism of carbohydrates by fish in aquaculture. In Chapter 9, Javier Sánchez-Hernández, María J. Servia, Rufino Vieira-Lanero, Fernando Cobo discuss the

variables that are involved in the feeding behaviour of brown trout as an example of a predatory freshwater fish species.

Finally, I would like to acknowledge the contributors for their cooperation. I also express my gratitude to Ms. Iva Simcic at Intech in assisting me with editing of this book.

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New Advances and Contributions to Fish Biology

Fish Cytokines and Immune Response

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Additional information is available at the end of the chapter

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1. Introduction

The immune system can be defined as a complex system that protects the organism against organisms or substances that might cause infection or disease. One of the most fascinating characteristics of the immune system is its capability to recognize and respond to pathogens with significant specificity. Innate and adaptive immune responses are able to recognize foreign structures and trigger different molecular and cellular mechanisms for antigen elimination. The immune response is critical to all individuals; therefore numerous changes have taken place during evolution to generate variability and specialization, although the immune system has conserved some important features over millions of years of evolution that are common for all species. The emergence of new taxonomic categories coincided with the diversification of the immune response. Most notably, the emergence of vertebrates coincided with the development of a novel type of immune response. Apparently, vertebrates inherited innate immunity from their invertebrate ancestors [1].

In higher vertebrates, the immune system consists of primary and secondary lymphoid organs with distinct compartments and morphology located in anatomically distinct sites. The thymus and bone marrow constitute the primary lymphoid organs, while the spleen, lymph nodes, and mucosal associated lymphoid tissue (MALT) comprise the secondary lymphoid organs [2].

Fish are a heterogeneous group divided into three classes: Agnatha (jawless fish such as the hagfish and lampreys), Chondrichthyes (cartilaginous fish such as sharks, rays and skates) and Osteichthyes (bony fish) [3]. As in all vertebrates, fish have cellular and humoral immune responses and organs, the main function of which is immune defence. Most genera-

tive and secondary lymphoid organs in mammals are also found in fish, except for lymphatic nodules and bone marrow [3].

The head kidney or pronephros has hematopoietic functions [3, 4], and unlike in higher vertebrates, it is the immune organ involved in phagocytosis [5], antigen processing, production of IgM [6, 7] and immune memory through melanomacrophagic centres [8, 9]. The thymus, another lymphoid organ situated near the opercular cavity in teleosts, produces T lymphocytes involved in allograft rejection, stimulation of phagocytosis and antibody production by B cells [10, 11]. The spleen is a large, blood-filtering organ that undergoes increasing structural complexity in order to augment its efficiency in trapping and processing antigens [12-15]. Melanomacrophage centres are present for clearance of ingested material and can be surrounded by immunoglobulin-positive cells, especially after immunization [8]. Proliferation of granular cells has also been observed in association with ellipsoids and melanomacrophage centres after immunization [16].

1.1. Innate and adaptive immune response

The development of an immune system is essential for the survival of living organisms. In vertebrates, immunity can be divided into two components, the innate immune response and the adaptive immune response. The innate immune response is the initial line of defence against infection, which includes physical barriers and cellular response. The adaptive immune response is capable of specific antigen recognition and is responsible for the secondary immune response.

The innate immune system recognizes conserved molecular structures common to pathogenic microorganisms such as polysaccharides, lipopolysaccharides (LPS), peptidoglycans, bacterial DNA, and double-strand viral RNA, among others, through their interaction with specific receptors like toll receptors (TLRs). These mechanisms of recognition may lead directly to successful removal of pathogens, for instance by phagocytosis, or may trigger additional protective responses through induction of adaptive immune responses [17]. Cells of the innate immune system have a diverse array of functions. Some cells are phagocytic, allowing them to engulf and degrade pathogenic particles. Other cells produce and secrete cytokines and chemokines that can stimulate and help guide the migration of cells and further direct the immune response [18].

The adaptive system recognizes foreign structures by means of two cellular receptors, the B cell receptor (BCR) and the T cell receptor (TCR). Adaptive immunity is highly regulated by several mechanisms. It increases with antigen exposure and produces immunological memory, which is the basis of vaccine development and the preventive function of vaccines [19, 20]. The adaptive response generally starts days after infection and is capable of recognizing specific protein motifs of peptides, which leads to a response that increases in both speed and magnitude with each successive exposure [21]. The main effector cells of the adaptive immune response are the lymphocytes, specifically B cells and T cells. When B cells are activated, they are capable of differentiating into plasma cells that can secrete antibodies. Upon activation T cells differentiate into either helper T cells or cytotoxic T cells. Helper T cells are capable of activating other cells of

the adaptive immune response such as B cells and macrophages, while cytotoxic T cells upon activation are able to kill cells that have been infected [22].

1.2. Fish immune response

Immune responses in fish have not been as well characterized as they have in higher vertebrates. Consequently, there is not enough information about the components of the fish immune system and its function and regulation. Key immune mammalian homologous genes have been identified in several fish species, suggesting that the fish immune system shares many features with the mammalian system. For example, the identification of α and β T cell receptor genes (TCR) [23], key T cell markers such as CD3, CD4, CD8, CD28, CD40L, and a great number of cytokines and chemokines [24-26] suggest that T helper (Th)1, Th2 and Th17 and the regulatory subset Treg are present in fish. Some cell subsets have been better studied mainly because their activity can be easily differentiated and measured, as in the case of cytotoxic cells [27] and macrophages [28, 29]. Finally, B cells have been much more studied due to the availability of monoclonal antibodies that have been isolated and identified by a number of techniques [30, 31]. Phenotypic characterization of leukocytes has been hampered mainly by the lack of membrane cell markers [32, 33]. Researchers anticipate developing antibodies for cell lineage markers of fish immunocompetent cells that can be used to isolate and characterize immune cells to obtain insights into their regulation and role in immune response [34-36].

Antibodies in teleosts play a key role in the immune response. In general, IgM is the main immunoglobulin in teleosts that can elicit effective specific humoral responses against various antigens. For IgM, one gene alone can generate as many as six structural isoforms. Therefore, diversity is the result of structural organization rather than genetic variability [37]. Recently, several reports have provided evidence for the existence of IgD/IgZ/IgT in fish [38-41]. Interestingly, B cells from rainbow trout and salmon have high phagocytic capacity, suggesting a transition in B lymphocyte during evolution in which a key cell type of the innate immunity and phagocytosis evolved into a highly specialized component of the adaptive immune response in higher vertebrates [42, 43].

1.3. Fish cytokines

Cytokines are secreted proteins with growth, differentiation, and activation functions that regulate the nature of immune responses. Cytokines are involved in several steps of the immune response, from induction of the innate response to the generation of cytotoxic T cells and the production of antibodies. In higher vertebrates, the combination of cytokines that are secreted in response to an immune stimulation induces the expression of immune-related genes through multiple signalling pathways, which contributes to the initiation of the immune response. Cytokines can modulate immune responses through an autocrine or paracrine manner upon binding to their corresponding receptors [44].

Cytokines have overlapping and sometimes contradictory pleiotropic functions that make their classification difficult. Cytokines are produced by macrophages, lymphocytes, granulo-

cytes, DCs, mast cells, and epithelial cells, and can be divided into interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines [45]. They are secreted by activated immune-related cells upon induction by various pathogens, such as parasitic, bacterial, or viral components [46]. Macrophages can secrete IL-1, IL-6, IL-12, TNF α , and chemokines such as IL-8 and MCP-1, all of which are indispensable for macrophage, neutrophil, and lymphocyte recruitment to the infected tissues and their activation as pathogen eliminators [47]. Meanwhile, cytokines released by phagocytes in tissues can also induce acute phase proteins, including mannose-binding lectin (MBL) and C-reactive protein (CRP), and promote migration of DCs [48].

Fish appear to possess a repertoire of cytokines similar to those of mammals. To date several cytokine homologues and suppressors have been cloned in fish species [24, 25, 49]. Some cytokines described in fish are TNF α , IL-1 β , IL-6 or IFN.

Current knowledge of fish cytokines is based on mammal models of the cytokines network and their complex interactions. In this review we included the pro-inflammatory cytokines associated with innate and adaptive immunity, regulatory cytokines and anti-inflammatory cytokines.

1.4. Pro-inflammatory fish cytokines

1.4.1. Tumour necrosis factor α (TNF α)

TNF α (tumour necrosis factor alpha) is a pro-inflammatory cytokine that plays an important role in diverse host responses, including cell proliferation, differentiation, necrosis, apoptosis, and the induction of other cytokines. TNF α can induce either NF- κ B mediated survival or apoptosis, depending on the cellular context [50]. TNF α mediates powerful anti-microbial responses, including inducing apoptosis, killing infected cells, inhibiting intracellular pathogen replication, and up-regulating diverse host response genes. Many viruses have evolved strategies to neutralize TNF α by direct binding and inhibition of the ligand or its receptor or modulation of various downstream signalling events [51].

TNF α has been identified, cloned, and characterized in several bony fish, including Japanese flounder [52], rainbow trout [53, 54], gilthead seabream [55], carp [56] catfish [57], tilapia [58], turbot [59] and goldfish [60]. These studies have revealed the existence of some obvious differences from their mammalian counterpart, such as the presence of multiple isoforms of TNF α in some teleost species [54, 56] the high constitutive expression of this gene in different tissues of healthy fish and its relatively poor up-regulation by immune challenge *in vitro* and *in vivo* [53, 55, 57]. However, the most unexpected and interesting difference between fish and mammal TNF α concerns the weak *in vitro* effects of TNF α on phagocyte activation in goldfish [60], rainbow trout [57], turbot [59] and gilthead seabream [61]. This weak *in vitro* activity of fish TNF α sharply contrasts with the powerful actions exerted by the i.p. injection of recombinant TNF α in gilthead seabream, which includes the recruitment of phagocytes to the injection site, with a concomitant strong increase in their respiratory burst [61]. Apparently endothelial cells are the main target cells of fish TNF α , suggesting that TNF α is mainly involved in the recruitment of leukocytes to the inflammatory foci rather than in their

activation [62]. Despite the above, differential expression has been observed in studies with rainbow trout leucocytes, which have shown increased response to different pro-inflammatory stimuli, as human recombinant TNF α [63], LPS [53, 64], zimosan and muramyl dipeptide as a peptidoglycan constituent of both gram-positive and gram-negative bacteria [64]. Moreover, it is known that Infectious Pancreatic Necrosis Virus (IPNV)-mediated up-regulation of TNF α regulates both the Bad/Bid-mediated apoptotic pathway and the RIP1 (receptor-interacting protein-1)/ROS-mediated secondary necrosis pathway [65].

1.4.2. Interleukin 1 family

In mammals, the 11 members of the Interleukin-1 family include IL-1 α (IL-1F1), IL-1 β (IL-1F2), IL-1 receptor antagonist (IL-1ra/IL-1F3), IL-18 (IL-1F4), IL-1F5-10 and IL-33 (IL-1F11). These molecules tend to be either pro-inflammatory or act as antagonists that inhibit the activities of particular family members [66]. Despite these semantic issues, to date only two clear homologues of these molecules have been discovered in fish, IL-1 β and IL-18 [24].

1.4.2.1. Interleukin 1 β

IL-1 β is one of the earliest expressed pro-inflammatory cytokines and enables organisms to respond promptly to infection by inducing a cascade of reactions leading to inflammation. Many of the effector roles of IL-1 β are mediated through the up- or down-regulation of expression of other cytokines and chemokines [67]. Mammalian IL-1 β is produced by a wide variety of cells, but mainly by blood monocytes and tissue macrophages. IL-1 β was the first interleukin to be characterized in fish and has since been identified in a number of fish species, such as rainbow trout [68], carp [69], sea bass [70], gilt head seabram [71], haddock [72], tilapia [73]. A second IL-1 β gene (IL-1beta2) has been identified in trout [74]

In mammals pro-IL-1 β remains cytosolic and requires cellular proteases to release the mature peptide. It is known that the peptide is cleaved by the IL-1 β converting enzyme (ICE) [75]. However, the aspartic acid residue for which this enzyme has specificity is not present in all fish genes sequenced to date. Nevertheless, using a combination of multiple alignments and analysis of the N-terminal sequences of known mature peptides, it is possible to predict fish gene cutting sites. In trout, this gives a mature peptide of 166 and 165 aminoacids for IL-1 β 1 and IL-1 β 2 [76].

Like its mammalian counterpart, teleost IL-1 β has been found to be regulated in response to various stimuli, such as LPS or poly I:C [68, 70-74, 77-81]. The biological activity of recombinant IL-1 β (rIL-1 β) has been studied in several fish species, indicating that fish IL-1 β is involved in the regulation of immune relevant genes, lymphocyte activation, migration of leucocytes, phagocytosis and bactericidal activities [77, 81-84].

1.4.2.2. Interleukin 18

In mammals, IL-18 is mainly produced by activated macrophages. It is an important cytokine with multiple functions in innate and acquired immunity [85-87]. One of its primary

biological properties is to induce interferon gamma (IFN γ) synthesis in Th1 and NK cells in synergy with IL-12 [88, 89]. It promotes T and NK cell maturation, activates neutrophils and enhances Fas ligand-mediated cytotoxicity [90-92]. Like IL-1 β , it is synthesized as an inactive precursor of approximately 24 kDa and is stored intracellularly. Activation and secretion of IL-18 is mainly effected through specific cleavage of the precursor after D35 by caspase 1, also termed the IL-1 β -converting enzyme (ICE), which is believed to be one of the key processes regulating IL-18 bioactivity [93, 94]. Some other enzymes, including caspase 3 and neutrophil proteinase 3, also cleave the IL-18 precursor to generate active or inactive mature molecules [95, 96].

IL-18 was discovered in fish by analysis of sequenced fish genomes (fugu) and EST databases (medaka) [97, 98]. An alternative splicing form of the IL-18 mRNA was discovered in trout that may have an important role in regulating IL-18 expression and processing in this species. This form shows a lower constitutive expression relative to the full length transcript, but unlike the full length transcript, it increases in response to LPS and polyI:C stimulation in the RTG-2 fibroblast cell line [98]. The expression level of the full length transcript can increase in response to LPS plus IL-1b in head kidney leucocyte cultures, and by IFN γ in RTS-11 cells [99].

1.4.3. Other pro-inflammatory cytokines

1.4.3.1. Interleukin 6

A number of other interleukins are considered pro-inflammatory, some of which are released during the cytokine cascade that follows bacterial infection. Of these IL-6 is one of the best known, and is itself a member of the IL-6 family of cytokines that includes IL-11 and IL-31, as well as cytokines such as mammalian CNTF, LIF, OSM, CT-1 and CT-2 [24]. Whilst the homology of known fish molecules with many of these IL-6 family members is not conclusive [100], true homologues appear to be present in at least in the cases of IL-6 and IL-11 [24]. IL-6 is produced by a diverse group of cells including T lymphocytes, macrophages, fibroblasts, neurons, endothelial and glial cells. The pleiotropic effects of IL-6 are mediated by a 2-subunit receptor [101] and include the regulation of diverse immune and neuro-endocrine processes. IL-6 has been implicated in the control of immunoglobulin production, lymphocyte and monocyte differentiation, chemokine secretion and migration of leukocytes to inflammation sites [102-104].

IL-6 was first discovered in fugu by analysis of the genome sequence [105] and subsequently in other species as part of EST analysis of immune gene-enriched cDNA libraries [106-108]. However, little is known about the function and signalling pathways of IL-6 in fish. Interestingly, trout IL-6 expression in macrophages is reported to be induced by LPS, poly I:C and IL-1 β in the macrophage cell line RTS-11, as well as in head kidney macrophages [109]. Moreover, IL-6 induces the expression of itself, so it can act in an autocrine and paracrine fashion to increase its expression, with the potential to both amplify and exacerbate the inflammatory response. However, IL-6 can significantly down-regulate the expression of

trout TNF α 1, TNF α 2, and IL-1 β , suggesting a potential role of trout IL-6 in limiting host damage during inflammation [109].

1.4.3.2. Interleukin 11

In mammals, IL-11 is produced by many cell types throughout the body. Basal and inducible IL-11 mRNA expression can be detected in fibroblasts, epithelial cells, chondrocytes, synoviocytes, keratinocytes, endothelial cells, osteoblasts and certain tumour cells and cell lines [110]. Viral [111] and bacterial infection [112] and cytokine stimulation (IL-1, TNF α and TGF- β 1) induce IL-11 expression. IL-11 acts on multiple cell types, including hematopoietic cells, hepatocytes, adipocytes, intestinal epithelial cells, tumour cells, macrophages, and both osteoblasts and osteoclasts. In the hematopoietic compartment IL-11 supports multilineage and committed progenitors, contributing to myeloid, erythroid, megakaryocyte and lymphoid lineages [113]. IL-11 is also an anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines from lipopolysaccharide (LPS)-stimulated macrophages [114]. In combination with its trophic effects on the gastrointestinal epithelium, IL-11 plays an important role in the protection and restoration of gastrointestinal mucosa [115, 116].

The teleostean IL-11 orthologue has been found to consist of duplicate IL-11 genes, named IL-11a and IL-11b [117], with expression patterns indicating that both divergent forms of teleostean IL-11 play roles in antibacterial and antiviral defence mechanisms of fish [117-119]. In trout, IL-11 molecule is grouped with IL-11a and is constitutively expressed in intestine and gills and is highly up-regulated at other immune sites (spleen, head kidney, liver) following bacterial infection. *In vitro*, the macrophage-like RTS-11 cell line has shown enhanced IL-11 expression in response to LPS, bacteria, poly I:C and rIL-1 β [118]. In carp, IL-11a is modulated by LPS, ConA and peptidoglycan in head kidney macrophages [117, 120] and cortisol has been found to inhibit IL-11 expression on its own and in combination with LPS [117]. In contrast to carp IL-11a, which shows low levels of constitutive expression in blood leucocytes, IL-11b in Japanese flounder shows higher expression at this site, and strong up-regulation was found in response to rhabdovirus infection in kidney cells [119]. This suggests that these paralogues have some complementarity of function related to their differential expression, although study of both forms in a single experiment is still required [24].

1.5. Chemokines

Chemokines are a superfamily of approximately 40 different small secreted cytokines that direct the migration of immune cells to infection sites. Their activity is coordinated by binding to G-protein-linked receptors with seven transmembrane domains. Four distinct subgroups make up the chemokine superfamily. These are designated as CXC (or a), CC (or b), C (or g) and CX₃C (or d), which are defined by the arrangement of the first two cysteine residues within their peptide structure. The CC subfamily can be further subdivided according to the total number of cysteine residues, as some members of this group contain four cysteines whilst the remainder possesses six (and are known as the C6-b group). Similarly, the

CXC subfamily contains two subgroups based on whether or not the first two cysteines are preceded by a Glu-Leu-Arg (ELR) motif associated with specificity to neutrophils [76, 121].

1.5.1. Interleukin 8

An important chemokine related to the pro-inflammatory process is CXCL-8, also called interleukin 8, this chemokine is a member of the CXC chemokine subfamily and attracts neutrophils, T lymphocytes and basophils *in vitro*, but not macrophages or monocytes [122]. Many cell-types, including macrophages, produce IL-8 in response to a variety of stimuli (LPS, cytokines and viruses). The neutrophil-attracting ability of IL-8 can be attributed to the presence of the ELR motif adjacent to the CXC motifs at its N-terminus, presumably by affecting its binding to specific receptors [123, 124]. In contrast, CXC chemokines lack an ELR motif and specifically attract lymphocytes but not neutrophils. The biological effects of IL-8 on neutrophils include increased cytosolic calcium levels, respiratory burst, a change in neutrophil shape and chemotaxis [125].

The fish IL-8 has been found in flounder [126], trout [125, 127], catfish [128], and lamprey [129]. *In vitro* stimulation of a trout macrophage cell line (RTS-11) [125] or *in vivo* intraperitoneal challenge [78] with either LPS or poly I:C did result in clear up-regulation of IL-8 expression. Moreover, induction of IL-8 expression in primary cultures of rainbow trout leukocytes stimulated for 24 hours with LPS and TNF α confirms that this fish chemokine is associated with inflammatory response, as has been suggested in mammals [127]. Interestingly, the ELR motif associated with the neutrophil-attracting ability is absent from the lamprey molecule and it is similar in flounder, where CXCL8 also lacks the ELR motif and appears to be regulated by a bacterial mechanism, since its transcript has only been detected in the major immune organs (spleen and head kidney) of an LPS stimulated flounder. The case of the trout is different, although there is also no ELR preceding the CXC motif, it has a very similar motif (DLR) in this position [130]. The human CXCL8 molecule, where the ELR motif has been mutated to DLR, retains neutrophil-attracting ability, albeit at lower potency [123]. Consequently, it is possible that the trout molecule has similar chemotactic activity to that of mammalian CXCL8 [130].

1.6. The interleukin 2 family

The IL-2 subfamily of cytokines signals via the common gamma chain (gC or CD132), a member of the type I cytokine receptor family expressed in most leucocytes. These cytokines in mammals include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-2, IL-4, IL-9 and IL-21 are all cytokines released from Th cells, which affect their responses [24], whilst IL-7 and IL-15 are particularly important for the maintenance of T cell memory [131]. To date molecules with homology to all of these have been found in fish, except IL-9 [24].

1.6.1. Interleukin 2

Interleukin-2 (IL-2 is an important immunomodulatory cytokine that primarily promotes proliferation, activation and differentiation of T cells [132]. IL-2, initially known as T-cell

growth factor (TCGF), is synthesized and secreted mainly by Th1 cells that have been activated by stimulation by certain mitogens or by interaction of the T-cell receptor with the antigen/MHC complex on the surface of antigen-presenting cells [133-135]. Although CD4 T cells are the major source of IL-2 production in response to TCR stimulation, transient induction of IL-2 mRNA and production of the protein has been detected in murine dendritic cells activated by gram-negative bacteria [136]. IL-2 can also be produced by B cells in certain situations [137, 138]. The produced IL-2 promotes the expansion and survival of activated T cells and is also required for the activation of natural killer (NK) cells [139] and for immunoglobulin (Ig) synthesis by B cells [140].

The IL-2 gene has been detected only recently in fish by analysis of the fugu genome sequence, which also identified IL-21 as a neighbouring gene, as in mammals, providing the first direct evidence for the existence of a true IL-2 homologue in bony fish [141]. The gene has a 4 exon/3 intron organisation, as in mammals, and showed no constitutive expression in a range of tissues examined. However, injection of Fugu with poly I:C induced expression of IL-2 in the gut and gills [141]. Moreover, IL-2 could be induced in head kidney cell cultures stimulated with PHA, and in T-cell enriched cultures isolated from PBL when stimulated with B7-H3 or B7- H4 Ig fusions proteins in the presence of PHA [24, 142]. IL-2 has since been cloned in rainbow trout [143, 144]. The trout IL-2 was significantly up-regulated in head kidney leucocytes by the T cell mitogen PHA and in classical mixed leucocyte reactions and *in vivo* following infection with bacteria (*Y. ruckeri*) or the parasite *Tetracapsuloides bryosalmonae*. More importantly, the recombinant trout IL-2 produced in *Escherichia coli* was shown to induce expression of two transcription factors (STAT5 and Blimp-1) known to be involved in IL-2 signalling in mammals [143], as well as interferon- γ (IFN γ) and IL-2 itself, and a CXC chemokine known to be induced by IFN γ , termed a IFN γ -inducible protein (γ IP) [145].

1.6.2. Interleukin 4

Interleukin-4 IL-4 is a pleiotropic cytokine produced by T cells, mast cells, and basophils and is known to regulate an array of functions in B cells, T cells, macrophages, hematopoietic and non-hematopoietic cells [146, 147]. IL-4 serves as a key cytokine in driving Th2 differentiation and mediating humoral immunity, allergic responses and certain autoimmune diseases [148]. The IL-4 gene is conserved evolutionally in the animal kingdom and has been isolated from various animals including humans [149], mice [150, 151] and bovines [152], in which the IL-4 locus has been mapped in a region adjacent to those of IL-5 and IL-13 on the same chromosome [153, 154].

Teleost fish have two genes of the IL-4/13 family, IL-4/13A and IL-4/13B, which are situated on separate chromosomes in regions that duplicated during the fish-specific whole genome duplication (FS-WGD) around 350 million years ago [155, 156]. A few IL-4-like genes have been found in fish to date. The first was discovered by searching the *Tetraodon nigroviridis* genome [157]. In this work, IL-4 was constitutively expressed in head kidney, spleen, liver, brain, gill, muscle and heart. The ubiquitous expression of IL-4 is consistent with a postulated role in immune cytokines regulation. Stimulating the fish with a mixed stimulant con-

taining ConA, PHA and PMA significantly up-regulated the expression of IL-4, which suggests that IL-4 is involved in the immune inflammatory responses triggered by mitogens [157], as in mammals, where it has been observed that this mitogen increases IL-4 expression [158]. However, the homology (amino acid identity) of this molecule was very low [12–15%], making it difficult to be sure it is an IL-4 homologue, although clearly related to Th2-type cytokines [24]. In fugu, T cell enriched PBL was found to express more IL-4/13A and IL-4/13B after stimulation with recombinant B7 molecules [142]. In zebrafish a recombinant IL4/13B was shown to increase the number of IgT-positive and CD209-positive cells in blood [159, 160], and in zebrafish spleen the expression of IL-4/13B and transcription factor related to Th2 immune response as GATA-3, and STAT6 was simultaneously enhanced after PHA stimulation [161]. The IL-4/13A gene was identified in trout and salmon [162], where the tissue distribution of salmonid IL-4/13A and GATA-3 expression were compared to the expression of IL-4, IL-13, and GATA-3 in mice. High levels of these transcripts were found in both salmonid and murine thymus, while constitutive IL-4/13A richness of skin and respiratory tissue was found in salmonids but not in mice. Experiments with isolated cells from gill and pronephros (head kidney) indicated that trout IL-4/13A is mainly expressed by surface IgM-negative cells, readily inducible by PHA but not by poly I:C, and regulated differently from the Th1 cytokine IFN γ gene. In mammals, IL-5 is also considered a Th-2 type cytokine and along with IL-3 and GM-CSF it signals through receptors with a common γ -chain (γ C). None of these cytokines have been discovered in fish to date [24].

1.6.3. Interleukin 7

The cytokine IL-7 plays several important roles during lymphocyte development, survival, and homeostatic proliferation [163]. It is produced by many different stromal cell types, including epithelial cells of the thymus and the intestine [164–166]. There is only one report on IL-7 in fish, for the fugu molecule that was discovered using a gene synteny approach by searching with the mammalian IL-7 gene neighbours C8orf70 and PKIA. Fugu IL-7 shows constitutive expression in head kidney, spleen, liver, intestine, gill and muscle, with expression shown to increase in head kidney cultures stimulated with LPS, poly I:C or PHA [24, 167].

1.6.4. Interleukin 15

The central action of IL-15 cytokine is on T-cells, dendritic cells and NK cells. IL-15 is an important regulator of the innate immune response to infection and autoimmune disease conditions. This gene shares activities with IL-2 and utilizes IL-2R β and γ units [45].

Two genes with homology to IL-15 have been discovered in fish. One shows similar gene organisation and synteny to mammalian and chicken IL-15, and has been termed IL-15. The second gene, which has a 4-exon structure and is in a different genome location, has been termed IL-15-like [168–170]. They show differential expression patterns in terms of the tissues where constitutive expression is apparent, and in terms of inducibility in PBL, with IL-15L being refractory to induction [168]. Two alternative splice variants of IL-15L (IL-15La and IL-15Lb) have also been described [170]. Trout IL-15, which has subsequently been

cloned and sequenced, was strongly induced by rIFN γ in two trout cell lines (RTS-11 and RTG-2). rIL-15 could up-regulate IFN γ expression in splenic leucocytes, suggesting a positive feedback loop exists in fish between these two cytokines. Interestingly, unstimulated head kidney leucocytes were not responsive to rIL-15, at least in terms of the IFN γ expression level [171].

1.6.5. Interleukin 21

Interleukin 21 (IL-21) is a newly recognized member of IL-2 cytokine family that utilizes the common γ -chain receptor subunit for signal transduction [172-174]. In humans and other mammals, IL-21 is produced by both Th1 and Th2 cells [172, 175, 176]. IL-21 has pleiotropic effects on both innate and adaptive immune responses and can act on CD4+ and CD8+ T cells, B cells, NK cells, dendritic cells (DC), myeloid cells, and other tissue cells. IL-21 enhances the proliferation of anti-CD3-stimulated T cells and acts in concert with other γ c cytokines to enhance the growth of CD4+ T cells [177]. IL-21-producing CD4+ T cells exhibit a stable phenotype of IL-21 production in the presence of IL-6 but retain the potential to produce IL-4 under Th2-polarizing conditions and IL-17A under Th17-polarizing conditions [178]. IL-21 stimulates CD8+ T cell proliferation and synergizes with IL-15 in promoting CD8+ T cell expansion in vitro and their antitumor effects in vivo [177, 179]. B cells that encounter IL-21 in the context of Ag-specific (BCR) stimulation and T cell co/stimulation undergo class-switch recombination and differentiate into Ab-producing plasma cells. In contrast, B cells encountering IL-21 during nonspecific TLR stimulation or without proper T cell help undergo apoptosis [180].

Since its discovery in fugu as a gene neighbour of IL-2 [141], IL-21 has been reported in tetraodon [181, 182] and rainbow trout [182]. Fugu IL-21 shows low constitutive expression. However, stimulation of isolated kidney leucocytes with PHA induced IL-21 expression. IL-21 was also up-regulated at mucosal sites as gill and gut when fish were injected with LPS or poly I:C [141]. Similarly, in tetraodon IL/21 expression is low but detectable in the gut, gonad and gills of healthy fish, and is induced in the kidney, spleen and skin following LPS injection [181]. In trout IL-21 expression is highest in gills and intestine, and is induced *in vivo* by bacterial (*Y. ruckeri*) and viral (VHSV) infection [182]. Relative to IL-2, induction of IL-21 expression in head kidney cells appears more rapidly but has shorter duration after stimulation. The trout rIL-21 has also been produced and shown to increase the expression of IL-10, IL-22 and IFN γ , and to a lesser extent IL-21, and to maintain the expression levels of key lymphocyte markers in primary cultures [182]. Thus, IL-21 may act as a survival factor for fish T and B cells [24].

1.7. The interleukin 10 family

Interleukin-IL-10 is an anti-inflammatory cytokine and a member of the class II cytokine family that also includes IL-19, IL-20, IL-22, IL-24, IL-26 and the interferons [183]. Although the predicted helical structure of these homodimeric molecules is conserved, certain receptor-binding residues are variable and define the interaction with specific heterodimers of

different type-2 cytokine receptors. This leads to diverse biological effects through the activation of signal transducer and activator of transcription (STAT) factors [184].

1.7.1. Interleukin 10

Interleukin-10 (IL-10) was discovered initially as an inhibitory factor for the production of Th1 cytokines. Subsequently, pleiotropic inhibitory and stimulatory effects on various types of blood cells were described for IL-10, including its role as a survival and differentiation factor for B cells. IL-10, which is produced by activated monocytes, T cells and other cell types like keratinocytes, appears to be a crucial factor for at least some forms of peripheral tolerance and a major suppressor of the immune response and inflammation. The inhibitory function of IL-10 is mediated by the induction of regulatory T cells [185].

IL-10 was discovered in fish by searching the fugu genome. The translation showed 42–45% similarity to mammalian molecules with very low constitutive expression in tissues [186]. IL-10 has since been cloned in several other fish species including carp [187] zebrafish [188], rainbow trout [189], sea bass [190, 191] and cod [79]. Such studies have shown that IL-10 expression can be increased by LPS stimulation, by bacterial infection, by bath administration of immunostimulants [192] and by IPNV infection which may be associated with mechanisms of immune evasion [78].

1.7.2. Interleukin 20 (IL-20Like)

In mammals, IL-20 was discovered as a new member of the IL-10 family of cytokines. IL-20 shares the highest amino-acid sequence identity with IL-10, IL-24 and IL-19. It is secreted by immune cells and activated epithelial cells like keratinocytes. A high expression of the corresponding IL-20 receptor chains has been detected on epithelial cells. In terms of function, IL-20 might therefore mediate crosstalk between epithelial cells and tissue-infiltrating immune cells under inflammatory conditions [193].

In fish, the gene of IL-20 has been described in pufferfish [183], zebrafish [194] and trout [195]. In the latter work, the IL-20 gene, called IL-20-like (IL-20L) has been described as having a high level of expression in immune related tissues and in the brain, suggesting an important role of the fish IL-20L molecule in both the immune and nervous systems. Although the exact cell types expressing IL-20L have yet to be defined, macrophages express IL-20L. Moreover, IL-20L expression in the macrophage cell line RTS-11 is modulated by pro-inflammatory cytokines, signalling pathway activators, microbial mimics and the immuno-suppressor dexamethasone. These data suggest that trout IL-20L plays an important role in the cytokine network. The increased expression of IL-20L was only detected at late stages (4–24 h) of LPS stimulation in RTS-11 cells and in spleen 24–72 h after infection with *Yersinia ruckeri*, which suggests that the increased expression of IL-20L by LPS and infection is via the rapid increase of pro-inflammatory cytokines (e.g., IL-1 β) and other factors known to occur [195].

1.7.3. Interleukin 22/26

In mammals, interleukin-22 is secreted by Th17 cells [196], as well as by a subset of NK cells, designated as NK22 [197]; and even by some Th1 cells [198]. Studies have suggested there is a distinct Th22 cell lineage [199, 200]. Many of the same cytokines that induce differentiation and proliferation of IL-17-producing cells also lead to the secretion of IL-22 by Th17 cells, NK22 cells, and putative Th22 cells, including IL-6, IL-23, IL-1 β , TGF- β , and TNF α [201]. IL-17 and IL-22 are therefore frequently produced together in response to infections [202]. Interleukin-22 interacts with a heterodimeric receptor, IL-10R2/IL-22R1 [203], which is expressed on a variety of non-lymphoid cells, especially epithelial cells. Ligation of this receptor leads to both protective and detrimental effects. In synergy with IL-17, IL-22 induces pro-inflammatory cytokines in human bronchial epithelial cells against *Klebsiella pneumoniae* infection [204] and in colonic myofibroblasts [205]. Independently or in synergy with IL-17, IL-22 acts in defence against intestinal infection of mice with *Citrobacter rodentium* [206]. Moreover, IL-22 has been implicated in intestinal homeostasis keeping commensal bacteria contained in anatomical niches, which is key to our symbiotic relationship and normal intestinal physiology. However, the mechanisms that restrict colonization to specific niches are unclear. David Artis and colleagues have described a crucial role for IL-22-producing innate lymphoid cells (ILCs) in preventing lymphoid-resident commensal bacteria from escaping their niche and causing inflammation [207].

IL-26 can be produced by primary T cells, NK cells and T cell clones following stimulation with specific antigen or mitogenic lectins. IL-26 was initially shown by several groups to be co-expressed with IL-22 [208]. IL-26 is co-expressed with IFN γ and IL-22 by human Th1 clones, but not by Th2 clones. It was subsequently found that IL-26 is co-expressed with IL-17 and IL-22 by Th17 cells, an important subset of CD4 $^+$ T-helper cells that are distinct from Th1 and Th2 cells [209-211]. More recently, a novel subset of CD56 $^+$ NKp44 $^+$ NK cells was identified that co-expresses IL-22 and IL-26, especially following treatment with IL-23 [212]. Furthermore, a different subset of immature NK cells was described that do not express CD56 or NKp44 but do express CD117 and CD161 and constitutively express IL-22 and IL-26 [213].

The mechanisms that regulate transcription of the human IL-26 gene are so far largely undefined. It is possible and perhaps likely that expression of the IL-26 gene is induced in an IL-23-dependent manner because IL-23 is known to induce differentiation of Th17 cells, and IL-23 amplifies expression of IL-17 and IL-22 by Th17 cells [214].

In fish, the IFN γ locus was discovered using a gene synteny approach, and was first reported for fugu [215]. It contained a homologue of IL-22/26, that later studies of the zebrafish genome revealed to be two genes, one with clear homology to IL-22 and one with somewhat less clear homology to IL-26 [216]. The IL-22 gene was expressed constitutively in intestine and gills in all the treated and non-treated tissues. The gene was also expressed in kidney and spleen in LPS and PolyI:C-treated tissues, respectively, while IL-26 was expressed only in intestine treated with PolyI:C without expression [216]. IL-22 expression has been correlated with disease resistance in haddock vaccinated against *V. anguillarum*, with a strong constitutive expression in gills in vaccinated fish

but not in control fish 24 hours post bath challenge, resulting in complete protection in fish vaccinated [217]. Moreover, IL-22, a cytokine released by Th-17 cells in mammals, is also interesting, and such responses are thought to be crucial for protection against extracellular microbes and at mucosal sites [218]. This coupled with the recent discovery of novel gill-associated immune tissue in fish [219] may provide a clue to a potential mechanism of resistance elicited by the *V. anguillarum* vaccination [24].

1.8. The interleukin 17 family

Interleukin-17 and a related family of genes are known to have pro-inflammatory actions and are associated with diseases [220]. After the discovery of the human IL-17 gene [221], five cellular paralogs of IL-17 were identified, namely IL-17B, C, D, E and F [222-227]. These paralogs, identified by ESTs, genomics and proteomic databases, share identities of 20–50% with IL-17A gene. Human IL-17 A and F are present in tandem in opposite transcriptional orientation on the same chromosome 6p12, while IL-17B (Chr 5q24), IL-17C (Chr 16q24), IL-17D (Chr 13q11) and IL-17E (Chr 14q11) are dispersed. The structural similarities lead to the classification of IL-17 A, B, C, D, E, and F genes to a larger IL-17 sub-family [45]. Several IL-17 family members have been discovered in teleost fish, but homology to mammalian genes has not always been easy to assign. Two IL-17A or F homologue genes (IL-17A/F) have been found on the same chromosome. However, it has been difficult to determine which gene codes IL-17A and F. This gene in zebrafish was named IL-17A/F1 and 2. Furthermore, another IL-17A or F homologue gene (IL-17A/F3) has been found in zebrafish localized on a chromosome different from that of IL-17A/F1 and 2 [228]. In addition to those in zebrafish, IL-17A or F homologue genes have been found in rainbow trout [229], Atlantic salmon [230], pufferfish (IL-17A/F1, 2 and 3) [231], and medaka (IL-17A/F1, 2 and 3) [232].

The tissue distribution of the fugu IL-17 gene family also differs. In particular, IL-17 family genes are highly expressed in the head kidney and gills. Moreover, expression of IL-17 family genes is significantly up-regulated in the lipopolysaccharide-stimulated head kidney, suggesting that Fugu IL-17 family members are involved in inflammatory responses [231]. In Atlantic salmon IL-17D expression is widely distributed in tissues, with the highest levels of expression in testis, ovary and skin. Infection with *A. salmonicida* by injection increases IL-17D expression levels in the head kidney (but not the spleen) in a time-dependent manner. Skin and kidney showed an increased IL-17D expression level in fish given a cohabitation challenge with *A. salmonicida* [230]. The two trout IL-17C genes show some degree of differential expression within tissues, with IL-17C1 being more dominant in the gills and skin, whilst IL-17C2 is more dominant in the spleen, head kidney and brain. Expression of both genes increases significantly with bacterial infection, although the increased expression of IL-17C2 is greater in terms of fold change. Similarly, both genes could be up-regulated in the trout RTS-11 cell line by LPS, poly I:C, calcium ionophore and rIL-1 β , with IL-17C2 showing higher fold increases in all cases [229].

1.9. Interleukin 12

IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits. It can mediate a number of different activities, including stimulation of IFN γ secretion from resting lymphocytes, NK cell stimulation and cytolytic T cell maturation. Perhaps most crucially, IL-12 also affects the progression of uncommitted T cells to either the Th1 lineage, which in general is characterized by secretion of lymphokines associated with cell-mediated rather than humoral immunity [233].

The p35 and p40 subunits were discovered in fish by analysis of the fugu genome [234]. The p35 locus is quite well conserved, with Schip1 being the immediate neighbour in all cases. This association has allowed p35 to be cloned by gene walking from Schip1 from fish species for which no genome sequence is available [24, 235]. The p40 subunit in fugu is constitutively expressed in all the tissues examined, except muscle, and no increases in expression were seen 3 h after injection with poly I:C or LPS. This constitutive and broad expression distribution of the p40 subunit suggests that it may be expressed in most cell types. The expression of the p35 subunit is more limited in its tissue expression and is induced after injection with poly I:C in the head kidney and the spleen, but not after injection with LPS. These results show that there are differences from the mammalian data in fugu IL-12 subunit expression. Further investigation will be required to show whether this is unique to fugu, if IL-12 is involved more in antiviral defence in fish and if the two subunits are regulated differently from their regulation in the mammalian system [234].

1.10. Transforming growth factor β (TGF- β)

TGF- β is a pleiotropic cytokine that regulates cell development, proliferation, differentiation, migration, and survival in various leukocyte lineages including lymphocytes, dendritic cells, NK cells, macrophages and granulocytes [236, 237]. In the mammalian immune system, TGF- β 1 is a well-known suppressive cytokine and its dominant role is to maintain immune tolerance and suppress autoimmunity [238, 239]. The potent immunosuppressive effects of TGF- β 1 are mediated predominantly through its multiple effects on T cells: TGF- β 1 suppress Th1 and Th2 cell proliferation, while it promotes T regulatory cell generation by inducing Foxp3 expression. On the other hand, TGF- β also promotes immune responses by inducing the generation of Th17 cells [236, 240, 241]. Therefore, the regulatory roles of TGF- β as a positive or negative control device in immunity are widely acknowledged in mammals [238, 240, 241].

In teleost, despite the lack of extensive investigation on the functional role of TGF- β , some recent studies have revealed that TGF- β 1 also exerts powerful immune depressing effects on activated leukocytes, as it does in mammals. For instance, TGF- β 1 significantly blocks TNF α -induced activation of macrophage in goldfish and common carp, but induces the proliferation of the goldfish fibroblast cell line CCL71 [242, 243]. In grass carp, TGF- β 1 down-regulates LPS/PHA-stimulated the proliferation of peripheral blood lymphocyte by contrast with the stimulatory effect of TGF-b1 alone in the same cells [244]. In red sea bream, similar phenomenon was observed during leukocyte migration under TGF- β 1 treatment, with or without LPS challenges [245]. These findings not only define TGF- β 1 as an immune regula-

tor in teleost, but also indicate that TGF- β 1 may have retained similar functions in immunity during the evolution of vertebrates [246].

1.11. Interferons

Interferons genes are involved in mediating cellular resistance against viral pathogens and modulating innate and adaptive immune systems. Broadly, IFNs are classified into two main groups called type I and type II [45]. Type I IFN includes the classical IFN α/β , which is induced by viruses in most cells, whereas type II IFN is only composed of a single gene called IFN γ and is produced by NK cells (NK cells) and T lymphocytes in response to interleukin-12 (IL-12), IL-18, mitogens or antigens [247]. Structurally both IFN types belong to the class II α -helical cytokine family, but have different 3-dimensional structures and bind to different receptors [248].

Two IFNs (IFN α 1 and IFN α 2) have been cloned from Atlantic salmon and characterized with respect to sequence, gene structure, promoter, antiviral activity and induction of ISGs [249-252]. Salmon IFN α 1 induces both Mx and ISG15 proteins in TO cells and thus has properties similar to mammalian IFN α/β and IFN λ [251, 252]. Furthermore, salmon IFN α 1 induces potent antiviral activity against the IPNV *in vitro* [251], but this protection has not been observed *in vivo*, despite a high level of expression of IFN α detected in spleen and head kidney of Atlantic salmon challenged intraperitoneally with IPNV [78].

At least three type I IFNs have been discovered in rainbow trout. The IFN1 (rtIFN1) and rtIFN2 show high sequence similarity to Atlantic salmon IFN α 1 and IFN α 2, which contains two cysteines. On the other hand, rtIFN3 contains four cysteines, which further confirms the relationship between mammalian IFN α and fish IFNs. Recombinant rtIFN1 and rtIFN2 have both been shown to up-regulate expression of Mx and inhibit VHSV replication in RTG-2 cells. In contrast, recombinant rtIFN3 has been found to be a poor inducer of Mx and antiviral activity. Interestingly, the three rtIFNs show differential expression in cells and tissues [253]. This suggests that the three trout IFNs have different functions in the immune system of fish, which is an interesting subject for further research [254].

IFN γ has been identified in several fish species, including rainbow trout and Atlantic salmon [215, 216, 248, 255-257]. In contrast to the type I IFNs, fish and mammalian IFN γ are similar in exon/intron structure and display gene synteny. However, some fish species also possess a second IFN γ subtype named IFN gamma rel, which is quite different from the classical IFN γ [258]. Rainbow trout and carp IFN γ have several functional properties in common with mammalian IFN γ , including the ability to enhance respiratory burst activity, nitric oxide production, and phagocytosis of bacteria in macrophages [257-259]. Far less is known about the antiviral properties of fish IFN γ . However, it has been reported that it induces antiviral activity against both IPNV and the Salmon Alpha Virus (SAV) in salmon cell lines [260].

1.12. Tools for fish cytokine analysis

The major strategy of functional genomics is to identify the types of responses to specific pathogens based on cytokines expression as a predictor of profile immune response, which began by using suppressive subtractive hybridization as major tools at the beginning of the immunogenomics and upgrade to platforms of wide screening that allow identify thousands of EST's that are differentially regulated in their expression and that allow identifying potential candidates as biomarkers in the progression of the immune response at differential environmental conditions, not only against pathogens, but also in captivity stress conditions that affect the fisheries production.

1.13. Suppressive subtractive hybridization (SSH)

One of the most important biological processes in higher eukaryotes against external stimuli is the response mediated by differential gene expression. To understand the molecular regulation of these processes, the relevant subsets of differentially expressed genes of interest must be identified, cloned, and studied in detail using specific molecular techniques. In this matter, subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNAs of differentially expressed genes [261-263]. Numerous cDNA subtraction methods have been reported. In general, they involve hybridization of cDNA from one population (tester) to excess of mRNA (cDNA) from another population (driver) and then separation of the unhybridized fraction (target) from the common hybridized sequences. One of these tools is a PCR-based technique called representational difference analysis, which does not require physical separation of single-strand (ss) and double-strand (ds) cDNAs. Representational difference analysis has been applied to enrich genomic fragments that differ in size or representation [264] and to clone differentially expressed cDNAs [265]. However, representational difference analysis has the problem of the wide differences in abundance of individual mRNA species so that multiple rounds of subtraction are needed [265]. Other strategies, such as mRNA differential display [266] and RNA fingerprinting by arbitrary primed PCR [267], are potentially faster methods for identifying differentially expressed genes, but both of these methods have high levels of false positives [268] that bias high-copy-number mRNA [269], which can be inappropriate in experiments where only a few genes are expected to vary [268]. One of the techniques most often used to establish differential expression pattern between two conditions is suppression subtractive hybridization (SSH), which selectively amplifies target cDNA fragments (differentially expressed) and simultaneously suppresses non-target DNA amplification. The method is based on the fact that long inverted terminal repeats attached to DNA fragments can selectively suppress amplification of undesirable sequences in PCR procedures [270]. This method overcomes the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics [271]. Two types of SSH are possible: forward SSH, when the reaction involves the hybridization of cDNA from one population indicated as the evaluated phenotype (tester) to excess of mRNA (cDNA) from a control phenotype (driver); and reverse

SSH, when the conditions described above are inverted. Together, the two processes are called reciprocal SSH.

Different works have been done with SSH to evaluate fish immune response at the gene expression level against challenges with bacteria-derived pathogen-associated molecular patterns (PAMP) like LPS [272, 273] and whole bacteria like *Aeromonas salmonicida* [274, 275], *Listonella anguillarum* [276], *Edwardsiella tarda* [277], and *Vibrio parahaemolyticus* [278] (Table 1).

A critical step in any immune response is the recognition of invading organisms. This is mediated by many proteins, including pattern recognition receptors (PRR), which recognize and bind to molecules present on the surface of microorganisms. LPS is an essential cell wall component of gram-negative bacteria and is recognized by PRR, triggering a series of responses that lead to the activation of the host defence system. These PRRs include a number of toll-like receptors, as well as other cell-surface and cytosolic receptors that, upon stimulation, modulate immunity [279, 280]. In LPS-stimulated yellow grouper spleen a subtracted cDNA library was constructed using SSH. The contigs and singlets obtained were analyzed and a low number of immune-related genes were found [272]. In Asian seabass the up-regulation of differentially expressed genes like pro-inflammatory cytokines and related receptors, such as TNF receptor super family member 14 (TNFRSF14), IL-31 receptor A (IL31RA), chemokine receptor-like 1 (CMKLR1), chemokine (C-X-C motif) receptor 3 (CXCR3), chemokine (C-C motif) receptor 7 (CCR7) and chemokine (C-C motif) ligand 25 (CCL25), was identified at 24h post-challenge by bacterial LPS in spleen. Complement components were also identified [273]. These genes are a solid basis for a better understanding of immunity in Asian seabass and for developing effective strategies for immune protection against infections in that species.

Infection of Atlantic salmon by *A. salmonicida* was observed to stimulate an acute-phase response (APR) as part of the innate immune defence system to infection, whose gene expression pattern was remarkably observed in liver at 7 days post-infection [275] indicating that the liver appears to be the main source of APPs in fish, as in mammals. Not surprisingly, the liver gene expression pattern observed in other fish species against *L. anguillarum* [276], *E. tarda* [277], and *V. parahaemolyticus* [278]. The APR is characterized by alterations in the levels of plasma proteins referred to as acute-phase proteins (APPs), as well as the secretion of some other innate defence molecules important for innate immunity, such as complementary systems [281-283]. In Atlantic cod stimulated with atypical *A. salmonicida* (formalin-killed) interleukin-1 β (IL-1- β), interleukin-8 (IL-8), CC chemokine type 3, interferon regulatory factor 1 (IRF1), ferritin heavy subunit, cathelicidin, and hepcidin were identified in the forward spleen SSH library. Atlantic cod IRF1 was constitutively expressed at low levels, and expression was significantly elevated in spleen and head kidney at 24 h following *A. salmonicida* stimulation, with the highest levels of induction observed in the spleen [274]. The target IRF1 genes, as well as their importance in innate immune responses in fish, have not yet been determined, although the expression of IRF1 in teleost macrophages can be induced by both IFN γ and IL-1 β , with IFN γ being a much more potent inducer of IRF1 than IL-1 β [99].

Microorganism	Fish	Pathogen	Tissue/Cell type	Infection route	Reference
Bacteria	Atlantic salmon	<i>Aeromonas salmonicida</i> A449	Liver, head kidney and spleen	Intraperitoneal	Tsoi et al., 2004
	Yellow grouper	<i>LPS (E. coli)</i>	Spleen	Intraperitoneal	Wang et al., 2007
	Atlantic cod	<i>Aeromonas salmonicida (formalin-killed)</i>	Head kidney, Spleen	Intraperitoneal	Feng et al., 2009
	Asian seabass	<i>LPS (E. coli)</i>	Spleen	Intraperitoneal	Xia and Yue 2010
	Ayu	<i>Listonella anguillarum</i>	Liver	Intraperitoneal	Li et al., 2011
	Japanese flounder	<i>Edwardsiella tarda</i> (NE8003) PBL		Intraperitoneal	Matsuyama et al., 2011
	Marine medaka	<i>Vibrio parahaemolyticus</i>	Liver	Intraperitoneal	Bo et al., 2012
Virus	Mandarin fish	ISKNV	Spleen	Intramuscular	He et al., 2006
	Sea bream	Nodavirus (strain 475-9/99)	Brain	Intramuscular	Dios et al., 2007
	Atlantic cod	poly I:C	Spleen	Intraperitoneal	Rise et al., 2008
	Sea bass	SBNNV	Head kidney	Intramuscular	Poisa-Beiro et al., 2009
	Atlantic cod	ACNNV	Brain	Intraperitoneal	Rise et al., 2010
	Orange-spotted grouper	SGIV	Spleen	Intraperitoneal	Xu et al., 2010

Table 1. Transcriptomics studies on fish after treatments with bacteria or virus *in vivo* analyzed with SSH. LPS: Lipopolysaccharide; ISKNV: Infectious spleen and kidney necrosis virus; poly I:C: polyriboinosinic polyribocytidylic acid; SBNNV: Sea bass nervous necrosis virus; ACNNV: Atlantic cod nervous necrosis virus; SGIV: Singapore grouper iridovirus; PBL: Peripheral blood leukocytes.

SSH has been in several investigations to evaluate fish gene expression patterns against challenges with PAMPs, such as polyriboinosinic polyribocytidylic acid (poly I:C) [284], Infectious Spleen and Kidney Necrosis Virus (ISKNV) [285], Nodavirus [286-288], and Singapore grouper iridovirus (SGIV) [289].

Spleen gene expression in mandarin fish at 4 days post-infection with ISKNV of Mx protein, interferon-inducible protein Gig-2, and viperin (interferon-inducible and antiviral protein) was up-regulated, suggesting IFN pathway stimulation after ISKNV infection [285]. Also, two inflammatory cytokine genes, CC chemokine and IL-8, were found in the forward SSH

library, whereas the CD59/Neurotoxin/Ly-6-like protein gene was down-regulated. In mammals, CD59 is a complement regulatory protein, which can inhibit complement activation and membrane attack complex (MAC) formation on autologous cells [290], suggesting that down-regulation in the ISKNV-infected host cells may make these cells more sensitive to complement attack, mounting an anti-virus mechanism of the host [285].

In orange-spotted grouper after 5 days of infection with Singapore grouper iridovirus (SGIV) novel genes were annotated as immune-related, such as C-type lectin, epinecidin, and complement components C3 and C9. Interestingly, the most abundant clone was C-type lectin, and the microarray results at 1, 5 and 9 days post-infection indicated that its expression was up-regulated in liver, spleen and kidney [289]. Lectins are multivalent carbohydrate-binding proteins that function as important pattern-recognition receptors (PRR) and have been isolated and characterized in fish [291-294]. C-type lectin represents a very large family, most members of which are able to bind PAMP and microorganisms themselves through sugar moieties and play important roles in non-self recognition and clearance of invading microorganisms. The up-regulation of C-type lectin in different organs with immunological functions confirmed as SSH as microarrays suggest an important role in the development of control strategies against SGIV infection.

The SSH method was used to generate a subtracted cDNA library enriched in gene transcripts differentially expressed after 1 day post-infection in the brains of sea bream infected with nodavirus. Most of the expressed sequence tags (ESTs) differentially expressed in infected tissues fell into gene categories related to cell structure, transcription, cell signalling or different metabolic routes. Other interesting putative homologies corresponded to genes expressed in stress responses, such as heat shock proteins (Hsp-70) and to immune-related genes such as the Fms-interacting protein, TNF α -induced protein, interferon-induced with helicase C domain protein (mda-5), which in mammals play an important role in the synthesis and secretion of IFN type I [295]. Another nodavirus, sea bass nervous necrosis virus (SBNNV) was studied to identify genes potentially involved in antiviral immune defence in sea bass head kidney using the SSH technique [287]. The results of up-regulated EST from sea bass head kidney SSH showed significant similarities with immune genes, such as β -2 microglobulin, heat shock protein 90 (Hsp-90), IgM, MHC class I and class II, and β -galactoside-binding lectin, identified as a member of the galectin family and closely related to the galectin-1 group (Sbgalectin-1). When the recombinant protein (rSbgalectin-1) was produced and functional assays were conducted, a decrease in IL-1 β , TNF α , and Mx expression was observed in the brain of sea bass simultaneously injected with nodavirus and rSbgalectin-1 compared to those infected with the nodavirus alone, suggesting a potential anti-inflammatory protective role of Sbgalectin-1 during viral infection. A similar nodavirus, the Atlantic cod nervous necrosis virus (ACNNV), was studied to evaluate the transcript expression responses in the Atlantic cod (*Gadus morhua*) brain to asymptomatic high nodavirus carrier state [288]. In the forward brain SSH library was identified with significant similarity to genes with immune-relevant functional annotations the interferon stimulated gene 15 (ISG15), IL-8 variant 5, DEXH (Asp-Glu-X-His) box polypeptide 58 (DHX58; LGP2), radical Sadenosyl methionine domain-containing 2 (RSAD2; viperin), β -2-microglobulin (B2M), che-

mokine CXC-like protein, signal transducer and activator of transcription 1 (STAT1), and CC chemokine type 2. Interestingly, ISG15, DHX58, RSAD2, and saccin (SACS) transcripts are all strongly upregulated by both high nodavirus carriage and intraperitoneal poly I:C stimulation, suggesting a similar host response is significantly induced in the brain by both nodavirus and poly I:C. This expression pattern is corroborated when the response of Atlantic cod spleen is evaluated against poly I:C stimulation, showing the up-regulation of ISG15, RSAD2, LGP2 and other transcripts such as MHC class I, and IRF1, 7, and 10, indicating that Atlantic cod recognize dsRNA and mount an interferon pathway response [284].

1.14. Microarrays

Microarray analysis measures the expression of large numbers of genes in parallel. This methodology, which combines hypotheses-driven and hypotheses-free research strategies, is used to infer molecular mechanisms, classify samples, and diagnose and search for novel biomarkers. With the use of standard platforms, laboratory protocols and procedures for processing of primary data, the results of microarrays analyses are well suited for database management and meta-analysis across multiple experiments, whilst data mining is based on powerful statistical procedures with support from functional and structural annotations of genes [296].

The Atlantic salmon is of particular importance to the global aquaculture industry. Salmonid cDNA microarrays were constructed shortly after large-scale sequencing of salmon and trout cDNA libraries by several research institutes. One of the projects related to salmon sequencing is GRASP (Genomics Research on Atlantic Salmon Project), an initiative funded by Genome Canada that is intended to improve understanding of physiological and evolutionary processes influencing the survival and phenotype of salmonids and other fish in natural and aquaculture environments. The first salmonid GRASP microarray platform (GRASP-1), containing 7356 salmonid elements representing 3557 different cDNAs (3.7K), was obtained from 80,388 ESTs, principally from cDNA libraries [298] of different salmon species such as Atlantic salmon, rainbow trout, Chinook salmon, sockeye salmon, and lake whitefish cDNA libraries. The second version of the GRASP microarray platform (GRASP-2) was developed and contained cDNAs representing 16,006 genes (16K). The genes identified in the array have been stringently selected from Atlantic salmon and rainbow trout EST databases representing a wide variety of different classes of genes [297]. Finally, a new expanded salmonid cDNA microarray (GRASP-3) of 32,000 features (32K) was created where 69% of the total EST collection used was from Atlantic salmon [298]. The Aleksei Krasnov's group designed the rainbow trout microarray (SFA1.0) by identifying a relatively small number of genes (1300 genes; 1.3K) using clones from normalized and subtracted cDNA libraries, as well as genes selected by the functional categories of Gene Ontology for inclusion in a microarray aimed at characterizing transcriptome responses to environmental stressors [299] to maximize the presence of transcripts related directly to immune response in rainbow trout, because of which this platform is also called Immunochip (SFA1.0 immunochip). The updated SFA platform (1.8K; SFA2.0 immunochip) was specially designed for stud-

ies of responses to pathogens and stressors and has substantially improved coverage of immune genes [300]. Another cDNA platform in commercial fish species has been designed in Japanese flounder [301] and European flounder [302], turbot [303], and sole [304]. However despite impressive achievements, cDNA platforms suffer from limitations and disadvantages. At present most research groups working with salmonids and other aquaculture species do not have full access to clones required for fabrication of cDNA microarrays. Maintenance and PCR amplification of large clone sets is expensive and time consuming, while the risk of errors is high [296]. Probably the most important drawback of cDNA microarrays is their limited ability to discriminate paralogs since long probes cross-hybridize with highly similar transcripts from members of multi-gene families [305]. In salmonids this problem is aggravated by the large number of expressed gene duplicates. These complications can be resolved with oligonucleotide microarrays (ONM) that also provide greater accuracy and reproducibility of analyses. Until recently, the use of ONM platforms was hampered by the cost, but they are now rapidly replacing cDNA platforms. Construction of ONM platforms begins with establishment of mRNA sequence sets for comprehensive coverage of transcriptomes with low redundancy. The next stage is identifying genes by searching protein databases and annotating them according to functions, pathways and structural features. For successful development and use of ONM, it is necessary to define the gene composition and optimum number of spot replicates and to choose criteria for quality assessment [296].

Because of the commercial importance of salmonid species, there is special interest gene expression pattern against different pathogens. Initially salmonid (rainbow trout) ONM contained 1672 elements, representing more than 1400 genes [306]. Currently, one of the most often used ONM platforms to evaluate the response against different conditions and pathogens is the custom salmon ONM (SIQ-3), based on the Agilent Technology system (21K in 4x44K format). Because limited availability of peripheral blood leukocyte (PBL) markers is a well-recognised problem of fish immunology, this platform compares the transcriptomes of PBL and other tissues to search for genes with preferential expression in leukocytes [296], making it a very significant tool to evaluate the response to pathogens in Atlantic salmon. Another ONM platform based on 500K ESTs Atlantic salmon and 250K ESTs rainbow trout [298] is the cGRASP 44K salmonid oligo array (Agilent eArray), although no studies employing this platform have been published yet. Another ONM has been designed in fish model organisms like zebrafish and in commercial fish species such as channel catfish and turbot [307].

Functional genomic studies based on evaluating immune responses, also called immunogenomics, have been conducted *in vivo* to evaluate the response to different pathogens at the systemic level in different organs, especially the liver and head kidney. The functional genomic approach has been used with *Salmo salar* and *Oncorhynchus mykiss*, where PAMPs, whole bacteria and viruses are the most studied pathogens. Here we present different works in fish challenged by bacteria or viruses where differential gene expression profiles were evaluated using microarray platforms with special emphasis on *in vivo* fish immune response (Table 2).

1.15. Studies with bacterial pathogens, PAMPs and cytokine network interactions

One of the most commonly studied bacterial pathogens is *Aeromonas salmonicida*, a gram-negative bacteria and the causative agent of furunculosis. In fact prior to the development of species-specific cDNA microarrays a preliminary study used a human microarray (GENE-FILTERS GF211) to explore the liver response in Atlantic salmon infected using a cohabitation model [275]. Only 4 mRNAs were consistently up-regulated ($p < 0.01$) from the 241 positively identified spots with a clearly detectable hybridization signal, none of them related to cytokine expression. This was probably due to the lack of sequence homology, a problem commonly associated with cross-species cDNA hybridization. Thus the creation of species-specific platforms was a key step in fish immunology. Using a custom Atlantic salmon cDNA microarray (NRC-IMB) consisting of over 4,000 different cDNA amplicons, the first results for challenge with *Aeromonas salmonicida* were reported in 2005 [275]. The study described a cohabitation challenge and identified 16 up-regulated mRNAs in all three tissues studied (spleen, liver and head kidney), whereas 2 and 19 mRNAs were identified as down-regulated in the head kidney and liver, respectively. The authors found that genes related to the acute phase response were up-regulated in spleen and head kidney of infected salmon, indicating that the infected fish underwent a typical acute phase response to infection.

The effects of an *Aeromonas salmonicida* infection were recently reported in turbot, *Scophthalmus maximus*, [307]. Using a custom designed oligonucleotide-microarray (8x15K), the authors identified a set of 48 differentially regulated mRNAs in the spleen of challenged fish at 3 dpi, mostly related to the acute-phase and the stress/defence immune response. A study using channel and blue catfish explored the effects of a gram-negative bacterial infection on the acute phase response (APR) [308]. The authors showed up-regulation of mRNA transcripts involved in iron homeostasis, transport proteins, complement components and inflammatory and humoral immune response, indicating that conserved APR occurs as part of the innate immune response in both catfish species. Interestingly, a more acute response was observed composed of several immune pathways in the blue but not the channel catfish. More studies are required to elucidate expression patterns resulting from gram-negative bacterial infection of phylogenetically similar and different fish are required to describe common and divergent responses. This could lead to the development of marker systems, consensus on the APR in fish and treatments tailored to certain species, all of which have significant applied interest.

The activity of LPS from gram-negative bacteria, a common membrane-associated PAMP used in immunological studies, has been explored in several fish species. These studies include effects on the spleen in channel catfish [285], rainbow trout head kidney [309], and liver in the Senegalese sole [310]. Using a 19K oligonucleotide microarray (ONM) it was observed that some pro-inflammatory mRNAs in the catfish spleen were up-regulated very quickly, principally between 2 and 4 hours post-injection with LPS, whereas immunoglobulin- (2h post-injection) and antigenic presentation-related mRNA transcripts were repressed 24h post-injection [311]. A similar inhibition was reported in head kidney of rainbow trout, where the suppression of major cellular processes, including immune function and an initial

stress reaction, was followed by a proliferative hematopoietic-type/biogenesis response 3 dpi [309]. However, in the Senegalese sole a clear up-regulation of transcripts related to the immune response was reported 24 hpi in the liver [310]. These results collectively highlight the diversity of responses observed at the tissue level and reflect the nature of the immune system that is diffusely located throughout many organ compartments.

Microorganism	Fish	Pathogen	Tissue/Cell type	Resource	Platform	Reference
Bacteria	Atlantic salmon	<i>Aeromonas salmonicida</i> (A449)	Liver	cDNA	GENE- FILTERS GF211	Tsoi et al., 2003
		<i>Aeromonas salmonicida</i> (A449)	Head kidney/liver/spleen	cDNA	NRC-IMB	Ewart et al., 2005
		<i>Aeromonas salmonicida</i>	Liver/spleen	cDNA	SFA-2	Skugor et al., 2009
		<i>Aeromonas salmonicida</i> (Brivax II)	Liver	cDNA	TRAITS/SPG	Martin et al., 2010
		<i>Piscirickettsia salmonis</i>	Head kidney	cDNA	GRASP-1	Rise et al., 2004
	Chinook salmon	<i>Vibrio anguillarum</i>	Head kidney	cDNA	GRASP-1	Ching et al., 2010
	Rainbow Trout	<i>Vibrio anguillarum</i> (FDKC)	Liver	ONM	OSUrbt	Gerwick et al., 2007
		LPS (<i>E. coli</i> 026:B6)	Head kidney	cDNA	SFA-1	MacKenzie et al., 2008
		LPS (<i>E. coli</i> 026:B6)	HK macrophages	cDNA	SFA-1	Mackenzie et al., 2008
		LPS, PGN (<i>E. coli</i> 0111:B4)	HK macrophages	cDNA	SFA-2	Boltaña et al., 2011
		PGN (<i>E. coli</i> 0111:B4, K12)	HK macrophages	cDNA	SFA-2	Boltaña et al., 2011
		LPS, PGN (<i>E. coli</i> 0111:B4)/poly I:C	Erythrocytes	cDNA	SFA-2	Morera et al., 2011
	Brook trout	LPS (<i>E. coli</i> 026:B6)	HK macrophages	cDNA	SFA-1	Mackenzie et al., 2006
	Channel catfish	LPS (<i>E. coli</i> 0127:B8)	Spleen	ONM	UMSMED-1	Li et al., 2006
		<i>Edwardsiella ictaluri</i> (MS-S97-773)	Gills/head kidney/liver/skin/spleen	ONM	UMSMED-2	Peatman et al., 2007

	Blue catfish	<i>Edwardsiella ictaluri</i> (MS-S97-773)	Liver	ONM	UMSMED-2	Peatman et al., 2008
	Japanese flounder	<i>Edwardsiella tarda</i> (NE9505)	Head kidney	cDNA	Japanese flounder custom-3	Yasuike et al., 2010
		<i>Streptococcus iniae</i> (02; FKC)	Head kidney cells	cDNA	Japanese flounder custom-3	Dumrongphol et al., 2008
		<i>Mycobacterium bovis</i> (TUMSAT-Msp001) FKC	Kidney cells	cDNA	Japanese flounder custom-3	Kato et al., 2010
	Zebra-fish	<i>Mycobacterium marinum</i> (M, E11)	Whole fish	ONM	MWG - Sigma Genosys - Affimetrix	Meijer et al., 2005
		<i>Mycobacterium marinum</i> (E11; Mma20)	Whole fish	ONM	ZF Agilent	Van der Sar et al., 2009
		<i>Streptococcus suis</i> (HA9801)	Whole fish	ONM	Affimetrix Zebrafish GeneChip	Wu et al., 2010
	Turbot	<i>Aeromonas salmonicida</i>	Spleen	ONM	Turbot custom Agilent	Millán et al., 2010
	Solea	LPS (<i>E. coli</i> 011:B4)	Liver	cDNA	GENIPOL-1	Osuna-Jimenez et al., 2009
Virus	Atlantic salmon	ISAV (Glesvaer 2/90)	Gills/heart/liver/spleen	cDNA	SFA-2	Jorgensen et al., 2008
		ISAV (Glesvaer2/90)	Heart/PBL	ONM	SIQ-3	Krasnov et al., 2011
	Chinook salmon	ISAV NA-HRP4 (970)	Head kidney	cDNA	GRASP-3	Leblanc et al., 2010
	Rainbow Trout	IHNV (32/87)/attenuated IHNV	Head kidney	cDNA	SFA-1	MacKenzie et al., 2008
		IHNV (strain 220-90)	Head kidney	cDNA	GRASP-2	Purcell et al., 2011
	Japanese flounder	VHSV (KRRV9822)	Head kidney cells	cDNA	Japanese flounder custom-1	Byon et al., 2005
		VHSV (KRRV9822)	Head kidney cells	cDNA	Japanese flounder custom-2	Byon et al., 2006

	HIRRV (8601H)	Kidney cells	cDNA	Japanese flounder custom-1	Yasuike et al., 2007
	HIRRV (8601H)	Kidney cells	cDNA	Japanese flounder custom-3	Yasuike et al., 2010
Zebra-fish	VHSV (07.71)	Fin/head kidney/ liver/spleen	ONM	ZF Agilent	Encinas et al., 2010
Turbot	Nodavirus (AH95)/poly (I:C)	Kidney	cDNA	Turbot custom	Park et al., 2009

Table 2. Transcriptomics studies on fish after treatments with bacteria or virus in vivo analyzed with microarrays. FDKC: Formaldehyde –killed cells; LPS: Lipopolysaccharide; FKC: Formalin-killed cells; ISAV: Infectious salmon anemia virus; PD: Pancreas disease; CMS: Cardiomyopathy syndrome; HSMI: Heart and skeletal muscle inflammation; IHNV: Infectious hematopoietic necrosis virus; VHSV: Viral hemorrhagic septicemia virus; HIRRV: Hiram rhabdovirus; poly I:C: polyribonucleosinic polyribocytidylic acid; PBL: Peripheral blood leukocytes; ONM: oligonucleotide microarray.

For gram-positive infections in fish at the level of transcriptome analyses, infection of zebra-fish with *Streptococcus suis* is the only model reported [272]. *Streptococcus suis* is a pathogen associated with zoonosis reported in several countries [312, 313]. The Affymetrix Zebrafish GeneChip was used to identify 125 up-regulated transcripts where the most significant pathways were antigen processing and presentation, leukocyte trans-endothelial migration and the proteasome. The authors suggested that the target list obtained could serve as infection markers for gram-positive infection in fish.

Undoubtedly, the identification of prognostic biomarkers for disease resistance is a major aim for aquaculture. Functional genomics has the potential to identify such potential tools. Disease resistance is normally measured by challenge with the pathogen of interest and assessing the cumulative mortalities. Surviving fish or non-challenged siblings from the same family are then considered 'resistant'. Because this process is costly there is a need for non-lethal methodologies of measuring resistance, ideally based on molecular determinants of resistance. An initial example of this approach used the GRASP 3.7K cDNA array to identify *in vitro* macrophage and *in vivo* head kidney biomarkers in response to *Piscirickettsia salmonis* infection, yielding a number of 11 regulated genes common to both challenges. The researchers proposed 19 highly regulated transcripts as potential biomarkers to evaluate the efficacy of vaccines against *Piscirickettsia salmonis* [314]. C-type lectin 2-1, a gene whose product is involved in endocytosis and the C/EBP-driven inflammatory response [315] was identified and has been identified in almost all reports in which bacterial preparations have been used to challenge live fish [275, 309, 314, 316, 317]. Another study aimed at identifying biomarkers at the transcriptional level described differences between triploid and diploid Chinook salmon under live *Vibrio anguillarum* challenge using the GRASP 3.7K cDNA microarray [318]. Twelve annotated mRNAs were identified as showing significant differences between diploid and triploid fish. The authors however were unable to provide a descrip-

tion of the underlying mechanisms to explain the observed reduced immune function of triploid salmon.

Individual variation is a major hurdle for the development of prognostic markers as both genetic and epigenetic factors must be taken into account. The utility of, for example, C-type lectin in salmon, and other potential biomarkers in other species for bacterial disease resistance, requires further development. The future publication of several fish genomes coupled to array platforms with a much increased transcript representation could provide an exciting route to further develop this strategy by combining both functional and structural genomics for species of commercial interest with a sequenced genome. Several studies have attempted to correlate gene expression profiles with the activity of bacterins (killed bacteria preparations) used to vaccinate fish in culture. Most studies have concentrated on the rainbow trout and Japanese flounder [262, 277, 319, 320]. In trout, intraperitoneal administration of killed *V. anguillarum* resulted in identifying 36 differentially expressed transcripts [320]. Most of the identified targets are involved in inflammatory response and respond to a broad range of stimuli. This suggests that these targets have little use as markers for vaccination, contrary to previous descriptions in other studies. Both the second and fourth versions of the Japanese flounder cDNA microarray have been used to address vaccination [262, 277, 319]. The results of experimental infection with Gram-negative *E. tarda* indicated that a formalin-killed preparation reduced mortality in vaccinated fish from 90% to 20% [277]. However, a correlation between the transcriptome and the efficacy of vaccination could not be identified.

The effects of a commercial vaccine for Atlantic salmon (a six-component oil-adjuvant vaccine from PHARMAQ) were evaluated to correlate vaccine protection to high and low resistance to furunculosis. The authors did not find any association between either group and suggested that “outcomes of vaccination depend largely on the ability of host to prevent the negative impacts of immune response and to repair damages” [305]. Although this study did not identify correlations between vaccination and gene expression profiles, the potential of a functional genomics approach to evaluate the efficacy and underlying mechanisms of vaccination is highlighted. In terms of the immune response and the resulting complexity in expression patterns resulting from multiple cell types and different tissue responses, the investigator has the potential to obtain a clearer ‘image’ of the biological response from global expression data. A key objective is therefore to increase the available genomic resources much facilitated by next generation sequencing technologies to form a more robust representation of the immune system among different fish species. Furthermore, the increasing use of ONM platforms will also improve comparison across species as data sets become more easily comparable.

It remains difficult to compare microarray experiments across distinct platforms. In this respect, Meijer et al. 2005, evaluated host transcriptome profiling to *Mycobacterium marinum* infection of adult zebrafish employing three oligonucleotides platforms (MWG, Sigma Genosys, and Affimetrix). At a significance level of $P < 1.00E-5$, there were differences among the platforms in the total number of more than 2-fold up-regulated genes, whereas the 2-fold down-regulated genes were in a similar range. Evaluation of the distribution of

infection-induced genes over different categories revealed some divergence in the set from MWG, probably due to the abundance of genes of the same UniGene cluster. As well, from the total overlap of 4,138 UniGene clusters among the three microarrays, only 66 and 93 genes were up- and down-regulated, respectively [321]. With this antecedent, the same group generated a new platform (Agilent 44K) that includes their 22K probes, a 16K set of probes similar to the Sigma-Compugen oligonucleotide library, and 6K set of probes for selected genes of interest identified by previous data mining of zebrafish transcript and genome databases [322], and they evaluated the transcriptome response to acute and chronic infection by *Mycobacterium marinum*. This important effort in combining different platforms makes it clear that not all relevant genes, including immune-related ones, are represented in all platforms. Consequently, new efforts are necessary to broaden our understanding of the immune response in fish challenged with a pathogen of interest.

1.16. Wide screening in fish challenged with viral pathogens

Two studies have reported host responses to IHNV with the SFA and GRASP platforms [309, 323]. The potential mechanisms responsible for host-specific virulence were assessed in rainbow trout infected with high (M) and low virulence (U) strains of IHNV. A marked down-regulation in biological processes, including the immune response, lymphocyte activation, response to stress, transcription and translation, together with a greater viral load (M), suggest that the higher virulence is due to the ability to suppress the immune response via the transcriptional and translational machinery of cell [323]. Furthermore, in rainbow trout was compared the expression profiles of IHNV and attenuated IHNV were compared in rainbow trout over a short time frame of one and three days post-challenge. At 3 dpi, a significant change in the transcriptional program of head kidney revealed an immunological shift orientated toward the activation of adaptive immunity. This shift was IHNV-dependent as determined by differences between the attenuated and virulent IHNV specific expression profiles. The rapid systemic spreading of IHNV inhibited TNF α , MHC class I, and several macrophage and cell cycle/differentiation markers and favored a MHC class II, immunoglobulin and MMP/TBX4-enhanced immune response [309].

Parallel studies were conducted with a cDNA microarray enriched with 213 immune-related genes to study the immune response and the efficacy of DNA vaccines containing the viral G proteins of VHSV and HIRRV administered intramuscularly in the Japanese flounder [301, 324]. As expected, all DNA vaccines containing the viral G glycoprotein conferred specific protection to the challenged fish one month after vaccination. It is suggested that the protection occurs via the IFN type I system due to the number of IFN-related genes up-regulated in both studies, ISG-15, interferon-stimulated gene 56kDa (ISG56) and the Mx protein. In both studies, VHSV and HIRRV in the Japanese flounder, the majority of differentially up-regulated genes were identified between 3 and 7 days post-vaccination (dpv), including the less effective DNA vaccine containing N protein of HIRRV. Interestingly, Mx, an antiviral protein commonly used as a marker for antiviral activity in animal species, was consistently up-regulated across vaccinations [301]. In a similar observation, IRF-3, Mx, Vig-1 and Vig-8 were up-regulated in trout at the site of DNA vaccination against IHNV at 7 dpv [323].

In turbot challenged with nodavirus, both Mx and IFN-inducible proteins were identified 24 hpi [303]. These and the previously described results suggest that both the host-expressed viral glycoprotein and the virulent rhabdovirus induce a systemic anti-viral state indicative of non-specific IFN type1 innate immune response and that this canonical response is conserved among all fish. However, the mechanisms to develop a specific cytotoxic T or B lymphocyte-mediated humoral response in fish vaccinated with plasmid DNA-IHNV G that confers protective immunity have not been identified [323].

In direct relation to the above, a significant increase in transcript markers for adaptive immunity was reported in Atlantic salmon during ISA virus (ISAV) infection [325]. Importantly, a progressive increase was observed in IgZ mRNA parallel to a decrease in IgM expression that peaked > 30 days post-infection. This coordinated increase in a group of genes related to B lymphocyte differentiation and maturation and activation of T lymphocyte-mediated immunity, including CD4, TGF- β , CD8 α and IFN γ , provides strong evidence for the coordinated regulation of the two arms of the immune system in response to viral infection. An important technological contribution derived from the above study was the development of an ONM for Atlantic salmon (SIQ-3). The first assessment of the performance of these arrays was carried out in Atlantic salmon for the study of virus-responsive genes from samples infected with ISA, salmonid alpha virus/PD-virus, cardiomyopathy syndrome (CMS) agent, heart and skeletal muscle inflammation (HSMI) and PBL from fish infected with ISAV. Some 95 up-regulated transcripts were identified. Most of the regulated transcripts are related directly to the immune response or associated with antiviral response [296]. As previously mentioned, the creation of species-specific platforms has been a key challenge for the study of the immune response against pathogens. Despite impressive achievements, cDNA microarrays suffer from limitations and disadvantages, the most important drawback being the limited ability to discriminate between paralogs as long cDNA probes cross-hybridize with highly similar transcripts from members of multi-gene families [305]. Furthermore this information needs to be supplemented to establish if the increased level of detected transcripts is consistent with specific protein synthesis. Recently, a study employed a combined proteomic and transcriptomic approach to evaluate the immune response against VHS [326]. In the fins of infected fish a series of mRNA transcripts principally related to complement components, immunoglobulin-related proteins, and macrophages were up-regulated (> 2-fold), whereas in parallel using two dimensional differential gel electrophoresis (2D-DIGE), enzymes of the glycolytic pathway and some proteins related to cytoskeletal remodelling and apoptosis (such as annexin A1a) increased with infection. However, very few proteins related to anti-viral response were identified.

2. Concluding remarks

A complex network exists to regulate the innate and adaptive immune responses of fish from the various cytokines that have been reported. The study of the functional activity of these cytokines is in progress and it will be interesting to know whether mammalian Th1, Th2, Th17 and Treg responses are present in fish, regulating specific cell-mediated immuni-

ty. The recombinant production of these cytokines and antibodies against them will be the next challenge in understanding the balance of such immune responses and aid in the effective design of therapeutic strategies to manipulate the fish immune system. towards humoral or cellular immunity in response to specific antigen stimulation, vaccine strategies, functional diets to increase the quality of fishery production and predict the health of cultured fish.

The study of functional genomics in fish has provided substantial data on species of commercial interest. The major aim has been to functionally identify the intensity of responses to specific pathogens and their associated molecular components and to identify transcripts in a whole organism or specific tissue that contribute to such responses. However, the complex biology of the immune response, in which different spatial-temporal expression occurs in multiple cell types at distinct body locations, makes complete mapping of a response difficult and expensive. Moreover, considering that arrays are only as good as the transcripts represented upon them. Thus, the representation of transcripts relevant to the immune response is intimately linked to gene discovery efforts through large-scale sequencing projects, where strategies like SSH contribute not only to understanding transcriptomic response against specific pathogens but also to gene discovery. In this area, access to high-throughput NGS technology has increased in recent years and promises to make an important contribution to understanding immune response in fish. The major task now is the meta-analysis of transcriptomic data to delineate responses common among fish species to specific pathogen groups and highly specific responses. This approach will reveal host specific expression profiles and facilitate the identification of prognostic markers for diseases.

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Cytokine Regulation of Teleost Inflammatory Responses

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Additional information is available at the end of the chapter

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1. Introduction

The inflammatory response is a highly regulated process initiated by tissue damage, infiltrating pathogens or both. The primary role of inflammation is the resolution of tissue damage, including the elimination of damaged or dead cells and any infiltrating pathogens, and restoration of homeostasis. The initial recognition of tissue damage and/or pathogens is mediated by tissue resident macrophages primarily through various sentinel pattern recognition receptors such as toll-like receptors. In response to and in accordance with distinct stimuli, macrophages become activated to produce a wide range of bioactive molecules, some of which attract other cells to the site of inflammation, and others that dictate the course of an inflammatory response and eventual tissue repair. Recent evidence suggests that there are at least two activation states of monocytes/macrophages [4, 130, 211, 214]. The classically activated monocytes/macrophages possess significant antimicrobial armamentarium, secrete a plethora of factors that propagate and enhance the microbicidal activities and in general mediate pathogen clearance. The non-classically or alternatively activated monocytes/macrophages secrete factors that ablate the destructive components of the inflammatory response and promote tissue healing, repair and angiogenesis and will not be addressed further here.

The processes involved in the onset, progression and resolution of inflammation are complex and remain to be fully elucidated in vertebrates. However, it is widely believed that myeloid lineage cells are intimately involved in inflammatory reactions and their function is controlled by cytokines. This review focuses on the recent advancements in the understanding of the biology of hallmark fish pro-inflammatory cytokines, tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ) and interleukin-1 beta (IL-1 β) and their receptors.

2. Antimicrobial responses of fish phagocytes

It is well established that fish phagocytes possess oxidative burst responses, comparable to those of mammals. Absence of readily available fish cytokines limited the early research of fish phagocytes to employing pathogen products and/or crude activated cell supernatants, presumed to contain hallmark “activating” agents. This fundamental work of fish phagocyte-mediated inflammatory processes has been comprehensively reviewed [138]. Since then the specific genes encoding the components of the fish NADPH oxidase complex (Fig. 1) have been cloned in various fish species [13, 84, 119] and their expression correlated with reactive oxygen radical production [13, 63, 141]. The priming of the fish phagocyte ROI responses by recombinant fish cytokines such as TNF α [67, 133, 217], IFN γ [62, 63, 215] and IL-1 β [54, 97, 144] has also been reported. Similar to the mammalian monocyte/macrophage paradigm [22], fish monocytes have greater ability to generate reactive oxygen intermediates (ROI) following short stimulation [67, 136, 152], whereas mature fish macrophages require relatively prolonged immune stimulations to achieve comparable magnitudes of this response [58, 59, 136, 171].

Phagocytes (primarily mature macrophages) also produce microbicidal/tumoricidal reactive nitrogen intermediates in a stimulus-specific manner. This response, catalyzed by the inducible nitric oxide synthase enzyme (iNOS, Fig. 2), involves the conversion of arginine to citrulline and results in the production of nitric oxide (NO) and other products including nitrite, nitrate, and nitrosamines [86, 134, 184]. The NADPH oxidase produced superoxide anion may also react with NO to form the peroxynitrite intermediate [ONOO $^-$] that also has potent microbicidal activity [35, 156, 191, 213]. The biology of the iNOS enzyme has been reviewed in references [2, 111, 126].

The ability of fish phagocytes to produce microbicidal NOs has been well established (reviewed in reference [138]). The iNOS gene transcript has been identified in several fish species [101, 102, 163, 200] and fish macrophages have been demonstrated to up-regulate iNOS expression and produce copious amounts of NO in response to a plethora of immune stimuli [62-64, 66, 67, 85, 88, 140, 158, 180, 210]. The inflammatory cytokine regulation of fish iNOS and NO production is described below.

3. Cytokine regulation of inflammatory responses

The onset, progression and resolution of the inflammatory responses are tightly regulated through soluble mediators (cytokines, monokines, chemokines) that orchestrate inflammation. Pro-inflammatory cytokines such as TNF α , IFN γ , and IL-1 β enhance antimicrobial functions of immune cells and facilitate the pathogen clearance, while anti-inflammatory cytokines such as TGF β and IL-10 down-regulate inflammatory processes and skew cell functions towards tissue repair mechanisms. It is important to note that in addition to the hallmark cytokines discussed in this review, other cytokines such as chemokines and growth factors also participate in the regulation of an inflammatory response.

The last decade has yielded significant advances in the understanding of inflammatory responses of lower vertebrates, such as bony fish. The genes encoding hallmark cytokines have been identified and characterized in a number of fish species. Interestingly, many of these exhibit structural similarities and gene synteny organization comparable to their higher vertebrate counterparts. Conversely, multiple isoforms of certain cytokines are present in distinct fish species.

3.1. Tumor necrosis factor alpha (TNF α)

Tumor necrosis factor alpha is a central inflammatory mediator, initially identified as a serum component capable of eliciting "hemorrhagic necrosis" of certain tumors [20]. Since discovery, TNF α has been found to be produced by many cell-types and confer an incredible range of immune processes [44, 203, 209]. In the context of inflammatory responses, TNF α promotes the chemotaxis of neutrophils and monocytes/macrophages [123, 207], enhances their phagocytic capacity [95, 105, 194], primes ROI and NO responses [42, 135], chemoattracts fibroblasts [168] and elicits platelet activating factor production [19, 73, 104].

The mammalian TNF α functions as a 26 kDa type II trans-membrane protein as well as a 17 kDa soluble moiety, released by the TNF α cleaving metalloproteinase enzyme (TACE)-mediated cleavage [98, 128, 148]. A homotrimerized TNF α (soluble or membrane bound) engages one of two cognate receptors, TNF-R1 or TNF-R2, which in turn trimerize around the ligand [7, 45]. Currently, there is no consensus as to the respective contribution of these receptors to the biological effects caused by TNF α . Some evidence suggests that TNF-R1 propagates the signal from the soluble TNF α , while the membrane-bound TNF α acts exclusively through TNF-R2 [70]. Other evidence suggest that TNF-R1 is primarily involved in induction of apoptosis while the TNF-R2 functions in proliferation and cell survival [129], while other contributions suggest cooperation between the two receptors [204]. The prevailing theory proposes that TNF-R1 confers signal propagation, while TNF-R2 binds and redistributes TNF α to TNF-R1 in a process coined "ligand passing" [28, 43, 197]. Despite this, more recent literature suggests that TNF-R2 is directly involved in many inflammatory processes including the activation of T lymphocytes [93, 94], stimulation of myofibroblasts [190], as well as tumor suppression [212]. The TNF-R1 and TNF-R2 utilize largely non-overlapping signaling mechanisms, relying on recruitment of numerous downstream signaling molecules, the relative abundance of which ultimately dictates signaling outcomes (for a current review of TNF α signal transduction see recent review [143]). It is likely that the roles of respective TNF receptors in the biological outcomes of TNF α stimulation are cell type and cell activation state dependent.

3.1.1. Identification of TNF α in fish

Presence of an endogenous bony fish TNF system was first suggested in the early 1990s where the human recombinant TNF α elicited ROI production in trout leukocytes while administration of a monoclonal anti-TNF-R1 antibody blocked this response [75, 87]. Hirono *et al.* [76] identified and characterized the first cDNA transcript encoding a Japanese flounder TNF α , which had only 20-30% amino acid identity with the mammalian TNFs, but had very

similar intron/exon organization. The expression of this flounder TNF α gene increased in PBLs following LPS, ConA or PMA stimulation, suggesting a conserved role for this cytokine in fish inflammatory responses. Shortly thereafter, the rainbow trout TNF α was identified, shown to be constitutively expressed in the gill and kidney tissues, and upregulated in LPS-stimulated or IL-1 β -treated kidney leukocytes and in the trout macrophage cell line, RTS11 [103]. The catfish TNF α was also constitutively expressed in healthy fish and several catfish immune cell lines including macrophage and T cell lines, but not in B cell or fibroblast cell lines [218]. Notably, all fish TNF α proteins possess the TNF family signature, [LV]-x-[LIVM]-x₃-G-[LIVMF]-Y-[LIMVMFY]₂-x₂-[QEKHL] [103], underlining the evolutionary conservation of this cytokine.

3.1.2. Isoforms of TNF α in fish

Trout were reported to possess an additional TNF α isoform [219] and the presence of multiple bony fish TNFs was confirmed in common carp, in which first two [162], and soon thereafter a third carp TNF α isoform [167] were identified. Interestingly, polymorphisms in the gene encoding the carp TNF α 2 isoform were linked to carp resistance to the protozoan parasite, *T. borreli* [162].

3.1.3. TNF α receptors of fish

Tumor necrosis factor alpha has now been identified in a number of fish species including flounder [76], trout [103, 114], catfish [218], carp [162, 167], sea bream [18, 56], tilapia [151], turbot [142], ayu [193], fugu [165], zebrafish [165], sea bass [133], goldfish [67] and tuna [89]. Additionally, novel TNF-like molecules with unique intron/exon organization have been identified in zebrafish and flounder [165]. Despite this, knowledge regarding the cognate receptors for the teleost TNF proteins has been relatively limited.

A death domain containing TNF receptor was identified in zebrafish ovarian tissues and coined the ovarian TNF receptor (OTR) [12]. Predicted zebrafish TNF-R1 and TNF-R2 sequences are in the NCBI database, with zebrafish TNF-R1 showing high sequence homology to the OTR. The goldfish TNF-R1 and TNF-R2 cDNAs were identified based on the zebrafish sequences [61] and display conserved regions included cysteine residues and predicted docking sites for downstream signaling. Also, the goldfish TNF-R1 possesses a conserved death domain including the highly conserved motif (W/E)-X₃₁-L-X₂-W-X₁₂-L-X₃-L (with R in the W/E position) and six conserved or semi-conserved residues, crucial for the mammalian TNF-R1-mediated cytotoxicity [189]. Interestingly, while the mammalian TNF-R1 and TNF-R2 and the fish TNF-R2 contain four TNF homology domains (THD, defined by specific cysteine residues), the fish TNF-R1 proteins exhibit only 3 full THDs [61].

Recombinant extracellular domains of goldfish TNF-R1 and TNF-R2 bound to both goldfish recombinant TNF α 1 or TNF α 2 in *in vitro* binding assays [61]. As with the sea bream TNF α [56], the goldfish recombinant TNF α ligands and receptors all adopted dimeric conformations and interacted as dimers rather than trimers *in vitro*. Interestingly, there have also been several reports indicating dimerization of the mammalian TNF-R1 [131, 132, 147]. Further-

more, the mammalian p75/NTR neurotrophin receptor, a member of the TNF superfamily of proteins with many structural similarities to teleost and mammalian TNF-R1, binds to its cognate NTR ligand as a dimer [26, 57].

By examining the TNF system in lower vertebrates such as teleost fish, we can gain insight into the evolutionary origins of our own immune systems and the selective pressures that shaped them. As in mammals, TNF α appears to be central to the regulation of inflammatory responses of bony fish. Further examination of biological effects of this molecule using different lineages of fish immune cells will yield a more concrete understanding of this system in teleosts.

3.1.4. Inflammatory roles of the fish TNF α

The first functional characterization of a fish TNF α was reported in 2003 when Zou et al. [217] demonstrated that recombinant trout TNF α 1 and TNF α 2 (isoforms) both induced the IL1 β , TNF α 1, TNF α 2, IL-8 and COX-2 gene expression in primary kidney leukocytes and in the RTS11 trout macrophage cell line [217]. These recombinant TNF α isoforms also elicited dose-dependent chemotaxis of trout kidney leukocytes and phagocytosis of yeast particles. SDS-PAGE analysis of the recombinant TNF α 1 and TNF α 2 suggested that both of the recombinant molecules existed in monomeric, dimeric and trimeric states. Together, these findings suggested conservation in the pro-inflammatory roles for teleost TNF α .

Intraperitoneal administration of a mature recombinant (cleaved) form of gilthead sea bream TNF α to fish resulted in rapid recruitment of phagocytic granulocytes to the sites of injection, granulopoiesis and the priming for enhanced ROI of peritoneal and primary kidney leukocytes [56]. Intriguingly, size exclusion chromatography of this recombinant sea bream TNF α suggested that this protein existed primarily in a dimeric state [56], in contrast to the trimeric state of the mammalian TNF α .

Subsequent reports indicated that the pro-inflammatory effects of sea bream and zebrafish TNF α s were not direct but resulted from the stimulation of endothelial cells [155]. When sea bream peritoneal and head kidney leukocytes were primed with recombinant TNF α (100 ng/mL) or bacterial DNA (*Vibrio anguillarum*, 50 μ g/mL) for 16 hours, TNF α elicited significant ROI responses, albeit modest when compared to those induced by *V. anguillarum* DNA. Intraperitoneal injections of the recombinant TNF α resulted in increased expression of several pro-inflammatory genes of sea bream peritoneal leukocytes, while TNF α treatments of sea bream endocardium endothelial cells (EECs) *in vitro* also increased their immune gene expression. Notably, while the *in vitro* TNF α stimulation of EECs and macrophages resulted in substantially elevated pro-inflammatory gene expression in both cell types, the stimulated macrophages exhibited significantly more robust transcriptional responses. Although this TNF α failed to chemoattract leukocytes, TNF α -stimulated EEC-conditioned medium and supernatants from TNF α -injected peritoneal exudate cells elicited leukocyte chemoattraction. Furthermore, the zebrafish TNF α conferred neutrophil recruitment but also increased fish susceptibility to bacterial and viral infections. Accordingly, the authors of these studies proposed that unlike the mammalian cytokine, the fish TNF α elicits inflammatory functions indirectly, through non-immune cells.

In similar studies, supernatants from recombinant carp TNF α -stimulated cardiac endothelial cells primed kidney phagocyte ROI responses while recombinant carp TNF α 1 and 2 did not enhance phagocyte antimicrobial functions [155]. Notably, the recombinant forms of the zebrafish, trout, sea bream and carp TNF α proteins exhibited lytic activity towards *Trypanoplasma borreli*, akin to the trypanolytic capacity of the mammalian TNFs [52], where the membrane form of the fish TNF α was thought to be responsible for these effects. In summation of the above observations, Florenza *et al.* (2009) suggested that while the trypanolytic roles of TNF α are evolutionarily conserved, the pro-inflammatory mechanisms elicited by this molecule were not [155].

Contrary to the sea bream and carp studies, other literature suggests that akin to the mammalian cytokine, teleost TNF α also directly elicits pro-inflammatory functions. The tilapia non-specific cytotoxic cells (NCCs) constitutively express membrane bound as well as soluble forms of TNF α (in addition to granzymes and Fas ligand) to confer cytolytic activity towards target cells, and when stimulated with recombinant tilapia TNF α , become protected from activation-induced apoptosis [151]. While the turbot recombinant TNF α did not enhance macrophage ROI, it elicited *in vitro* NO production and *in vivo* inflammatory cell recruitment and activation [142]. The Ayu fish, recombinant TNF α induced ROI production by kidney cells [193] and the blue fin tuna recombinant TNF α 1 and 2 enhanced the phagocytic responses of tuna PBLs [89].

Zebrafish TNF α elicited cell signaling and conferred increased resistance to *Mycobacterium marinum* [30] while knockdown of the TNF-R1 led to enhanced mycobacterial disease progression, increased fish mortality, accelerated bacterial growth, granuloma breakdown and necrotic macrophage cell death [30]. Thus, it appears that the zebrafish TNF α is pivotal in the maintenance of encapsulated *M. marinum* granulomas and the restriction of the growth of this pathogen. Our recent work supports these findings, where the pre-treatment of goldfish macrophages with recombinant goldfish TNF α 2 ablated the *M. marinum*-mediated down-regulation of NO production by these cells and reduced the survival of intracellular bacteria [65].

We recently identified two isoforms of the goldfish TNF α and functionally characterized a recombinant goldfish TNF α 2 (rgTNF α 2) in the context of primary kidney-derived goldfish macrophage cultures (PKMs) [67]. This rgTNF α 2 induced dose-dependent chemotaxis of goldfish macrophages, enhanced their phagocytic abilities, NO production and primed the ROI responses of PKMs.

The extent of the conservation in the biology of teleost TNF α will become more evident with increased availability of tools, reagents and cell culture systems. While some literature proposes a lack of conservation of the inflammatory roles of teleost TNF α , others strongly implicate this molecule in the regulation of fish antimicrobial functions. It is also possible that the discrepancies in the above findings stem from the culture systems employed. For example, both the sea bream and carp studies that failed to observe direct effects of fish TNF α utilized freshly isolated adherent kidney phagocyte populations [52, 155]. Notably, freshly isolated mammalian myeloid populations are highly variable in their antimicrobial capabilities [116]. Similarly, goldfish PKMs exhibit temporal gain and loss of antimicrobial capabili-

ties after prolonged cultivation [67, 136]. Specifically, ROI is primarily mediated by PKM-monocytes while NO is predominantly produced by mature macrophages [67, 136].

3.2. Interferon-gamma (IFN γ)

Interferon gamma is a pleiotropic, pro-inflammatory and anti-viral cytokine, identified in the supernatants of PHA-activated lymphocytes for its unique anti-viral properties [205]. IFN γ is primarily produced by activated Th1 phenotype CD4⁺ cells [127], CD8⁺ cells [161] and natural killer (NK) cells [149] and is of central importance in host defense against intracellular and extracellular pathogens [8, 91, 99, 179, 182]. For example, IFN γ gene knock-out mice are incapable of controlling infections with *Leishmania major* [202], *Listeria monocytogenes* [81], and *Mycobacterium* [32], underlining the importance of this cytokine in the regulation of antimicrobial responses [8, 10, 21, 51, 115].

The mammalian IFN γ dimer ligates the interferon gamma receptor 1 (IFNGR1), which then associates with IFNGR2, forming a signaling complex and activating the Janus kinases (Jak) 1 and 2, associated with the receptor chains 1 and 2, respectively [83]. Jak1 and Jak2 in turn activate the IFNGR1-associated signal transducer of activation-1 (Stat1) transcription factor [33]. The IFNGR ligation may also activate and utilize Stat2 [187], albeit to lesser extent than Stat1. Subsequent transcriptional regulation of several other genes then ensues through homodimeric Stat1, heterodimeric Stat1: Stat2, through the transcription factor complexes ISGF3 and Stat1-p48, composed of Stat1: Stat2:IRF-9 and Stat1: Stat1:IRF-9, respectively [11, 118, 187, 188]. These confer transcriptional changes through recognition of IFN γ -activated sequences (GAS) in the promoter regions of target genes [188]. Within 30 minutes of IFN γ receptor ligation, there are increased transcript levels for several interferon regulatory factors (IRFs), which then modulate subsequent waves of gene expression in the IFN γ signaling cascade [208].

3.2.1. Identification of IFN γ in bony fish

Trout mitogen-simulated leukocyte supernatants possess macrophage activating capabilities (MAFs) akin to the mammalian IFN γ [58, 59], suggesting the existence of an IFN γ counterpart(s) in fish. It was also established that downstream signaling factors employed by the mammalian IFN γ (Stats), were present in fish [160] where the antibody-purified fish Stat-like factor was shown to bind the mammalian IFN γ promoter [160].

The initial fish IFN γ homolog discovery came from examination of fugu gene scaffolds [220]. Fugu homologs of mammalian genes syntenic to IFN γ were also present on the same fugu gene scaffold. This fugu IFN γ had 4 exon / 3 intron organization similar to its mammalian counterpart and shared 32.3-32.7 % and 34.9-43.3% identities with bird and mammalian IFN γ sequences, respectively. An identified trout IFN γ sequence exhibited low sequence identity with other vertebrate IFN γ proteins, but possessed the conserved signature motif ([IV]-Q-X-[KQ]-A-X₂-E-[LF]-X₂-[IV]) and C-terminal nuclear localization signal (NLS), characteristic of the mammalian IFN γ proteins [215].

3.2.2. Inflammatory roles of the fish IFN γ

The recombinant trout IFN γ (rtIFN γ) elicited increased immune gene expression in the RTS-11 monocyte-macrophage cell line [199], while this response was pharmacologically abrogated with ERK (transcription factor) or protein kinase C (PKC) inhibitors as well as by deleting the C-terminal NLS on the rtIFN γ [215]. Also, rtIFN γ stimulation of trout kidney leukocytes primed their ROI production, suggesting functional similarities between the trout and mammalian type II IFNs [215].

Adult zebrafish microinjected with recombinant IFN γ did not exhibit increased immune gene expression or enhanced protection against *Streptococcus iniae* and the spring viremia carp virus (SVCV) [110]. However, this lack of response could stem from IFN γ being bound up by cells expressing only the IFNGR1 without eliciting a detectable level of response from the relatively few cells expressing both IFNGR1 and IFNGR2. If this is the case, these results underline the localized nature of the zebrafish IFN γ function rather than the efficacy of regulation of antimicrobial functions by this cytokine.

We [62] and others [3] have functionally characterized the cyprinid IFN γ . A recombinant goldfish IFN γ (rgIFN γ) primed goldfish monocytes ROI response in a concentration dependent manner [62] and, as in mammals [72, 157], at lower concentrations rgIFN γ conferred additive ROI priming effects. The rgIFN γ also enhanced the expression of the ROI enzyme, NADPH oxidase, catalytic subunits, p67^{phox} and gp91^{phox}. Similarly, the recombinant carp IFN γ (rcIFN γ) also primed carp kidney phagocytes for enhanced ROI production [3].

The goldfish rIFN γ elicited modest but significant enhancement of phagocytosis and NO production by goldfish monocytes and macrophages, respectively [62]. This was paralleled with increased iNOS gene expression in rgIFN γ -stimulated macrophages. In contrast, carp kidney phagocytes only displayed significant iNOS gene expression and NO production when treated with a combination of carp recombinant IFN γ and 30 μ g/mL LPS, but not following rcIFN γ treatments alone [3].

Since carp and goldfish are closely related species, the above discrepancies presumably stem from differences in experimental systems. Akin to bone-marrow-derived macrophages, which acquire antimicrobial capabilities with culture time, we have observed that the antimicrobial capabilities of goldfish kidney-derived phagocytes are dynamic [136, 137]. For this reason, we used culture-derived cells, while the carp studies used freshly isolated phagocytes. Additionally, it is well established that in mammals the production of ROIs is seen as early as 1 hour after immune stimulation, whereas the production RNIs is first detected approximately 24 hours post stimulation [169]. This is very similar to what we have observed with rgIFN γ -elicited iNOS expression and NO responses.

The treatment of mature goldfish macrophages with rgIFN γ resulted in increased expression of TNF α and IL-1 β isoforms; IL-12 p35 and p40; IFN γ ; IL-8 (CXCL-8); CCL-1; and viperin (an anti-viral molecule) [62]. The treatment of carp phagocytes with a combination of carp IFN γ and LPS resulted in increased expression of TNF α ; IL-1 β ; IL-12 subunits p35 and isoforms of IL-12 p40 subunit [3]. The carp IFN γ also induced the expression of the CXCL-10 like chemokine, CXCLb, while inhibiting the LPS-induced expression of CXCL-8 isoforms,

CXCL-8_L1 and CXCL-8_L2 [3]. The latter is reminiscent of the mammalian IFN γ and CXCL-8 relationship, where shorter treatments of mammalian granulocytes with IFN γ causes decreased CXCL-8 production [23, 90, 120], while prolonged treatments increase the CXCL-8 mRNA and protein levels [90]. Additionally, mammalian blood monocytes and macrophage cell lines stimulated with IFN γ up-regulate CXCL-8 mRNA transcripts and protein levels [14, 36] due to post-transcriptional stabilization of the CXCL-8 mRNAs rather than gene expression [14]. Together, these findings suggest that the pro-inflammatory roles of IFN γ , including synergism with LPS, are conserved in cyprinid fish, while it is probable that the discrepancies in gene expression profiles may be due to distinct immune cell model systems used (primary kidney phagocytes versus cultured mature macrophages).

3.2.3. Interferon-gamma-related (IFN γ rel) cytokine

Using gene synteny analysis, Igawa *et al.* (2006) discovered two tandem IFN γ isoforms next to the fish IL-22 and IL-26 genes [82]. The corresponding IFN γ sequences, coined IFN γ 1 and IFN γ 2, were later renamed IFN γ -related (rel) and IFN γ respectively [166]. These proteins share only 17 % amino acid identity, but exhibit exon/intron organization similar to that of mammalian and fugu IFN γ , and have the IFN γ signature motif ([IV]-Q-X-[KQ]-A-X₂-E-[LF]-X₂-[IV]). Interestingly, only IFN γ but not IFN γ rel has a C-terminal nuclear localization signal (NLS).

The existence of two IFN γ isoforms, sharing all of the above characteristics was soon confirmed in siluriformes and other cypriniformes when IFN γ and IFN γ rel were identified in the catfish [122] and common carp [183], respectively. Catfish and carp were both reported to possess two distinct, alternatively spliced IFN γ transcripts, as well as a single transcript of IFN γ rel [122, 183]. The pattern of tissue gene expression of catfish IFN γ and IFN γ rel differed while both cytokines were expressed in various cell lines and immune cells, with highest expression observed in macrophages, T cells and NK cells [122]. Interestingly, low transcript levels of IFN γ rel but not IFN γ were also reported in a catfish B-cell line. Expression of the carp IFN γ increased in T cells after stimulation with PHA, while the mRNA levels of carp IFN γ rel increased in IgM⁺, B lymphocyte-enriched immune cell fractions, stimulated with high doses of LPS [183]. Furthermore, increased transcript levels of the carp IFN γ , but not IFN γ rel, were observed in the head kidneys of fish infected with *T. borreli*. The goldfish IFN γ and IFN γ rel exhibited similar mRNA levels across tissues and immune cell types [63].

3.2.4. Inflammatory roles of fish IFN γ rel

While the siluriforme IFN γ rel has yet to be functionally characterized, there has been some insight into the functions of cyprinid IFN γ rel. Zebrafish IFN γ rel mRNA is detectable in freshly laid eggs, suggesting maternal supply of this transcript [174]. The IFN γ rel mRNA levels persist throughout embryonic development, while the expression of IFN γ is not detected until later stages of development. Embryos injected with *in vitro* transcribed IFN γ or

IFN γ rel mRNAs individually, exhibited similar immune gene expression changes, while combined injections further increased certain expression profiles, suggesting non-overlapping roles for the respective IFN γ proteins.

Morpholino knock-downs of either IFN γ or IFN γ rel alone had negligible effects on zebrafish embryo survival following *Escherichia coli* challenge, while knock-down of both IFN γ s resulted in substantially diminished survival following infection [174]. Interestingly, individual morpholino knockdowns of IFN γ or IFN γ rel caused decreased survival rates of embryos infected with *Yersinia ruckeri*, while double knockdowns had a further deleterious effect on embryo survival. Presumably IFN γ and IFN γ rel elicit some overlapping and some distinct antimicrobial mechanisms such that the presence of one cytokine may be sufficient for dealing with certain pathogens but not others. It is noteworthy that while the *E. coli* (strain DH5 α) is not a natural fish pathogen, the *Y. ruckeri* is [159].

A comprehensive functional investigation of recombinant goldfish (rg) IFN γ rel and rgIFN γ revealed that these molecules differed in respective capacities to modulate pro-inflammatory responses of goldfish PKMs [63]. While rgIFN γ conferred long-lasting ROI priming effects, rgIFN γ rel induced short-lived ROI priming, causing subsequent unresponsiveness to ROI priming by other recombinant cytokines (rgIFN γ or rgTNF α 2). While rgIFN γ elicited modest phagocytosis and nitric oxide responses in goldfish monocytes and macrophages, respectively [62, 63], rgIFN γ rel was a highly potent inducer of both responses. Interestingly, rgIFN γ and rgIFN γ rel induced different gene expression profiles in goldfish monocytes, where rgIFN γ rel elicited significantly greater expression of key inflammatory genes. Notably, while both cytokines induced the phosphorylation of Stat1, its nuclear translocation was only observed following rgIFN γ treatment. Together, these findings suggest a functional segregation of the goldfish type II interferons in the regulation macrophage antimicrobial functions.

Further confirmation of this functional dichotomy between the fish type II IFNs is warranted using *in vivo* and other *in vitro* fish models. Notably, the zebrafish IFN γ rel has recently also been demonstrated to elicit more robust pro-inflammatory gene expression than IFN γ in larvae microinjected with respective IFN expression constructs [110]. Furthermore, these zebrafish IFN γ rel-mediated effects were dependent on the myeloid transcription factor SP1, underlying the specificity of this cytokine for macrophage-lineage cells.

3.2.5. IFN γ receptors in fish

Despite the growing knowledge regarding the teleost type II IFNs, the receptor systems employed by these fish molecules remain poorly understood. The IFNGR1 and IFNGR2 chains were recently identified in the rainbow trout [55]. The expression of the IFNGR1 was generally greater than that of IFNGR2 and was subject to decrease following rIFN γ or rIL1 β stimulation. Furthermore RTG-2 trout fibroblast cells transfected with an IFNGR1 construct, or CHO cells transfected with constructs expressing IFNGR1 and IFNGR2, both bound rIFN γ , where, as in mammals, the expression of the IFNGR2 chain was essential for the IFN γ -induced activity. It should be noted that a reliable trout IFN γ reporter cell line has been estab-

lished and was effectively demonstrated to specifically increase luciferase reporter expression following IFN γ stimulation but not in response to a range of other stimuli [24, 25]. Hence, a system is now in place to elucidate the distinct binding and signaling mechanisms involved in the salmonid IFN γ biological processes.

In light of the functional differences between IFN γ and IFN γ rel, we postulated that these cytokines might function through distinct receptors. When we performed gene synteny analysis of IFNGR1, we observed that while some genes were localized to the chromosome bearing the known zebrafish IFNGR1 gene, other syntenic neighbours of the mammalian IFNGR1 were present on a distinct zebrafish chromosome. Further analysis of the chromosomal region flanked by these genes revealed a second gene, encoding a distinct IFNGR1 protein [60], (these genes were denoted IFNGR1-1 and IFNGR1-2). The corresponding goldfish receptor cDNA transcripts were identified. The fish IFNGR1 sequences displayed putative Jak1 and Stat1 binding sites, pivotal for the biological functions of the mammalian IFN γ [49, 68, 69]. While the zebrafish receptors displayed comparable tissue expression, the goldfish IFNGR1-1 exhibited substantially greater mRNA levels than the IFNGR1-2 in all tissues and immune cell types examined. In order to elucidate possible binding partners for the goldfish IFNGR1-1 and IFNGR1-2, recombinant forms of their extracellular domains were produced and *in vitro* binding assays were performed. While IFNGR1-1 bound exclusively to IFN γ rel (IFN γ 1), IFNGR1-2 bound strictly to IFN γ (IFN γ 2, receptors were named after the fact). It has recently been reported that morpholino knockdowns of IFNGR1-1, IFNGR1-2 or a putative IFNGR2 abolished zebrafish IFN γ -induced gene expression [1]. In contrast, only the knockdown of IFNGR1-1, but not the knockdown of IFNGR1-2 or IFNGR2, abrogated gene expression elicited by IFN γ rel. It was suggested that IFN γ might signal through a heterodimer of IFNGR1-1 and IFNGR1-2 and a homodimer of IFNGR2 while the IFN γ rel would ligate with a homodimeric IFNGR1-1 and an as of yet unidentified receptor 2 chains. Alternatively, since IFN γ rel is present in cyprinids early in development, the knockdown of its putative receptor, IFNGR1-1 in embryos might effect development of the components required for IFN γ function. A biological relationship of such nature would be phenotypically manifest as a loss of IFN γ function (as seen in the above zebrafish study).

It would appear that certain teleost species possess receptor signaling systems to facilitate a dichotomy of type II IFN functions. Presumably, the cyprinid IFNGR1 genes arose from duplications of an ancestral IFNGR1 and subsequently diverged in respective signaling mechanisms used, where the IFN γ rel-induced Stat1-phosphorylation might be an artifact, remnant of the ancestral gene. Indeed, the importance of the C-terminal NLS of fish (and mammalian) IFN γ has been demonstrated [217] while the lack of this NLS on IFN γ rel proteins is the key distinguishing feature of the latter cytokine. The leading model for mammalian IFN γ signaling [185] suggests that after IFN γ receptor ligation, Stat1 is delivered into the nucleus via the IFN γ NLS in a complex consisting of Stat1:IFNGR1:IFN γ . Therefore, due to a lack of an NLS, IFN γ rel may have evolved to utilize distinct signaling mechanism.

3.3. Interleukin-1 beta (IL-1 β)

3.3.1. Interleukin-1 cytokine family

The interleukin-1 cytokine family is becoming increasingly diverse, with new family members being discovered and/or assigned to this family. In addition to the well characterized IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra) and IL-18 the new members of the family now include the IL-1F5-10, and IL-1F11 [34, 192]. Accordingly, it has been proposed that IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra) should be renamed IL-1F1, IL-1F2, IL-1F3 and IL-1F4, respectively [177]. The exact biological roles of these new IL-1 cytokines (IL-1F5-11) have not been fully elucidated, and it was reported that concentrations of 100-1000 fold greater than those of IL-1 β are required to them to induce biological effects [177, 178]. These new family members will not be discussed further, instead the remainder of this section will deal with the classical IL-1 cytokines and primarily IL-1 β .

The IL-1 α [106] and IL-1 β [6] were initially identified as monocyte transcripts with only 23% amino acid identity but with structural similarities. Both of these cytokines are produced as leaderless 31 kDa pro-peptides that are cleaved to generate mature 17 kDa molecules, which mediated their respective effects by binding to the IL-1RI [37, 38, 40]. The synthesis of IL-1 has been reported in several cell types, including keratinocytes, Langerhan's cells, synovial fibroblasts, mesangial cells, astrocytes, microglia, corneal cells, gingival cells, thymic epithelial cells, in addition to myeloid and some lymphoid cell types (reviewed in references [38, 40]). Although IL-1 α and IL-1 β signal through the same receptor, they induce different biological functions. It has been suggested that IL-1 α is produced primarily by epithelial cells and keratinocytes [125] and is involved in mediating local inflammatory processes. In contrast, IL-1 β is synthesized by cells such as monocytes, macrophages, Langerhan's cells and dendritic cells [38], and mediates systemic inflammatory responses [37]. This is corroborated by the fact that while IL-1 α may act as a membrane-bound pro-IL-1 α through myristylation of the protein [100, 181], IL-1 β requires intracellular processing for activation.

As mentioned, IL-1 α and IL-1 β are both produced as pro-peptides and while IL-1 α can mediate biological effects as pro-IL-1 α or as a mature IL-1 α following enzymatic cleavage (e.g. with calpain) [96], only the proteolytically cleaved mature IL-1 β , but not the pro-IL-1 β can elicit immune functions. In fact, the release of IL-1 β appears to be a two-step process, whereby the first stimulus such myeloid cell encounter of a pathogen results in increased synthesis and cytosolic accumulation of pro-IL-1 β , while a second (as of yet poorly defined) stimulus induces the proteolytic processing of pro-IL-1 β by caspase-1/IL-1 β -converting enzyme (ICE), and the subsequent release of the biologically active mature IL-1 β [39]. This processing event is further enhanced by the presence of extracellular ATP, which is recognized by the ATP receptor P2X₇R, causing an efflux of K⁺ and a concomitant activation of ICE [50]. A number of mechanisms have been proposed for the release of the mature IL-1 β into the extracellular milieu. These include: exocytosis of IL-1 β containing lysosomes; through microvesicular budding of the plasma membrane; release in exosomes by fusion of multivesicular bodies with the plasma membrane; export through specific membrane transporters; and cell-lysis-mediated release (reviewed in [46]). Possibly, these mechanisms are mutually ex-

clusive and/or cell type dependent. Further research in multiple model organism and cell types is warranted before specific mechanism(s) can be defined.

Both IL-1 α and IL-1 β bind to IL-1RI, resulting in recruitment of the IL-1R associated protein (IL-1RAcP), which amplifies the signal transduction [176]. The IL-1RI is structurally related to the toll-like pattern recognition receptors where the signal propagation through the IL-1RI involves many of the same downstream signaling components (MyD88, IL-1R associated kinase (IRAK), and NF κ B) as those employed in TLR signaling [139]. IL-1 α and IL-1 β also bind to the IL-1RII, but because this receptor lacks the intracellular signaling components of IL-1RI, it functionally serves as a “decoy” receptor by dampening the IL-1 α/β signal transduction [31]. Additionally, the IL-1 receptor antagonist (IL-1Ra) exhibits competitive inhibition of the IL-1 α/β signaling by interacting with the IL-1RI without eliciting the downstream signaling events [31].

Unlike most other cytokines and growth factors IL-1 α and IL-1 β (primarily IL-1 β) target nearly every cell type and induce a range of biological processes (reviewed in references [37, 38, 41]). Some of the numerous pro-inflammatory roles of IL-1 β include increases in collagen and pro-collagenase synthesis; increase of chondrocyte protease and proteoglycan release; increase in osteoclast-activating factor release and hence bone resorption; induction of the synthesis of lipid mediators such as PGE₂; and enhancement of the proliferation of fibroblasts, keratinocytes, mesangial, glial cells and smooth muscle cells. IL-1 β also induces chemotaxis of T and B cells, the synthesis of thromboxane by neutrophils and monocytes, basophil histamine release and eosinophil degranulation. Additionally, IL-1 β induces synthesis of type I IFNs, endothelial plasminogen activator inhibitors, and expression of leukocyte adherence receptors on endothelial cell surfaces. Based on the above, it is not surprising that IL-1 β has been implicated in numerous disorders including cardiac disease [15], rheumatoid arthritis [92], and neurodegenerative diseases [71].

3.3.2. Identification of IL-1 in fish

Fish were first suspected of possessing an IL-1 homolog when the mammalian PBL-derived IL-1 was demonstrated to enhance the proliferation of catfish T-lymphocytes in response to ConA [74], while carp epithelial cells [175], carp macrophages and granulocytes [195] and catfish monocytes [47], were shown to produce factor(s) with properties similar to those of the mammalian IL-1. Gel filtration analysis of catfish monocyte revealed two distinct bands of approximately 70 kDa and 15 kDa, recognized by polyclonal antibodies raised against mammalian IL-1 α and IL-1 β . Paradoxically, the catfish 70 kDa molecule activated catfish (but not mouse) T cells, while the 15 kDa molecule activated mouse (but not catfish) T cells [47].

The first fish IL-1 β cDNA sequence was identified in trout and exhibited 49-56% amino acid identity to the mammalian IL-1 β [170]. Notably, the trout IL-1 β did not possess a putative ICE cleavage site required for the maturation-cleavage of the mammalian IL-1 β , while the gene expression of this trout cytokine could be induced in tissues and head kidney leukocytes following LPS stimulation [170, 216], suggesting its pro-inflammatory nature.

Although there have been no reports of a fish IL-1 α , recently a novel IL-1 family member, nIL-1F has been identified in trout and *Tetraodon* spp. and was reported to have IL-1 family signature motif as well as a putative ICE cleavage site [198]. The expression of nIL-1F increased after activation of macrophages with LPS or recombinant IL-1 β . Furthermore, a recombinant form of the C-terminal nIL-1F abrogated the rIL-1 β -induced immune gene expression in RTS-11 trout macrophage-like cells, suggesting a possible competitive inhibition through the trout IL-1RI. This mechanism would be analogous to the mammalian IL-1Ra, which also competes with IL-1 β for IL-1RI binding, without inducing downstream events.

3.3.3. Isoforms of IL-1 β in fish

Because many fish species are tetraploid and have undergone genome duplication events, it is not surprising that additional isoforms of IL-1 β exist in certain fish species. An identified second trout IL-1 β , denoted IL-1 β 2, also has the 6 exon/5 intron organization, 82% amino acid identity to the trout IL-1 β 1 and no putative ICE cleavage site [150]. In catfish, two IL-1 β genes have been described and shown to undergo distinct expression patterns following challenge with *Edwardsiella ictaluri* [201]. Additionally identified cDNA of another carp IL-1 β , IL-1 β 2, exhibited 74% identity with the carp IL-1 β 1 and 95-99% identity across individual IL-1 β 2 transcripts [48]. Since several distinct IL-1 β 2 sequences were identified in a homozygous individual, it was suggested that there might be multiple IL-1 β 2 genes. The expression of the carp IL-1 β 1 and IL-1 β 2 differed following immune stimuli where the expression of IL-1 β 1 gene was on average at least ten fold greater than that of IL-1 β 2. It was also reported that transcripts of IL-1 β 2 had high substitution numbers in the coding regions, including key areas predicted to be involved in receptor binding. In light of the above, it was suggested that the IL-1 β 2 may be a pseudogene [48]. Our observations in the goldfish, a close carp relative, support this theory. Notably, the predicted goldfish IL-1 β 2 protein is truncated compared to the IL-1 β 1, and while the expression of both IL-1 β isoforms is subject to change following immune stimuli, specific treatments elicit more robust changes in the expression of IL-1 β 1 compared to IL-1 β 2 (unpublished observations).

3.3.4. Maturation cleavage of the fish IL-1 β

As alluded to above, despite the functional similarities of the fish IL-1 β s and their mammalian counterpart, all fish IL-1 β proteins identified to date lack the typical ICE cleavage site necessary for the functional maturation of the mammalian IL-1 β 1. Despite this, evidence suggests that indeed the fish IL-1 β proteins undergo cleavage. Early fish IL-1 studies using anti-mammalian IL-1 α/β serum to surveys supernatants of activated fish cells demonstrated the recognition of distinct, multiple IL-1 protein species in these supernatants. While the catfish monocyte supernatants contained 70 kDa and 15 kDa molecules with IL-1-like activity [47], carp macrophages secreted 22 kDa and 15 kDa IL-1-like factors that were recognized by mammalian anti-IL-1 antibodies [195]. The immunoprecipitation experiments using the anti-mammalian IL-1 polyclonal antibodies recognized the 15kDa factor from the macrophage supernatants, suggesting possible maturation events of the carp IL-1 [195]. In an independent survey of PHA-stimulated carp leukocytes using a monoclonal anti-carp IL-1 β antibody,

the 15 kDa protein was confirmed to be a mature carp IL-1 β , with the putative carp IL-1 β cleavage site situated approximately 15 amino acids downstream of the mammalian ICE site [117]. Analysis of the supernatants of the trout macrophage cell line, RTS-11, using an anti-trout IL-1 β polyclonal antibody detected putative native, as well as potentially cleaved trout IL-1 β proteins of 29 kDa and 24 kDa, respectively [79]. When the RTS-11 cells were transfected with a plasmid encoding a C-terminally His⁶ tagged trout IL-1 β , and the RTS-11 supernatants assayed using an Ni-NTA-column specific for the His⁶ tagged IL-1 β , the authors detected primarily a mature, 24 kDa IL-1 β protein, confirming that the trout IL-1 β underwent maturation cleavage.

In an elegant set of studies it was demonstrated that combined immune stimuli of sea bream head kidney leukocytes resulted in an accumulation of a 30 kDa, pro-IL-1 β protein, which, unlike the mammalian counterpart [113], did not exhibit a maturation cleavage or secretion following stimulation of the cells with extra-cellular ATP [146]. Furthermore, sea bream peritoneal acidophilic granulocytes and peripheral blood leukocytes accumulated the 30 kDa form of IL-1 β following challenge with *Vibrio anguillarum* [27]. In contrast, the sea bream SAF-1 fibroblast cell line shed a mature, 22 kDa IL-1 β protein through microvesicular plasma membrane budding within 30 minutes of treatment with extracellular ATP [113]. Interestingly this IL-1 β maturation/shedding process could be ablated with a pharmacological inhibitor of the mammalian ICE, suggesting a presence of an orthologous sea bream enzyme responsible for this process.

The P2X₇R receptor is the primary receptor responsible for the recognition of extracellular ATP and the concomitant release of mature IL-1 β [50]. Upon ATP treatment and activation of the HEK 293 mammalian cell line expressing the rat P2X₇R, human ICE and sea bream IL-1 β , a non-cleaved (30 kDa) sea bream IL-1 β was secreted by these cells [109]. Interestingly, neither sea bream nor zebrafish P2X₇R expression in ATP-stimulated HEK 293 cells resulted in sea bream or mammalian IL-1 β secretion. In contrast, the expression of a chimeric P2X₇R bearing the sea bream ATP-binding and rat intracellular domains led to maturation/secretion of the mammalian IL-1 β , while the outcomes of this combination on the sea bream IL-1 β were not addressed [109]. The authors of this work suggest that the mechanisms involved in IL-1 β secretion are conserved across vertebrates while the distinct stimuli that elicit the maturation events are not. We propose that alternatively, functional specificity may stem from the P2X₇R intracellular signaling, activation of species specific ICE (or alternative maturation mechanisms), and the substrate specificity of the fish Caspase1/ICE. Notably, the above group also identified the sea bream Caspase 1 and demonstrated that the recombinant form of this fish enzyme cleaved a commercially available substrate, for which the mammalian counterpart holds specificity [107]. Additional studies are needed to establish the association between the sea bream Caspase I/ICE, P2X₇R and IL-1 β .

3.3.5. IL-1 receptors of fish

Majority of the IL-1 receptor signaling components have been described in fish. A fish IL-1RI was first described in Atlantic salmon, with 43-44% similarity to chicken and 31% similarity to the human IL-1RI, respectively [186]. The expression of this gene increased in fish tissues

following LPS injection. The identification of a carp IL-1R1 was also described and the receptor characterized in the context of acute stress conditions [121].

The trout IL-1RII was identified through a selective subtractive hybridization of genes up-regulated following immune stimulation [164]. This receptor displayed low sequence identity with the mammalian IL-1RII, but exhibited surprisingly similar overall gene organization including a very short intracellular domain. The gene expression of the identified sea bream IL-1RII increased in stimulated macrophages to levels 15 times greater than IL-1 β expression [108], suggesting a conservation in the roles of the sea bream IL-1RII as a “decoy” receptor. Furthermore, sea bream IL-1RII expressed on HEK 293 cells bound the recombinant IL-1 β , confirming the specificity of this receptor-ligand pair.

Enhanced understanding of the evolutionary mechanisms responsible for shaping the vertebrate IL-1 biological pathways will be achieved through studies that examine the IL-1 receptor-ligand relationships and biological outcomes of these interactions in teleosts.

3.3.6. Inflammatory roles of the fish IL-1 β

Using the selective subtractive hybridization technique, a carp IL-1 β was identified [53] and the C-terminus of the cytokine produced as a recombinant protein [206]. This recombinant carp IL-1 β dimerized and enhanced carp antibody responses to *Aeromonas hydrophila*, where sera from carp co-injected with the IL-1 β and *A. hydrophila* had a greater agglutinating capacity than the respective controls.

A number of studies have since utilized recombinant technology to investigate the functions of the fish IL-1 β . A recombinant form of the mature trout IL-1 β (rtIL-1 β) was produced by Hong and co-workers [80] and shown to enhance the expression of the MHCII β chain, IL-1 β and COX-2 genes in trout head kidney leukocytes and the macrophage cell line, RTS-11. Functionally, rtIL-1 β elicited the proliferation of trout head kidney cells as well as the proliferation of a murine cell line, D10.G4.1, known for its dependence on the mammalian IL-1 β [80]. Also, rtIL-1 β enhanced the phagocytosis of yeast particles by trout head kidney cells [80] while peritoneal administrations of rtIL-1 β induced migration of trout leukocytes to the site of injection, enhanced phagocytosis of peritoneal cells and increased the systemic expression of IL-1 β , COX-2 and lysozyme II [77]. The injection of fish with rtIL-1 β also enhanced trout resistance to infection with the fish pathogen *A. salmonicida*. Additionally, the rtIL-1 β and the recombinant sea bass IL-1 β were demonstrated to induce Ca²⁺ mediated downstream signaling events, abrogated by leukocyte trypsin-treatments and indicating a requirement for receptor engagement [9]. Interestingly, these authors reported that IL-1 β of trout, sea bass and humans were highly species specific, which is in contradiction of the early fish IL-1 β carp and catfish work, where cross-reactivity was observed [9, 47, 74, 175, 195].

The distinct biological roles of individual sub-domains of the trout IL-1 β were also examined by generating appropriate peptides [78, 144, 145]. While a control scrambled peptide (P2) had no effect and the peptide corresponding to the putative trout IL-1 β receptor binding region (P1) had little effect on its own, a peptide (P3) corresponding to an alternative receptor binding area of the mammalian IL-1 β [5, 196], had chemotactic properties towards

head kidney cells alone or in combination with the P1 peptide (at low concentrations) [145]. Intraperitoneal injections of the rtIL-1 β P3 peptide induced peritoneal leukocyte migration and enhanced phagocytosis and reactive oxygen production by peritoneal cells. Additionally, the rtIL-1 β P3 conferred fish resistance to viral hemorrhagic septicemia virus (VHCV) early after injection. Surprisingly, injection of rtIL-1 β P1 caused an increase in *in vivo* expression of the antiviral gene Mx and inhibition of trout TNF α expression, while having no effects on the expression of a panel of other pro-inflammatory genes [78]. The injection of the rtIL-1 β P3 peptide resulted in a more robust, widespread pro-inflammatory response with increased expression in trout rtIL-1 β , IL-8 and lysozyme. This work suggests that the fish IL-1 β utilizes a highly complex receptor-ligand system, possibly unique to that of mammals.

The functional roles of the trout IL-1 β have been corroborated in others fish species. For example, injection of carp with a plasmid encoding the carp IL-1 β gene caused enhanced PHA-induced proliferation of carp lymphocytes, increased carp macrophage reactive oxygen production, enhanced phagocytosis and improved protection against *A. hydrophila* challenge [97]. A sea bass recombinant IL-1 β exhibited immuno-adjuvant properties when combined with rsIL-1 β in immunization trials against the pathogen, *V. anguillarum*. The sea bass IL-1 β also induced the proliferation of the murine IL-1 β reporter cell line (D10.G4.1), sea bass thymocytes, enhanced kidney leukocyte phagocytosis and activated peritoneal macrophages when administered i.p. [16, 17, 29]. The orange spotted grouper rIL-1 β stimulated the proliferation of grouper head kidney leukocytes and increased the gene expression of IL-1 β and COX-2 through p38 MAPK and Jnk signaling pathways [112]. Together, the above findings suggest that the functions of IL-1 β have been evolutionarily conserved in vertebrates.

4. Concluding remarks

A successful inflammatory response is defined by the presence and proficient coordination of cytokine networks consisting of hallmark mediators such as TNF α , IFN γ and IL-1 β . The synchronized involvement of these pleiotropic yet functionally distinct agents in the recruitment, regulation and functional polarization of inflammatory cells dictates the outcome of the mounted response. Thus, it can be argued that the inflammatory processes are largely defined by the efficacy of the individual and interdependent cytokine pathways.

The regulation of the vertebrate inflammatory response is complex, involving numerous mechanisms, some of which are poorly understood while others remain to be identified. This is particularly true for the teleost model systems, where lack of specific reagents for different fish species hampers our ability to examine different aspects of the regulation of inflammation at a mechanistic level. However, there is growing evidence that the key immune components required for effective inflammatory responses are present in teleosts. Notably, certain fish species possess additional pathways that regulate inflammatory processes (for example IFN γ rel and its receptor IFNGR1-1, novel chemokines and PRRs) that are distinct from those reported in mammals. The elucidation of the coordination of inflammatory responses by these factors may shed new light on the evolution of innate host defense mechanisms in lower vertebrates.

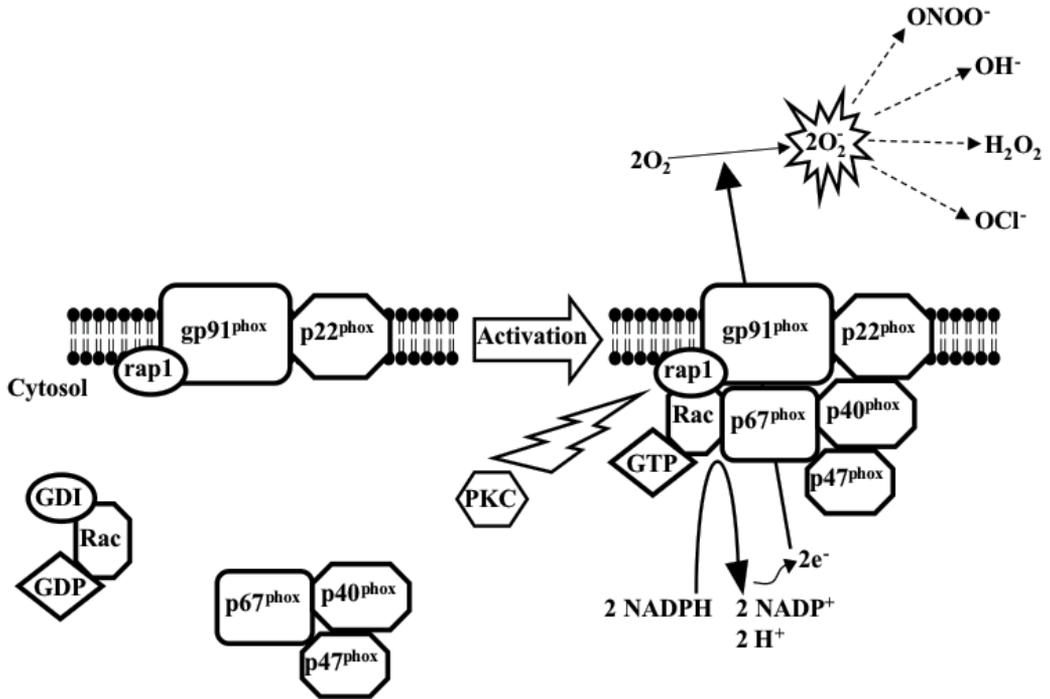


Figure 1. Schematic representation of the NADPH oxidase complex mobilization and reactive oxygen production following phagocyte activation. In a resting state, the gp91^{phox} and p22^{phox} components are membrane bound while the p40^{phox}, p47^{phox} and p67^{phox} components are located in the cytosol and the small G-protein Rac is GDP bound. Upon cell activation, Rac is rapidly converted from a GDP- to a GTP-bound state and facilitates the translocation and assembly of the cytosolic NADPH oxidase components at the cell membrane. Following PKC activation, Rap1 is thought to serve as the final switch in the activation of the NADPH complex. The activated NADPH oxidase complex accepts the electrons from the reduced NADPH and transfers these to molecular oxygen, forming the superoxide anion (O₂⁻). The generated O₂⁻ can subsequently be converted to other reactive oxygen species. Reviewed in references [124, 153, 154, 173].

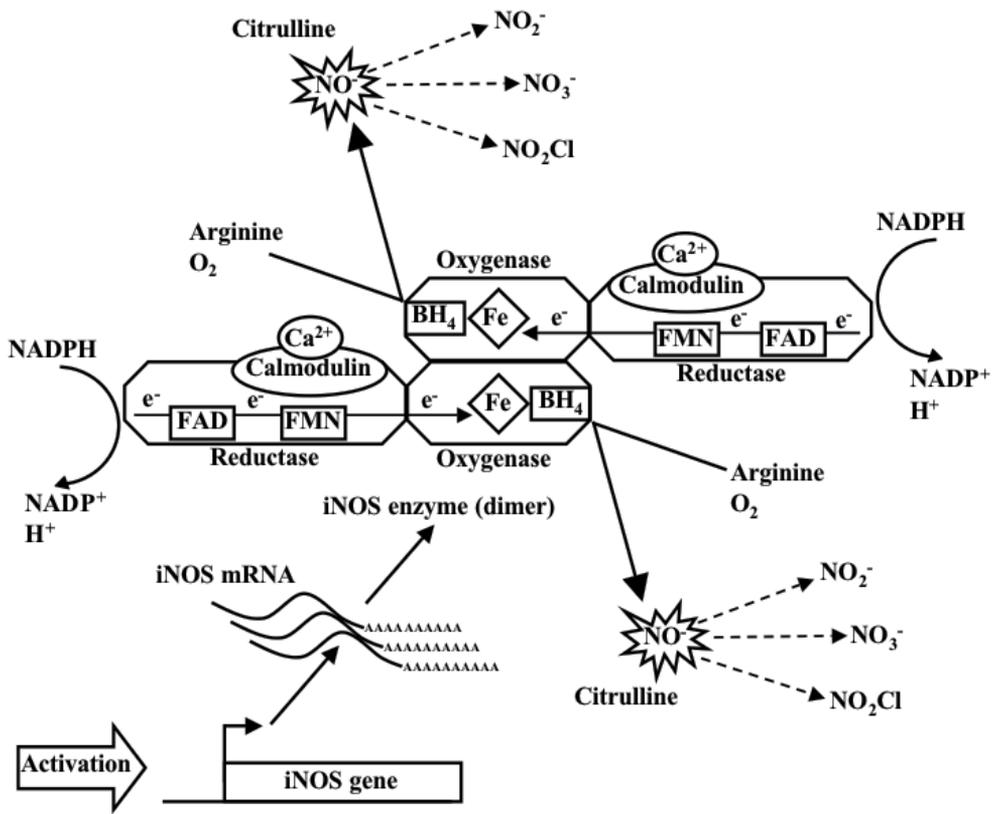


Figure 2. Schematic representation of iNOS gene expression, iNOS protein synthesis and enzymatic production of nitric oxide by the iNOS dimer complex. Upon phagocyte activation, there is a substantial increase in the expression from the iNOS gene and de novo synthesis of the iNOS enzyme. The iNOS enzyme forms a dimer and associates with a Ca²⁺ bound calmodulin, stabilizing the structure and facilitating the function of the enzymatic complex. The iNOS dimer catalyzes the transfer of electrons from a reduced NADPH, through a series of cofactors (FAD, FMN, Fe, BH₄) in the oxidation of L-Arginine to L-Citrulline with a concomitant production of nitric oxide (NO). The generated NO[•] can subsequently be converted to other reactive nitrogen species. Reviewed in references [2, 111, 126, 172].

Abbreviations

ConA: concanavalin A; **GAS:** γ -IFN-activated sequence; **Jak:** janus activated kinase; **ICE:** IL-1 cleaving enzyme; **IFN:** interferon; **IFNGR:** interferon gamma receptor; **IL:** interleukin; **IL-1R:** interleukin-1 receptor; **IL-1RAcP:** IL-1R associated protein; **iNOS:** inducible nitric oxide synthase; **IRAK:** IL-1R associated kinase; **IRF:** interferon regulatory factor; **MAF:** macrophage activating factor(s); **NK:** natural killer; **NLS:** nuclear localization signal; **NO:** nitric oxide; **NTR:** neurotrophin receptor; **PBL:** peripheral blood leukocyte; **PHA:** phytohemagglutinin; **PKC:** protein kinase C; **PKM:** primary kidney macrophage; **PMA:** phorbol myristate acetate; **PRR:** pattern recognition receptor; **rg:** recombinant goldfish; **RNI:** reactive nitrogen intermediates; **ROI:** reactive oxygen intermediates; **Stat:** signal transducer of activation transcription factor; **TACE:** TNF α cleaving enzyme; **TLR:** toll-like receptor; **TNF:** tumor necrosis factor; **TNFR:** tumor necrosis factor receptor.

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Regulation of Teleost Macrophage and Neutrophil Cell Development by Growth Factors and Transcription Factors

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Additional information is available at the end of the chapter

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1. Introduction

Macrophages and neutrophils are the sentinel cells of the innate immune response of vertebrates, such as bony fish (teleosts). As phagocytic myeloid cells, they are involved in homeostatic mechanisms, wound healing, and the detection, elimination and clearance of foreign entities including tumors, virus-infected cells and invading pathogens. Furthermore, macrophages and neutrophils are responsible for producing hundreds of bioactive molecules that are important in pathogen recognition and destruction, cellular communication and activation, initiation of an adaptive immune response and later, resolution of an inflammatory response and tissue repair. Neutrophils and macrophages, while essential to survival, have a finite lifespan. Therefore, a manufacturing centre, the hematopoietic niche, is needed for the production of myeloid cells. The hematopoietic niche must maintain basal myeloid cell production levels during homeostasis, yet retain the flexibility to ramp-up cell production in response to physiological demands, such as pathogenic insult. The development of macrophages (monopoiesis) and neutrophils (granulopoiesis) is collectively known as myelopoiesis, and is regulated by the complex interaction of colony-stimulating factors (CSFs), their receptors, and intracellular transcription factor machinery that control lineage fate decisions and terminal differentiation events.

Over the past 50 years, research using the mouse model system has culminated in the identification of the site(s) of myelopoiesis, the progenitor cell types that give rise to mature myeloid cells, the extracellular and intracellular cues required, and a detailed understanding of the complex intracellular and extracellular milieu of factors that drive this tightly controlled

process. From these studies we understand hematopoiesis as an exquisitely fine-tuned, highly regulated, process whereby all blood cells develop from a small number of hematopoietic stem cells (HSCs). HSCs are characterized as long-term repopulating, pluripotent, quiescent cells that undergo symmetrical self-renewal to sustain the population of HSCs within the hematopoietic niche, or asymmetrical division to give rise to hematopoietic progenitor cells (HPCs) [1]. HPCs can develop along the lymphoid lineage, termed lymphopoiesis, to give rise to B-cells, T-cells, natural killer (NK) cells and dendritic cells (DCs). Alternatively, HPCs can develop along an erythroid lineage, termed erythropoiesis, to give rise to erythrocytes and megakaryocytes, or develop along a myeloid lineage to give rise to granulocytes (neutrophils, basophils, eosinophils, mast cells), mononuclear phagocytes (monocytes and macrophages), and DCs. The lymphoid lineage represents the adaptive arm of the immune response, while the myeloid lineage represents the innate arm of the immune response. Regardless of lineage, the decisions made to commit and develop along a given lineage are controlled by extracellular growth factors and intracellular transcription factors that act in concert to regulate gene and protein expression to achieve the desired outcome.

When compared to the mechanisms of myelopoiesis in the mouse, studies using lower vertebrates, such as teleosts, have identified both evolutionary conservation as well as divergence in the mechanisms of myelopoiesis. With over 30,000 identified species, teleosts are the most expansive class of vertebrates, and represent an excellent model system to study the evolution of vertebrate myelopoiesis as they are one of the ancient classes of vertebrates to retain the production of myeloid cells. Within the teleost system, much research surrounds the characterization of teleost cytokines and receptors involved in inflammation and their cellular targets (primarily macrophages). In comparison, little is known about the mechanisms that govern myeloid cell production. Research on teleost myelopoiesis is hampered by the lack of reagents, the difficulty in isolating appreciable numbers of relatively pure populations of HSCs/HPCs, and in identifying key growth factors important for myeloid cell development due to evolutionary selection pressures. As such, the focus of this review is to provide an overview of the current knowledge of the fish model systems used and the growth factors, receptors and transcription factors involved in teleost myelopoiesis, using information from the mammalian model systems as a scaffold to put the advances into context.

2. Teleost model systems of myelopoiesis

2.1. Zebrafish model system

The zebrafish model has been instrumental in advancing our knowledge of the sites of hematopoiesis/myelopoiesis in teleosts, the development, differentiation and migration of HSCs, and through genetic manipulation, the characterization of the early acting growth factors, receptors and transcription factors involved in hematopoiesis. By far, the major advantage provided by zebrafish is the ease of generating transgenic zebrafish, morphant zebrafish (morpholinos) and knockout zebrafish (zinc finger nucleases), as well as many others, due to

genetic manipulation. In conjunction, the rapid generation of embryos, embryonic transparency, and small embryo size allows for mass screening strategies. The advantages of zebrafish as a model system, and their contributions to hematopoiesis have been extensively reviewed elsewhere [2-5], and thus will not be covered in this review. While the zebrafish is an excellent *in vivo* model, it does not lend itself to *ex-vivo* studies due to the small size of the fish and the difficulty of isolating sufficient number of cells for *in vitro* studies.

2.2. Ginbuna crucian carp model system

In vivo transplantation to test the repopulation activity of donor cells has been the gold standard for the characterization of HSCs and HPCs [6-9]. In cyprinid fish, there is a unique transplantation model system for detecting HSCs and HPCs using clonal ginbuna crucian carp (*Carassius auratus langsdorfii*, S3n strain) and ginbuna-goldfish (*Carassius auratus*) hybrids (S4n strain). Ginbuna crucian carp have advantages for transplantation experiments because they are easily maintained, tolerate handling and are large enough to allow for the collection of sufficient hematopoietic cells. Clonal ginbuna are unisexual triploid fish (all female, $3n = 156$) that principally reproduce gynogenetically. A unique clone (S3n) can reproduce by not only gynogenesis but also bisexual reproduction. When eggs from the S3n clone are inseminated with UV-irradiated goldfish sperm, triploid clones result. In contrast, when the eggs are inseminated with normal goldfish sperm, tetraploid hybrids (S4n) are obtained [10]. These S4n fish possessed four sets of chromosomes, three from the S3n clone and one from the goldfish. Therefore, when the cells from S3n clones are transferred into S4n recipients, transplants are accepted, whereas the reverse transplants are rejected [11, 12]. Moreover, the donor cells in the recipient tissues are easily distinguished by their difference in DNA content by flow cytometric analysis (ploidy analysis) [13].

The ginbuna crucian carp model system has been instrumental in serving as a close parallel to the mouse model system in terms of hematopoietic reconstitution experiments to demonstrate the existence of HSCs in teleosts. Identification of donor and recipient HSCs/HPCs and characterization of their progeny by ploidy analysis is useful for assessing the multipotency of different progenitor cell populations. Furthermore, the use of this model system has allowed for the determination of the location of HSCs within the hematopoietic organs of cyprinids. Use of the ginbuna crucian carp system will be particularly important for future work as antibodies are developed against markers on the surface of fish HSCs and HPCs to allow for the analysis of the potency of progenitor cell subpopulations.

2.3. Goldfish model system

The goldfish model system represents a unique opportunity to study myelopoiesis *in vitro*. Firstly, teleost monopoiesis can be examined using the previously developed primary kidney macrophage (PKM) culture system [14, 15] and has provided information on the growth factors, receptors, and transcription factors involved. Secondly, large numbers of relatively pure neutrophils can be isolated from the goldfish kidney [16] and represents a starting point for studying granulopoiesis in goldfish and will be discussed in the following sections.

Together, these two model systems will prove instrumental in understanding the factors that regulate teleost myelopoiesis.

In the *in vitro* PKM system, small mononuclear cells isolated from the goldfish kidney proliferated and differentiated over 8-10 days giving rise to three cell sub-populations, R1-, R2- and R3-gated cells [14, 15]. The cytochemical, molecular and functional characterization of these cell sub-populations demonstrated the presence of putative progenitor cells (R1 gate), monocytes (R3 gate), and mature macrophages (R2 gate). These three cell sub-populations in PKM cultures represent distinct junctures of macrophage development simultaneously occurring *in vitro* [14, 15].

The spontaneous proliferation and differentiation of PKMs suggested the production of endogenous growth factors and prompted the examination of the target cell sub-population(s) upon which they acted and their effects on cell proliferation and differentiation. The putative progenitors (R1 cells) and macrophages (R2 cells), but not monocytes, were determined to be responsible for the production of endogenous growth factors that act in an autocrine and paracrine fashion [15]. Addition of cell-conditioned medium (CCM) to sorted cell populations demonstrated the capacity of putative progenitors and monocytes to proliferate and differentiate in response to endogenous growth factors. However, treatment of macrophages (R2 cells) with CCM demonstrated their apparent terminal differentiation, while their capacity to proliferate suggested they were capable of self-renewal [15, 17]. Clearly, different endogenous growth factors present in CCM exert distinct actions on macrophage cell sub-populations.

Two pathways of macrophage development were proposed to occur in the PKM cultures. The predominant pathway was classical macrophage development in which progenitor cells differentiated into monocytes and then macrophages [17]. The second was an alternative pathway of macrophage development in which progenitor cells differentiated into macrophages without a prominent monocytic stage [17]. The possible retention of the alternative pathway of macrophage production in addition to the classical pathway may provide a mechanism for rapid generation of macrophages during injury or infection *in vivo*.

The observed kinetics of the PKM cultures suggested three phases of growth. Initially, there is a lag phase (days 1-4) where many cells die, followed by a proliferative phase (days 5-9) where cell numbers rapidly increase [14], and finally, a senescence phase (days 10-14) characterized by cell clumping and cell apoptosis [17, 18]. Differential cross screening of proliferative versus senescence phase PKMs identified a number of differentially expressed genes including those involved in hematopoiesis, signal transduction, transcription, translation and protein processing [19]. The involvement of the identified transcripts in the regulation of cell development [20-22] will be discussed in the following sections.

These seminal observations from PKM cultures established three important ideas regarding goldfish myelopoiesis: (1) kidney leukocytes produce their own endogenous growth factors important for driving proliferation and differentiation [14, 15]. (2) Within the population of small leukocyte R1 cells, a population of macrophage progenitor cells must exist. (3) Unlike mammalian systems, the progenitor cell population gives rise to fully differentiated macro-

phages *in vitro* in the absence of exogenous growth factors. Thus, the goldfish PKM model system allows for comprehensive analysis of the interactions between developing macrophage subpopulations *in vitro*.

3. Site of hematopoiesis/myelopoiesis

3.1. Two waves of hematopoiesis in vertebrates

There are two waves of hematopoiesis in vertebrates. The first wave is primitive hematopoiesis and occurs during embryonic development. Definitive hematopoiesis follows primitive hematopoiesis and occurs in the post-natal or adult animal. Primitive and definitive hematopoiesis are different on a temporal scale, a spatial scale, and in the types of cellular progeny generated. With the exception of T-cells, that undergo maturation in the thymus, lymphopoiesis and myelopoiesis occur in the major hematopoietic organs. The major hematopoietic organ of teleosts is the kidney, akin to that of mammalian bone marrow.

3.2. Primitive myelopoiesis in teleosts

The development of myelopoiesis in fish has primarily been studied using the zebrafish model system. Primitive myelopoiesis is predominated by HPCs with primarily erythroid and myeloid development potential. Initially, primitive hematopoiesis is initiated in the anterior lateral mesoderm (ALM), that gives rise to the rostral blood island (RBI), and in the posterior lateral mesoderm (PLM), that gives rise to the intermediate cell mass (ICM). The RBI is the site of primitive myeloid cell development, generating primarily primitive macrophages that undergo rapid differentiation, lacking or having a very short monocytic stage [23] and a few neutrophils [24], while the ICM is the site of primitive erythroid cell development [25]. This stage of primitive hematopoiesis occurs early during development of zebrafish, approximately 11 hours post fertilization (hpf). Following the onset of circulation, at around 24 hpf, the site of hematopoiesis then switches to the posterior blood island (PBI) [26] and produces multi-lineage progenitor cells capable of producing both primitive erythroid and myeloid cells [27]. Primitive macrophages act as phagocytes during tissue remodeling throughout embryonic development and in clearance of bacterial pathogens [23]. While primitive neutrophils also migrate to a site of infection, they were not observed to phagocytose bacteria [24]. The temporal, spatial and transcriptional control of zebrafish primitive hematopoiesis has been reviewed by [28-30]. Differences in the initial site of hematopoiesis occur between fish species, however, the production of erythrocytes and macrophages during primitive hematopoiesis is consistent [31, 32].

3.3. Definitive myelopoiesis in teleosts

The onset of definitive myelopoiesis occurs around 36 hpf in the zebrafish. Here, HSCs seed the aorta-gonad-mesonephros (AGM) and the caudal hematopoietic tissue (CHT) [33, 34]. By 48 hpf, the HSCs seed the kidney [33], the final hematopoietic site equivalent to mammalian bone marrow [35-37].

The existence of teleost kidney HSCs and HPCs capable of generating all hematopoietic lineages was demonstrated using transplantation studies in zebrafish and gimbuna crucian carp. Transplantation of whole kidney marrow from *gata1^{eGFP}* zebrafish into pre-thymic *vlad tepes* (*gata1^{-/-}*) zebrafish [37] or whole kidney marrow from *β-actin^{eGFP}* zebrafish into lethally irradiated zebrafish [38], resulted in rescue of the phenotype and produced lymphoid and myeloid cell types suggestive of the presence of HSCs capable of long-term reconstitution. However, these studies were complicated by the use of whole kidney marrow during transplantation. Using gimbuna crucian carp, HSCs, found to be associated with the trunk kidney renal tubules, were identifiable by their ability to efflux Hoechst 33342 using the ATP-binding cassette (ABC) transporter, ABCG2a, and HPCs were identified by their ability to efflux rhodamine 123 by another ABC transporter, P-glycoprotein [39-42]. HSCs, consisting of 0.33% ± 0.15 of the total body kidney cells, were capable of engraftment and long-term production (>9 months) of all hemopoietic progeny, including erythrocytes, granulocytes, monocytes, thrombocytes and lymphocytes [40, 41, 43]. HPCs, while they could also give rise to all hemopoietic progeny, were only capable of short-term reconstitution [42]. However, engraftment of donor HSCs and HPCs only occurred in anemia-induced or gamma irradiated recipients [40, 43, 44] suggesting that space within the hematopoietic niche is required for successful engraftment of HSCs to occur [40, 43]. Experiments using zebrafish and gimbuna crucian carp provide strong evidence that the teleost trunk kidney contains HSCs and HPCs capable of multi-lineage differentiation, including myelopoiesis [45].

4. Commitment to the myeloid lineage

4.1. Progression of cell development

From the mouse model we know that the commitment of a pluripotent, self-renewing HSC to a common myeloid progenitor (CMP) is a progression of lineage fate decisions controlled by extracellular cues, such as growth factors, within the hematopoietic niche [46-48], as well as the modulation of intracellular transcription factors [49-52]. The process of committing to a CMP begins with long-term HSCs (LT-HSCs), capable of self-renewal and multi-lineage differentiation. LT-HSCs give rise to short-term HSCs (ST-HSCs) with limited capacity for self-renewal, which then differentiate into multipotent progenitors (MPPs) with no ability to self-renew, reviewed by [53]. The MPPs can give rise to the CMP or the lymphoid-myeloid primed multipotent progenitors (LMPPs) [54-57]. The CMP can differentiate into megakaryocyte/erythroid progenitor (MEP) or to a granulocyte/macrophage progenitor (GMP) [58] (Figure 1). The LMPPs can differentiate into a common lymphoid precursor (CLP) that gives rise to T- and B-lymphocytes, or can also give rise to GMPs [54-57, 59, 60], and reviewed in [61].

On the other hand, the “myeloid-based model” of hematopoiesis, in which myeloid potential is retained in erythroid, T, and B cell branches even after these lineages have segregated from each other, has been proposed [62]. Notably, there is no CLP in this model [63-65]. According to this model, hematopoiesis can be understood as follows: specification toward er-

ythroid, T, and B cell lineages proceeds on a basis of a prototypical developmental program to construct myeloid cells [66, 67]. Indeed, several findings in teleosts are supportive of the myeloid-based model [68, 69]. In the future, the myeloid-based model may bring a paradigm shift in the concept of blood cell lineage development. In the following sections the key growth factors and transcription factors studied in the teleost system will be discussed.

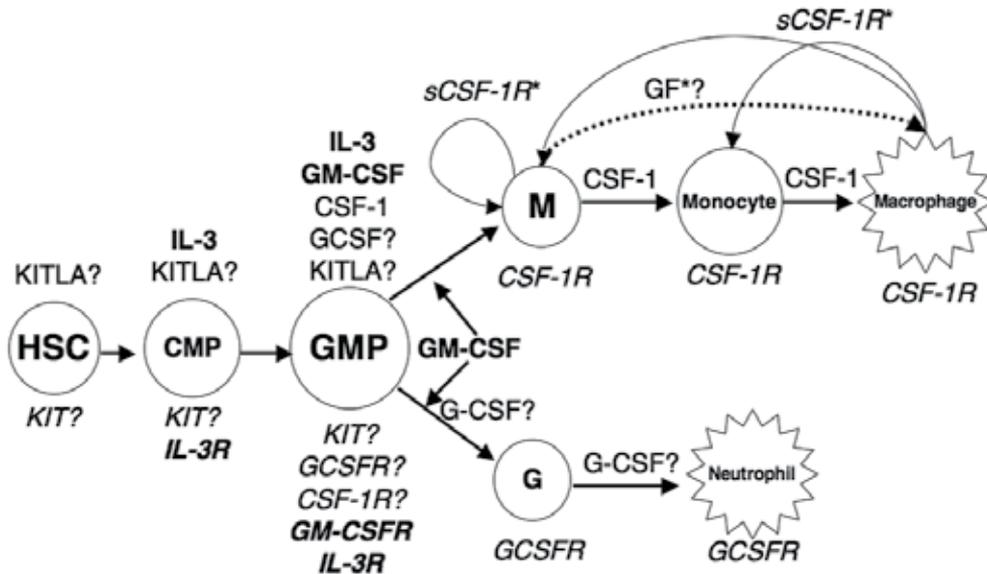


Figure 1. Growth factors and their receptors involved in goldfish myelopoiesis. Goldfish growth factors are shown in uppercase lettering, goldfish growth factor receptors/surface receptors are shown in uppercase *italics* lettering, and growth factors and their receptors important in mammalian myelopoiesis, but have yet to be identified in teleosts are shown in uppercase **italics**. The dashed arrow denotes the alternative pathway of macrophage development in goldfish, the solid curved arrows denote negative regulation of macrophage development by *sCSF-1R*. Question marks denote the hypothesized role of growth factors or receptors and further studies are required to test the hypothesis. Asterisks mark differences between teleosts and mammals. Abbreviations used: (1) **Cellular stages**: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocytic precursor. (2) **Growth factors**: KITLA, kit ligand a; IL-3, interleukin 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; CSF-1, colony-stimulating factor 1 (macrophage colony-stimulating factor); G-CSF, granulocyte colony-stimulating factor; GF, growth factor. **Receptors**: IL-3R, interleukin 3 receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; CSF-1R, colony-stimulating factor-1 receptor (macrophage colony-stimulating factor receptor); *sCSF-1R*, soluble colony-stimulating factor-1 receptor; GCSFR, granulocyte colony-stimulating factor receptor.

4.2. Receptors and growth factors

4.2.1. Mammalian stem cell factor and Kit receptor

Stem cell factor (SCF) was identified [70-72] as short-chain four-helix bundle [73] encoded by the *Steel* locus in the mouse [74]. Mutations in the *Steel* locus were associated with defects in stromal cells, and resulted in reduced numbers of HSCs and HPCs [75]. The *SCF* gene

produces two alternatively spliced mRNAs that differ in the presence or absence of exon 6 [71]. Although the two SCF splice variants can be expressed in the same tissues, they have tissue specific regulation of expression [71, 76]. Both SCF isoforms are produced as extensively glycosylated [77, 78] membrane bound forms (mSCF) that can undergo proteolytic cleavage to produce a soluble form of SCF (sSCF) [79, 80]. In human blood, sSCF is at a concentration of 3.0 ± 1.1 ng/mL [77]. Alternatively, mSCF may provide a means for cell-to-cell contact with the stromal cells in the hematopoietic niche [71], and may act to increase the signal strength provided to the HSC/HPCs, reviewed in [81]. Both mSCF and sSCF are capable of forming dimers [78, 82] and signal through their receptor, c-KIT.

The SCF receptor, c-KIT (CD117), was first identified as the cellular oncogene (*c-onc*) equivalent of the viral oncogene (*v-onc*), *v-Kit*, isolated from the Hardy-Zuckerman 4 feline sarcoma virus [83]. Based on structural analysis, the c-KIT protein was grouped within the Type III tyrosine kinase receptor family that includes colony-stimulating factor-1 receptor (CSF-1R), platelet derived growth factor receptor (PDGFR), and FLT3/FLK2 receptor [84-87]. Studies mapped *c-KIT* to the *White* locus (*W*) in the mouse [74, 83], and demonstrated that mice with mutations in the *White* or *Steel* loci exhibit hypopigmentation, mast cell deficiency, macrocytic anemia, and sterility, while the complete loss of either of these genes was lethal [74, 88].

The c-KIT protein is primarily found on hematopoietic cells and is a marker of long-term reconstituting HSCs in humans [89] and mice [90-92]. c-KIT is expressed on pluripotent and multipotent HSCs and myeloerythroid precursors, but not on differentiating or mature cell types [90-92], with the exception of mast cells [93]. Approximately 2×10^4 c-KIT receptors are found on normal human HPCs [94], and can undergo proteolytic cleavage to release a soluble form of c-KIT [95-97]. The soluble c-KIT receptor is thought to regulate membrane bound c-KIT activity, *in vivo*, by blocking SCF binding [95, 98].

Binding of homodimeric SCF to c-KIT results in receptor homodimerization, conformational changes in the extracellular and intracellular domains and autophosphorylation of the intracellular tyrosines (reviewed extensively in [73, 78, 99-105]) leading to a number of downstream signaling pathways that mediate the action of SCF through c-KIT. These signaling pathways include phosphatidylinositol-3-kinase (PI3K), phospholipase C γ (PLC γ), members of the Janus family of protein tyrosine kinases (JAK) and signal transducers and activators of transcription (STATs), Src family members, the Ras/Raf/MAP kinase pathway, and others. The signaling pathway initiated depends on the cell type, and the strength and duration of the signal, reviewed in [106-108].

4.2.2. Biological functions of stem cell factor

SCF and its type III tyrosine kinase receptor c-KIT, are involved in hematopoiesis [81, 107, 108], spermatogenesis [109-111], and development of melanocytes [110, 112-114] and mast cells [93, 96, 115-120]. Within the hematopoietic niche, one role of SCF/c-KIT is to mediate HSC and HPC survival, important for the generation of spleen, interleukin-3 (IL-3), granulocyte/macrophage, and macrophage colony-forming units (CFU-S, CFU-IL-3, CFU-GM, and CFU-M) [121]. Further studies have confirmed SCF/c-KIT to mediate the survival of long-

term HSCs by blocking cell cycling or by inhibiting apoptosis [122, 123]. Furthermore, SCF can synergizing with other growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) [124], granulocyte colony-stimulating factor (G-CSF), IL-1, IL-3 [98], IL-6, and IL-7, among others, to promote the proliferation and differentiation of HPCs [125, 126] and reviewed in [101]. Often, the progeny of HPC differentiation depends on the particular growth factor and SCF. Lastly, SCF acts as a homing signal to HPCs, such as CFU-GEMM (granulocyte-erythrocyte-macrophage-megakaryocyte), CFU-GM, CFU-Meg (megakaryocyte) and burst forming units-erythrocyte (BFU-E) [127]

4.2.3. Teleost Kit and Kit ligand

Whole genome duplication has resulted in two orthologues of *c-KIT* and *SCF* in teleosts. Teleost orthologues of *c-KIT*, termed kit a (*kita*) and kit b (*kitb*), were first identified in zebrafish and have subsequently been predicted from genomic analysis of *Takifugu rubripes* and *Tetraodon nigroviridis* [128, 129]. The *kita* orthologue has also been identified and characterized in *Carassius auratus* [130]. The two orthologues of mammalian *SCF* are termed kit ligand a (*kitla*) and kit ligand b (*kitlb*) [128, 131]. The *kitla* and *kitlb* have been identified in zebrafish, and predicted in fugu, medaka, and stickleback genomes [131]. The *kitla* orthologue has been identified and characterized in goldfish [130].

Zebrafish *kita*, located on chromosome 20, and *kitb*, located on chromosome 1, are the orthologues of human and mouse *c-KIT* [128, 129]. Both *kita* and *kitb* genes contain 21 exons, however, their respective proteins only retain 55% identity to each other [129]. The partitioning of gene distribution and function was proposed to explain the retention of *kita* and the duplicated gene, *kitb* [128, 129]. From studies on developing zebrafish, *kita* is expressed in hematopoietic progenitors, melanoblasts and melanocytes derived from the neural crest, along the lateral line, the notochord and pineal gland [128, 129]. The expression of *kitb* occurs by 9 hpf and does not overlap that of *kita*. Instead, *kitb* expression is restricted to the Rohon-Beard neurons, trigeminal ganglia, and otic vesicle [129]. Together, the expression of *kita* and *kitb* approximates that of *c-KIT* in the mouse model system, with the notable exception of *c-KIT* expression in primordial germ cells (PGCs).

The *kitla* gene is located on chromosome 25 and the *kitlb* gene is located on chromosome 4 of the zebrafish genome [132]. *Kitla* has 9 exons while *kitlb* has 8 exons [131]. The nine *kitla* exons correspond to the 9 exons of mammalian *SCF* isoform 1, including exon 6 which allows for cleavage of membrane bound *SCF* into a soluble form [131]. However, *kitlb* appears to correspond to *SCF* isoform 2, in which exon 6 has been spliced out. The expression of *kitla* is first observed at 19 hpf in the zebrafish and is found in the developing tail bud, pineal gland, sensory epithelium of the ear, ventral otic vesicles, and in the somites [131]. Similar to the expression of goldfish *kita*, *kitla* showed constitutive mRNA levels in tissues [130] and this expression pattern was similar to what was observed in adult zebrafish tissues [132]. Goldfish *kitla* showed high levels of mRNA in isolated putative progenitor cells and monocytes compared to macrophages [130]. Zebrafish *kitlb* mRNA expression was observed in the brain ventricles, ear and cardinal vein plexus and at lower levels in the skin as zebrafish development progressed [131].

4.2.4. Biological functions of teleost *kit* ligands and receptors

Based on the non-overlapping expression of *kita* and *kitb*, the functional roles of c-KIT in mammals may be partitioned between teleost KITA and KITB. The zebrafish mutant *sparse*, shown to map to *kita* [128], or *kit^{w34}* mutants [133] show defects in their pigmentation pattern. Zebrafish KITA was shown to be involved in the dispersion and maintenance of melanocytes [128], and may play a transient role in melanocyte differentiation when melanoblast development is perturbed [134]. Furthermore, knock-down of zebrafish *kitla* or *kitlb* using morpholinos supported the involvement of KITLA in the migration and survival of melanocytes [131]. Teleost *kit* expression in melanocytes has been implicated in the pigment pattern formation in a number of fish species [128, 135-137] and suggests that the functions in myelocyte development have been partitioned to the *kita* orthologue.

The role of teleost *kita/kitla* and *kitb/kitlb* during hematopoiesis is not clear. Examination of hematopoiesis in zebrafish *sparse* mutants revealed no obvious defects in hematopoiesis during development. Although, slight decreases in promyelocyte and neutrophil cell numbers, and slight increases in band cells and monocytes were observed in the kidney [128]. In addition, zebrafish injected with *kitla* morpholinos or *kitlb* morpholinos also did not show defects in hematopoiesis. However, studies in the goldfish model system demonstrated the expression of *kita* mRNA in isolated kidney progenitor cells, and the functional role of goldfish KITLA in progenitor cell chemotaxis, proliferation, and maintenance [130]. Taken together, these data suggest that KITA and KITLA proteins play a central role in myelopoiesis (Figure 1). However, redundancy between the two ligands and receptors may account for the absence of hematopoietic defects in the zebrafish system, or there may be redundancy with another tyrosine kinase receptor. Additionally, the absence of hematopoietic defects in the zebrafish may represent KIT-independent and KIT-dependent stages of hematopoiesis. The function of KITLB and KITB during hematopoiesis in teleosts remains to be determined.

Lastly, c-KIT plays a role in the development of primordial germ cells (PGCs) in mice. Examination of primordial germ cell development in fish revealed that *kita* and *kitb* expression was not detected in PGCs, and suggests teleost KITS do not play a role in the development of PGCs [128, 129]. However, it appears that *kita*, *kitb*, *kitla* and *kitlb* play a role in ovarian folliculogenesis in zebrafish and provides evidence of neofunctionalization of these genes [132].

4.2.5. Interleukin-3 and Interleukin-3 receptor

Interleukin-3 (IL-3) is a multi-lineage colony-stimulating factor (multi-CSF) that acts through the IL-3 receptor alpha and common beta chain on multipotent erythro/myeloid HPCs to promote their self renewal, proliferation and differentiation [138-140]. IL-3 can also act on committed myeloid progenitors to promote their proliferation and differentiation [138-142]. Interestingly, *IL-3*, *IL-4*, *IL-5* and *GM-CSF* are all found on chromosome 5q in humans. The close proximity of the CSFs on the chromosome, along with their similar structure and function may suggest they arose from a common ancestral gene [143]. However, genes encoding IL-3 and the specific IL-3 receptor alpha (IL-3R α) have not been identified in any teleosts to date, despite genome sequencing (Figure 1). The lack of IL-3 in teleosts may be due to diffi-

culties in identifying the IL-3 orthologue in teleosts due to the low sequence conservation of IL-3 observed between mammals, or may represent the evolutionary loss of IL-3 in teleosts. As IL-3 and IL-3R have not been identified in teleosts, IL-3 and IL-3R will not be discussed here. The structure, function and regulation of mammalian IL-3 and its receptor have been extensively reviewed elsewhere by [144-146].

4.2.6. *Granulocyte-macrophage colony-stimulating factor/Granulocyte-macrophage colony-stimulating factor receptor*

GM-CSF shares redundancy with IL-3 in terms of its function. However, GM-CSF acts on a more mature population of HPCs and has been associated with the formation of both granulocyte and macrophage colonies from CFU-GM [147, 148]. GM-CSF is produced by activated T-lymphocytes [147, 149], endothelial cells [150], and lung fibroblasts [151] and suggests the importance of GM-CSF during emergency hematopoiesis. GM-CSF promotes the survival, proliferation and differentiation of GMPs [147, 148, 152]. Furthermore, GM-CSF is chemoattractive to immature and mature neutrophils *in vitro* and *in vivo* [153, 154] and enhances neutrophil anti-microbial functions and neutrophil survival [155]. GM-CSF can also promote monocytes to differentiate into inflammatory dendritic cells [156, 157]. The GM-CSF receptor (GM-CSFR) is composed of heterodimeric alpha and beta chains as described for IL-3. Since the βc chain is common to IL-3, IL-5 and GM-CSF, the βc chain signals through JAK/STAT, MAPK, and PI3K pathways [145, 158].

Similar to that of *IL-3*, *GM-CSF* has not been identified in teleosts (Figure 1). The close proximity of *IL-3* and *GM-CSF* on the same chromosome may suggest that a genomic deletion occurred on this chromosome, subsequent to the divergence of fish and mammals. The hemopoietic CSFs that compensate for the loss of IL-3 and GM-CSF in teleosts are not known.

4.3. Transcription factors

Commitment of LT-HSCs to the myeloid lineage is an intricate regulation of the transcription factors expressed, their relative levels to one another, and their expression on a temporal scale. Transcription factors (TFs) can act antagonistically or co-operatively. Thus, the presence or absence of a TF partner, or the relative levels of a TF to its antagonistic counterpart, determine lineage fate decisions. Furthermore, the expression of a transcription factor in an HSC does not exert the same effect as when it is expressed in a committed progenitor cell. The transcriptional regulation of mammalian hematopoiesis/myelopoiesis has been extensively reviewed elsewhere [159-162], and will only be briefly described here for the purpose of putting advances in the teleost model systems into context. A visual representation of which stages these transcription factors are important is shown in Figure 2.

4.3.1. *MafB*

MAFB, a bZIP transcription factor family member, is highly expressed in LT-HSCs, but not in MPPs, CMPs, or GMPs and was recently found to be involved in restricting proliferation and myeloid lineage differentiation of LT-HSCs [163]. *MAFB*^{-/-} LT-HSCs showed increased

proliferative activity and gave rise to large numbers of primarily myeloid progeny in a mouse repopulation assay [163]. The *MAFB*^{-/-} HSCs had higher proliferative ability and gives rise to greater numbers of myeloid progeny in response to CSF-1 compared to wild type HSCs, *in vitro*. Furthermore, *in vitro* studies demonstrated that treatment of *MAFB*^{-/-} HSCs with CSF-1 led to the rapid activation of *PU.1* transcription that suggested *MAFB* must be down-regulated to allow expression of *PU.1* in MPPs [163]. It appears that *MAFB* plays an important role in antagonizing the expression of *PU.1* and the commitment of MPPs to CMPs. Furthermore, *MAFB* has been shown to bind ETS-1 though its zipper-binding domain and can act to repress erythroid lineage commitment in CMPs [164].

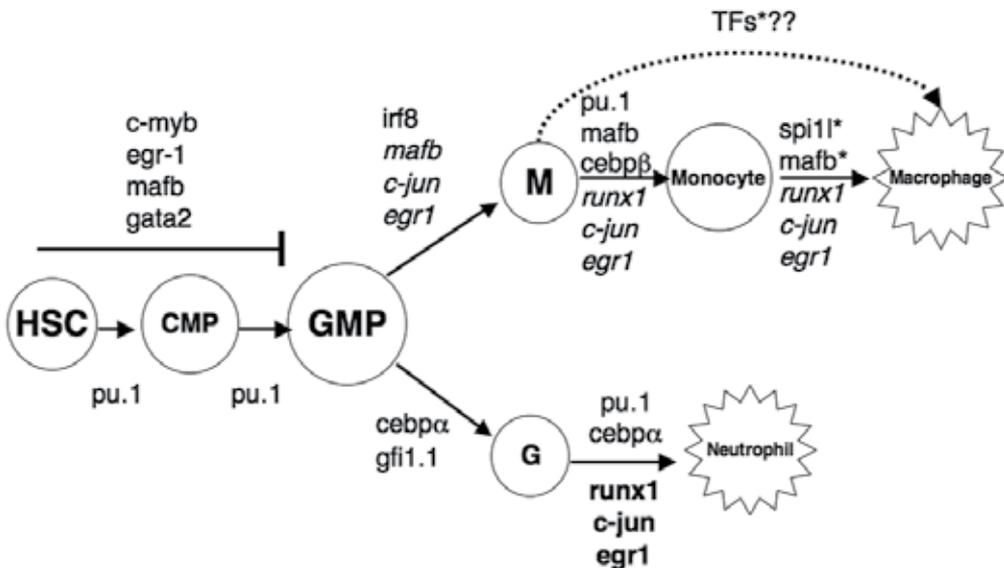


Figure 2. Transcription factors involved in goldfish myelopoiesis. Goldfish transcription factors shown in lower case lettering are up-regulated, goldfish transcription factors shown in bold are down-regulated, transcription factors that are important in cellular differentiation in mammalian systems but have yet to be studied in the teleost system are shown in italics. The dashed arrow denotes the alternative pathway of macrophage development in teleosts. Question marks denote unknown transcription factors involved in the alternative pathway of macrophage development. Asterisks mark differences between teleosts and mammals. Abbreviations used: (1) Cellular stages: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocyte precursor. (2) Transcription factors: c-MYB, cellular myeloblastosis oncogene; EGR-1, early growth response-1; MAFB, musculoaponeurotic fibrosarcoma oncogene homologue B; GATA2, GATA binding protein 2; IRF8, interferon regulatory factor 8; CEBPα, CCAAT/enhancer-binding protein alpha; GF11, growth factor independent 1; RUNX1, runt-related transcription factor 1.

In zebrafish, the *mafb* orthologue has been identified and mRNA was found expressed in the blood forming regions of the developing embryo [165]. However, the role of *MAFB* in zebrafish HSCs has not yet been assessed.

4.3.2. C/EBPs

CCAAT/enhancer binding proteins (C/EBPs) are members of the family of transcription factors that contain a C-terminal basic leucine zipper domain (bZIP) comprised of a basic region involved in DNA binding and a leucine zipper domain involved in protein interactions [166]. Six members of the C/EBP family have been identified in mammals: alpha, beta, gamma, delta, epsilon and zeta [167]. Orthologues of the C/EBP family of transcription factors have been identified in teleosts [168-171], corresponding to C/EBP α , C/EBP β , C/EBP γ , C/EBP ϵ , and C/EBP δ .

Expressed in HSCs, CMPs and GMPs [172, 173], C/EBP α has been shown to be involved in directing granulocyte cell fate and terminal differentiation of neutrophils, along with C/EBP ϵ . Mice deficient in C/EBP α show diminished numbers of CFU-GM, CFU-M, CFU-G, macrophages and neutrophils [174, 175]. The loss of myeloid cells in C/EBP α deficient mice is reflective of the role that CEBP α plays in determining the fate of a CMP to a GMP lineage versus an MEP lineage [176]. C/EBP α is capable of binding to the *PU.1* promoter [175] and up-regulating *PU.1* expression, to dictate a GMP cell fate [175, 177] (see discussion on PU.1 below). The increase in C/EBP α in GMPs has been shown to inhibit monocyte/macrophage differentiation [178] and initiate differentiation along the granulocyte lineage by regulating *GCSFR*, elastase and myeloperoxidase gene expression [179-181].

The zebrafish CEBP α orthologue showed 66% amino acid identity to human C/EBP α , while the bZIP domains showed 99% amino acid identity [168]. In zebrafish, *cebpa* was expressed in myeloid cells on the surface of the yolk sac during embryogenesis [168]. At 16 hpf, a population of blood cells co-expressed the transcription factors *gata1*, *pu.1* and *cebpa*, and by 22 hpf, the majority of the *cebpa*⁺ cells co-expressed *pu.1*, however, not all *pu.1*⁺ cells expressed *cebpa* [182]. Furthermore, *cebpa* was co-expressed with myeloperoxidase (*mpo*), a marker for granulocytes, but *cebpa*⁺ cells did not always express *mpo* [182]. These three cell sub-populations likely represent distinct junctures in myeloid cell development: erythromyeloid cells, GMPs and committed neutrophils and their precursors, respectively. The expression of *cebpa* with these additional markers mirrors the importance of C/EBP α in the mammalian system in which C/EBP α is important for commitment to a myeloid lineage versus an erythroid lineage, to a granulocyte lineage over a macrophage lineage, and in terminal differentiation of neutrophils. An orthologue of *cebpa* was also identified in Japanese flounder and mRNA was observed in the head and posterior kidney, spleen, liver, gill, heart, brain, skin, intraperitoneal cells, and weakly in the intestine, muscle and PBLs [171]. However, expression of *cebpa* in isolated cells populations was not performed.

Two studies have examined the function of CEBP α in zebrafish primitive myelopoiesis. The injection of a deletion mutant of *cebpa* into zebrafish embryos functioned as a dominant-negative mutation and blocked the production of full-length CEBP α . These embryos exhibited an increase in *gata1*⁺ expression in the posterior lateral plate mesoderm at 22 hpf and in the intermediate cell mass at 26 hpf, reflective of an erythroid progenitor cell expansion. This expression corresponded to a subsequent increase in circulating erythrocytes based on the increase in α -hemoglobin expression, indicative of erythrocytes [182]. However, the expressions of the myeloid specific genes, *mpo* and *l-plastin*, were normal [182]. Based on the pat-

tern of expression, it was suggested that PU.1 acts upstream or in parallel with C/EBP α during zebrafish primitive myelopoiesis [182]. Recently, it has been shown that the sumoylation (a post-translational protein modification) of zebrafish CEBP α inhibited CEBP α transcriptional activity and its ability to interact with and repress GATA1, thus driving lineage commitment of a myelo-erythroid progenitor to that of the erythroid lineage [183]. Taken together, these studies demonstrate the conserved role of CEBP α in the commitment of a CMP to a GMP. However, due to the toxicity of *cebpa* morpholinos to zebrafish embryos, knock-down experiments could not be performed.

Cebpb was identified in rainbow trout as a single intron-less gene and the predicted CEBP β protein showed 30-34% amino acid identity to mammalian C/EBP β [169]. The *cebpb* mRNA was detected in the head and posterior kidney, spleen, liver, gill, intestine, muscle and peripheral blood leukocytes (PBLs) [169]. Japanese flounder CEBP β also showed a low (33-38%) amino acid identity to other vertebrate sequences, but retained 95% amino acid identity in the bZIP domain. The *cebpb* mRNA was expressed in the head and posterior kidney, liver, gill, brain, peritoneal cavity fluid and PBLs, with low mRNA levels in the heart, intestine, mucus, eye and spleen [170]. In zebrafish, CEBP β showed 49% amino acid identity to human C/EBP β and *cebpb* mRNA was detected in cells on the surface of the yolk sac, corresponding to the myeloid cells that normally spread over the yolk sac early in embryogenesis [168]. A *cebpb* transcript was also identified in a differential cross-screen of goldfish proliferative phase and senescence phase PKMs, and was up-regulated in goldfish monocytes, and expressed in low levels in progenitors and macrophages [19]. However, the functional role of CEBP β has not been examined in teleost myelopoiesis.

The orthologues of C/EBP δ , C/EBP γ and C/EBP ϵ exist in teleosts. The *cebpd* and *cebpg* transcripts were identified in zebrafish and show a ubiquitous expression pattern in embryos [168]. CEBP δ and CEBP γ showed 57 and 50% identity to their human counterparts on the amino acid level. However, their bZIP domains showed higher conservation to their human counterparts, with 86% and 76% amino acid identity, respectively [168]. The *cebpe* orthologue was identified in Japanese flounder and its corresponding predicted protein had a 27% overall amino acid identity and a 90% amino acid identity in the bZIP domain compared to the mammalian counterparts, but failed to cluster with other *cebpe* sequences in phylogenetic analysis [170]. The *cebpe* mRNA was detected in the head and posterior kidney, spleen, brain, peritoneal cavity fluid and at low levels in the PBLs. However, the functional role of these C/EBPs in teleost myelopoiesis is unknown.

4.3.3. PU.1

The Ets transcription family member PU.1 is well known as the master transcriptional regulator of mammalian myelopoiesis through an antagonistic relationship with GATA1, recently reviewed by [184]. At the N-terminus, PU.1 comprises of an acidic domain and a glutamine rich domain that are involved in activation of transcription, and a PEST domain important for protein interactions [184]. At the C-terminus, PU.1 has an Ets domain important for binding the DNA consensus sequence AAAG(A/C/G)GGAAG [185]. Mice deficient in PU.1 (*PU.1*^{-/-}) have reduced CLPs, and GMPs, increased numbers of MEPs, and lack B-

cells, T-cells, monocytes/macrophages as well as severely reduced numbers of granulocytes [186-190]. *PU.1* is expressed in HSCs, CLPs and at varying levels in CMPs, increasing as these progenitors are induced to differentiate into monocytes/macrophages and neutrophils [191]. At the CMP stage, PU.1 antagonizes with GATA1 to determine whether the CMP commits to a GMP or a MEP. PU.1 binds to GATA1 and inhibits GATA1 from binding to and initiating transcriptional activation of a number of erythroid genes that are important for commitment to an erythroid lineage [184, 192, 193]. The reverse is also true; GATA1 can bind to PU.1 and inhibit the binding of PU.1 and transcriptional activation of a number of myeloid genes [184, 192, 193], including to the promoters of *CSF-1R* [194-196] and *GCSFR* genes [181, 196, 197]. Therefore, the lineage fate decision along a GMP or a MEP fate is a balancing act in timing and relative protein levels of PU.1 and GATA1.

PU.1 also plays a role at the GMP stage to regulate commitment to a granulocyte or macrophage lineage. Increased levels of PU.1 at the GMP stage, along with AP-1 association, drives a monocyte cell fate, while lower levels of PU.1 drives granulocyte cell fate [175, 177]. Furthermore, PU.1 induces *EGR-2* and *NAB-2* expression [177]. The *EGR-2/NAB-2* transcription factors function to repress neutrophil genes by antagonizing GFI1, an important transcription factor in the initiation of neutrophil differentiation [177], discussed in section 5.3.2.

An orthologue of PU.1 has been identified in teleosts. In the Japanese flounder, *pu.1* mRNA was detected in the head and posterior kidney, spleen, heart, PBLs, intraperitoneal cells, and weakly in the intestine and gill, but was absent from the liver, skin, muscle and brain [171]. In zebrafish, *pu.1* was identified as a single gene copy and analysis of the predicted protein sequence showed the conserved transactivation, PEST, and DNA-binding domains. Although the overall amino acid identity to other PU.1 proteins was 48-53%, the DNA-binding domain of zebrafish PU.1 showed 83% amino acid identity to mammalian PU.1 [198]. Examination of the zebrafish *pu.1* promoter region predicted potential binding sites for PU.1 and CEBP α [199]. The expression of *pu.1* is first detected at 12 hpf in blood cells from the PLM, later in the ICM, and finally in the kidney, and these *pu.1*⁺ blood cells give rise to myeloid cells [198-200]. The population of *pu.1*⁺ cells represents myeloid HPCs, myeloid precursors, monocytes/macrophages and neutrophils during both primitive and definitive myelopoiesis in the zebrafish [24, 200].

Knockdown of *pu.1* in zebrafish using morpholinos showed a large reduction in the number of cells positive for *mpo* and *l-plastin* mRNA, markers of granulocytes and monocytes/macrophages [201, 202]. In addition to the loss of myeloid cells, an increase in *gata1* expression was observed, and these *gata1*⁺ cells gave rise to mature erythrocytes [201]. Conversely, *gata1* morphants failed to develop mature erythrocytes and showed an increase in the number of *pu.1*⁺, *mpo*⁺ and *l-plastin*⁺ cells [201, 202]. Ectopic expression of *pu.1* or *gata1* was observed in *gata1* or *pu.1* morphants, respectively, suggesting the conversion of progenitors to an alternate lineage [201, 202]. Microarray analysis of genes regulated by PU.1 revealed the regulation of ~250 genes, including *cebpa*, *csf-1r* and myeloid-specific peroxidase (*mpx*), among others [203]. Taken together, PU.1 has a conserved role in dictating a myeloid lineage, opposing GATA1 and the transcriptional activation of erythroid genes.

A *pu.1*-like gene (*spi-1* like, *spi-1l*) was also identified in zebrafish. The predicted amino acid sequence of SPI-1l showed 45% amino acid identity to zebrafish PU.1, and retained all three domains [204]. *In situ* hybridization revealed a population of blood cells positive for *pu.1* and *spi-1l*, in addition to a population of single positive *pu.1* cells [204]. However, only a few single-positive *spi-1l* cells were observed. *Spi-1l* morphants showed a loss of *mpx* and *l-plastin* positive cells, indicative of a loss in granulocytes and monocytes/macrophages [204]. Unlike *pu.1* morphants, no change in *gata1* expression was observed, suggesting that SPI-1l acts downstream of PU.1, and plays an important role in myeloid cell differentiation [204].

5. Commitment of bi-potent myeloid progenitors to the macrophage or neutrophil lineage

5.1. Macrophage development

5.1.1. Progression of cell development

In mammalian systems, the progression of macrophage development proceeds from a committed macrophage progenitor, monoblast, promonocyte, monocyte and then to a mature tissue macrophage, reviewed by [205-207] (Figure 1). While the presence of a uni-potent committed macrophage progenitor has yet to be unequivocally demonstrated in the teleost systems, progenitor/precursor cells that give rise to monocytes and macrophages have been demonstrated. *In vitro*, a spontaneous proliferating trout RTS-11 cell line has two predominant cell types; a round non-adherent cell type that appears to be a pre-monocyte or myeloid precursor and an adherent macrophage-like cell, arising from the non-adherent cell type [208]. The cultivation of trout kidney progenitor-like cells developed a trout primary kidney monocyte culture that contained progenitor cells, promonocyte-like cells, and monocytes [209]. Furthermore, the generation of goldfish primary kidney macrophage cultures demonstrated that small mononuclear cells became monocytes and mature macrophages, *in vitro*. In the zebrafish model system, whole kidney marrow was added to a kidney fibroblast layer and was shown to maintain HPCs and precursor cells that then differentiated into myeloid and lymphoid cells [210]. Recently, the development of a zebrafish methylcellulose colony forming unit assay suggested the presence of a common erythro-myeloid HPC [211]. *In vivo* studies, primarily in the zebrafish, have demonstrated that monocytes/macrophages arise from the hematopoietic organ [45, 212-215], migrate to various tissues [216], and both primitive and definitive macrophages are motile, migrate to the site of insult, and readily phagocytose particles or pathogens [23, 45, 217-219]. The identification of progenitor cells that are capable of differentiating into monocytes and macrophages suggests a conserved macrophage differentiation pathway in vertebrates.

5.1.2. Receptors and growth factors

5.1.2.1. Colony-stimulating factor-1

The central growth factor that regulates the survival, proliferation, and differentiation of macrophages and their precursors is colony-stimulating factor-1 (CSF-1) [220-223]. Alternative splicing of *CSF-1* transcripts leads to production of a secreted glycoprotein, a secreted proteoglycan, or a membrane-bound glycoprotein that can be proteolytically cleaved from the surface, reviewed by [144, 221]. However, only the first 149-150 aa of the N-terminal portion of the CSF-1 core protein has shown to be important for biological function [224, 225]. CSF-1 homodimers, are covalently linked by an interchain disulphide bond to form a dimer [226] that then binds the CSF-1 receptor. CSF-1 is produced by an array of cell types including fibroblasts, endothelial cells, and bone marrow stromal cells, reviewed by [144]. In addition, activated T-cells [227-229], monocytes, macrophages [230, 231], fibroblasts, and endothelial cells [144] can produce CSF-1. CSF-1 production by activated cell types suggests a role for CSF-1 at the site of inflammation, which may be necessary for the rapid recruitment, differentiation and activation of macrophages and their precursors.

5.1.2.2. Interleukin-34

Recently, IL-34 was identified as another growth factor involved in mediating macrophage development in mammals, in addition to CSF-1 [232-234]. The IL-34 protein does not show homology to any other human protein and or contain any known conserved structural motifs [232]. Homodimeric IL-34 binds to CSF-1R, although with a different affinity than that of CSF-1, and to different sites on the receptor [232, 233] [235]. The hierarchy in binding of the CSF-1R ligands may provide a mechanism for differential signaling depending on the bound ligand. To date, IL-34 has not been identified in teleosts.

5.1.2.3. Colony-stimulating factor-1 receptor

The *CSF-1R* gene, shown to map to the proto-oncogene *c-fms*, is a member of the type III tyrosine kinase family of receptors [236, 237], reviewed by [238]. The binding of homodimeric CSF-1 to CSF-1R, triggers receptor homodimerization and activation [239]. Receptor activation triggers autophosphorylation of the intracellular tyrosine residues and activation of JAK/STAT, PI3K/Akt, and MAPK pathways, as well as pathways for receptor-mediated internalization and destruction, reviewed by [162, 238, 240]. Within the hematopoietic system, CSF-1R protein is primarily found on macrophages and their precursors and has been used as a marker of cells along the macrophage lineage in mammalian systems [222, 237]. CSF-1R progressively increased with macrophage differentiation [144].

5.1.2.4. Biological functions of colony stimulating factor-1

In addition to the regulation of survival, proliferation, and differentiation of macrophages and their precursors [220-223], CSF-1 has been shown to exert pro-inflammatory effects on monocytes and macrophages. These effects include the enhancement of macrophage chemo-

taxis, phagocytosis of pathogens, and the production of antimicrobial agents, reviewed by [162, 238]. CSF-1 is a pleiotropic cytokine and functions in a number of other biological systems such as regulation of macrophage and osteoclast numbers, bone remodeling, tooth production and fertility and breast development [241-245]

5.1.2.5. Teleost colony stimulating factor-1

Teleost *csf-1* (*mcsf*) was first identified in the goldfish as a 600 bp mRNA transcript that was present at high levels in spleen tissue, monocytes, and phorbol ester-activated monocytes [246]. The *csf-1* transcript encoded for a 199 aa precursor protein, with the mature CSF-1 protein predicted to have a molecular weight of 22 kDa. The goldfish CSF-1 has 27% aa identity to human CSF-1 [246]. Alignment of goldfish CSF-1 with mammalian CSF-1s showed conservation of four cysteine residues required for protein folding, similar to that of mammalian CSF-1 [246]. Ligand-receptor binding studies demonstrated that homodimeric CSF-1 could bind to soluble CSF-1R (see teleost CSF-1R section below, Figure 1). Functional characterization of a recombinant goldfish CSF-1 was shown to induce monocyte proliferation and differentiation (Figure 1), which was abrogated in the presence of sCSF-1R or in monocytes transfected with *csf-1r* RNAi oligos [246, 247]. Recombinant goldfish CSF-1 also aided in the long-term survival of mature macrophages *in vitro* [247]. The recombinant CSF-1 protein was chemoattractive to PKMs, and promoted their ability to perform phagocytosis and produce antimicrobial compounds [248], suggesting a pro-inflammatory role for CSF-1 in goldfish.

Two *csf-1* genes were later identified in trout and zebrafish, termed *mcsf-1* and *mcsf-2*, and a second goldfish *mcsf* transcript was identified [249]. The trout and zebrafish *mcsf-1* genes encoded for proteins of 593 and 526 aa, the trout and zebrafish *mcsf-2* genes encoded for proteins of 276 and 284 aa, respectively, while the goldfish *mcsf* gene encoded for a 544 aa protein [249]. All of the identified transcripts possessed a signal peptide, a CSF-1 domain, a transmembrane domain, and a short cytoplasmic domain [249]. However, the N-terminal region of all teleost CSF-1 proteins showed high homology (46-88%), consistent with the important role of the CSF-1 N-terminal portion for biological function.

The genomic structure of the identified *mcsfs* also differed. The zebrafish *mcsf-1*, found on chromosome 11, possessed seven exons and *mcsf-2*, found on chromosome 8, possessed nine exons. Based on syntenic analysis, the two *mcsf* genes appeared to have arose through a chromosomal or genome duplication [249]. Examination of the intron-exon structure of trout *mcsfs* showed *mcsf-1* to possess 10 exons and 9 introns, and *mcsf-2* to have 9 exons and 8 introns [249].

Along with differing genomic organizations, trout *mcsf-1/2* are differentially expressed in tissues. The *mcsf-1* transcript was predominantly expressed in the spleen, intestine and brain, while *mcsf-2* was predominantly expressed in the head kidney, gills, muscle and liver [249]. While a recombinant trout MCSF-1 protein was produced and demonstrated to induce the proliferation of head kidney macrophages, a recombinant trout MCSF-2 protein was not produced to examine whether there was differential regulation of macrophage function by

the MCSFs [249]. Whether MCSF-1 and MCSF-2 are functionally redundant or functionally partitioned (sub-functionalization), remains to be determined.

5.1.2.6. Teleost colony-stimulating factor-1 receptor

The *csf-1r* sequences have been identified in a number of teleost species including puffer fish [250, 251], zebrafish [252], rainbow trout [253], gilthead seabream [254] and goldfish [20]. CSF-1R protein appears to be a marker of monocytes and macrophages in teleosts [20, 254, 255] (Figure 1). Analysis of the puffer fish *csf-1r* gene shows a 21 exon gene structure in fish, same as in mammals. However, the puffer fish *csf-1r* gene only spans 10.5 kbp versus the mammalian 55 kbp, due to the decrease in the size of the intronic sequences [250]. The *csf-1r* mRNA open reading frame encodes for a 975 aa protein, with a signal peptide, an extracellular domain with 10 conserved cysteine residues characteristic of immunoglobulin domains, transmembrane domain, and an intracellular kinase domain with an interruption of 70 bp [250]. While CSF-1R of puffer fish is only 39% similar to human CSF-1R, the kinase domain is considerably more conserved, particularly in the motifs associated with signaling. The fish *csf-1r* gene was linked with *pdgfrb-1* [250].

A second *csf-1r* gene (*csf-1r-2*) was also identified in puffer fish, and linked with a second *pdgfrb* (*pdgfrb-2*). The *csf-1r-2* gene was comprised of 22 exons and had a different intron-exon organization than *csf-1r-1* [251]. Despite the similar protein structure of the two CSF-1Rs, the amino acid sequences were only 39% identical. The *csf-1r* mRNAs were differentially expressed in tissues. The *csf-1r-1* was expressed in blood, brain, eye, gill, heart, kidney, ovary, skin, and spleen, while *csf-1r-2* was expressed in the blood, brain, eye, gill, heart, kidney, liver, muscle, skin, spleen and testis. [251].

The duplication of *csf-1r* genes was also observed in cichlids, the green-spotted pufferfish, medaka, and *Tetraodon* (found on chromosomes 1 and 7), with the *csf-1r-2* duplicated genes appearing to have undergone evolutionary selection or diversification while the *csf-1r-1* gene appeared to resemble that of the ancestral gene [256]. It was proposed that the fish specific whole genome duplication generated the two paralogues of *csf-1r* in fish, as well as two *pdgfrb* and *kit* genes, and that *kit* and *csf-1r-2* may have been retained to play a role in the survival, migration and differentiation of melanocytes and xanthophores, important pigment cells involved in fish coloration patterns [256].

The *panther* (*fms*) mutant zebrafish have a defect in the *csf-1r* gene, and mutant fish fail to develop their characteristic pigment pattern of black and yellow stripes. The CSF-1R was found to be important in the survival, migration and differentiation of precursors to yellow xanthophores in zebrafish [257, 258]. However, unlike that of the CSF-1R^{-/-} mice, there were no reports of hematopoietic defects in *panther* zebrafish. The lack of hematopoietic defects may be due to the presence of another *csf-1r* gene, a low level of *csf-1r* expression, or a differential requirement for CSF-1R during embryonic macrophage development versus adult macrophage development in teleosts. However, CSF-1R was shown to be important in the migration of primitive macrophages to tissues, such as the brain, retina and epidermis upon comparing primitive macrophage distribution and migration in wild-type and *panther* zebrafish [252]. Furthermore, *csf-1r* mRNA was detected in inflammatory macrophages from 3

dpf zebrafish embryos [219]. Taken together, these results support a role for CSF-1R in teleost macrophage biology.

A full-length *csf-1r* cDNA sequence was identified in trout, with an open reading frame of 2904 bp encoding for a 967 aa protein, predicted to be ~109 kDa. Trout CSF-1R had 40% aa identity to that of human and mouse, and 54% and 52% identity to that of puffer fish and zebrafish CSF-1R [253]. The trout *csf-1r* gene was similar to that of the ancestral gene, and mRNA was found in the head-kidney, spleen, blood, ovary, and showed lower mRNA levels in the liver, brain, heart, muscle, gill, and skin [253]. Southern blotting revealed two bands in each lane, suggestive of a second *csf-1r* gene in trout. However, a second *csf-1r* gene was never identified.

CSF-1R was also identified in goldfish as a 975 aa integral membrane bound protein (mCSF-1R) that possessed the five Ig extracellular domains with multiple N-linked glycosylation sites, a transmembrane domain, and an intracellular tyrosine kinase domain [20]. The mRNA of mCSF-1R could be detected in progenitor, monocyte and macrophage subpopulations, and an antibody produced against the first two Ig domains of CSF-1R was able to recognize monocytes and macrophages [20]. However, unlike mammalian neutrophils, zebrafish and goldfish neutrophils do not appear to express mRNA for *csf-1r* [16, 219]. Additionally, alternative splicing of the *csf-1r* transcript encoded for a soluble form of the CSF-1R (sCSF-1R), possessing only the D1 and D2 Ig domains, important for binding of CSF-1. The *scaf-1r* mRNA was expressed by leukocytes within the progenitor and macrophage populations, but not in the monocyte subpopulation [20]. Furthermore, addition of a recombinant purified sCSF-1R dampened the proliferation of spontaneously growing and differentiating PKMs [20]. The increased production of the sCSF-1R by PKMs during senescence phase suggested that sCSF-1R was involved in the negative regulation of CSF-1 signaling through mCSF-1R [20, 246] (Figure 1).

5.2. Neutrophil development

5.2.1. Progression of cell development

Following the commitment of the CFU-GM to a committed granulocyte progenitor cell, terminal differentiation through a promyelocyte, myelocyte, and metamyelocyte stages occur to give rise to a mature neutrophil, and are regulated through growth factor and transcription factor signaling, reviewed by [259] (Figure 1). Similar to that of mammals, the differentiation of fish neutrophils appears to occur through various stages, based on morphological and cytochemical characteristics, and include the promyelocyte, myelocyte, metamyelocyte and the mature neutrophil, which sometimes had a segmented nucleus [45, 212, 213, 215, 260]. These neutrophils were shown to migrate from the hematopoietic organ to the site of wounding, pathogen injection, or transformed cell injection [24, 45, 261], in response to a hydrogen peroxide attractant produced by cells at the site of damage [217]. However, the responding neutrophils had low phagocytic activity [24], or engulfed small fragments of the pathogen [217]. *In vitro*, treatment of zebrafish kidney marrow cells with G-CSF gave rise to CFU-GM in a methylcellulose assay [211]. However, there is a lack of *in vitro* culture sys-

tems for studying progenitor cell to neutrophil differentiation. The identification of functional neutrophils and their precursors suggests the presence of a committed granulocyte progenitor cell in teleosts.

5.2.2. Receptors and growth factors

5.2.2.1. Granulocyte colony-stimulating factor

Neutrophils contribute to both innate and adaptive immune responses. They are capable of chemotaxis, phagocytosis, antimicrobial molecule production, and formation of extracellular traps [262-267]. Upon activation, neutrophils produce a number of chemokines, pro-inflammatory and anti-inflammatory cytokines, as well as the colony-stimulating factors G-CSF, CSF-1, GM-CSF, IL3 and SCF, reviewed by [268, 269]. However, neutrophils are short lived, 6-90 hrs, and need to be continuously replaced.

GCSF, a member of the class I cytokine family, is the primary CSF that mediates the proliferation, differentiation, survival and activation of neutrophils and their progenitors, and has been reviewed extensively by [144, 270]. The transcription of *GCSF* is controlled by an upstream promoter region with a tumor necrosis factor alpha response region that is bound by NF-kB p65 and NF-IL6, reviewed elsewhere by [144, 271]. As such, GCSF can be produced by activated monocytes/macrophages, neutrophils, fibroblasts and endothelial cells in response to a number of pro-inflammatory stimuli, reviewed elsewhere by [144, 270, 271]. In humans, the normal GCSF concentration in blood ranges from 30-162 pg/mL, and can be massively up-regulated during infection up to 3200 pg/mL [272-274].

5.2.2.2. Granulocyte colony-stimulating factor receptor

The protein structure of GCSFR is comprised of a signal peptide, an immunoglobulin-like domain, a cytokine receptor homology (CRH) domain containing the class I cytokine receptor superfamily motif W-S-X-W-S, three fibronectin domains, a transmembrane domain, and an intracellular cytoplasmic signaling domain containing three motifs termed Box 1, Box 2, and Box 3, important for signal transduction [270, 275]. Based on their protein structure and conserved motifs, the human and mouse integral membrane GCSFR proteins were placed in the type I cytokine receptor family.

While there are reports of GCSFR on other hematopoietic cells such as monocytes [276] and lymphocytes, as well as some non-hematopoietic cells, GCSFR is primarily found on neutrophils and their precursors [270, 277]. Neutrophils up-regulate their levels of GCSFR as they differentiate from progenitor cell to mature neutrophil, with 50-500 GCSF receptors per cell [278]. Structural analysis showed GCSF forms a homodimer, binds two GCSFRs, and leads to receptor homodimerization in a 2:2 complex [279-281]. Binding of a homodimeric GCSF to two GCSF receptors triggers intracellular signaling through the JAK/STAT, Ras/Raf/Erk, or PI3K pathways [275, 277, 282]. These signaling pathways ultimately lead to the migration, survival, proliferation, and differentiation of neutrophils. Control of GCSFR signaling in neutrophils is modulated through (1) transcriptional activation of the *GCSFR* by AP-1, AP-2,

C/EBP α , NF-IL6, GATA-1, and PU.1/SPI1 transcription factors [181, 197], (2) the production of a soluble receptor through alternative splicing [275], and (3) cleavage of surface GCSFR by elastase [283].

5.2.2.3. Biological activity of granulocyte colony stimulating factor

The targeted gene disruption of *GCSF* and *GCSFR* has demonstrated the important functional roles of GCSF *in vivo*. GCSF and GCSFR deficient mice display severe neutropenia (70%-88% reduction in circulating neutrophils), reduction in monocyte and macrophage numbers, and ~50% reduction in the numbers of neutrophil precursors present in the bone marrow [284, 285] [282, 286] and are unable to control *Listeria monocytogenes* infections [284, 285]. GCSF treatment of bone marrow cells, *in vitro*, induced CFU activity to produce mainly neutrophil colonies [287] and promoted the proliferation of neutrophil precursors [270]. The release of mature neutrophils, their terminal differentiation, survival, and activation, is also mediated by GCSF *in vitro* and *in vivo*, reviewed by [270]. Lastly, GCSF has been used in the clinical setting to increase peripheral blood neutrophil numbers for treatment of disease and for stem cell mobilization from the bone marrow into the peripheral blood, reviewed by [288, 289].

5.2.2.4. Teleost granulocyte colony-stimulating factor

The teleost *gcsf* gene was first identified in Japanese flounder, fugu, and the green-spotted pufferfish [290]. Both the fugu and green-spotted pufferfish have two *gcsf* genes, termed *gcsf-1* and *gcsf-2*, while only an orthologue of *gcsf-2* was identified in flounder [290]. Phylogenetic analysis of vertebrate *gcsfs* predicted fish *gcsf-1* to be the ancestral gene, while *gcsf-2* was predicted to be the duplicated gene. Alignment of the fish GCSFs with human and mouse GCSF showed low identity, ranging from no significant identity to 34% amino acid identity [290]. Despite the low amino acid identity of fish to mammalian GCSF, all fish *gcsf* genes retained a 5 exon/ 4 intron structure with a conserved tumor necrosis factor alpha response element in the promoter region. Furthermore, the predicted transcripts have an open reading frame of 561-636 bp, corresponding to a predicted protein of 20-23 kDa, and 4-5 AU rich sequences in their 3' UTRs shown to be involved in mRNA instability and degradation [290]. Determination of the ratio of synonymous to asynonymous nucleotide substitutions (Ks/Ka) in fish *gcsf* genes ranged from 0.467 to 0.961 with an average of 0.793, demonstrating that positive selection was occurring in GCSFs of fish (and chicken) [290]. Two *gcsf* genes were also identified in the black rockfish (*Sebastes schlegelii*) [291] and in zebrafish [292] (O. Svoboda and P. Bartunek, personal communication), while only one *gcsf* gene has been identified in trout (NM_001195184).

Flounder *gcsf-2* mRNA levels were highest in the spleen, kidney, and gill. However, *gcsf-2* mRNA was still detected in the brain, eyes, heart, peripheral blood leukocytes, ovary, skin, and stomach, but was not detected in intestine, liver, or muscle tissue [290]. As expected, *gcsf-2* mRNA levels were up-regulated in kidney and peripheral blood leukocytes following treatment with lipopolysaccharide (LPS) or a mixture of concanavalin A and phorbol esters (ConA/PMA) [290]. The black rockfish *gcsf-1* showed expression in the peripheral blood leu-

kocytes, spleen, gill, intestine and muscle [291]. However, black rockfish *gcsf-2* was ubiquitously expressed in the peripheral blood leukocytes, head and trunk kidney, spleen, gill, intestine, muscle, liver and brain [291]. Although both *gcsf-1* and *gcsf-2* black rockfish mRNA levels were upregulated in PBLs treated with LPS or ConA/PMA, differential kinetics and levels of expression were observed between the two *gcsfs* [291]. It appears that *gcsf-1* may be rapidly induced with sustained levels following stimulation, whereas *gcsf-2* is only slightly upregulated and showed a drastic increase in mRNA levels after ConA/PMA treatment for 24 hrs [291]. Taken together, these data suggest that GCSF-1 may play an important role during inflammation, although functional studies are required to determine the roles of GCSF-1 and GCSF-2 in teleost granulopoiesis and inflammation.

Functional studies on fish GCSF-1 are limited. Only two manuscripts report on the function of GCSF-1 and both utilize the zebrafish model system. *In vitro*, precursor cells from whole kidney marrow were sorted, plated in a methylcellulose colony forming unit assay and treated with either GCSF or a combination of GCSF and erythropoietin (EPO). While both treatments led to CFUs containing granulocytes and macrophages, the combination of GCSF and EPO also supported the formation of erythroid CFUs [211]. *In vivo*, morpholino mediated knockdown of *gcsfr* in zebrafish showed a decrease in numbers and migration of cells expressing both neutrophil and macrophage specific transcripts, during both primitive and definitive hematopoiesis in the zebrafish embryo. However, a population of myeloid cells remained, despite morpholino mediated knockdown of *gcsfr*, suggesting the presence of a GCSFR-independent pathway of myeloid cell development and migration [292]. Injection of wild-type zebrafish with *gcsf* mRNA increased the number of myeloid and *gcsfr*⁺ cells, while injection of *gcsf* mRNA into *gcsfr* morpholino zebrafish did not result in an increase in myeloid cell numbers [292]. These studies suggested GCSF-1 participates in myeloid cell development, similar to that observed in mammalian systems (Figure 1). No functional studies have been performed using GCSF-2, and the role(s) of GCSF-2 in myelopoiesis remain to be elucidated.

5.2.2.5. Teleost granulocyte colony-stimulating factor receptor

The *gcsfr* has been identified in zebrafish [292], goldfish [293], and trout (AJ616901). Only one gene copy has been identified, although Southern blotting for goldfish *gcsfr* suggested the presence of more than one gene [293]. Analysis of the upstream promoter region of the 16 exon zebrafish *gcsfr* gene showed conserved putative sites for binding of the transcription factors HOXA5, PU.1 and CEBP family members [292], similar to the human *gcsfr* promoter region. These data suggest the conserved regulation of *gcsfr* gene expression in teleosts.

The predicted protein structure of zebrafish and goldfish GCSFRs is conserved across vertebrates. The teleost GCSFR extracellular domain is comprised of a signal peptide, an Ig-like domain, a cytokine homology domain containing the WSXWS motif and four cysteine residues, and three fibronectin domains. Following the transmembrane region, the intracellular region contains predicted Box1, Box2, and Box 3 signaling motifs and 6 tyrosine residues [292, 293], shown to be involved in receptor activation and internalization in higher vertebrates.

In zebrafish, *gcsfr* mRNA is expressed by 14 hpf in the RBI, followed by the yolk sac, the ICM, and finally in the kidney by 96 hpf, consistent with the production of neutrophils during primitive and definitive hematopoiesis. In adult goldfish, *gcsfr* mRNA levels were highest in kidney and spleen, followed by the gill, intestine, heart, brain and blood [293]. The *gcsfr* mRNA was highly expressed in goldfish neutrophils and was up-regulated in response to mitogens or pathogens [293] (Figure 1).

5.3. Transcription factors

In addition to the transcription factors described in section 4.3, there are a number of transcription factors downstream that participate in determining GMP fate decisions and that play a role in macrophage and neutrophil cell development, reviewed by [51, 294]. A visual representation of the stage(s) in which these transcription factors are important are shown in Figure 2.

5.3.1. Early growth response (*Egr*)

The four *Egr* proteins, *EGR1* [295, 296], *EGR2* [297], *EGR3* [298] and *EGR4* [299], are members of the zinc finger transcription factor family and have an N-terminus activation domain, a repressor domain capable of binding to NAB1/2, and a DNA binding domain comprised of three zinc fingers that bind to the GC rich sequence, 5'-GCGGGGGC'3' [300]. *EGR1* promotes commitment to the macrophage lineage at the expense of granulocytic lineage [301, 302] and has been shown to be essential for myeloblast differentiation into monocytes/macrophages [303, 304]. Treatment of mouse bone marrow cells with CSF-1 has been shown to induce *EGR1* mRNA levels by 6-7 fold three hours post treatment, as well as *EGR2* and *EGR3* mRNA levels by 2-4 fold [305]. Although *EGR*^{-/-} mice display normal macrophage development [306], it is thought that there is redundancy amongst the *Egr* transcription factors. Consistent with this idea, *EGR2* is also abundant in monoblasts and monocytes [307], and may be involved in monocyte differentiation. Although a zebrafish orthologue of *EGR1* has been identified [308], the role of *egr1* in teleost macrophage development has not been examined.

5.3.2. Growth factor independence 1 (*Gfi1*)

Growth factor independence 1 (*GFI1*) is a zinc finger transcription factor comprised of an N-terminal Snail/*Gfi1* (SNAG) domain that is involved in recruiting proteins to modify histones, and a C-terminal domain containing six zinc fingers involved in DNA recognition [309]. *GFI1* is expressed in T-cells, B-cells, mature granulocytes and activated macrophages [310, 311]. *GFI1*^{-/-} mice showed slight defects in lymphocyte development, increased monocyte and monocyte precursor numbers, an absence of granulocytes and were highly susceptible to infections [310, 311]. Furthermore, myeloid progenitors from *GFI1*^{-/-} mice did not differentiate into mature granulocytes in the presence of GCSF *in vitro* [310] or *in vivo* [311]. C/EBP α can up-regulate *GFI1* expression, promoting a neutrophil cell fate, and *GFI1* also acts as a negative regulator on *PU.1* to decrease its expression [177, 180]. This lower level of *PU.1* drives granulocyte cell fate [175, 177]. *GFI1* is important for neutrophil differentiation [177,

310, 312] and acts by activating Ras guanine nucleotide releasing protein 1 (RasGRP1) which is necessary for activating Ras in the Ras/MEK/Erk pathway that is initiated during GCSF signaling [313]. The expression of GFI1 is sustained during differentiation and the transcription factor functions by blocking the expression of *EGR-2/NAB-2*, effectively antagonizing the EGR1/2 transcription factor and preventing initiation of a monocytic differentiation pathway, thereby promoting neutrophil differentiation [177, 312]. Like that of PU.1 and GATA1, GFI1 and EGR-1/EGR-2 act as an antagonistic pair to regulate neutrophil versus macrophage lineage fate.

In zebrafish, two *gfi1* genes have been identified, termed *gfi1* and *gfi1.1*. *gfi1* is primarily expressed in neural tissues, and not in the hematopoietic system [314], suggesting that this is not the functional orthologue of mammalian GFI1. However, *gfi1.1* was expressed in the different hematopoietic organs of the developing zebrafish embryo, suggesting that *gfi1.1* is expressed in hematopoietic cells [315]. Zebrafish *gfi1.1* morphants displayed a three-fold increase in the number of *pu.1*⁺ cells, along with an increase in *l-plastin* expression and a decrease in *mpo* expression [315]. These data are consistent with the known functional role of mammalian GFI1, suggesting that zebrafish GFI1.1, and not zebrafish GFI1, is the functional orthologue of mammalian GFI.

5.3.3. Interferon response factor-8 (IRF-8)

Interferon response factor-8 (IRF-8, also known as ICSBP) is one out of nine members of the IRF transcription factor family and is characterized by an N-terminal DNA binding domain and a C-terminus IRF association domain that can associate with other IRF or Ets transcription family members [316, 317]. IRF8^{-/-} mice and BXH-2 mice with a mutation in their IRF association domain show a drastic expansion of granulocytes at the expense of macrophages [318, 319]. Enforced expression of *IRF8* in myeloid progenitor cells *in vitro* led to the induced expression of a number of macrophage lineage differentiation transcripts including *CSF-1R* and *EGR1*. Additionally, enforced expression of *IRF8* in myeloid cell lines prevented their differentiation into granulocytes when treated with GCSF [320]. It is clear from the *in vivo* and *in vitro* studies that IRF8 promotes the commitment of myeloid progenitors along the macrophage lineage at the expense of the granulocyte lineage.

The homologue of *irf-8* was identified in rainbow trout [321] and zebrafish [322] with 53-55% amino acid identity to human IRF8 [321, 322]. In trout tissues, *irf8* mRNA was detected in the spleen, head kidney, gill, brain, intestine, skin, muscle, and liver [321] and mRNA levels could be up-regulated in splenocytes upon treatment with Poly I:C, PMA, PHA and recombinant IL-15. However, the role of IRF8 in GMP fate decisions or during macrophage development was not assessed. In zebrafish developing embryos, *irf8* mRNA was first detected in the rostral blood island, the site of primitive myelopoiesis, and was co-expressed with *csf-1r* mRNA, but not in cells positive for *mpx*, suggesting that *irf8* is expressed in cells committed to the macrophage lineage [322]. In zebrafish *irf8* morphants, *csf-1r*⁺ cells were absent, while *mpx*⁺ cells and mature neutrophils were increased by approximately three-fold, suggesting IRF8 is required for macrophage development. This phenotype could be rescued by injecting embryos with *irf8* mRNA.

Conversely, the over-expression of *irf8* mRNA in zebrafish resulted in an increase in macrophages by approximately 50% and a decrease in neutrophil numbers by about 40% [322]. These data are similar to those of the mammalian system and suggest a conserved role for IRF8 in determining macrophage over neutrophil cell lineage during primitive myelopoiesis. However, whether IRF8 plays the same role during definitive myelopoiesis in teleosts remains to be determined.

5.3.4. *MafB*

In addition to the previously described role of MAFB in HSCs and CMPs (see section 4.3.1), *MAFB* is highly expressed in monocytes and macrophages [164, 323] and has been shown to induce differentiation of myeloblasts into monocytes and macrophages [163, 307, 324, 325]. Furthermore, *MAFB* and c-MAF double knockout mice displayed differentiated macrophages that were capable of proliferating in response to CSF-1 in semi-solid and liquid culture [325]. Therefore, it appears that *MAFB* expression is sustained in monocytes and macrophages in order to prevent proliferation in these terminally differentiated cell populations.

Studies examining the role of MAFB in teleost myelopoiesis are limited. In the goldfish PKM system, a *mafb* transcript was identified and showed increasing mRNA levels with macrophage development [19]. The increasing mRNA levels of *mafb* during macrophage differentiation are similar to what has been observed in mammalian systems and suggest that MAFB may play a role in teleost macrophage differentiation.

6. Conclusion

Myelopoiesis is an orchestration of a multitude of growth factors and transcription factors that control cell fate decisions and differentiation along a chosen cell lineage. It is evident that there exists some functional redundancy in the action of myelopoietic growth factors, most likely put in place to ensure the production of these critical innate immune cells. Studies have focused on examining the regulation of myelopoiesis in the mouse model system, and have only just begun in the teleost model system.

The divergence of teleosts and mammals occurred approximately 400-450 Mya, thus teleosts represent one of the most basal groups of vertebrates [326]. Comparison of soluble factors and their receptors in teleosts and mammals show retention of many of important hematopoietic growth factors and receptors, including PDGFR [250, 251, 327], c-KIT [128-130], FLT3 (accession number DQ317446), CSF-1R [20, 250-254], GCSFR [292, 293], and their ligands PDGF [328], KIT ligand [128, 130, 131], CSF-1 [246, 249], and GCSF [290-292], although FLK2, the ligand to FLT3, has not yet been reported. However, it appears that teleosts do not possess the key myeloid growth factors IL-3 and GM-CSF, and their cognate receptors. In addition, teleosts possess all of the TF families required for hematopoiesis in higher vertebrates, reviewed in [28, 30]. Based on studies performed to date, the regulation of hematopoiesis is largely similar between mammals and teleosts. However, teleosts often possess a

number of gene duplications for many of the soluble factors, receptors, and to some extent, transcription factors as a result of a teleost-specific whole genome duplication predicted to have occurred approximately 350 Mya, and is believed to be responsible for the radiation of teleosts [329, 330]. Many of these teleost genes are rapidly evolving, often undergoing sub-functionalization or neo-functionalization making the identification of teleost orthologues difficult. By developing an understanding of the soluble mediators, receptors, and the intracellular machinery that govern teleost myelopoiesis, we may be better equipped to develop strategies to promote host defense against pathogens, particularly in aquaculture in which fish are predisposed to infection.

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List of abbreviations:

ABC ATP-binding cassette; **AGM** aorta-gonad-mesonephros; **ALM** anterior lateral mesoderm; **ATP** adenosine triphosphate; **BFU-E** burst-forming unit-erythrocyte; **bZIP** basic leucine zipper domain; **CCM** cell-conditioned medium; **C/EBPs** CCAAT/enhancer binding proteins; **CFU** colony forming unit; **CFU-G** colony-forming unit-granulocyte; **CFU-GEMM** colony-forming unit granulocyte/erythrocyte/macrophage/megakaryocyte; **CFU-GM** colony-forming unit-granulocyte/macrophage; **CFU-M** colony-forming unit-macrophage; **CFU-Meg** colony-forming unit-megakaryocyte; **CFU-S** colony-forming unit-spleen; **CHT** caudal hematopoietic tissue; **CLP** common lymphoid progenitor; **CMP** common myeloid progenitor; **ConA** concanavalin A; **CRH** cytokine receptor homology; **CSFs** colony-stimulating factors; **CSF-1** colony-stimulating factor 1; **CSF-1R** colony-stimulating factor-1 receptor; **DCs** dendritic cells; **EGR/egr** early growth response; **EPO** erythropoietin; **GCSF** granulocyte colony-stimulating factor; **GFI1** growth factor independence 1; **GM-CSF** granulocyte/macrophage colony-stimulating factor; **GM-CSFR** granulocyte/macrophage colony-stimulating factor receptor; **GM-CSFR α** granulocyte/macrophage colony-stimulating factor receptor alpha chain; **GMP** granulocyte/macrophage progenitor; **HPCs** hematopoietic progenitor cells; **hpf** hours post fertilization; **HSCs** hematopoietic stem cells; **IL** interleukin; **IL-3R α** interleukin-3 receptor alpha; **IRF** interferon response factor; **JAK** Janus family of protein tyrosine kinases; **Ks/Ka** ratio of synonymous to asynonymous nucleotide substitutions; **LMPPs** lymphoid-myeloid primed multipotent progenitors; **LPS** lipopolysaccharide; **LT-HSCs** long-term hematopoietic stem cells; **MCSF** macrophage colony-stimulating factor; **mCSF-1R** membrane-bound colony-stimulating factor-1 receptor; **MEP** megakaryocyte/erythroid progenitor; **MMP** matrix metalloprotease; **mpo** myeloperoxidase; **MPPs** multipotent progeni-

tors; **mpx** myeloid-specific peroxidase; **mRNA** messenger ribonucleic acid; **mSCF** membrane-bound stem cell factor; **multi-CSF** multi-lineage colony-stimulating factor; **NF- κ B** nuclear factor kappa B; **NK** natural killer; **PBI** posterior blood island; **PBL** peripheral blood leukocytes; **PDGFR** platelet-derived growth factor receptor; **PGCs** primordial germ cells; **PKM** primary kidney macrophages; **PI3K** phosphatidylinositol-3-kinase; **PLC γ** phospholipase C γ ; **PLM** posterior lateral mesoderm; **PMA** phorbol esters; **Poly I:C** polyinosinic:polycytidylic; **RasGRP1** Ras guanine nucleotide releasing protein 1; **RBI** rostral blood island; **SCF** stem cell factor; **sCSF-1R** soluble colony-stimulating factor-1 receptor; **SOCS** suppressor of cytokine signaling; **sSCF** soluble stem cell factor; **STATs** signal transducers and activators of transcription; **ST-HSCs** short-term hematopoietic stem cells; **TFs** transcription factors; **UTR** untranslated region;

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Freshwater Fish as Sentinel Organisms: From the Molecular to the Population Level, a Review

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Additional information is available at the end of the chapter

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1. Introduction

Fish around the world are found occupying almost any aquatic habitat. In particular, freshwater fish are severely threatened as the freshwater ecosystems are considered the most endangered of the world [1]. The ultimate destination of most contaminants is water; rivers, lakes, aquifers, or sea, are receptors of wastewaters with a complex mixture of xenobiotics. The variety of contaminants and their mixtures that daily reach the water bodies coupled with a multitude of irresponsible water management practices and destructive land uses, are currently threatening freshwater ecosystems [2], such is the case of discharge of municipal and industrial wastewaters, deforestation, increase of land crops, and water extraction from water bodies to human consumption and other uses. The impact of contaminants in an aquatic ecosystem is complex, therefore has increased the need for determining the ambient status in order to provide an indication of changes induced by anthropogenic activities and their influence on aquatic organisms. As physicochemical analyses shed no light on the biological status of ecosystems, a biological approach is needed to evaluate environmental health; moreover, the biological effects of contaminant interactions cannot be expressed by physicochemical investigations [3]. The aquatic ecosystem health is often reflected by the health of organisms that reside in that system. Fish in their natural environments are typically exposed to numerous stressors including unfavorable or fluctuating temperatures, high water velocities and sediment loads, low dissolved oxygen concentrations, limited food availability, and among other types of natural episodic variables. In addition, anthropogenic stressors such as contaminant loading can add to the insults that fish may already experience in many systems. All these factors, individually or together, can impose considerable stress on physiological systems of fish and impair their health [4, 5]. Environmental contaminants are known

to induce measurable biochemical changes in exposed aquatic organisms [6]. Likewise, stressors can load or limit physiological systems, reduce growth, impair reproduction, predispose fish to disease, and reduce the capacity of fish to tolerate additional stressors. Many species of fish, in particular those species near the top of the food chain, are generally regarded as integrators of environmental conditions and may reflect, therefore, the health of aquatic ecosystems [4] and therefore, they are excellent indicators of the relative health of aquatic ecosystems and their surrounding watersheds [7]. Thus, effects of contaminants on aquatic organisms may be manifested at all levels of biological organization (in a hierarchical scale that can be at cellular level, organisms, populations, communities, and ecosystems). In this way, the measuring of a suite of indicators across such levels of organization is often necessary to assess ecological integrity; these indicators also should include molecular, biochemical, physiological, population, community, and ecosystem responses.

The indicators allow us to isolate key aspects of the environment from an overwhelming array of signals [8]. Ecological indicators have been defined as measurable characteristics of the structure (e.g. genetic, population, habitat, and landscape pattern), composition (e.g., genes, species, populations, communities, and landscape types), or function (e.g., genetic, demographic/life history, ecosystem, and landscape disturbance processes) of ecological systems [9]. On the other hand, other authors [10], established that bioindicators are organisms or communities of organisms, which reactions are observed representatively to evaluate a situation, giving clues for the condition of the whole ecosystem; Gerhardt also indicate that bioindicators are species reacting to anthropogenic effects on the environment, concluding that a biological indicator would be: a species or group of species that readily reflects the abiotic or biotic state of an environment, represents the impact of environmental change on a habitat, community or ecosystem or is indicative of the diversity of a subset of taxa or the whole diversity within an area. In this sense, the primary role of ecological indicators is to measure the response of the ecosystem to anthropogenic disturbances [9]. A sentinel species can be defined as any domestic or wild microorganism, plant or animal, that can be used as an indicator of exposure to and toxicity of a xenobiotic that can be used in assessing the impact on human and/or environmental health because of the organism's sensitivity, position in a community, likelihood of exposure, geographic and ecological distribution or abundance [11].

The specific objective of this review is to provide a short framework of effects of xenobiotics on the responses of freshwater fish across molecular to population level when have been exposed to environmental stressors. Likewise, the present review considers the use of fish as sentinel organisms to assess the anthropogenic impacts over the freshwater ecosystems. The review asks whether fish can be able to reflect the environmental damage from molecular to population levels. Also, the present review offers a selection of examples of studies employing fish as sentinel organisms in ecological, toxicological and environmental risk assessments.

2. Suborganismal responses

When an organism is exposed to stressors like contaminants or a mixture of them, energy is demanded to deal with that stress [4]; stressors tend to impact ecosystems at lower levels of organization first [12]. One of the methods to quantify the exposure to xenobiotics and its potential impact on living organisms is the monitoring by the use of the so-called biomarkers [13]. Biomarkers have been defined by several authors, all of them, in reference to biological responses to contaminants exposure, as a) measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response [8]; b) a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) which can be related to exposure to or toxic effects of environmental chemicals [14]; c) any biological response to an environmental chemical at the subindividual level, measured inside an organism or in its products (urine, faeces, hair, feathers, etc.), indicating a deviation from the normal status that cannot be detected in the intact organism [15]; d) a xenobiotically induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or samples [16]; e) contaminant-induced physiological, biochemical, or histological response of an organism, and f) as functional measures of exposure to stressors expressed at the sub-organismal, physiological or behavioural level. Considering these definitions of biomarkers, we could adopt our own definition: *“any biological measurable response from an organism, induced by the exposure to a xenobiotic or complex mixture of them”*. Biomarkers can provide valuable information in field or semifield testing and be used to measure a wide range of physiological responses to chemicals at the biochemical, cellular, or tissular level [17].

In concordance with [18] and other authors [19, 20], biomarkers have been classified in three different categories: a) biomarkers of exposure, which represent responses such as induction or inhibition of specific enzymes involved in biotransformation and detoxification as a consequence of chemical exposure [21], b) biomarkers of effect, are any changes in a biological system that reflects qualitative or quantitative impairment resulting from exposure [20], including responses measurable at level biochemical, physiological or some other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease [19], and c) biomarkers of susceptibility, which serve as indicators of a particular sensitivity of individuals to respond to the challenge of exposure to a effect of a xenobiotic or to the effects of a group of such compounds, in this case, individual changes included genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure [19]. However, other authors have been subdivided the biomarkers in exposure biomarkers, effects biomarkers and predictive biomarkers.

Responses of fish at suborganismal level to xenobiotic exposure are complex and varied and depending of type of contaminant and time of exposure. The most general effect of xenobiotics on fish is oxidative stress, which is experienced when antioxidant defenses are overcome by prooxidant compounds. Oxidative stress include a variety of oxidative reactions, usually started by free radicals and propagated by molecular oxygen, which results in the oxidation of lipids, proteins, and nucleic acids [22]. Free radicals are atoms, molecules, or ions with un-

paired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive due to which radicals are likely to take part in chemical reactions. Very often free radicals are confused with reactive oxygen species (ROS) such as molecular and singlet oxygen, superoxide anion, hydroxyl radical and some their derivatives; however, hydrogen peroxide is not a radical, but it is a reactive species because has higher activity than molecular oxygen [23]. Hydroxyl radical is the most important free radical of biological importance, because of its potent oxidative potential and indiscriminate reactivity with cellular components of enzymes and DNA [24, 25]; likewise, being oxidant, all ROS are agents which at high concentrations are toxic to cells. Oxidative stress is a risky condition in which increases in free radical production, and/or decreases in antioxidant levels can lead to potential damage. The antioxidant system in aerobic organisms includes several biochemical safety mechanisms such as antioxidant enzymes and other compounds like vitamins, glutathione, metallothioneins, and others.

Antioxidant defense enzymes are induced by various environmental pollutants under prooxidant conditions among these enzymes we can find superoxide dismutase (SOD), Catalase (Cat) and Glutathion Peroxidase (GPx), Glutathion reductase and Glutathion S Transferase (GST, catalyze the nucleophilic conjugation of different biologically and potentially carcinogenic compounds [20]). The role of SOD is to catalyze the reaction of superoxide radical (O_2^-) to peroxide (H_2O_2). CAT detoxifies H_2O_2 to H_2O and O_2 . GPx detoxifies mainly organic peroxides. CAT is an enzyme with high biological relevance because reduce the concentration of peroxide, a precursor of OH^- , which is a highly reactive toxic form of ROS [26]. Cytochrome P450 monooxygenases (CYPs) are a multi-gene family of enzymes that play a key role in the biotransformation of pollutants, such as dioxins, pesticides, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). One of the most common and highly conserved is the CYP1A subfamily. The CYP1A biomarker is widely used as a biomarker of effect both in vertebrates and invertebrates for environmental biomonitoring, especially in marine bivalves and fish. The induction of CYP1A is triggered via the cytosolic aryl hydrocarbon (Ah) receptor due to exposure to pollutants, such as PCBs, dioxins, and numerous PAHs. CYP1A activity is typically measured using the substrate ethoxyresorufin, which is o-deethylated by ethoxyresorufin-O-deethylase (EROD) to a fluorescent product, resorufin, which can be easily measured. Because EROD activities are generally measured using liver homogenates that also tend to accumulate numerous CYP1A substrates, activity may be inhibited by residual substrates or metals [20].

3. Individual responses

Morphological alterations is one of the most individual level parameters that are measure to identified damage in sentinel organisms [27, 28]. In reference [28] identified severe histological damage in gills and liver of *Goodea atripinnis* a goodeid fish from Central Mexico, after the chronic exposure to Yerbimat, an herbicide with glyphosate. After 75 days of exposure to pesticide, they found lamellar hypertrophy and leukocyte infiltration in gills, and hepatocytes with vacuolization in the cytoplasm and piknotic nuclei in liver, concluding that Yerbimat

induces histological alterations in the gills and liver that might impair normal organ functioning that could lead to health damage in fish because of the important physiological roll of these organs.

Also, haematological parameters, such as erythrocyte and leucocyte count, erythrocyte indices and thrombocyte number vis-a-vis coagulation of blood has been considered bioindicators of toxicosis in fish following exposure to xenobiotics [29]. Any alteration in normal cellular components (morphology and number), and level of fluids of blood is named dyscrasia. Some authors [29] carry out an extensive review over dyscrasia in fish. They show several examples over alterations in morphological changes in blood cells, total count, haemoglobin content, thrombocytes and clotting time, concluding that haematological parameters are not specific to faced acute and chronic exposure of fish to xenobiotics, mainly organochlorinated, organophosphonate, pyrethroid and carbamate pesticides.

4. Population responses

The status of a fish population is a reflection of the overall condition of the aquatic environment in which that population resides. As such, fish population characteristics can be used as indicators of environmental health. Although changes in population structure may act as a sensitive indicator of changing environmental conditions, the timing, degree and nature of the feedback response to altered conditions will vary with the intensity, identity and the number of stressors, as well as the availability of energy [30].

Bioindicators are responses to environmental effects that occur at higher levels of biological organization than sub-organism (biomarkers); This kind of responses can be measured at different high levels of biological organization, from individual, through population (reproductive success, mortality, size distribution, reduction in abundance and biomass), community (primary production, disruption of the nutrient cycle) to ecosystem levels [31, 32], whose main characteristic is that the measure change with exposure to negative environmental factors. Organosomatic indices are common approaches for assessing fish health [4]. In this review we consider three organosomatic indices: Condition factor (CF), hepatosomatic index (HSI) and gonadosomatic index (GSI).

Since the nutritional status of fish can change according to different factors, such as season, food availability, and among they the exposure to xenobiotics, is important to have a measure of corporal condition of fish. The chronic exposure to contaminants may cause changes in feeding behavior, leading to a deterioration of the health. The CF is a frequently used index for fish biology study, as it furnishes important information related to fish physiological state [33]. It is a measure of corporal condition since measuring the body mass associated with the body length. A fish that is heavier for a given length (higher CF) is considered to be a healthier fish, because extra weight means extra energy reserves. While a lighter fish lack energy reserves and therefore, tend to be more susceptible to environmental stressors. A low body condition may also suggest muscle wasting (proteolysis) indicating a starvation response [34]. It has also

been suggested that females with a lower body condition reduce reproductive investment yet still have an increased risk of mortality.

The liver plays a major role in the metabolism of xenobiotic compounds with biochemical alterations occurring under some toxic conditions; likewise, the liver is a primary detoxification organ in fish [35]. Therefore, this strong activity can lead to an increase in liver size, from hypertrophy (an increase in size) to hyperplasia (an increase in number) of hepatocytes [35], or both. Studies evaluating the relative liver size of fishes from contaminated and reference sites often utilize the Hepatosomatic Index (HSI), which expresses the ratio of liver weight to body weight as a percentage.

Gonadosomatic index (GSI) is also a percentage relationship between the gonad weight and fish weight. Depending on the severity of exposure to xenobiotics, the sublethal effects can be to limit physiological capacity, reduce growth, and impair reproduction, therefore GSI is a convenient organosomatic index [36].

5. Specific case studies about fish as sentinel organisms

In México some studies have analyzed the use of freshwater fish as sentinels. These studies include the use of biomarkers and sentinel fish and the use of the whole fish population as indicators of environmental change. Furthermore, these studies are in areas of contrasting environmental conditions, in the case of biomarkers studies are in: the course of a river in the Atlantic slope (Río Champotón), a Lake in the Central Plateau (Yuriria Lake), a spring and a reservoir in the upper portion of a river of the Pacific slope (Ameca river). These studies make evident the utility of freshwater fish as sentinels and are briefly exposed.

5.1. Case of study of *Astyanax aeneus* in the Champotón river

The Champotón river, located in the humid subtropics of southeastern Mexico in terrain with a high content of karstic material, is the main surface stream in the Yucatán Peninsula; is a coastal river with 48 km in length to its outlet with a drainage basin surface area of 650 km². The fish studied inhabit the fresh water zone of the river with salinity up to 1.2 practical salinity units [37]. The climatic regime is hot subhumid with summer rain (June to September) and occasional winter precipitation as a result of the windy (northerly) and hurricane seasons. The main anthropogenic activity in the basin is agriculture and livestock raising [38]. During the study period (2007-2008) the region was affected by several hurricanes (mostly from August to October), that caused the river overflow. This study assessed the effects of the environmental conditions along the freshwater portion of the Champotón river on the native fish *A. aeneus*, analyzing responses between lower and higher levels of organization, and linking spatial and seasonal fish responses with water quality features. A Water Quality Index (WQI) was employed as an indicator of environmental conditions, a set of sub-organismal biomarkers in *A. aeneus* (lipid peroxidation (LPO), GST, EROD, and lactate dehydrogenase (LDH)) was monitored to determine the Integrated biomarker response (IBR), and organosomatic indices: GSI, HSI and CF were characterized. Three study sites were analyzed: San Juan Carpizo (SJC) in

the upper portion of the river, San Antonio del Río (SAR) in the middle portion of the river (where there are a rustic swimming spot lacking of sanitary facilities), and downstream Ulumal (U); the study periods were: April, July, and November 2007 and February 2008.

The WQI scores exhibited spatial and temporal variations (from 53.21 to 78.49) on a scale of 0 to 100. The lowest value was recorded in July at site SAR, where the lack of sanitary facilities provoke fecal materials are swept away by runoff during the rainy season (July), increasing coliform numbers and lowering the WQI. In addition, this river flows through a region in which calcareous substrates predominate; with a high content of calcium carbonate and increase in conductivity both provoked decreases WQI scores, particularly during the drought. However, WQI scores were higher in November and February, following the hurricane season that brought large amounts of precipitation and increased river flow favoring dilution. As a result, the values of several WQI parameters (including hardness and conductivity) decreased while the river was in flood. Several studies reported similar WQI fluctuation patterns. WQI scores in the Lerma-Chapala Basin, Mexico, indicated severe degradation of the basin, particularly during the dry season, when its rating ranged from contaminated to highly contaminated; however, WQI improved during the wet season, [39, 40].

Although the WQI scores indicated that the Champotón river had acceptable water quality, some pollutants (residues of persistent organic compounds (POCs), such as PCBs, hexachlorocyclohexanes, aldrin-related pesticides, heptachlor, and dichlorodiphenyltrichloroethane) have been detected in the Champotón river [41]. In reference [42] reported that sediments from several Champotón river sites contained two or more of the 16 PAHs considered by the Environmental Protection Agency United States as priority pollutants that represent a potential threat to exposed organisms. Seasonal variations in POCs were found by [41]; PCBs and hexachlorocyclohexanes reached their highest values during the rainy season, while dichlorodiphenyltrichloroethane, drines, and heptachlor peaked during the dry season. Additionally, high episodic loadings of contaminants have been detected in aquatic ecosystems following flooding events [43].

Regarding biomarkers in the sentinel fish *A. aeneus*, the highest LPO values were detected in November and February (post-hurricane and windy seasons), while the lowest values were detected in July at all study sites. GST activity was highest in November at site U and lowest in July and April at all study sites. EROD activity in general was highest in April, while the lowest means occurred in July at all sites. Spatial analysis revealed that SAR recorded the highest mean EROD activity, while the lowest occurred at sites SJC and U. LDH values peaked in November and February following the hurricane and windy seasons, while minimum LDH levels occurred in April during the dry season. Spatial variability occurred; sites in the middle reaches (U, SAR) had the highest LDH values, while the lowest were recorded at headwater site SJC [44].

The increased LPO values in the post-hurricane season (November) and in the windy season (February) was associated with the hurricane season in the Champotón river which provokes flooding of adjacent areas where field crops are treated with agrochemicals that, along with the POCs and PAHs detected in sediments by [41], may be incorporated into the aquatic system. Similar results were found by [45] that detected the mobilization of agriculture-related

xenobiotics, during the flooding of the river Elbe. Oxidative stress in fish after extensive flooding was also detected by [43] in the Pamlico Sound estuary. The observed increase in LPO levels in *A. aeneus* may be related to climate-induced stress and by exposure to the mixture of xenobiotics that may be mobilized during that period.

Produced ROS are detoxified by antioxidant defense mechanisms, which are essential for protection of cellular systems against xenobiotic-induced oxidative stress [46]. GST is particularly interesting, since it is involved in elimination of reactive compounds and is the transport system for glutathione [22]. GST activity in *A. aeneus* peaked during the same period that LPO increased, following the hurricane season, which may reflect a defense of the fish against oxidative stress, as reported by [47] in *Prochilodus lineatus* in Argentina and by [48] in three subspecies of *Salmo trutta* in Turkey. The liver usually contains high levels of antioxidant enzyme activity, which may be due to high rates of free radical generation in this tissue [49].

A. aeneus exhibited seasonal fluctuations in EROD activity, peaking in April at the SAR site. Several authors [43, 50] point out that POCs (including PAHs) typically cause elevated levels of EROD activity, and fossil fuel spills and infiltrations are a major source of PAH input [51]. In *A. aeneus* EROD activity was highest at the SAR site, a recreational spot reached only by unpaved road where motor vehicles park on the riverbank. EROD induction may thus be due to surface runoff carrying PAHs.

LDHs are cytoplasmic enzymes that catalyze the reversible reduction of pyruvate to lactate [52], an important step in the energy processes of many animal groups. Its use as a biomarker is based on the assumption that organisms subjected to chemical stress must obtain additional energy rapidly, thus increasing anaerobic glycolysis. The LDH response is apparently time-dependent and may vary with the pollutant and organism involved [41]. LDH in *A. aeneus* increased at all sites in November and February, coinciding with the period of maximum LPO as well as the post-hurricane season and highest river flow.

The IBR enables evaluation of the global variations of biomarkers, taking into account the contributions and variations in the biomarkers assessed [17]. IBR data revealed seasonal fluctuations, the maximum total IBR values occurred in April and November (April, 15.73; November, 14.73) and the minimum values in February (2.53). Response in April and November suggested that *A. aeneus* was exposed to greater stress during this period, one at the end of the dry season and the other coinciding with the post-hurricane season that also led to high LPO values (oxidative stress), high GST levels (antioxidant responses), and high levels of LDH (high energetic need). Multiple stressors are involved during flooding, such as altered habitat, changes in hydrological regime, and mobilization of pollutants. Responses of biota to environmental stressors are the integrated result of natural and anthropogenic stressors that can be ultimately manifested in biotic changes at several levels of organization [53]. IBR differences among the study periods may reflect a compensatory mechanism by which the fish regains homeostasis following the period of highest stress. Fish display a large variety of physiological stress responses that manifest as an increase in certain biomarkers after a stressful event [54]. These responses are considered adaptations of the fish to adjust itself to the disturbance and regain homeostasis [55]. Fish have also been found to exhibit recovery responses dependent on stress duration and magnitude. If the stressors are too severe or persistent and the fish is

unable to regain homeostasis, the responses may become maladaptive and may pose a risk to the health and wellbeing of the fish [54].

Regarding somatic indices, GSI displayed the reproductive period of *A. aeneus* occurred from April (end of the dry season) to July (early rainy season), with the reproductive peak in July. HSI was highest in *A. aeneus* during periods of reproductive inactivity, which is interpreted as an increase in liver-stored reserve materials for later use during gametogenesis. Furthermore, low HSI values prior to and during reproduction may result from the transfer of liver energy reserves toward gonadal maturation and the reproductive event, with consequent depletion of these reserves and a decrease in HSI values [56]. CF remained constant among sites and study periods, with maximum values during July. CF reflects the interactions between abiotic and biotic factors in the physiological conditions of the fish [57]. Observations demonstrate that *A. aeneus* maintains a stable, robust condition. CF and HSI trends may reflect physiological conditions in *A. aeneus* that enable oocyte maturation and release, suggesting that oocyte production relies on energy stored in the liver and not on energy stored in the musculature [58, 59]. GSI, HSI, and CF revealed that the reproductive success of the sentinel species had not been affected, since the GSI values concurred with those reported for other species in the genus *Astyanax*. HSI values showed the transfer of energy during the reproductive period, and CF remained stable throughout the study, evidencing no effects on the general condition of the fish during periods of higher stress and indicating that the stress was temporal and the fish were able to compensate for it.

Despite WQI scores suggest that the Champotón river water is not highly polluted, the set of *A. aeneus* biomarkers constitutes a more sensitive and effective tool for identifying periods of environmental conditions adverse to fish health. Markers of oxidative damage (LPO), energy processes (LDH), detoxification (EROD), and antioxidant activity (GST) suggested that two stress periods affected the health condition of *A. aeneus* in different ways. These biomarkers can be used as early warning signals of environmental change prior to the onset of irreversible damage at the population level. Indeed, the IBR values highlighted the two periods of high biomarker response. Overall, this study provides evidence supporting the use of a biomarker set in assessing the health of aquatic systems, corroborating the suitability of *Astyanas aeneus* as a sentinel species.

5.2. Study case of *Chirostoma jordani* in Yuriria Lake

Chirostoma jordani is an Atherinopsid fish endemic of Central México. Data presented are from a population living in Yuriria Lake, one of the most important lakes in México, located in the Central Plateau (hydrologic region Lerma-Chapala-Santiago 20 ° 20'24" -19 ° 04'48" N and 101 ° 55'48" -100 ° 48 '36" W). It is an artificial, small and shallow lake (area= 66 km² and maximum depth of 3.2 m) feed by a diversion of Lerma river (their main tributary), that carries wastes from mining activities, livestock, industrial, urban and rural areas [60]. In the western end the lake receives water from two small an intermittent tributaries. Yuriria Lake supplies water for the surrounding farming areas, harbors migratory bird populations, and supports fisheries and tourism of several human settlements on the littoral zone.

This study shows an assessment of water quality by means of a WQI and a battery of oxidative stress: LPO, and the activity of antioxidant enzymes, SOD, CAT, and GPx along with somatic indices, such as the GSI, HSI, and CF were analyzed to assess the health condition of *C. jordani* in Yuriria Lake. The study was carried on in a period with scarce pluvial precipitation and prolonged drought (May, August, November 2009 and February and May 2010).

Yuriria Lake is characterized by a high deterioration in their water quality, WQI values ranged from 55 to 70, with a global mean of 65.85. The lake has spatial differences in water quality, the limnetic zone has higher scores (63 to 70), and the tributaries have the lower quality (55 to 58). Yuriria Lake being located in the Central Plateau, one of the most highly populated areas in México, displays the general problem of water quality of the basin (the Lerma-Chapala basin), where urban and industrial wastewater discharges, and leachates of agrochemicals are the main pollutants that diminish the water quality [61]. Particularly the middle Lerma (where Yuriria Lake is located) is recognized as the most affected area, with WQI scores between 41.1 and 54.2 in 1999 [40, 61, 62]. Furthermore, previous studies have recognized the entry of pollutants in Yuriria Lake [61]; however, the effect of the mixtures of these pollutants on the aquatic biota inhabiting the lake had not been analyzed.

The biomarker assessment suggests that the lake conditions exert stress on the fish *C. jordani*. The biomarker response showed pronounced seasonal variations. The gills presented higher values of LPO. May 2009 displayed the highest levels and November the lowest. In the liver the higher levels of LPO were detected during November and February and May 2010 (the end of the rainy season and the dry season). In muscle, the highest level of LPO was observed during February and May 2010. Gills being the first organ of contact with water are exposed directly to any xenobiotic in the aquatic environment and their biomarker responses are the result of the exposure to stressors. In addition, toxics can also enter via the intake of water and food and be absorbed and transported by the portal system to the liver before entering the general circulatory system; in consequence, the liver is one of the most sensitive organs to environmental stressors [63].

The activity of the antioxidant enzymes in liver and gills also showed a marked seasonal variation. SOD and GPx significantly increased during November, mainly in the gills, compared to the rest of the seasons. CAT also showed higher values in activity during November; however, its highest value was found in gills during February. In general, the activity of antioxidant enzymes decreased from November to May 2010.

Exposure to various xenobiotics, such as metals and organic compounds that enter water bodies, can promote the formation of ROS and induce oxidative stress [12]. The increase in the level of LPO in liver observed in November 2009 and February and May 2010, suggests the existence of pro-oxidant agents in Yuriria Lake and indicates increased oxidative stress in these seasons. Seasonal variation in LPO values could be related to the rainy and the dry seasons; the rainy season can promote dilution of xenobiotics that induce less stress during this season. Rainfall also increases leaching and runoff that enhance the entry of xenobiotics (chemicals), in this study results show that the damage generated in the fish liver became evident from November (the end of the rainy season) until February and May 2010 (dry season), when the processes of evaporation and consequent concentration of xenobiotics could be higher. Fur-

thermore, in May 2009, there were higher levels of LPO in gills, which may indicate that water in the lake at the beginning of the rainy season provoke oxidative stress in gills.

An increase in LPO levels in fish can trigger an antioxidant response as a defense mechanism to prevent cell damage caused by pro-oxidant agents [19, 64] and could be expressed as increased or depleted CAT, SOD, and GPx activities [12]; in both cases, the result is damage to the antioxidant system. In *C. jordani* inhabiting Yuriria Lake, both responses were detected: 1) stimulation of the activity, when LPO levels in liver and muscle were higher (November and February) and antioxidant activity showed the highest values; and 2) depletion in antioxidant activity during May 2009 with high levels of LPO in the gills, and May 2010 with increased LPO in liver and muscle. These highly variable responses of the antioxidant system depend on the type and concentration of contaminant to which fish are exposed, as well as on the intensity and duration of exposure [65]. The clear decrease in the activities of CAT, SOD, and GPx in *C. jordani* is extremely important because it suggests severe damage to the antioxidant system of fish. These damages have been previously documented by [66], who recorded a collapse of the antioxidant defense system of *Liza aurata* in the Ria de Aveiro, Portugal, with exposure to mercury. In addition, [67] reported damage to the antioxidant system of *Oreochromis niloticus* from acute and chronic exposure to Cd, Cr, Cu, Zn, and Fe.

At the population level the assessment can reveal changes in the fish biology and ecology resulting from natural fluctuations and/or ecosystem changes caused by environmental degradation. The standard length of *C. jordani* ranges from 21.16 to 77.61 mm. Three size classes were determined: Class I with a mean size of 26 mm, represented only in the month of May 2010; Class II with a mean size of 56 mm; and class III with a mean size of 62 mm. There was a gap in the size frequencies in the class of 32–50 mm from May to August 2009, whose abundances were not sufficient to form this cohort. The lack of smaller sizes from May to November 2009 can be interpreted as a possible reproductive failure during that year, resulting in low recruitment and consequently precluding estimation of the cohort [68]. These gaps or missing cohorts have been previously documented for other fish as a result of overfishing and/or environmental degradation [69]. Several causes could explain this event. According to [70], the hydrological cycle plays an important role in the development of different biological attributes such as gonadal maturation, migration, spawning, larval development, growth, and feeding. Prolonged periods of drought are also associated with failures in the recruitment and subsequently reduced adult stocks and serious effects on fisheries.

The somatic indices revealed that K displayed small variation between sites and between periods. HSI showed significant differences between sites in August, and between periods in May, values were significantly lower than those of other periods. The GSI showed the greatest variation, with a clear reproductive peak during May 2010. There was a positive correlation between the GSI and HSI. By size class, only the GSI showed variations between seasons. Class I was significantly lower than the rest of the classes. Classes II and III the GSI showed a reproductive peak in May 2010.

In fish, the cost of reproduction may be considerable; thus, fish can express different patterns of energy storage and depletion in relation to reproductive cycles, with an alternation in energy storage (56). The comparison of K and the HSI with the GSI could therefore be useful for

estimating the possible balances or energy transfer between the reproductive period and nutritional status [71]. *C. jordani* in Yuriria Lake show that K was maintained at stable levels. Furthermore, there was no alternation of energy storage between the liver and gonads; GSI and HSI correlated positively, indicating that the reproductive period did not compromise energy reserves or the liver or the soma.

According to [72], fish living in waters contaminated with domestic sewage exhibit higher K and GSI, these authors suggest it is because these sites have more available food for fish, enabling them to compensate for the environmental impairments. This scenario is likely occurring in Lake Yuriria, where *C. jordani*, having enough food, can complete reproductive cycles and reach larger sizes despite the presence of stressors in the lake.

According to the results critical periods in the health of *C. jordani* occur from November, February to May 2010 (end of the rainy season and the dry season) because in this period, the higher LPO, the lowest antioxidant response, and the lowest K were observed in contrast to the higher WQI scores detected in the same period. This result indicates that fish health assessed by biological indicators as oxidative stress biomarkers and the lack of a cohort, are highly sensitive to environmental conditions imposed by the dry season. The drought has been recognized as one of the critical periods in fish health because during this period, the dilution capacity of aquatic ecosystems is low, which increases the risk of exposure to high concentrations of pollutants [73].

The findings suggest that *C. jordani* faces oxidative stress resulting from the presence of pro-oxidant agents in Yuriria Lake. At the population level, *C. jordani* has adapted to the conditions in Yuriria Lake, with mean values of HSI and GSI greater than those in other sites and K values stable throughout the year. Changes were observed in recruitment and reproductive success associated with low water levels in the lake in 2009 which shows that the fish population is highly dependent on water levels in the lake and climate changes. Previous studies indicate that Yuriria Lake receives various xenobiotics with levels that vary both spatially and temporally. Biomarkers of oxidative stress, somatic indices, and monitoring of the size classes in the fish *C. jordani* are appropriate indicators of Yuriria Lake conditions.

5.3. Study case of *Ameca splendens* and *Goodea atripinnis* in Ameca river

The Ameca River is located in the western slope of Mexico which drains to the Pacific Ocean. It is a river characterized by their great fish biodiversity, however, the upper portion of Ameca River, is affected by several environmental disturbers: the construction of a reservoir, the inputs of wastewater from a sugar-processing facility and water extraction, which have resulted in a drastic reduction in fish biodiversity [74, 75]. In addition, some endemic fish species such as *Ameca splendens*, have suffered a reduction in their range, and have become more prone to extinction (NOM-059, 2002) than those with a broad distribution (like *Goodea atripinnis*).

The authors of this paper [32] analyze biomarkers and bioindicators of two viviparous fish species, *A. splendens* and *G. atripinnis* living in a reservoir of the upper portion of the Ameca River, which receives wastewater, and in a spring of the same river that is free from such polluting water. In this study a comparison of the biomarker responses and bioindicators in

two fish species were assessed, according to the main objectives: a) to assess water quality of a spring (ER reference site) and De LaVega reservoir (LV impacted site) where *G. atripinnis* and *A. splendens* coexist; b) to examine the health of both fish species in the reference and impacted sites by means of a battery of biomarkers; and c) to analyze physiological condition indices and population level assessment by mean of bioindicators (population measurements). A WQI was assessed. The set of biomarkers was composed by: enzyme activities of gamma-glutamyl-transpeptidase (γ -GTP), acetylcholinesterase (AChE), EROD and the LPO were determined. Additionally, somatic index were analyzed: CF, HSI and GSI.

Data of WQI scores showed spatial and temporal variations, the spring ER achieved the highest scores in all months over the course of the study and the highest values occurred in March in both sites.

Regarding biomarkers highest values of LPO were found in September (rainy season); the highest values were detected for female livers and gills of *A. splendens* in the impacted site LV and for female livers and male gills of *G. atripinnis* in LV. The LPO activity in the upper Ameca River displayed the stress to which organisms are subjected, since in the reservoir LPO showed the highest values, being *A. splendens* the most affected species. Many environmental pollutants and their metabolites have shown to exert toxic effects associated to oxidative stress, producing free radicals that initiate the LPO and cause damage to membrane proteins [76].

Results of γ -GTP showed less marked seasonal differences than LPO. In this study, in most comparisons between sites, γ -GTP activity was slightly higher at the spring; the inhibitory effect of this activity at LV could indicate a diminution in the amount of membrane proteins caused by LPO [77]. On the other hand, an increase of γ -GTP activity towards March in LV could be to prevent increases in LPO [76]; this increase coincides with the rainy season. Several authors have found seasonal variations in the response of this enzyme as a result of exposition to alkylphenols, the final degradation products of pesticides, detergents and other formulated products [78].

There was a seasonal variation in AchE activity. Organophosphates and carbamates, as well as PAHs, have been widely recognized for causing AchE inhibition, through their reaction with the serine at active site of the enzyme [79]. In nervous tissue AchE is responsible for the breakdown of acetylcholine (Ach) during transmission of an impulse; if the enzyme is inhibited, Ach is accumulated and thus a prolonged transmission of impulses could result in tetani and often in respiratory failure and death. In September in *A. splendens* living at LV, AchE exhibited lower values related to reference site that could have resulted from fish exposure to diverse pesticides used in the adjacent agricultural lands that run off in the rainy season. In March during the dry season, in *G. atripinnis*, living at LV, AchE also exhibited lower values related to ER, may be the effect of evaporation and consequently the concentration of total solid dissolved at LV, including pesticides and other xenobiotics.

Regarding EROD, the hydrocarbons discharged by the sugar-processing facility and other effluents into LV from December to June, could be responsible for activating EROD detoxification mechanism in fishes living there [80]. This mechanism is considered as the main measure of the CYP1A activity, which in turn constitutes a part of the enzyme complex of

the Mixed-Function Oxidase (MFO). Since MFO facilitates the excretion of aromatic contaminants from the body induction of this complex is an effective biomarker of exposure [81, 82]; there are several studies reporting elevated levels of MFO activity in liver fishes as a result of exposure to organic contaminants, such as PAHs, dioxins, PCBs and agricultural and urban wastewater [83]. Also, there are other factors, such as UV radiation, that causes increase in MFO activity [84], moreover, damaged livers, like those of LV organisms, are less capable to MFO induction [85].

Responses to environmental stress also were reflected in bioindicators in both species studied. The major HSI values in LV concurs with [86] who reported major HSI values, related with higher EROD induction, at contaminated sites in comparison with a reference site. Moreover, in our study, higher LSI in LV concurs with higher IBR values. High values of HSI could have resulted from exposition to hydrocarbons which cause hypertrophy in liver [86].

Species may differ in the nature of their physiological response and reproductive consequences to stressors [87]. Tolerant species to environmental stress, like *G. atripinnis*, are more abundant in more disturbed environments, like LV [74, 88]; on the other hand, *A. splendens* is more abundant in a more stable environment, like ER. In LV females/male ratio for *A. splendens* could be affected by environmental estrogens, like pesticides [89]; these could act by merging receptor binding properties of estradiol, alteration of estradiol/testosterone ratios or estrogen receptor levels [12].

The higher SL, weight and CF values in LV are in concordance with [71], that found higher CF and GSI in fishes living in waters polluted with untreated domestic sewage; they suggested that fishes in these sites could find abundant food availability, and they are able to compensate for environmental changes caused by untreated domestic sewage discharges. Fishes could have major GSI values, higher fecundity and lower maturity age even under conditions of high pH [89]; in the present study, *A. splendens* in LV presented these features; only in *G. atripinnis* organisms GSI was higher in the ER. [90] found a decrease in GSI as a result of the exposition of chubs to effluents carrying out organic pollutants and metals. [71] revealed a negative relation between CF and HSI with GSI, but this relation was observed only in *G. atripinnis*. The larger size, higher growth, longevity and reproductive success of organisms living at LV suggest a tactic to compensate for the stress to which the populations of both species studied are subjected; however, offspring is smaller and has a lower weight.

Throughout this study, water quality was higher in ER than in LV due to human activities; but in both sites there were different spatial and temporal factors that produced stress on fishes living there. Therefore, fishes had responses at biomarker and population levels of biological organization. Every biomarker and IBR in this study showed seasonal variation and they were useful environmental tools to demonstrate that, as consequence of pollution, LV is a more stressing place to organisms living them in comparison with ER. In general, organisms in LV presented oxidative stress by the LPO levels, and then neurotoxic impacts by the AchE and some detoxification mechanisms were evident by the γ -GTP and EROD activities. Bioindicators showed evidences of physiological changes due to contaminants exposure and make evident the plasticity of the organisms to survive in this site, in turn the responses should be considered as tactics to survive under stress condition. Moreover, both biomarkers and bio-

indicators revealed that *A. splendens* is a less tolerant species than *G. atripinnis* to environmental stress. Differences in biological response could be attributed to different physiological status of each fish species during the wet and dry season as well as to differences in the type and quantity of the xenobiotics that input at LV due to the period of maximum and minimal activity of the sugar industry; the lixiviation of the agrochemicals from the adjacent lands to the water bodies and also to the complexity of the mixtures of pollutants that are conform at LV that provoke several biological responses.

6. Holistic approach

Environmental stressors can cause several and different damages over aquatic organisms. These damages could be from molecular to population levels, likewise community and ecosystem levels. Through the biomarkers such as defined in this document (any biological measurable response from an organism, induced by the exposure to a xenobiotic or complex mixture of them) we can determine only some of possible causal relationships. Therefore, it is necessary always, measure a set of biomarkers to identify different stressors or damage on sentinel organisms. Several indices have been proposed to try to integrate the multi responses of different biomarkers in a single number that is indicative of the severity of the damage or stress. Such is the case of the IBR proposed by [17]. In this index, the biomarker data must first be normalized and standardized; then the score is represented by the area of a star plot. IBR considers the responses of activation or inhibition of the biomarkers assessed. IBR is an exploratory tool and should be appropriate only if an a priori justification exists for each biomarker used and if the physiological significance of the changes to each biomarker is well known [17].

An other case of index based on a battery of biomarkers was proposed by [91]. This biomarker index was obtained by summing the biomarker values expressed in term of classes. Classes were determined by a distribution-free approach derived from the theory of rough sets. No synergistic or antagonistic assumptions were incorporated into this index.

In [4] the authors proposed a quantitative health assessment index for rapid evaluation of fish condition in the field named Health Assessment Index (HAI). This index is not based on a battery of biomarkers; however, it is a quantitative index that allows statistical comparisons of fish health among data sets. Index variables are assigned numerical values based on the degree of severity or damage incurred by an organ or tissue from environmental stressors.

The Bioeffect Assessment Index (BAI), is based on the integration of several pathological end-points measured in the liver of fish [21]. The BAI represents a modification of the HAI since it includes solely validated biomarkers reflecting toxically induced alterations at different levels of biological organisation in order to quantify the effects of environmental pollution. BAI is able to reflect deleterious effects of several classes of xenobiotics such as heavy metals, organochlorines, pesticides, PAHs, and therefore is also considered as an integrative index of health in aquatic ecosystems.

When we use sentinel organisms, a key point is the study of baseline or natural variation of responses of the sentinel organism selected, or characterizing the response of the same sentinel organism in reference sites.

The use of fish as sentinel organism is feasible for pollution monitoring in aquatic systems; however, the survey should consider the application of a suite of measurable responses (biomarkers and bioindicators) to identify potential sources of stress and damage to which organisms are exposed, as shown in the case studies presented above. The set of biomarkers or bioindicators should also, consider several levels of biological organization in order to identify effects of environmental stressors, spatio temporal trends in environmental conditions and to identify early warning signals to prevent that damage continue from low biological organizing levels to higher levels of organization.

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The Potential Impacts of Global Climatic Changes and Dams on Amazonian Fish and Their Fisheries

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Additional information is available at the end of the chapter

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1. Introduction

The Amazon River Basin, which encompasses the world's largest remaining tropical rainforest, has the highest diversity of fish species of any region in the world [1]. Some of these species represent highly abundant fish stocks that have supported an important fishery for many decades, or many centuries if the history prior to European colonization is included. The importance of fishing in the Amazon River Basin can easily be observed from the high fish consumption, which is mainly attributed to people who live in rural areas near rivers and lakes (Table 1). Regardless of this importance for food, there is no integrative strategy for fishery management, and the activity in this basin as a whole is highly vulnerable to externalities, including those resulting from environmental changes and man-made interventions.

There is a consensus that fishery production is directly related to biological productivity, which is a function of a set of environmental characteristics in the aquatic system. The majority of the Rio Amazonas and its tributaries are accompanied by large floodplains, which is where most of the biological production occurs. A key factor for biological production in the floodplains of large Amazonian rivers is the flood pulse [9], which generates tremendous variation in the input of nutrients over the course of the year, primarily at river headwaters located in the pre-Andean areas, such as Madeira, Purus, Juruá, and Solimões. The flood pulse is the driving element that structures the landscape of the floodplains adjacent to

the river channels, forming a mobile ecotone that is referred to as ATTZ or the aquatic-terrestrial transition zone [9].

Reference	Sub-basin	Social group	g/per capita.day	Kg/per capita.year
[2]	Rio Negro	urban	53.95	19.69
[3]	Rio Negro	urban	121.70	44.42
[4]	Rio Amazonas	rural	369.00	135.00
[5]	Rio Solimões	rural	510.00 to 600.00	186.00 to 219.00
[6]	Rio Solimões and Rio Japurá	rural	509.00 to 805.00	186.00 to 294.00
[7]	Rio Madeira	rural	243.00	88.00
[8]	Rio Amazonas	rural	511.00 to 643.00	187.00 to 235.00

Table 1. Fish consumption in the Amazon River Basin.

The Amazonian hydrological cycle is annual and quite predictable. The flood intensity and timing is controlled by several factors, including those that act on a global scale. The cyclical phenomenon of warming in the Pacific Ocean near the coast of Peru, termed *El Niño*, is related to severe drought in the Amazon Basin. Alternatively, *La Niña* is associated with strong floods. The simultaneous occurrence of other climatic phenomena, such as the warming of the Tropical North Atlantic Ocean, has been used to explain extreme climatic events [10].

Despite the lack of models describing the relationship between flood intensity and timing and fishing success, the life strategy of many species of Amazonian fish is synchronized with the hydrological cycle. For example, several species of Characiforms, including *Colossoma macropomum*, *Brycon amazonicus*, *Prochilodus nigricans*, *Semaprochilodus insignis*, *S. taenirus*, *Piaractus brachypomum* and others, begin their reproductive migration at the beginning of the rainy season when the waters begin flooding [11, 12, 13]. This life strategy was most likely developed to ensure the colonization of the floodplain with newly hatched larvae. The availability of food and places of refuge in the colonized floodplain may determine the strength of the annual recruitment of these species.

There is ample evidence that the Earth's climate is changing more rapidly now than it has in the past [14], with potential effects on the Amazon basin [15, 16]. These effects include physical alterations and changes in nutrient flow [14]. Although uncertainties associated with how local climates change in response to global climate change exist, global circulation models employed by the Intergovernmental Panel on Climate Change (IPCC) have found an increased likelihood of a significant increase in the mean global temperature [14]. A rise in sea level is also predicted, with estimates varying between 0.75 m to 1.90 meters by the end of twenty-first century [17]. Other environmental changes in freshwater systems, such as stratification, productivity reduction and acidification, have no consistent patterns. These changes are very difficult to generalize based on the available evidence; however, there is a

consensus that the impact on fish will be species-specific, that is directly related to the biological characteristics of each species.

Nevertheless, the effects on fisheries should be a result of a series of effects that start at the organism level. At an individual level, all fish have an optimal thermal interval that is limited on the upper and lower boundaries by their critical thermal maxima and minima, respectively [18], thus reducing the analysis of the warming effects. Therefore, fish exposed to temperatures within the sub-lethal interval, excluding the optimal thermal interval, may be affected by warming, and the consequences of this temperature effect should be evident by physiological responses. The high energetic cost necessary to compensate for these unfavorable environmental conditions may affect the growth rates or reproduction success of the fish (Figure 1). Realistically, general effects from water warming can be expected. Because biochemical reaction rates are a function of body temperature, all aspects of an individual fish's physiology, including growth, reproduction and activity, are directly influenced by changes in temperature [19].

When the environment changes, these temperature effects should continue to increase and may be perceptible at the population and community levels. Although different species are affected by environmental change in different ways, the abundance patterns of the entire community, and thus the fishing production, should be influenced (Figure 1).

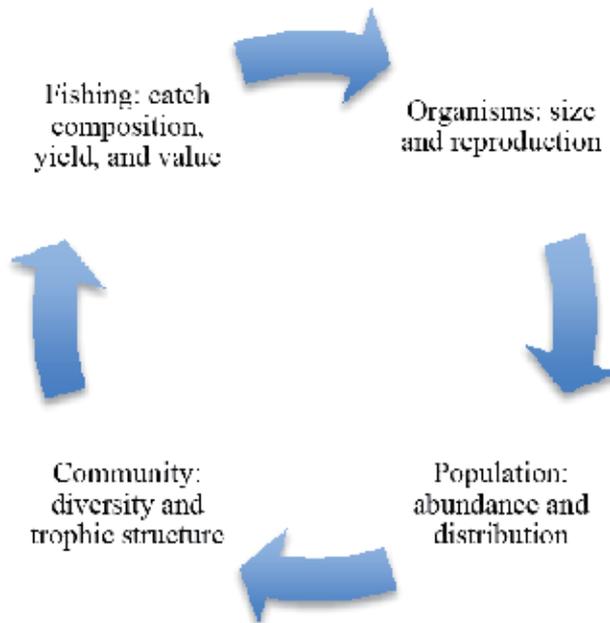


Figure 1. The effects of global climatic changes on different levels.

Dams cause local changes at the sub-basin level but also have the potential to exhibit regional effects. In essence, the impact of dams on the hydrological river cycle, which primarily

involves flood timing, is the most important effect of change in freshwater fisheries because the flood regime is the most important determining force in Neotropical rivers [20]. Dam construction can affect environments and fisheries by changing the timing and quantity of river flows; altering the water temperature, nutrient and sediment transport; reducing adjacent floodplains and other wetlands; and blocking fish migrations.

Currently, there is a large proliferation of hydroelectric dams within the Amazon region. At the western boundary near the Andean and Pre-Andean areas, there are plans for 151 new hydroelectric dams with greater than 2 MW of power over the next 20 years, which is more than a 300% increase [21]. Similarly, in the Brazilian region near the south and southeast boundaries of the Amazon, there are several hydroelectric dam projects that have the potential to completely fragment the river basins with headwaters on the Brazilian Plateau. Similar to climate change, the impact of dams should be associated with the life strategies of different fish species.

The most important species captured by small-scale fisheries in the Amazon basin belong to three groups: Characiforms, which are primarily from the Prochilodontidae, Characidae, and Serrasalminidae families; Siluriforms, which are primarily from the Pimelodidae family and include piramutaba (*Brachyplatystoma vailantii*), dourada (*B. rouseauxii*) and piraíba (*B. filamentosum* and *B. capapretum*); and Perciforms, which are primarily from the genus *Cichla*. Over evolutionary time, members of these groups have developed specific life strategies designed to optimize the survival of Amazonian environmental conditions. Alterations induced by global changes or man-made interventions may directly influence these strategies, with negative effects on both the recruitment and stock abundance of these species, as well as on the socio-economic conditions of the Amazonian people that exploit these fish stocks for food and income.

Therefore, the goals of this chapter are as follows:

1. Review the main scenarios for environmental alterations in the Amazon Basin, which is predicted to be a function of global climatic changes and dams.
2. Identify the potential impacts of different scenarios of environmental alterations in the Amazon Basin on Amazonian freshwater fish populations.
3. Identify the consequences of the predicted impacts on the Amazonian freshwater fish populations, taking into account the main characteristics of the population dynamics.
4. Illustrate the potential social and economic consequences for the local and regional fisheries and the people who depend on these fisheries.

2. Global climatic changes and dams: What we can expect and what are the potential effects

Despite the uncertainties at the local level, it is highly likely that there will be more frequent occurrences of extreme climatic events. Most of the global climate models (GCMs) proposed by

the IPCC [14] project significant Amazonian drying during the 21st century. Pacific sea surface temperature (SST) variation, which is dominated by the El Niño–Southern Oscillation (ENSO), is the main driving force for wet-season rainfall. However, dry-season rainfall is strongly influenced by the Tropical Atlantic north-south SST gradient. Therefore, an intensification of this gradient from the warming of northern SSTs relative to those of the south would move the Inter-Tropical Convergence Zone north and strengthen the Hadley Cell circulation. This change would enhance the duration and intensity of the dry season in much of southern and eastern Amazonia, which already has occurred in 2005. Studies indicated that the most extreme droughts in Amazonia were a result of the strong events of the El Niño–Southern Oscillation (ENSO), the large temperature increase of the sea surface in the Tropical North Atlantic or a combination of these events [22]. Changes in precipitation during the dry season are likely the most critical determinant of the climatic fate of the Amazon [14].

These extreme droughts, even if short in duration, can be catastrophic for aquatic organisms because of the strong reduction in the area of the aquatic environments. Floodplain lakes are the most impacted, and the areas of these lakes can be reduced by several orders of magnitude (Figure 2). Although several species of fish are able to relocate to the river channel during the dry season, some lake resident species remain in the lakes and are unable to survive if the drought is severe. Some studies have observed that fish assemblages seemed to recover rapidly from normal drought seasons [23], but there are indications that extreme droughts occasionally alter fish assemblages. Some species that are vulnerable to these catastrophic events may disappear at a local level [23].



Figure 2. A floodplain area of the Rio Amazonas during the flood season (A) and dry season (B) when an extreme drought occurred.

Another likely climatic change is global warming [14]. Over the next two decades, a warming of approximately 0.2°C per decade is projected for a range of SRES emission scenarios. Even if the concentrations of all greenhouse gases and aerosols had been maintained at the

levels present in the year 2000, a further warming of approximately 0.1°C per decade would be expected [14]. This warming represents an increase of 1-7°C in the mean global temperature within the next one hundred years.

Freshwater fish may explore habitats within an optimal thermal interval and thermo-regulate behaviorally and physiologically. Temperature tolerance ranges are species-specific and range from stenothermal species that support only a narrow thermal range to eurythermal species that are able to live in a wide thermal range. Fish populations subjected to changing thermal regimes may increase or decrease in abundance, experience range expansions or contractions or face extinction [19]. There is also an inverse relationship between the temperature and concentration of dissolved oxygen in water. Thus, an increase in the temperature can exacerbate the hypoxia or anoxia conditions naturally observed in some lentic habitats of freshwater fish.

A direct consequence of global warming is a rise in sea level. Despite the uncertainties related to the dynamics of ice sheets and glaciers, there are models that predict a rise in sea level, which were summarized previously [14]. One model proposed a relationship between global sea level variations and the global mean temperature and predicts a rise in sea level ranging from 75 to 190 cm for the period 1990-2100 [17]. However, some scenarios [14] predict a rise of 4.0 meters (Table 2).

What are the potential effects of a rise in sea level for the Amazon Basin? With regard to its physical characteristics, we can anticipate that the sea will be a hydraulic barrier and will flood areas that are not currently flooded but which are primarily within the floodplain adjacent to the river channel. Other environmental consequences of this barrier can also be expected: a reduction in water flow, an increase in the sedimentation rate and an increase of the flooded area. It is possible that the hydrological cycle will also be affected.

These changes to the hydrological cycle can be magnified by the fragmentation of the environment that will occur as a result of the introduction of hydroelectric dams. We can identify at least four phenomena associated with the introduction of dams:

1. Blockage of the sediment flow in whitewater systems (e.g., Rio Madeira).
2. Change of the flood pulse.
3. Blockage of fish migration.
4. Reduction in oxygen levels both above and below the dams.

Blockage of the sediments might have a large effect on fish communities. Whitewater rivers originate in Pre-Andean areas and are heavily loaded with volcanic soil sediment. The dams act as a barrier and result in a reduction of water speed, thus improving the rate of decantation. The end result is an impoverishment of the river below the dam. Thus, the species that have evolved in the presence of high levels of nutrients will not be able to adapt to the rapid loss in primary productivity that is associated with the reduction of nutrient content. This result will favor a change in the composition of local species and will have serious impacts on fishing activity.

	Temperature change ((C at 2090-2099 relative to 1980-1999)		Sea level rise (m at 2090-2099 relative to 1980-1999)
	Best estimate	Likely range	Model-based range excluding future rapid dynamic changes in ice flow
Constant Year 2000 concentration	0.6	0.3 - 0.9	NA
B1 scenario	1.8	1.1 - 2.9	0.18 - 0.38
A1T scenario	2.4	1.4 - 3.8	0.20 - 0.45
B2 scenario	2.4	1.4 - 3.8	0.20 - 0.43
A1B scenario	2.8	1.7 - 4.4	0.21 - 0.48
A2 scenario	3.4	2.0 - 5.4	0.23 - 0.51
A1F1 scenario	4.0	2.4 - 6.4	0.26 - 0.59

Table 2. Projected global average surface warming and associated sea level rise at the end of the 21st century. Source: [14]

Similarly, Amazonian fish species evolved in a system regulated by an annual and predictable flood pulse, developing life strategies to explore the several habitats available during the hydrological cycle. The elimination or change in the timing or duration of this pulse can destroy signals that trigger reproduction and other life cycle events, which will potentially influence fish recruitment.

The blockage of the fish migration can be critical, with significant impacts for some species that participate in long-distance migrations from the estuary to the headwaters of whitewater rivers to spawn. As a result, some populations may be locally extinct.

The fourth phenomenon concerning the fall in oxygen levels is relatively self-explanatory. The large amount of organic material in the reservoirs will remove a great deal of oxygen from a system that is already low in oxygen content due to the water temperature. The synergy between this phenomenon and global warming is quite evident. The results may include the loss of species with less tolerance for low oxygen conditions.

Clearly, these phenomena can be completely integrated and synergistic, and their effects on fish communities can be magnified and strongly disruptive. As is most often the case with multiple sources of environmental stress, the combined stress resulting from several sources is greater than the sum of the individual stresses. This point is emphasized by [21], who stated that the impact of hydroelectric dams in the Amazon Basin should be considered in a broad perspective, including the planned projects of other Amazonian countries, such as Bolivia, Colombia, Ecuador and Peru. The fragmentation of Amazonian rivers originating in Pre-Andean areas may result in severe nutrient depletion of the rivers because the mountains and associated uplands are the main source of sediments that form the basis for the high primary productivity observed in the Amazonian floodplains.

An unavoidable effect of dams is the shift in species composition and abundance. This shift includes the extreme proliferation of some populations and a reduction, or even elimination, of others [25]. The obstacles in the migratory routes, the loss of natural nursery areas placed upstream of dams and the modification of the hydrological regime downstream of dams, in addition to the rheophilic behavior of the community, are factors directly linked to failures in recruitment and the limited distribution of adults in reservoirs [25], which strongly affect fisheries [26].

3. Life strategies of freshwater amazonian fish

In the Amazon Basin, the life strategies associated with migratory and reproductive processes can be employed to distinguish three fish groups. First, groups can be distinguished by their migration length. The fish species that participate in long-distance migrations are from the family Pimelodidae and belong to a unique genus: *Brachyplatystoma rousseauxii*, *B. vailantii*, *B. filamentosum*, *B. capapretum* and *B. platynemum* [13]. These species migrate up to 3,000 km to complete their life cycle. They migrate from the Amazonian estuary to the border of the Andean mountains in Bolivia, Colombia and Peru [27]. The estuary is the nursery area, and the fish remain there approximately one year prior to beginning their migration. The floodplain areas of the Central Amazon Basin are feeding habitats where the immature fish grow up and store fats prior to their reproductive migration toward the Pre-Andean areas [13, 27, 28, 29]. This process is synchronized with the hydrological cycle. The gonads of *B. rousseauxii* are in an advanced stage of development starting at the beginning of the flood season, while *B. filamentosum*, *B. platynemum* and *B. vailantii* show the highest reproductive activity at the end of the flood season [28].

The short-distance migratory species belong to several groups, including Siluriforms such as *Pinirampus pirinampu*, *Calophysus macropterus*, *Hypophthalmus marginatus*, *H. edentates*, *H. fimbriatus*, *Phractocephalus hemiliopterus*, *Pseudoplatystoma punctifer*, *P. fasciatum* and *P. tigrinum* [13, 30, 31]. These species are also called floodplain migratory fish because they participate in short-distance migrations between the main stem of the Amazon River and its tributaries and floodplain lakes. Despite the absence of published studies, evidence from field research indicates that the migrations of this group do not appear to exhibit a pattern associated with reproductive events. Because these fish are predator species, these short-distance migrations have trophic causes and are developed to find prey in general small and medium size characins.

Another short-distance migratory species is a highly diverse group of Characiformes, which are extensively exploited by the small-scale fishing fleet from the Amazon Basin. Species such as *Colossoma macropomum*, *Prochilodus nigricans*, *Semaprochilodus insignis*, *S. taenirus* and several Myleinae and Curimatidae evolved for a life strategy strongly associated with the hydrological cycle of the Amazon Basin [13, 32, 33]. These species build large schools at the beginning of the rainy season and participate short-distance migrations from their feeding habitat, which is generally within black water tributaries, to white water rivers where

spawning occurs [13, 32, 33, 34, 35]. The parental schools are very large, containing hundreds of thousands of individuals, and there is no parental care after spawning [13, 36]. Adult fish move toward flooded areas, which are rich in food, aiming to store energy for a new reproductive cycle. There are some differences between the timing of migration for these species; however, the schematic in Figure 3 shows the synchronism with the rainy season when the water starts to rise and the importance of the newly inundated floodplains as a place of refuge and feeding for the young fish [37].

Lastly, there are species that do not need to migrate to complete their life cycle. These fish are a diversified group with species from several orders; however, some cichlids from the genus *Cichla* are highly important for regional fisheries. These cichlids are called peacock bass and are the main target of recreational fisheries that are located primarily in black water rivers. The peacock bass is also a top predator that moves in several environments for trophic reasons [37]. Ornamental fish compose another group of non-migratory species that are exploited by fishing. This group is highly diverse, with species belonging to several orders, including Characiforms, Siluriforms, Perciforms, Osteoglossiforms and Gymnotiforms. In general, these are small sized fish with high levels of endemism.

4. Potential effects of global climatic changes and dams on freshwater amazonian fish

The intensity and direction (positive or negative) of the potential effects of environmental changes will vary among populations and species in the Amazonian fish fauna. Some global scenarios are catastrophic [38], proposing that 75% of global freshwater fish will become extinct before the end of the 21st century due to a reduction in river discharge. Nevertheless, the possible effect is local extinction, which would be a critical event for endemic species. Two species of the small fish *Paracheirodon*, which are exploited as ornamental species, exist in the middle to upper Rio Negro in Brazil and in the upper Rio Orinoco in Colombia and Venezuela. A study conducted at an inter-fluvial palm camp of the Middle Rio Negro found that these two species are rarely observed in the same habitat. The *P. simulans* habitat water temperature ranged from a low of 24.6 to a high of 35.2 °C, while the *P. axelroldi* habitat temperature varied between 25.1 and 29.9 °C [39]. The authors propose that because inter-fluvial areas flood as a function of rainfall, a decrease in regional precipitation could alter the hydrologic balance of these wetlands, especially during dry periods, which would lower water levels and increase the water temperature. This scenario would be extremely adverse for *P. simulans*, which exists only in very shallow inter-fluvial areas. A decrease in precipitation could dry out these areas completely, ultimately leading to the local extinction of this species.

At the beginning of rising waters season, the adults move down river from tributaries of black and clear waters to spawning in the turbid and rich environment of white water rivers. After breeding event, these fish move toward the flooded forest for feeding. The larvae

are carried by drift toward flooded areas of the floodplain. After six months, when the water starts to recede, large schools of adults and young fish move toward tributaries.

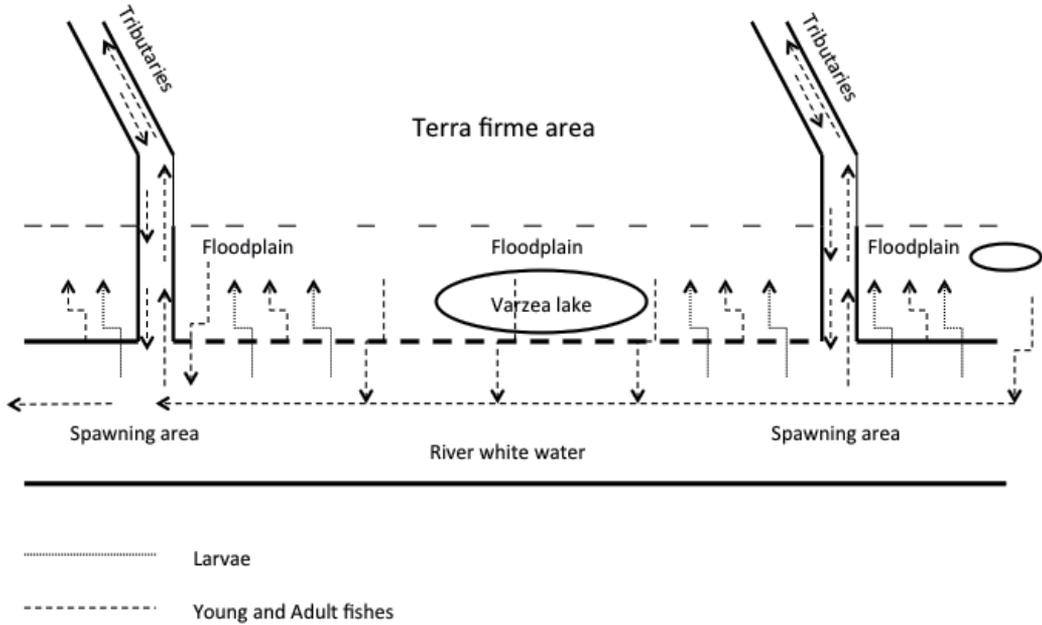


Figure 3. A general description of the Characiforms migrations.

The survivorship or abundance of fish species in a dynamic environment is dependent on three factors: the intensity of change, the velocity at which change will occur and the ability of organisms to adapt in the midst of these changes. Plasticity is a characteristic inherent to each species of fish. However, some common characteristics are useful for classifying the fish into groups and for discussing the most probable effects of environmental changes. For example, the impact of changes in the water temperature should be related to the lethal, sub-lethal and optimal thermal limits of each species. Despite of a scarcity of data on the physiology, life history and behavior of the Amazonian species, some information about population dynamics is available and can be used to hypothesize the effects of the environmental changes resulting from climate change and new dams.

The inverse relationship between temperature and dissolved oxygen in the water may result in an expansion of hypoxia zones. Although the Amazonian fish exhibit a variety of strategies related to oxygen intake in response to hypoxia [40], there are limits to these adaptive strategies that are a result of a long evolutionary time.

Another example of the types of analyses that can be conducted involves the use of the match-mismatch hypothesis (originally proposed by Cushing [41, 42] to describe the relationship between starvation and recruitment), which has clear connections to climate variability. This hypothesis recognizes that early-stage fish need food to survive and grow. It also

recognizes that periods of strong food production in the ocean can be variable and are often controlled by climate, which depends on the strength of the wind, the frequency of storms and the amount of heating or fresh water supplied to the surface layers in the ocean. The hypothesis examines the timing match or mismatch between when and where food is available and when and where early-stage fish are able to encounter and consume this food. Assuming that there is a synchrony between the flood pulse and the spawning season of the Amazonian Characiformes, this cycle can be modified by climatic changes with substantial consequences on species that are the most important sources of protein consumption in the Amazon Basin.

In fact, some species or groups of species may be positively affected. A study of the reproduction of fish from the Rio Cuiabá in Upper Pantanal indicated that both the reproductive dynamics and the hydrological regime were closely related. The authors of the study showed that the intense events of floods were positively related with gonadal development of species that participate in long-distance migration and parental care [43].

A potentially useful approach to develop strategies to study the effects of global climate change could be classifying their effects by the type of relationship between the phenomenon and the impact. [44] classified the range of effects that climate change will have on freshwater, estuarine and marine fish into primary, secondary and tertiary categories. These authors found that the primary impacts are climate-related changes that directly affect the behavior, physiology, fitness and survivorship of fish without intermediary causal drivers. Secondary effects are primarily related to changes in the quality or quantity of habitats. Lastly, the tertiary impacts are related to the interactions between several causal factors.

In contrast, impacts from dams are generally related to the fragmentation of the area in which the individual members of a species live, creating obstacles for migration. The decline in the abundance of long-distance migratory species is the most distinct consequence of such filters. The community of these species undergoes seasonal migrations toward spawning habitats located upstream and consequently requires free-flowing stretches of river. Therefore, recruitment success depends on the presence of and accessibility to spawning areas, which are located in the upstream stretches of the main channel and its tributaries, as well as nursery habitats, which are located in flooded areas downstream [25, 45]. The loss of nursery habitat can critically impact several species, including several species of Characins and Perciforms. Dams also alter water temperature and quality [26, 46], which affects the community structure as a whole.

In addition, there are predictable changes in the species composition of the fish assemblages above the dam in the altered environment of the reservoir, and pre-adapted species could become abundant in this location. However, an impoverishment in the fish diversity as a whole would be expected [25, 26]. A study developed to analyze the alterations of the fish communities due to pollution and the damming of highly impacted rivers from Southeast Brazil, which were fragmented and polluted in their upper stretches, and also detected a synergic effect due to these two impact sources [47]. These authors observed a noticeable decrease in species richness in the polluted stretches of the river, with one or two species dominating. However, the artificial control of floods and discharge levels should have direct

impacts on recruitment success. An analysis on the influence of the mean annual water level (m), the amplitude (maximum water level of the river in a given year; m) and the flood duration (number of days above 3.5 m; yearly total and for each season; summer and autumn were considered together) on the recruitment of *Prochilodus scrofa* for the fishery conducted at the Itaipu Reservoir and observed that flood duration is more important than flooding amplitude [48].

Table 3 summarizes the effects of global warming, sea level rise and dams on freshwater Amazonian fish, taking into account our level of knowledge. Fish faced with a changing environment must adapt, migrate or perish [19]. In addition to the high level of uncertainty at the species level, some evidence is available to predict that the resulting stress of a temperature increase will affect fauna as a whole, including fish. The effects of the higher energy demand to compensate the stress would start at the physiological level and would include size reduction and reproductive failure. This evolution affects the community structure when the dominant species has more adaptive capacity. Therefore, another possible effect of climate change is the loss of biodiversity through the extinction of specialized or endemic fish species [48]. This pattern of environmental change inducing effects will initiate from a rise in sea level and the introduction of dams.

5. Social and economic effects of climate changes and dams on amazonian fisheries

Fisheries are very important activity worldwide. Gross revenues from marine capture fisheries worldwide are estimated between US\$ 80 billion and 85 billion annually [49]. However, some authors stated that the global marine fisheries are underperforming economically due to overfishing, pollution and habitat degradation [50]. As is the case in many regions of the world, fish are a key source of animal protein, essential amino acids and minerals, mainly for low-income population who live in the Amazon basin [3, 5, 6]. A recent paper examines if marine fisheries and aquaculture can supply fish demand for a growing human population, taking into account climate change [51]. The authors claim that an effective management of fisheries is necessary to assure sustainability for world fish stocks. The authors also called for a reduction in the amount of wild fish employed to produce animal feed.

In general, Brazilian fish production followed the world tendency, with mean rates of growth of 2.48% and 10.82%, for fishing and aquaculture, respectively [52]. Analyzing just Amazonas State, the main producer of fish exclusively from freshwater, we can see that the state follows the same trend of the region, for the last ten years. On average, the Amazonas State contributed 29% of the region's fisheries production.

A closer analysis of the data shed some light on the impacts of environmental changes, as a result of climate changes or dams, on fisheries and its consequences for well being. Figure 5 shows the Amazonas state Gross Domestic Product (GDP) per capita and an index of fish production growth, for the period between 1992 and 2010, taking 1992 as base year.

Impacts	Effects
Global Climate Change – Global warming	Siluriformes–Pimelodidae: long-distance migrations – Alterations in physiological functions to survive in environmental conditions out of an optimal specific interval; – Medium size reduction; – Size of adult stock reduced due to reductions in prey abundance. Characiformes– Short-distance migrations – Alterations in physiological functions to survive in environmental conditions out of an optimal specific interval; – Failure in recruitment due to the mismatch between young fish and food; Perciformes–Cichlidae – Alterations in physiological functions to survive in environmental conditions out of an optimal specific interval; – Failure in recruitment due to impacts on reproductive functions. – Failure in recruitment due to loss of habitat;
– Sea level rise	Siluriformes–Pimelodidae: long-distance migrations – Strong recruitment due to expansion of nursery area at estuary. Characiformes– Short-distance migrations – Reduction of the abundance of less-adapted species for the altered environment; – Changes in the community structure as a response to the alterations in the environment. Perciformes–Cichlidae – Alterations in physiological functions to survive in environmental conditions out of an optimal specific interval; – Failure in recruitment due to impacts on reproductive functions.
Dams	Siluriformes–Pimelodidae: long-distance migrations – Stock abundance reduced due to reductions in the livable area; – Blockage of fish migrations, which create obstacles for freshwater species to complete their life cycles. Characiformes– Short-distance migrations – Change in species abundance because of alterations in the habitat; – Loss of important habitats for young fish (e.g., areas that are seasonally flooded); Perciformes–Cichlidae – Failure in recruitment due to the loss of spawning habitat.

Table 3. The potential impacts of global climate change and dams and their effects on freshwater Amazonian fish

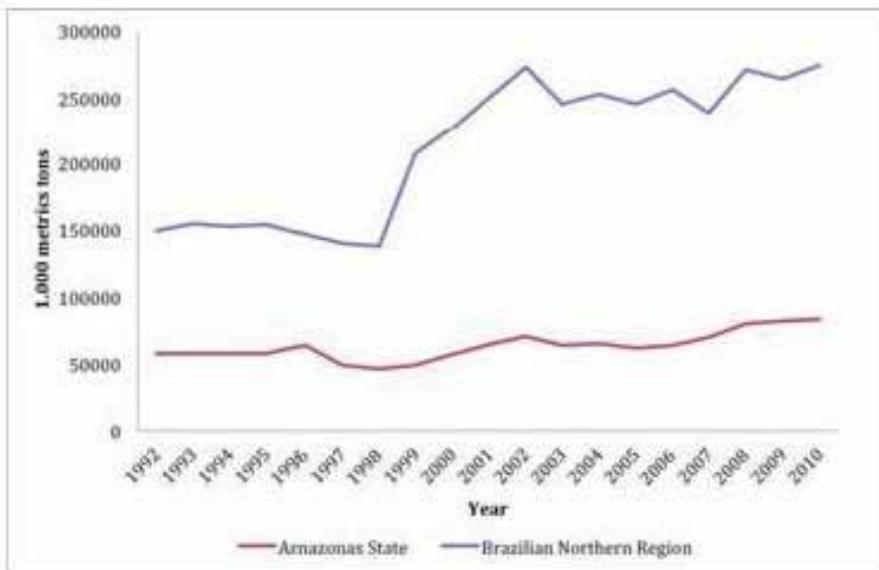


Figure 4. Amazonas State and Brazilian Northern region fisheries production between 1992-2010. (Source: 53, 54).

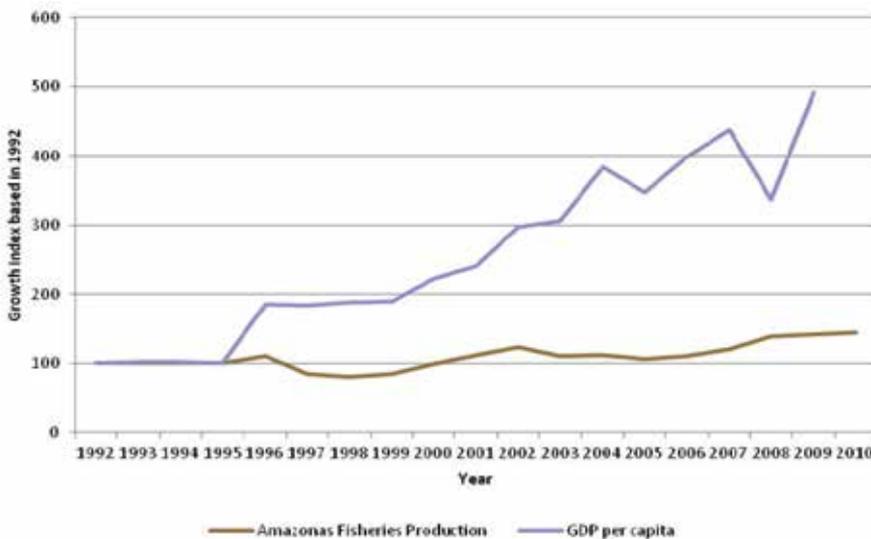


Figure 5. Amazonas State GDP per capita and an index of fish production growth [53, 54].

Taking 1992 as the base year both for a fish production index and GDP, it is possible to see that starting in 1995, GDP per capita grew continuously with fluctuations around the trend due to business cycles. At the same time, the index of fish production also grew but with

much less intensity. It is clear from the graph that while GDP per capita presented a strong growth trend, increments in fish production were very slow.

What relationships do these trends have with global warming? It is clear that fish production does not drive GDP growth, so global climate change is not likely to have much impact on the GDP of Amazonas through the impact of global change on fisheries. This is not to imply that there would not be critically important impacts through other sectors of the economy. Of course, impacts on fisheries would have a large impact on the income of those participating in the commercial fishing industry, and it could have a significant impact on the GDP of small cities in Amazonia (5 to 10 thousand inhabitants) that do not have alternative sources of income. This is particular true for small cities that are sufficiently close to Manaus to sell their catch in this large urban market.

It should be noted that GDP is a measure of market output, not economic benefits and there are a number of ways in which fisheries impact social welfare, both within the urban/industrial area and within the rural communities. For example, the output of subsistence fisheries is explicitly excluded from the measure of GDP because the output is not traded in formal markets. Clearly, small communities will suffer immensely if fisheries are highly impacted by climate change. Moreover, Amazonas has a fish culture, as opposed to the beef culture of the rest of Brazil. As Table I indicates, fish consumption is extraordinarily high, even in urban centers. If fish become scarcer and more expensive, the welfare of the urban centers will be diminished as they are forced to substitute meat and poultry for their traditional fish dishes.

The social welfare impacts of global climate change induced impacts on fisheries are difficult to calculate. The reason for this is that the direct impacts on fisheries may change economic behavior, which could then lead to a series of indirect impacts that could compound the impacts of climate change. These reactions could occur between urban and rural communities, within the fishery sector, or within the subsistence communities.

If the impacts of global climate change on fisheries reduce the quality of life of small communities, it could spur additional migration from the small communities to the urban centers. This would increase the urban externalities associated with population increases as a whole, and those associated with immigration of a group of people without training to participate in the service or industrial sectors of the urban center. Moreover, the introduction of more people with a high preference for fish consumption into the urban center will increase the urban demand for fish, putting more pressure on the fisheries near the urban centers, which are already stressed and showing evidence of decline.

People that remain in small communities will continue to be negatively impacted by the decreased fish populations because of the impacts of global climate change. This could lead to several negative impacts. First, they may react to the change by fishing more intensively to try to compensate for the decline in populations. This will further stress the populations that are already stressed by global climate change. Second, they may switch species, trying to capture species that previously were not high priority, but remain more abundant. In general, these species will be smaller, and the trophic cascading associated with the decline in for-

age fish is difficult to predict. Third, they may turn to more hunting to supply their protein needs, leading to other negative impacts on the ecosystem. In particular, the hunting of caiman could lead to impacts on biodiversity as the controlling predators are eliminated from the ecosystem. This would be in addition to the impacts of reductions in intermediate level aquatic predators such as peacock bass (*Cichla* spp.) which would suffer from the negative impacts of global climate change.

Worse impacts could potentially occur if the rural populations increased their participation in other extractive activities, including agriculture, timbering and non-timber forest products. Although the collection of non-timber forest products, such as fruit and fibers is likely to have a relatively benign direct impact, areas of the forest that were previously not the subject of economic activity could become the subject of economic activity. The heavy presence of people in these previously unharvested areas could lead to impacts on fisheries and wildlife, interfering with the ability of these areas to serve as a reserve for repopulating depleted areas.

Increased participation in timbering and agriculture will lead to deforestation, which has a negative impact on the biodiversity of the forest. Moreover, it will have a negative impact on the aquatic systems. If communities are successful in developing markets for these extractive products, it could lead to a reverse migration of people from urban areas back to the forest, leading to an increasing cycle of degradation.

Both the direct effects of global climate change and the indirect effects associated with the reaction to global climate change will have negative impacts on the social welfare of both urban and rural populations. It is likely that a feedback cycle could develop where the reaction to degradation is more degradation, dramatically reducing both social welfare and ecosystem function.

6. Conclusions

Climate changes and dams are likely to represent the most important threats to freshwater fish around the world. The effects of climate change on the ecosystem will include alterations of the timing, distribution and form of precipitation, as well as the timing of the flood pulse, and the intensity and frequency of floods and droughts. The impacts of these changes on the fish fauna and fisheries are, at that moment, unpredictable at both the species and ecosystem level. Actually, the degree of uncertainty and the low level of knowledge about the biology of the most of Amazonia fish species, make it hard to determine the current impacts of climate changes for each species and for the ecosystem as a whole, and even harder to predict future impacts. In addition to regional impacts, it will be very difficult to predict the impacts of dams for each fish species from the Amazon basin. As we discussed earlier, dams block fish migration, which could be very critical for many freshwater fish species that need to do migrations to complete their life cycles.

One pertinent question is what we can do to minimize the impacts of global climate changes and dams. Actually, we need to identify clearly the possible strategies to avoid human con-

tribution to the magnitude of both sources of impacts. We realize that any intervention on the crescendo of climate change needs action of a very large scale or a very large package of small scale actions. Both of these necessitate development strategies arising from a coordinated source, such as a global agreement. Unhappily, the global negotiations on this issue have made little progress, and we remain distant from an agreement. On the other hand, the effect associated with potential new dams are in the sphere of national decision-making and these impacts could be avoided if the proposed construction does not take place. Thus, it is not too late to find alternatives to Amazonian hydropower for power supply. A global goal to minimize the impacts of both climate change and dams on freshwater fisheries is needed in order to avoid these severe impacts.

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Evaluation of Toxicity in Silver Catfish

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Additional information is available at the end of the chapter

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1. Introduction

The global population growth observed in the recent decades coupled with continual technological advancement and increase in the generation of new industrial products, including the manufacture of chemicals such as fertilizers and pesticides, has led to an expansion in the levels of xenobiotic compounds in aquatic ecosystems (Jesus; Carvalho, 2008).

The pollution of rivers and lakes with chemicals of anthropogenic origin may have adverse consequences: the waters become unsuitable for drinking and other household purposes, irrigation, and fish cultivation, and the animal communities living in them may suffer seriously. Massive fish kills are recorded rather frequently, and changes in the population of the fauna as a consequence of sublethal effects on ecologically important species have also been described (Koprocu; Aydin, 2004).

Insecticides are used extensively in agriculture and industry because it is easy to apply, cost effective, and in some situations, it is only a practical method of control. However, benefits of pesticides are not derived without consequences. They are one of the most potentially harmful chemicals and are released into the environment by direct applications, spraying, atmospheric deposition, and surface runoff. Given the fact that, insecticides are not selective and affect non target species as readily as target organisms, it isn't surprising that a chemical that acts on the insect's different systems will elicit similar effects in higher forms of life (Dogan; Can, 2011).

Levels of insecticides in superficial waters generally range far below lethal concentrations for aquatic organisms. However, sublethal adverse effects may result from expo-

sure of aquatic organisms to insecticides at environmentally relevant concentrations (Das; Mukherjee, 2003).

Pesticides in the environment may be used as a model for the study of ecotoxicology, because they contaminate air, land and water, causing adverse effects that affect from bacteria to humans. It is well proven that these chemicals are toxic to aquatic arthropods, bees and fish (Santos et al., 2007). The effects of the use of pesticides are recognized worldwide and aggravated by misuse since part of this material is accumulated in plants and soil and much of it is transported to the rivers by rain (Tsuda et al., 1995; Wilson; Tisdell, 2001).

As a result of a great variety of human activities, the aquatic environment is becoming increasingly threatened by an alarming number of foreign chemicals or xenobiotics. Fish populations living in highly polluted areas often have high incidences of gross pathological lesions (Malins et al., 1988), associated with elevated levels of toxic contaminants in the sediments. However, pesticides applied to the land may be washed into surface waters and may kill or at least adversely influence the life of aquatic organisms.

Contamination of water with large amounts of pesticides leads to fish mortality or starvation by destruction of food organism, many toxicants have been shown to affect growth rate, reproduction and behavior, with evidence of tissue damage (Van Der Oost et al., 2003; Srivastav et al., 2002).

The poisoning of fish by pesticides can be acute or chronic and in general acute poisoning causes mass mortality. However, pollution is an often chronic process, apparently without any visible damage but sometimes producing several sublethal effects (Rodrigues, 2003).

The aim of this chapter was present a review based on some aspects of silver catfish's toxicology.

2. The fish

Silfvergrip (1996) conducted extensive taxonomic revision of the genus based on characters of internal morphology, and concluded that the genre *Rhamdia* consists of only 11 species among 100 previously described. According to the same author, quelen taxonomic division belongs to the following: Class: Osteichthues, Series: Teleostei, Order: Siluriformes, Family: Pimelodidae. Genre: *Rhamdia*, Species: quelen.

Rhamdia quelen, popularly known, as silver catfish is a species of fish found from southern Mexico to Argentina that displays the absence of teeth and scales with variable-length cylindrical wattles. This is an omnivorous species with an eating preference for fish, crustaceans, insects, plants and organic debris (Silfvergrip, 1996).

The silver catfish (*Rhamdia quelen*, Quoy & Gaimard) is an endemic South American fish species that with stands cold winters and presents fast growth rate in summer. These characteristics make catfish a suitable species for fish production in southern South America or any region with a temperate or subtropical climate. In aquaculture systems, at a density of two

to four fish/m² catfish reach a 600-800 g body weight in eight months. Our unpublished observations in experimental field trials and at fish farms have shown that this weight is easily reached, but high mortality rates (40-50%) might occur if small fish (1-3 g) are used to initiate the culture. However, when beginning with heavier juveniles (30-60 g), the final weight will still range from 600 to 800 g, but with mortality rates not exceeding 5-10%. Thus, the more reasonable order in silver catfish culture is: hatchery, larviculture (1-6 g), nursery (from 5-6 g to 30-60 g), and termination (from 30-60 g to 600-800 g) (Barcellos et al., 2001). This species can be considered eurythermal because the fry acclimated to 31°C withstand temperatures from 15 to 34°C (Barcellos et al., 2003).

3. Chemical contaminants in aquatic systems

Toxic compounds or natural anthropogenic are called xenobiotics. With the onset of the epidemiological investigations was to confirm the hypothesis of many xenobiotics to be dangerous to living things, as well as their respective offspring, exerting toxic effects in the short, medium or long term (Reys, 2001). These substances are persistent in the environment eventually absorbed and accumulated by living organisms, toxic effects on various organs and systems. Thus, it was noted that the use of xenobiotics without evaluation of risks to the ecosystem, constituted a potential threat to the health of people, animals and plants (Sanches, 2006).

The introduction of toxic substances in the aquatic environment causes local and immediate effects, but can also lead to contamination of watersheds and commitment of underground reservoirs by infiltration through the soil. Numerous compounds have been detected in surface water, groundwater and water supply relating to agricultural activities and human cases of environmental contamination (Sanches, 2006).

3.1. Insecticides

The growing use of synthetic insecticides is intensifying global pollution risks. Insecticides are toxic and were designed to repel or kill unwanted organisms and when used for their different purposes they may be brought to water bodies killing or influencing the lives of aquatic organisms (El Sayed et al., 2007). The effects of the use of insecticides are recognized worldwide and compounded by their improper use (Tsuda et al., 1995; Wilson; Tisdell, 2001).

Organophosphates comprise a group of chemical compounds extensively used in farming as insecticides, which cause accidental poisoning in animals and men. The toxicity of these compounds is due especially to the respiratory and cardiac impairment in consequence of autonomic nervous system disorders.

The primary effect of organophosphates (Ops) on vertebrate and invertebrate organisms is the inhibition of the enzyme Acetylcholinesterase (AChE), which is responsible for terminating the transmission of the nerve impulse. OPs block the hydrolysis of the neurotransmitter

acetylcholine (ACh) at the central and peripheral neuronal synapses, leading to excessive accumulation of ACh and activation of ACh receptors (Peña-Llopis et al., 2003). The overstimulation of cholinergic neurones initiates a process of hyperexcitation and convulsive activity that progresses rapidly to *status epilepticus*, leading to profound structural brain damage, respiratory distress, coma, and ultimately the death of the organism if the muscarinic ACh receptor antagonist atropine is not rapidly administered (Shih; McDonough, 1997).

Melo (2004) showed that *Rhamdia quelen* (silver catfish) juveniles exposed for 96 hours to a sublethal dose (0.01 mL/L) of Folidol® 600, was target for the toxicant action and some alterations became evident after 4 hours of exposure. The alterations observed in the liver were reduction in the density of melanomacrophages, focuses of necrosis, enhancement in the density of hepatocytes, loss of the cellular contour of the hepatocytes, cytoplasmic granulation, reduction of the cytoplasmic vacuolization, mitochondrial disruption, disorganization of the rough endoplasmic reticulum, nuclear heterochromatization and decharacterization of the endothelium. These alterations could diminish the liver metabolism, and as a consequence, they could cause damages to the health of the *R. quelen* juveniles.

Deltamethrin (DM) and other pyrethroids have proven to be toxic to aquatic organisms, mainly to fish. Due to its lipophilic characteristics it can be highly absorbed by the fish gills, which partially explains the high sensitivity of these animals to DM exposure in concentrations up to a thousand times lower than in mammals (Rodrigues, 2003).

Galeb et al. (2010) and Montanha (2010) studied the behavior of silver catfish exposed to sublethal concentrations of DM and CM, respectively, and they have presented loss of balance, swimming alteration, dyspnea (they kept their mouths and opercula open). *Post-mortem* signs observed in the animals exposed to DM and CM were mainly darkening of the surface of the body, tail and wattles erosion and hemorrhagic spots on the body surface.

The main behavioral changes observed are represented by respiratory and neurological manifestations. Such results corroborate with Polat et al. (2002) and Ylmaz et al. (2004), who have tested Cypermethrin in guppies (*Poecilia reticulata*); Borges (2007), who have tested CM in silver catfish (*Rhamdia quelen*). These changes can be attributed to the neurotoxic effect of DM/CM by blocking sodium channels and inhibiting the GABA receptors in the nervous filaments which results in an excessive stimulation of the central nervous system that sometimes can lead to brain hypoxia (El-Sayed et al., 2007).

Galeb et al. (2010) studied the haematological response of silver catfish exposed by DM, it was a significant increase in the total leukocyte counts. Similar results were reported by El-Sayed et al. (2007) in Nile tilapia (*Oreochromis niloticus*) and Pimpão et al. (2007) in catfish (*Ancistrus multispinis*). Montanha (2010) showed similar results in silver catfish exposed to CM. The leukocytosis showed that these pesticides can generate inflammatory or stress responses.

It was observed decrease of serum levels of ALT (alanine aminotransferase), AST (aspartate aminotransferase) and FA (alkaline phosphatase) were observed in silver catfish exposed to DM (Galeb, 2010) and CM (Montanha, 2010).

Fipronil is a non-systemic, chiral phenylpyrazole insecticide registered for use to control ants, beetles, cockroaches, fleas, mole crickets, ticks, termites, thrips, and other insects in a variety of agricultural and residential uses. Its mechanism of action involves non-competitive binding to the GABA receptor, effectively blocking the chloride channel and resulting in paralysis (USEPA, 2011).

Their toxicity to fish varies with species and it is highly toxic to *Lepomis macrochirus* ($LC_{50} = 85$ g/L), the *Oncorhynchus mykiss* ($LC_{50} = 248$ g/L), the *O. niloticus* ($LC_{50} = 42$ mg/L), *Poecilia reticulata* (LC_{50} less than 100 g/L) and *Cyprinus carpio* ($LC_{50} = 430$ g/L). Low concentrations of fipronil are lethal to most species of fish that were tested, and is especially toxic to young fish. Studies indicate that the dose 15 mg/L reduced the growth of trout. In addition, this compound is also bioaccumulated fish.

3.2. Drugs

The occurrence of pharmaceuticals for human and veterinary use has been detected in surface waters, sediments and drain in worldwide. Various substances such as anti-inflammatories, analgesics, antibiotics, hormones and antidepressants represent them. Although they have been subjected to pharmacokinetic studies, little information exists about the environmental fate and toxic effects in several organisms of aquatic fauna and flora, certainly affected (Stumpf et al., 1999; Fent et al. 2006).

Chemical toxicity distribution approaches have been employed for identifying Thresholds of Toxicological Concern for many industrial chemicals (Kroes et al., 2005), comparing the sensitivities of in vitro and fish models for estrogenicity (Dobbins et al., 2008), and predicting aquatic concentrations of ecotoxicological concern for chemical with common MOAs in plant models and invertebrates and fish (Dobbins et al., 2008).

4. Assessment of toxicity in fish by biomarkers

The biological response of an organism to xenobiotics following absorption and distribution starts with toxicant induced changes at the cellular and biochemical levels, leading to changes in the structure and function of the cells, tissues, physiology and behavior of the organism. These changes can perhaps ultimately affect the integrity of the population and ecosystem. For the biomonitoring and management of the aquatic ecosystems, these biological responses (biomarkers) have been proposed to complement and enhance the reliability of the chemical analysis data (Parv ez; Raisuddin, 2005).

There are very few pollutants that have been confirmed to cause adverse effects. In most cases, casual relationships have not been established to a large group of persistent pollutants, due the complex chemical contamination of environmental compartments, which difficulty to attribute harmful effects to any particular pollutant or category of pollutants.

Several definitions have been given for the term 'biomarker', which is generally used in a broad sense to include almost any measurement reflecting an interaction between a biologi-

cal system and a potential hazard, which may be chemical, physical or biological (WHO, 1993). A biomarker is defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) that can be related to exposure to or toxic effects of environmental chemicals (Van Der Oost, R et al., 2003).

A bioindicator is defined as organism giving information on the environmental conditions of its habitat by its presence or absence or by its behavior, and an ecological indicator is an ecosystem parameter, describing the structure and functioning of ecosystems.

According to the WHO (1993), biomarkers can be subdivided into three classes:

- **Biomarkers of exposure:** covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;
- **Biomarkers of effect:** including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease;
- **Biomarkers of susceptibility:** indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure.

4.1. Histopathology

Histopathological characteristics of specific organs express condition and represent time-integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organization. Therefore, histological changes occur earlier than reproductive changes and are more sensitive than growth or reproductive parameters and, as an integrative parameter, provide a better evaluation of organism health than a single biochemical parameter. Histopathological biomarkers signal the effects of exposures both acute and chronic to toxic agents in high or low levels. The pathological changes may be adaptive or degenerative and will determine the survival or death of the organism. The organs most commonly damaged by toxic agents are gills, liver and kidneys, which may be also affected by bacteria, viruses and parasites. That is why a health assessment of the animal is important to differentiate damage induced by toxic agents and diseases. Pharmaceuticals of many categories may be detected on the environment contaminating water flows. In a study for the evaluation of the effect of the analgesic dipyrone in surface waters, Pamplona (2009) observed important histological disorders in the kidneys of silver catfish. The fish were exposed to three concentrations of dipyrone (0.5, 5 and 50 µg/L) in the water for 15 days and then evaluated. According to the author, the histological parameters of the intoxicated animals showed important tissue damage in the silver catfish kidneys, such as necrosis, fibrous deposition, vacuolization and constriction of blood vessels of the renal parenchyma, hyperplasia, and even necrosis of great renal vessels and glomeruli in the higher concentration of dipyrone. Such alterations were not observed in the control group.

Pesticides are the substances most extensively researched in the aquatic ecotoxicology due to the large amounts used in agriculture and livestock in the whole world. Melo (2004) exposed the silver catfish to the organophosphate methyl parathion at the sublethal concentration of 6 mg/L for 96 hours with the aim of histological and ultrastructural analysis of liver tissue. Melo (2004) suggested that the histological changes are related to the intoxication by methyl parathion that is likely to bring the individuals metabolic, cellular and subcellular problems. It was observed in *Rhamdia quelen* loss of the contour of the hepatocytes and endothelial cells, as well as changes in the nuclear chromatin. According to Melo (2004), changes in the organization of rough endoplasmic reticulum and mitochondrial disruption within four hours of intoxication and strongly eosin stained granulation in the cytosol within 24 hours of exposure could be noticed. Ultrastructurally, a high incidence of lipid droplets was observed in the cytosol of hepatocytes when compared with control within 48 hours of exposure to fipronil. Increased foci of necrosis in the liver of silver catfish after 48 and 72 hours of exposure in relation to the control group, which presents only occasional foci of necrosis, were noted, among other structural changes. After 96 hours of exposure, Melo (2004) describes cells indistinguishable contour, presence necrosis focus, in addition of damaged blood vessels, vacuolization of the cytosol and the presence of an unknown material strongly eosin stained in the cytoplasm of the hepatocytes. Blood leukocyte infiltration and congestion were observed in all animals analyzed. Melanomacrophage were found in various locations between the hepatocytes as well as pyknotic nuclei cells.

Ghisi (2010) studied the effects of the phenyl pyrazolefipronil in the gills of the silver catfish after 60 days of intoxication in the sublethal concentrations 0.05; 0.10 and 0.23 µg/L. The author reports hyperplasia, lamellar fusion and aneurysms in all groups treated, including the control group that can impair the gill function. However, Ghisi (2010) considers the injuries of low severity and possible regression if the source of stress is eliminated, since the concentration of fipronil used was very low.

Cattaneo (2009) studied the effects of 2,4 - dichlorophenoxyacetic acid (2,4-D) herbicide in *Rhamdia quelen* fingerlings in an acute toxicity assay. After acclimation, silver catfish were intoxicated with concentrations of 0, 400, 600 and 700 mg/mL of 2,4 - D for 96 hours. The author states that the histological analysis showed alterations in the liver of silver catfish after exposure to 2,4-D herbicide, such as abnormal arrangement of hepatocytes cords, cell membrane rupture and hepatocytes vacuolation.

The effects of the herbicide clomazone in teleost fish *Rhamdia quelen* were studied by Crestani et al. (2007). The silver catfish were exposed to the concentrations 0.5 and 1.0 mg/L of clomazone for 12, 24, 48, 96 and 192 h. Histological analysis showed vacuolation in the liver after herbicide exposure, some of that were completely restored after a recovery period. Ferreira (2010) studied the sublethal effects of glyphosate (1.21 mg/L), methyl parathion (0.8 mg/L) and tebuconazole (0.88 mg/L) herbicides to silver catfish. The fingerlings were intoxicated during 96 hours and then sampled after anesthesia. The mapathologicalcal changes in liver histology were: diffuse hepatocyte degeneration, bile stagnation, granules on the cytoplasm, hyperemia and vacuoles in the cytoplasm and nucleus of fingerlings exposed to

methyl parathion and tebuconazole. The liver of fish exposed to glyphosate did not show any visible histological changes.

4.2. Hematological and biochemical analyses

Hematological analysis in fish is not routinely used for fish diseases diagnosis. Hematology of fish lags behind that of other classes of vertebrates, but analysis of blood still can be informative about disease processes in teleosts and elasmobranchs (Clauss et al., 2008). The most important factor limiting accurate hematological analysis in different fish species is the variation in types, numbers, and appearance of leukocytes. In addition, the considerable variation in reported leukocyte values from healthy fish, even within a species, is also partly caused by differences in the methodology used as well as by subjective interpretation of cell types by the investigator. Nevertheless, some effort should be made to try to surpass these difficulties since these parameters can be a useful tool for evaluation of the effects of pesticides in the cellular components of blood and even in the immune system. Therefore, the analysis of hematological and biochemical parameters in fish can contribute to the assessment of the animal's health and also the habitat conditions (Pimpão et al., 2007).

Table 1 provides an overview of some hematological and blood chemistry results in catfish (*Rhamdia quelen*) exposed to toxic chemicals.

4.3. Enzymatic analyses

One of the more sensitive effect biomarkers is alteration in levels and activities of biotransformation enzymes. The activity of these enzymes may be induced or inhibited upon in fish exposure to xenobiotic. Enzyme induction is an increase in the amount or activity of these enzymes, or both and inhibition is the opposite of induction. In this case, enzymatic activity is blocked, possibly due to a strong binding or complex formation between the enzyme and the inhibitors.

The according by Van Der Oost, R et al. (2003) enzymatic analyzes to fish are used more:

- Phase I enzymes: Total cytochrome P450 (cyt P450); Cytochrome P450 1A ; Cytochrome b5 (cyt b5); Ethoxyresorufin O-deethylase (EROD) and arylhydrocarbon hydroxylase (AHH); NADPH cytochrome P450 reductase (P450 RED);
- Phase II enzymes and cofactors: Reduced and oxidized glutathione (GSH and GSSG); Glutathione S-transferases (GSTs); UDP-glucuronyltransferases (UDPGTs);
- Oxidative stress parameters: Superoxide dismutase (SOD); Catalase (CAT); Glutathione peroxidase (GPOX); Glutathione reductase (GRED); Non-enzymatic antioxidants
- Biochemical indices of oxidative damage: Lipid peroxidation; DNA oxidation;

Table 2 provides an overview of some enzymatic results in catfish exposed to toxic chemicals.

Chemical substance	Diets containing aflatoxins *				Treatment with diflubenzuron in 24-hour immersion baths **				
	Control	41ppb/ aflatoxin/ kg	90ppb/ aflatoxin/ kg	204ppb/ aflatoxin/ kg	Control	0,01 mg/ I ¹ diflubenzuron	0,1 mg/ I ¹ diflubenzuron	1,0 mg/ I ¹ diflubenzuron	None
Erythrocytes (x 10 ⁶ /μL)					2,71 ± 0,3	2,67 ± 0,4	2,56 ± 0,3	2,6 ± 0,4	1,95 ± 0,40
Hemoglobin (g/dL)	8,2 ± 0,5	5,5 ± 0,3	4,0 ± 0,3	3,5 ± 0,2					6,73 ± 1,15
Hematocrit (%)	24,51 ± 1,51	13,16 ± 1,47	11,75 ± 0,41	11,16 ± 0,88	32,7 ± 3,62	32,9 ± 2,61	31,1 ± 2,8	30,1 ± 1,7	26,5 ± 5,3
Leucocytes (x 10 ³ /μL)					13,4 ± 2,7	13,2 ± 2,9	12,6 ± 2,1	12,2 ± 2,4	
Lymphocytes (%)					62,1 ± 5,7	61,9 ± 6,8	62,4 ± 6,3	62,6 ± 6,4	11,61 ± 6,57
Neutrophils (%)					25,3 ± 5,5	26,9 ± 5,8	25,9 ± 6,7	25,9 ± 6,7	6,02 ± 3,33
Granulocytic especial cell (%)					2,7 ± 1,2	2,0 ± 0	2,2 ± 0	4,0 ± 2,0	1,24 ± 1,96
Monocytes (%)					11,6 ± 2,9	10,9 ± 4,5	11,1 ± 3,5	10,6 ± 4,3	1,13 ± 1,09
Eosinophils (%)					2,0 ± 0	2,0 ± 0	2,0 ± 0	2,0 ± 0	0,02 ± 0,14
Glucose (mg/dL)	45,00 ± 6,28	36,40 ± 2,42	24,40 ± 6,69	23,20 ± 5,93					

* Vieira, et al. (2006).

**Mabilia; Souza (2006)

***Tavares-Dias et al. (2002)

Table 1. Some hematological and blood chemistry results in catfish (*Rhamdia quelen*) exposed to toxic chemicals.

Species	Pollutants	cyt P450	CYP1A	AHH	EROD	cyt b5	P450 RED	References
Channel catfish	PCB Aroclor1254	=		++	++	=	=	Ankley et al., 1986
<i>Ictalurus punctatus</i>	BKME	=			++			Mather-Mihaich; DiGiulio, 1991
	PAH (BNF)				++			Hasspieler et al., 1994
	2,4D+ Picloram							Gallagher and Di Giulio, 1991
	2 Aminocanthracene (AA)				-			Watson et al., 1995
	PAH (BaP)		+	+				Ploch et al., 1998
	PCB126			++		++		Rice and Roszell, 1998
Species	Pollutants	SOD	GPOX	GSSG	CAT	LPOX	GSH	References
Catfish/ <i>Ictalurus nebulosus</i>	OPP (dichlorvos)	=	=		=	+	=	Hai et al., 1995
Channel catfish <i>Ictalurus punctatus</i>	BKME	=	=		+	=	-	Mather-Mihaich and Di Giulio, 1991
	PAH			+	+		+	Di Giulio et al., 1989

Van Der Oost, R et al. (2003)

Symbols: --, strong inhibition (<20% of control); -, inhibition; =, no (significant) response; +, induction; ++, strong induction (>500% of control);

Table 2. Laboratory studies on responses of organic trace pollutants on fish enzymes

4.4. Genetic analyses

Genotoxic substances produce chemical or physical changes in DNA, commonly measured as breaks or DNA adducts, respectively (Nacci et al., 1996). The exposure of an organism to genotoxic substances can induce a cascade of events such as changes in the structure of DNA and the expression of damaged DNA using modified products. Thus, biological consequences may be triggered in cells, organs, body and finally in community and population (Lee; Steinert, 2003; Van Der Oost et al., 2003).

Many biomarkers have been used as tools for the detection of exposure and to evaluate the genotoxic effects of pollution. Among the major genetic biomarkers, we can mention the evaluation of the frequency of chromosomal aberrations, analysis of the frequency of sister chromatid exchanges, formation of DNA adducts, DNA breakage assessed by measuring the comet assay and micronucleus frequency and nuclear morphological abnormalities (Bombail et al., 2001).

According to the studies of Pamplona (2009) by the Comet Assay, widely used to measure DNA damage in aquatic organisms, after the intoxication of silver catfish by dipyrone, the

genetic damages occurred in the lowest concentration of dipyrone (0.5 µg/L). DNA fragmentation may be triggered by the release of free radicals in response to a stressor such as a xenobionte that may damage the nucleus chromatin, or by lipoperoxidation that causes the loss of cellular membrane integrity, subjecting the attack of the nucleic acid by oxygen radicals.

In a study of genotoxicity by copper sulphate in four different doses (5, 30, 50 and 500 mg/Kg) using the catfish, Costa (2011) induced DNA breakage triggered by comet assay. The fish were intoxicated by trophic copper sulphate during 60 days and suffered genetic damage in brain tissue, kidney, liver and blood tissues. Blood was less sensitive tissue to levels of copper used in this study. The piscine micronucleus test developed by Costa (2011), however, showed no significant changes in relation to the negative control, as well as the polychromatic erythrocytes frequency in relation to the total number of erythrocytes. There was an increase in the frequency of viable cells in blood tissue, according to the author.

Piancini (2011) performed the piscine micronucleus test and the comet assay in erythrocytes and gill cells of the silver catfish to evaluate the genotoxic effects of atrazine in concentrations 2, 10 and 100 µg/L for 96 hours. The author observed an increased frequency of nuclear morphological changes at all concentrations, a dose-dependent effect of atrazine on DNA through the comet assay in erythrocytes: damage was significantly higher on 10 and 100 µg/L concentrations compared to control. In gills, there was no difference between treatments. Piancini (2011) observed a dose-dependent effect of atrazine in relation to genotoxicity and its capacity of damaging the DNA of the silver catfish in concentration considered safe by the Brazilian legislation. The same author evaluated the genotoxic effects of copper on *Rhamdia quelen* through the piscine micronucleus test and the comet assay in erythrocytes and gills. The silver catfish were exposed to concentrations of 2.0; 9.0 and 18 µg/L of cuprous chloride for 96 hours. Dose-dependent breaks to the DNA were detected by the comet assays in all treatments, as well as in gills.

Ghisi (2010) did not observe micronuclei in the catfish cells, but did confirm nuclear morphologic changes, after 60 days of intoxication in the sublethal concentrations 0.05; 0.10 and 0.23 µg/L of fipronil. The control group was not significantly different from the group intoxicated by the lower concentration of the insecticide. The higher concentrations were showed significantly higher among of micronuclei compared to the control group, not intoxicated by fipronil, suggesting that the pesticide in higher concentrations (0.10 e 0.23 µg/L) may induce damages to the DNA of *Rhamdia quelen*. The comet assay, also performed by Ghisi (2010) in the same study, showed no significant difference between the intoxicated groups and the control, corroborating the results of histopathological analysis.

The aim of the study performed by Salvagni et al. (2011) was to determine, by micronucleus testing on silver catfish, the risk of genotoxic impact by the use of pesticides such as glyphosate, cyhalothrin, atrazine, simazine and azoxystrobinon in farms located in the Lamedor River watershed in Guatambu, State of Santa Catarina. Blood samples were collected from several species of fish, including *Rhamdia quelen*, captured in 10 different dams existing in agricultural rural properties. Micronucleate erythrocytes were found in blood samples of silver catfish close to the overall average of the other species. Salvagniet at. (2011) believe through *in vivo* piscine micronucleus testing, that water from the Lamedor watershed can

be considered genotoxic, with emphasis on the degree of genotoxicity from pollution in the area, since spontaneous formation of micronuclei in fish is normally very rare. Ferraro (2009) also exposed the silver catfish to different molecules of pesticides and the combinations of them in the aim to determine the bioindicator potential of this teleost species. After the exposure of the fish to the pesticides glyphosate (1.58 e 3.16 mg/L), tebuconazole (0.4 e 0.8 mg/L) and a mixture of them (3.16 e 0.8 mg/L) for 5, 10 and 15 days, were proceeded the micronucleus test and the comet assay in blood samples collected from the fish. DNA damage was confirmed by both techniques in all treatments, pointing the suitability of the species for biomonitoring. In a similar way, Ramsdorf (2011) studied the genotoxic effects of fipronil (0.05; 0.10 and 0.23 $\mu\text{g/L}$), lead nitrate (0.01; 0.03 and 0.10 mg/L) and naphthalene (0.005; 0.06 and 3 mg/L) in the water for *Rhamdia quelen* during 60, 30 and 28 days, respectively. In silver catfish the concentrations 0.10 and 0.23 $\mu\text{g/L}$ of fipronil increased the frequency of micronucleus, nuclear morphological changes, and damages to DNA observed after comet assay. After the intoxication by lead nitrate, it was observed that the concentrations 0.03 e 0.1 mg/L increased the levels of DNA breaks, as well as in all concentration of naphthalene tested for the species.

5. Impact of toxic agents in the reproductive system

The use of fish embryos is gaining popularity for research in the area of toxicology and teratology. Particularly embryos of the zebrafish, catfish and rainbow trout offer an array of different applications ranging from regulatory testing to mechanistic research.

Recently, much attention has been given to possible adverse effects resulting from exposure of aquatic animals to chemicals during the prenatal and perinatal (Tramuja et al., 2006; Montanha; Pimpão, 2012).

The impairment of reproductive function in humans and animal species has been of particular concern in recent years. Many factors can interact with the components and the reproductive function and cause infertility and other functional and structural changes. Illness, stress, hormonal changes and exposure to chemicals, are some factors that contribute to the emergence of reproductive system disorders (Sanchez, 2006).

Fish are one of the most thoroughly studied organisms in terms of effect of substance with estrogenic activity in the development of abnormalities in the reproductive system. According to Sumpter (1998), research on how estrogenic substances affect the sexual system of fish began in the 1980s (Bila; Dezotti, 2003).

The fecundity, the spawning period and type-specific characteristics are essential for the maintenance of any species of fish (Gomiero et al., 2007; Montanha; Pimpão, 2012). Reproduction in fish, as in other vertebrates, is affected by environmental factors, social and nutritional (Parra et al., 2008; Montanha, 2010). Reproductive parameters are more complex indicators of exposure and accumulation of chemicals, hampered for several reasons, the two main effects of pollutants on reproduction caused directly and indirectly and physiological process (Bernardi et al., 2008).

Few data exist regarding the potential sublethal effects of pesticides on reproduction and long-term viability of fish populations (Moore; Waring, 2001).

In 60 years, Rachel Carson published her famous book considered a landmark in the history of environmental pollution, *Silent Spring*, calling attention to the reproductive failure in birds and fish caused by bioaccumulation of persistent organochlorinepesticides such as dieldrin (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane) (DDT). This work brought out the fears of modern society in relation to the introduction of synthetic substances in the environment and renewed public interest in science and government toxicology (SANCHEZ, 2006).

In the mid-70s, researchers found that other chemicals, such as the Kepone and PCBs (polychlorinated biphenyls), also had hormonal effects. Thereafter the toxicological effects of the mixture of individual congeners have been studied mainly in fish, mammalian cells and even humans (Sanchez, 2006).

The structural integrity of the gonads can be altered by xenobiotics (Mayon et al., 2006). Chemical agents that can affect the endocrine system are called endocrine-active chemicals (ECAs), expression adapted to Portuguese as "endocrine disruptors" (Sanchez, 2006).

The Environmental Protection Agency of the United States (USEPA) alternatively defines endocrine disrupters as chemicals that lead to toxic results as various types of cancer and a wide range of adverse effects on the reproductive system (Sanchez, 2006).

The pollution in the aquatic environment can affect the reproductive potential, thus reducing the spread of fish species. This can occur by the possible interaction between the gametes and water pollutants, such as the blockage of the micropyle, which prevents the entry of sperm in the fertilization process (Hilbig et al., 2008). In addition, solutions containing certain levels of pollutants can directly interfere with sperm motility and morphology, and subsequently at fertilization (Hilbig et al., 2008; Witeck et al., 2011).

In teleost fish, such as quelen (catfish), with external fertilization, which occurs when spawning, the gametes are released into the environment for fertilization to occur. At that moment, the gametes are exposed to various contaminants in the water, including heavy metals mercury, zinc, lead, copper and cadmus. These trace elements, at certain levels, affect the motility of spermatozoa and fertilization of oocytes (Witeck et al., 2011).

The gonadotropins stimulate gonadal maturation and release of steroid hormones from the gonads. The steroid hormones and the pituitary determine the development of sexual characteristics and various influencing courtship and parental care (SANCHEZ, 2006).

In teleosts, there are two gonadotropin: R gonadotropin (GTH I) and II gonadotropin (GtH II). In females, the GTH I stimulates gonadal growth, gametogenesis and the entry of vitellogenin in the oocyte. GTH II is important for the final maturation of oocytes and spawning. In males, GTH II acts in the testis, acting on testosterone production (Sanchez, 2006).

Endocrine disrupters are chemicals, natural or synthetic compounds also contained in fungicides, pesticides and insecticides, which have estrogenic and antiandrogenic action. Some of these compounds can maintain their chemical nature for many years contaminating the wa-

ters, being accumulated in fish. Many are potentially harmful to health since they are difficult excretion, may accumulate in the body and cause changes in all systems, especially in the endocrine system (Lara et al., 2011).

Recently, the monitoring of drug residues in the environment has been gaining great interest due to the fact that many of these substances are frequently found in effluents from sewage treatment plants (STPs) and natural waters. Some groups of residual drugs deserve special attention, among them are estrogen, because of its potential to adversely affect the reproductive system of aquatic organisms such as the feminization of male fish found in rivers contaminated with effluent disposal TEE. This was observed in fish species such as *Cyprinus carpio* and *Rutilus rutilus*. Similar effects (induction of hermaphroditism or complete feminization) were also observed when fish species *Oryzias latipes* were exposed to estrogen 17 β -estradiol (Bila; Dezotti, 2003).

Currently, there is growing concern about the presence of pharmaceuticals in aquatic environments and their possible environmental impacts. Ternes et al. (1999) identified the presence of various estrogens in sewage effluent and wastewater treatment plant (WWTP) in Germany, Brazil and Canada (Bila; Dezotti, 2003).

These compounds are characterized by mimicking the structure and function of natural sex steroids leading to endocrine disorders, which can result in abnormal sexual and reproductive function in animals and humans (LARA et al., 2011).

Deltamethrin, a pyrethroid pesticide widely used, is listed by the USEPA as endocrine disruptor, interfering with the reproductive system (Tramuja et al., 2006). Concentrations of 0.01 and 0.1 mg/L of deltamethrin presented toxic to fertilization of eggs of silver catfish (*Rhamdia quelen*). Since the concentrations 0.1, 0.5 and 1.0 mg/L proved to be toxic in the hatching and survival rates of 12 and 24 hours for eggs and larvae of silver catfish (Montanha, 2010). Tramuja et al. (2006) reported histological changes in levels in the gonads of aquatic organisms subjected to different concentrations of deltamethrin.

In recent years, monitoring the impacts of pesticides and other pollutants on sperm production of various kinds of research has been aimed (Mabilia et al., 2008). According to Moore and Waring (2001), even at low levels, cypermethrin (synthetic pyrethroid) in water caused a negative effect on reproductive functions in Atlantic salmon (*Salmo salar*) (Begum, 2005; Mabilia et al., 2008; Montanha, 2010; Montanha; Pimpão, 2012), causing a reduction in fertilization rate (Moore; Waring, 2001). Tramuja et al. (2006) reported a reduction in egg hatching rate of zebrafish (*Danio rerio*) exposed to deltamethrin. Rodrigues (2007) reported hatching in early zebrafish (*Danio rerio*) exposed to dichlorodiphenyltrichloroethane (DDT), with consequent reduction in survival rate. Aydin et al. (2005) and Rodrigues (2007) reported mortality of larvae of carp (*Cyprinus carpio*) exposed to cypermethrin and zebrafish (*Danio rerio*) exposed to DDT, respectively after 12 hours of exposure.

DDT at concentrations of 50 $\mu\text{g/L}$, and cause premature death hatching of larvae of *Danio rerio*, as well as parathion, from which 20 $\mu\text{g/L}$ cause a decrease in the growth rates of fish of the same species (Rodrigues, 2007).

Cypermethrin proved toxic to the catfish during embryonic development in concentrations of 0.001, 0.01 and 0.1 mg/L relative to the fertilization rate and hatching. As regards the survival rate at 12 hours of exposure to cypermethrin after hatching, the concentration 10 mg/L performed toxic to larvae of catfish and 24 hours, concentrations of 0.001 to 10 mg/L (Montanha, 2010).

Some studies have reported the influence of heavy metals in fish breeding, referring to the influences of contaminants on the quality of the gametes on the fertilization of the oocytes and the quality of the larvae (Hilbig et al., 2008).

The water contamination by lead, the moment of fertilization to the first 8 hours of incubation eggs artificially catfish gray (quelen) at a concentration greater than 0.25 mg/L cause a deleterious effect on the percentage of fertilized eggs (Hilbig et al., 2008).

Water contamination fertilization with cadmus at levels up to 28.65 mg/L does not reduce rates of fertilization and hatching. However, concentrations above 20 mg/L cause reduction in sperm motility catfish (quelen) (Witeck et al., 2011).

In the study by Routledge et al. (1998), two fish species, *Oncorhynchus mykiss* and *Rutilus rutilus*, were exposed for 21 days at concentrations of 17 β -estradiol and estrone environmentally relevant. According to these researchers, the results confirmed that estrogens identified in effluents are present in sufficient amounts to cause the synthesis of vitellogenin, a protein that plays an important role in the reproductive system of female oviparous vertebrates, in fish species. Kang et al. (2002) clearly show that exposure to estrogen concentration of 17 β -estradiol environmentally relevant (in the range of 30-500 ng/L), for three weeks, induces high levels of vitellogenin hermaphrodite and the incidence of male fish species *Oryzias latipes* (Bila; Dezotti, 2003).

According to studies by Panter et al. (1998), low concentrations of 17 β -estradiol and estrone caused effects in male fish species *Pimephales promelas*, as vitellogenin synthesis and testicular inhibition (Bila; Dezotti, 2003).

Exposure to pesticides and other toxic substances during the prenatal and perinatal, can alter the reproductive system of fish without compromising growth and viability of offspring, but cause functional changes that become apparent later in adulthood (Tramuja et al., 2006; Montanha; Pimpão2012). Many xenobiotics are known to affect reproduction in many organisms (Mayonet al., 2006; Montanha, 2010).

6. The fish and the fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research

Biological and ecological responses to contaminant stressors may range from changes at the molecular level, where genetic integrity and subcellular processes are evaluated, to population and community levels where dynamics and structure of entire foodwebs can be affect-

ed. Indicators of stress at several levels of biological organization have been used to evaluate effects of contaminants at the organism level.

A variety of *in vitro* and *in vivo* assays with fish are being used as model systems for toxicological, biochemical and developmental studies (Powers, 1989). Fish species such as rainbow trout (*Oncorhynchus mykiss*), carpas, catfish and Japanese medaka (*Oryzias latipes*) have been used extensively as test organisms for studies of carcinogenesis. A variety of teleost species have been used to study the development of early life stages of fish and the teratogenic effects of environmental contaminants (Wisk; Cooper, 1990). Fish respond in a manner similar to mammalian test species to chemicals that induce peroxisome proliferation in hepatocytes (Yang et al., 1990), and oxidative damage in hepatocytes (Washburn; Di Giulio, 1989). Hepatic cytochrome P-450 monooxygenases in fish also metabolize many carcinogens in a manner analogous to mammalian organisms (Stegeman; Lech, 1991). The advantages of using fish as model organisms include the ease with which teleosts, especially small aquarium species, can be held in the laboratory and exposed to toxic chemicals. Since fish often respond to toxicants in a manner similar to higher vertebrates, they can be used to screen for chemicals that have the potential to cause teratogenic and carcinogenic effects in humans. However, the main application for model systems with fish is to determine the distribution and toxic effects of chemical contaminants in the aquatic environment. Industrial and urban discharges are responsible for high concentrations of toxic substances in the aquatic environment, although in many countries, regulatory activity is beginning to limit point-source discharges of toxic chemicals to our water resources.

Animal alternatives research has historically focused on human safety assessments and has only recently been extended to environmental testing. This is particularly for those assays that involve the use of fish. A number of alternatives are being pursued by the scientific community including the fish embryo toxicity (FET) test, a proposed replacement alternative to the acute fish test. Discussion of the FET methodology and its application in environmental assessments on a global level was needed. With this emerging issue in mind, the ILSI Health and Environmental Sciences Institute (HESI) and the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) held an International Workshop on the Application of the Fish Embryo Test as an Animal Alternative Method in Hazard and Risk Assessment and Scientific Research in March, 2008.

7. Conclusion

Environmental pollution is of current environmental concern. It is therefore necessary to find solutions to determine environmental contamination, because the organisms are continuously exposed to a variety of anthropogenic toxicants daily released to the environmental.

The silver catfish have been used for bioindicator of environmental contamination for many researches and can be used to aquatic biological systems. Moreover, a considerable expansion of knowledge in using various model species which are easily cultured and have lesion induction times of relatively short duration, such silver catfish.

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Functional and Structural Differences in Atria Versus Ventricles in Teleost Hearts

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Additional information is available at the end of the chapter

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1. Introduction

Across vertebrates, the fish heart is structurally relatively simple. The heart of teleosts is unique in structure, composed of four chambers in series: venous sinus, atrium, ventricle and bulbus arteriosus. The two chambers acting as pumps are the atrium and ventricle, a simplified version of that seen in tetrapods. These chambers develop from a simple linear tube [1] and differ not only morphologically but also physiologically with different characteristic rates of contractility [2]. Fish heart design is reflective of the needs for oxygen delivery to working skeletal muscle in an often oxygen poor environment. The heart is the first definitive organ to develop and become functional, as embryological survival depends on its proper function. The vertebrate-specific development of multiple chambers with enough muscle to generate higher systemic pressures allowed for perfusion of larger and more complex tissues.

Fish show great variability in the development of the heart and modification of heart performance capacities to meet tissue perfusion demands imposed by differences in life history. Research has tended to focus on inotropic or chronotropic responses of the heart to stress such as temperature in larger fish such as trout, or on development through the use of zebrafish mutants. Between these sources much has been revealed about the contractile properties of the two chambers. This research will be reviewed in the context of roles of the atrium and ventricle in fish in achieving variability in myocardial contractility.

2. Gene expression

In mammals, 23% of cardiac-specific genes show differences in expression between the atria and the ventricle [3]. Key morphogenetic events during heart ontogenesis are conserved

across vertebrates [4]. Gene expression programs specific to each chamber are likely regulating key aspects of chamber formation. The focus on fish heart developmental patterns has primarily been due to the use of zebrafish as a model organism of vertebrate development. The morphological differences created during chamber development are guided by changes in gene expression patterns [1], a process well conserved in vertebrates, regardless of whether there are one or two atria or ventricles. Genetic screens in zebrafish have revealed genes and pathways underlying development in the heart. Since many stages of development are conserved across vertebrates, this information has generated considerable insight into congenital heart defects in humans. In the context of this chapter, timing and localization of gene expression can also contribute to our understanding of the differentiation of chambers both in morphology as well as through variation in contractility.

The T-box genes (Tbx) are responsible, in large part, for patterning that distinguishes chamber myocardium from non-chamber myocardium [5]. Subregionalization is important for atrial or ventricular identity, either via selective transcription or repression of genes. The physical interaction of Nkx2.5, Gata4 and Tbx5 in particular activates the expression of chamber-specific genes to stimulate the differentiation of primary myocardial cells into chamber myocardium [1]. By the time cells are restricted to cardiac lineage, they are already destined for either the atrium or the ventricle. During early gastrulation, myocardial progenitors already have axial coordinates that lead to chamber assignment [6]. There are two sources of these myocardial progenitors, or two heart fields. In the first heart field, cardiomyocyte differentiation initiates in the ventricle and allows continuous addition of new cardiomyocytes to the venous pole of the heart tube progressing towards the atrium [7]. Differentiation at the arterial pole in the second heart field occurs only in the later stages of cardiac development. By 72 hours post-fertilization (hpf) individual heart chambers are morphologically differentiated with the ventricular wall being thicker and ventricular cardiomyocytes being larger than those of the atrium [8].

The chambers express different subsets (or paralogs) of sarcomeric proteins such as myosin heavy chains (MHC) and myosin light chains (MLC) [9]. Variation in paralog expression (frequently referred to in the literature as isoforms) may confer differing contractile properties to the atrium and the ventricle, such as with altered MHC expression being correlated with altered myofibrillar ATPase activity (crucian carp - [10]). MHC expression is chamber-specific by 22 hpf, preceding any obvious chamber formation but corresponding to the initiation of the heart beat [6]. These isoforms are not only divided spatially but also by differences in timing of expression. Atrial-specific myosin gene expression is induced after ventricle-specific myosin gene expression in zebrafish [11]. This time-dependent expression may be due to repression of the promoters of these genes [1].

The timing of these developmental pathways is critical, as the development of one chamber can influence the other. Ventricular morphology can change with developmental atrial dysfunction seen with zebrafish mutations in atrial-specific MHC [11]. For example, in the *wea* mutant, a lack of a functional atrial MHC results in defects in atrial myofilament organization and contractility. Despite the mutation being seen only in a chamber-specific protein, the ventricle changes as well in response to these atrial defects. Effective embryonic circula-

tion requires appropriate relative sizes of the atrium and ventricle. Bone morphogenetic protein (BMP) signaling has been shown to be involved with the regulation of chamber size in zebrafish [12]. However, mutations affecting BMP signaling only reduce atrium size but not the ventricle. During this stage of development, the size of chambers may be relatively independent of one another.

3. Morphology

In fish the heart is arranged in series with the ventricle primarily filled by contraction of the atrium [13], rather than the mammalian system in which the thin-walled atrium contributes only a small amount to ventricular filling under resting conditions. While the atrium and ventricle compose the main muscular components of the fish heart, there are other chambers with important roles in guiding appropriate blood flow through the heart. Generally in fish blood flows through the sinus venosus to the atrium to the ventricle and then out to the bulbus arteriosus. More recently, the atrioventricular region and conus arteriosus have been identified as morphologically discrete segments present throughout modern teleosts as well [14, 15].

3.1 General morphology

As with most vertebrates, the cardiac tube is S-shaped with a dorsally positioned atrium and ventrally positioned ventricle to allow for appropriate momentum of the bloodstream towards the arterial pole. The morphology of the heart sets up an alternating arrangement of slow conducting segments (e.g. inflow tract, atrioventricular region and outflow tract) with fast conducting contractile segments (atrium and ventricle). Blood first enters the thin-walled sinus venosus. This is an independent, but non-muscular chamber in fish that acts as a drainage pool for the venous system. The composition of the sinus venosus varies greatly between species, from primarily connective tissue in *Danio rerio* (zebrafish) to mainly myocardium in *Anguilla anguilla* (European eel) to even smooth muscle cells in *Cyprinus carpio* (carp) [16, 17, 18]. The presence of the sinus venosus has thus been suspected to have allowed the development of the atrium as the principal driving force for ventricular filling [19]. Blood then moves into the atrium, an active contractile chamber in fish that again shows considerable variability in size and shape between species [18]. A general trend throughout teleost species is the similarity in volume of the two main muscular chambers is indicative of the importance of the role of the atrium in filling the ventricle. While atrial volume is closer in size to that of the ventricle in fish, the atrium is still less developed muscularly and possibly representative of a more primitive heart [20-22].

Separating the atrium and the ventricle is the atrioventricular region, a ring of cardiac tissue supporting the AV valves. The degree of isolation of this region from the surrounding musculature varies between species [23] but in all species it plays a critical role in regulating the pattern of electrical conduction. The ventricle has a mass and wall thickness up to five times that of the atrium [18]. The thickly muscular ventricle varies in terms of structural organiza-

tion, histology, coronary distribution and relative mass between species as well. This variation reflects the fact that different modes of heart performance are attributed to different ventricle types since this chamber is responsible for the active cardiac output [18]. This classification will be discussed further in this section. From the ventricle, blood moves into the outflow tract leading into the beginning of the dorsal aorta. In more basal fish there are two segments: the proximal muscular conus arteriosus and the distal arterial-like bulbus arteriosus [24]. While later teleosts were thought to have lost the conus arteriosus, more recent evidence shows it is a distinct segment in many families [14, 15]. The bulbus arteriosus is more prominent and serves as the main component of the outflow tract. This elastic chamber expands to store large portions of the stroke volume, and gradually releases a steady non-damaging flow to gill vascular [18]. The role of these segments is variable between species and has been reviewed in more detail [25].

The AV-region, bulbus arteriosus and conus arteriosus are unique to fish though homologous regions may be seen in early embryological stages of higher vertebrates. While, the atrium and ventricle are present across vertebrates, their diversity in morphology and function and how they contribute to contractility will be the focus of the remainder of this Chapter.

3.2. Myocardial arrangement

The teleost myocardium is composed of two distinct layers: compact and spongy. The atrium tends to be primarily spongy, with more open space between fibers and greater variability in orientation [26]. The external rim is myocardium surrounding a complex network of pectinate muscles. Collagen provides support, being predominant both in surrounding the myocardium and the internal trabecular network [15]. Meanwhile, while the ventricle composition varies greatly between species, the periphery of the ventricle tends to be primarily compact tissue with the luminal being primarily spongy [15, 27]. The compact layer is composed of typically well-organized fibers composing well-arranged bundles covering the inner ventricle. The spongy layer is composed of long finger-like branching cells in close proximity to luminal fluids. Not all fish species have distinct layers in the ventricle to the same degree as the salmonids and scombroids in which most of the experiments characterizing the morphology of the chambers has been done. For example, the relatively sedentary flatfish has a heart with virtually no lumen and is composed almost entirely of spongy tissue [28]. These phenotypes have been subdivided into four subclasses (Types I-IV). A Type I ventricle consists of an entirely spongy ventricle, while Type II has both compact and spongy myocardium but with coronary vessels restricted to the compact layer. Type III and Type IV have vascularized trabeculae, with coronary vessels penetrating spongy and compact myocardium. Types III and IV are distinguished from one another by Type III having less than 30% of the heart mass composed of compact myocardium (exemplified by many elasmobranchs) and Type IV representing ventricles with greater amounts of compact myocardium [29].

3.3. Cardiomyocytes

On the cellular level, cardiomyocytes are more cuboidal in the ventricle compared to the more squamous shape seen in the atrium [30]. Ventricular myocytes from ectothermic vertebrates more closely resemble myocytes from neonatal mammals rather than adults in terms of structure and function. They are long ($>100\ \mu\text{m}$), thin ($<10\ \mu\text{m}$) and generally lacking in t-tubules (trout - [18]; trout - [31]; neonatal rabbit - [32]). These myocytes appear in electron microscopy images to have less extensive sarcoplasmic reticulum (SR) than mammals [33] but some peripheral couplings with sarcolemmal membrane are observed [34]. Atrial muscle cells contain well-developed sarcomeric reticulum, peripheral couplings but no t-tubules [35]. The narrow extended shape of teleost cardiomyocytes from both chambers decreases the diffusion distance from the sarcolemma to the center of the cell [36-38]. This high surface-to-volume ratio of the cells may be related to increased efficacy of E-C coupling. These morphological distinctions contribute to the chamber-specific contractile properties which will be discussed further in this Chapter.

3.4. Lifestyle demands

These morphological differences between species are reflective of a diversity of lifestyle demands. Active fish have a larger relative heart mass than that of less active species; the relative heart mass in the skipjack tuna is 10 times larger than in the sluggish flounder [39]. However, a similar atrium to ventricle mass ratio exists in species with very different lifestyles (0.2 in active Atlantic salmon, Atlantic cod and inactive winter flounder - [28]). More active fish also show a difference in whole heart shape with active fish (e.g. salmonid and scombrid families) exhibiting pyramid-shaped hearts whereas inactive fish hearts are more rounded and sac-like in shape [40]. The pyramidal shape normally correlates with a thicker myocardial wall and high cardiac output. This allows for high levels of stroke work and the maintenance of relatively high blood pressures [18].

The amount of compact myocardium is generally increased in fish with greater cardiac output demands. With pyramidal ventricles, the myocardial wall is normally formed primarily by the compact layer, which is complex and thicker in these higher performance fish [18] although this phenotype is also found in the relatively sedentary Antarctic teleosts [26]. This suggests that shape and degree of compact myocardium do not always correlate with lifestyle and in fact the shape of the chamber does not always correlate with the inner architecture [41]. This active phenotype may also display plasticity, as trout that are rendered inactive will display an abnormal rounded shape [42]. This may also be the case in aquaculture conditions, where fish become less active and may be overfed [43]. The proportion of compact myocardium has also been shown to vary with seasons [18] and growth [44].

Active fish rely on coronary circulation to allow for increased cardiac output. More compact tissue in the ventricle generally requires greater O_2 perfusion. In general, sluggish species lack this coronary circulation and can rely completely on O_2 contained in the venous circulation [45]. Hence thicker ventricles seen in more active fish have a more developed coronary distribution to the compact myocardium, and a greater percentage of compact myocardium. Very active fish like scombroids and elasmobranchs also have a coronary supply to the

spongy myocardium and atrium [46]. However, this increased demand for O₂ supply for cardiac function means that these species are at a disadvantage in hypoxic water. The more primitive hagfish heart composed almost entirely of spongy myocardium can meet cardiac ATP requirements for resting conditions even in anoxic water [47].

4. Filling patterns

Atrial filling occupies a substantial proportion (up to 75%) of the cardiac cycle and as discussed above, the atrial chamber volume in fish may be comparable to that of the ventricle. Substantial debate exists in the literature as to the mechanism of both atrial and ventricular filling. Though noninvasive echocardiography has greatly contributed to research on flow patterns, it does require anaesthetization and restraint of the fish. Because the heart is acutely sensitive to venous pressure, and venous pressure is acutely sensitive to gravitational forces and extramural pressure, considerable variation may be seen from study to study. Certain studies have shown a more monophasic filling of both atrium and ventricle via a vis-a-tergo mechanism (e.g. trout - [48]). In this model, ventricular pressure is derived from arterial flow and venous capacitance is the primary determinant of cardiac output [13]. Other studies have demonstrated a more biphasic filling pattern via a vis-a-front mechanism in which cardiac suction plays a prominent role in ventricular pressure (e.g. elasmobranchs - [49]). Even triphasic patterns have been observed, with a third wave of filling possibly involving sinus contraction (e.g. leopard shark - [49]).

These filling patterns of the chambers may be both a reflection of phylogeny and external conditions. Less active fish such as the sea raven require above-ambient filling pressures that may require vis-a-tergo filling [50]. The venous pressure control mechanisms are not seen to be as developed in elasmobranchs as in teleosts resulting in a greater necessity of vis-a-front filling [51]. Meanwhile with increased stroke volumes during exercise resulting in more positive filling pressures, teleosts may further shift to a vis-a-tergo cardiac filling mode for increased activity [52]. However, the relative proportion of early to late phase filling (or passive vs. atrial active) suggests that the late phase is greater than the early phase in biphasic [49] and the third phase in triphasic does not change this pattern. With late phase (active atrial contraction) playing such an important role regardless of filling mode, the trend still holds that fish hearts are still more dependent on atrial contraction for ventricular filling than mammals.

5. Physiological differences between chambers

It has been proposed that a key regulator of cardiac performance is the end diastolic volume (EDV) in both mammals and fish. Many of the morphological differences between chambers may have evolved in order to meet the high physiological demands of variation in metabolic rate and increased tolerance to a wide range of environmental conditions. Several of these will be highlighted in this section.

5.1. Force generation

Cardiac output may be modulated through variations in stroke volume and heart rate, but the relative contributions vary substantially among fish species. In general fish respond to greater hemodynamic loads by increasing cardiac output mainly through increased stroke volume rather than heart rate. Stroke volume can vary up to 2-3 fold with exercise (salmonids - [53]) but not all species exhibit the same ability [54-55]. End systolic volume (ESV) of the fish heart relative to stroke volume is very small [56-57] unlike that of the mammalian heart. However, end diastolic volume in fish is greatly determined by atrial contraction rather than central venous pressure seen in mammals [13]. The modulation of atrial contractility and Frank-Starling mechanisms are thus particularly important in determining ventricle filling.

The whole heart displays a greater sensitivity to filling pressure than in mammals, in part attributed to greater myocardial extensibility coupled with an increase in myofilament calcium sensitivity over a large range of sarcomere lengths [58]. The Starling principle dictates that the strength of contraction is, in part, a function of the length of the muscle fiber (and hence sarcomere length) prior to contraction (i.e. preload). Stroke volume increases as a function of end diastolic volume since muscle fibers and sarcomeres within the fibers are stretched towards their optimum. Between various species of fish there is considerable variation in length-dependent sensitivity. For example, flounder heart is very sensitive to filling pressures, needing smaller increases in preload to achieve higher values of stroke volume. This more pronounced Starling curve is further impacted by the high distensibility of its chambers based on pressure-volume curves [28]. This length-dependent strength of contraction is also directly affected by temperature in certain teleost species (e.g. trout- [58]). With fish cardiac myocytes showing enhanced length-dependent activation, and calcium sensitivity increasing with sarcomere length [59], calcium handling is an important consideration in the basis of the length dependence of the strength of cardiac contraction in fish. The unique properties of E-C coupling in the chambers of the fish heart will be discussed in greater detail further in this Chapter. The reduced relative duration of diastole seen with active atrial contraction in fish may have evolved in response to the relatively slow rate of the excitation-contraction coupling processes.

The importance of strong atrial contraction is reflected in the fact that atrial muscle generates greater maximal force (F_{max}) than ventricular muscle in a variety of teleost species (e.g. rainbow trout- [60]; yellowfin tuna - [61]; brown trout - [62]). Although systolic blood pressure measurements show the atrium to be a weaker pump, the higher force generated in excised strips suggests the relative weakness of the atrium may simply be reflective of the anatomical difference in wall thickness between the chambers. The greater F_{max} of the atrial cardiomyocytes individually does not account for the difference in overall force generation by the greater quantity of cardiomyocytes in the thicker ventricle wall.

Cardiac performance is directly related to the contractile properties of cardiomyocytes. As mentioned previously, ventricular and atrial muscles express different paralogs (or isoforms) of myosin heavy chain (MHC) [63]. The maximum rate of force produced by muscle fibers is determined to a large degree by MHC paralog content. Alterations in paralog composition therefore are associated with changes in myofibrillar function as well as myosin

ATPase activity [64]. Because of this, the myosin ATPase reaction is higher in atrial than in ventricular cardiomyocytes [65]. Across vertebrates, from the primitive dogfish right up to mammals, the atrium has a higher concentration of MHC protein content than the ventricle [4]. The increased levels of MHC may be contributing to the increased F_{\max} of individual atrial cardiomyocytes relative to ventricular. However, MHC expression pattern differences between chambers may also be species-specific. In goldfish, both chambers have electrophoretically similar MHC while in other fish species such as pike, ventricular myocardium exhibited electrophoretically faster MHC [65]. Linking chamber-specific isoform usage with functional differences across a broader phylogenetic range as well as temperatures may help to clarify the importance of this heterogeneity.

5.2. Action potential

As in other vertebrates, the teleost action potential can be divided into a rapid upstroke of the action potential (phase 0), a rapid initial repolarization of the AP (phase 1) followed by a plateau phase (phase 2). The plateau phase is followed by repolarization of the action potential (phase 3) returning it to the resting membrane potential (phase 4). Teleost atrial and ventricular myocytes also have distinct AP morphologies with the atrial action potential having a shorter plateau phase and a slower repolarization of the AP. Among the ionic currents contributing to the differences in fish atrial and ventricular AP morphologies, ventricular myocytes have prominent inwardly rectifying potassium current I_{K1} and a small I_{Kr} while the opposite is true for atrial myocytes [66-67]. Other currents expected to affect the AP plateau such as the L-type calcium current (I_{Ca}) or the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) current (I_{NCX}) have not been compared systematically in the teleost heart.

Action potentials have been recorded in a number of teleost species (carp - [68]; trout - [34]; zebrafish - [69]) with a considerable variability in AP morphology with the atrial AP duration ranging from 197 ms in carp atrium at 27 °C to 281 ms in trout atrium at 15 °C, 601 ms in carp at 11 °C and 1284 ms at 4 °C and ventricular AP duration ranging from 407 ms at 10 degrees to 143 ms at 20 °C in trout ventricle [71-72] and from 1042 ms at 11 °C to 737 ms at 18 °C in Burbot ventricle [70,72], likely reflecting species-dependent differences as well as variations in physiological-experimental conditions such as temperature or salinity. Although these variations in AP-duration are larger than 10-fold, they allow the teleost heart to adapt to a wide temperature range [18] while the mammalian heart becomes refractory when temperature is lowered. The greater adaptability of the teleost heart to temperature is also reflected in the properties of ionic channels and ion transporters the determine excitability and the action potential shape. For example the change in trout NCX activity is only weakly modified by temperature changes ($Q_{10} = 1.2 - 1.5$) as compared to mammals ($Q_{10} = 2.5$) [73-74] and calcium uptake and leak from the sarcoplasmic reticulum is slowed in parallel, resulting in preservation of calcium release from the SR in trout [75-76]

In recent years, action potentials have also been recorded in myocytes isolated from teleost atrial and ventricular myocytes with several reports showing that the resting membrane potential in atrial myocytes is significantly more depolarized than in ventricular myocytes [66,

77]. It was proposed that this could be due to the low I_{K1} density in trout atrial myocytes [66]. However, membrane potentials recorded in atrial tissue preparations [71] are comparable to values recorded in trout ventricular preparations [70, 78] under comparable experimental conditions. Studies have showed that the resting membrane potential recorded with current-clamp technique in trout atrial myocytes was highly sensitive to the holding current applied [70]. Moreover, recordings of potassium conductance in these myocytes suggested that the loss of the resting membrane potential in isolated trout atrial myocytes is likely due to the lack of activation of the acetyl choline-activated potassium current (I_{KACh}).

5.3. Excitation-contraction coupling

Calcium handling is an important consideration as the primary basis for regulation of cardiac contraction in all species, including fish, since it is determined by delivery and removal of calcium to and from cardiac troponin C (cTnC), the thin filament protein which senses changes in cytosolic Ca^{2+} concentrations and initiates the contractile process. Therefore, the amplitude and kinetics of the cytosolic calcium transient will modulate the kinetics and force of contraction, and this in turn will set the limit for the contraction frequency. Mammalian cardiac muscle has intracellular calcium stores in the SR in close proximity to T-tubules that provide the majority of the calcium that binds to cTnC. In ectotherms, the activation of calcium release from the SR and its contribution to contractile activation is controversial with some reports showing that the majority of calcium is derived from extracellular space [79, 36, 34, 58] while others report a considerable contribution of the SR both to the activation of contraction [38, 80] and to calcium removal from the cytosol during relaxation [58, 80-81]. Interestingly, calcium handling by the SR has also been reported to differ between fish atrial and ventricular myocytes and these differences as well as species dependent differences are likely to account for part of this controversy on the role of the SR in the teleost heart. Additionally, a significant number of reports examining the role of the SR in the teleost heart relied heavily on pharmacological inhibition of the SR calcium release channel (also termed ryanodine receptor or RyR2) in multicellular tissue preparations. As discussed below, this approach may not be optimal to evaluate the contribution of the SR to the activation of contraction if other calcium delivering mechanisms are capable of compensating for inhibition of the RyR2. Instead, quantification of the contribution of each mechanism involved in the delivery and removal of calcium to and from cTnC is necessary to estimate its capacity and contribution.

5.4. Activation of contraction

In mammalian cardiomyocytes the key contributors to the activation of contraction are the L-type calcium channel that mediates calcium entry across the sarcolemma and the SR, which releases the majority of the calcium from the SR. During the upstroke of the action potential, reverse mode NCX may also contribute [82]. Since the initial reports on I_{Ca} measurements in fish cardiomyocytes [73, 83-84] there is now substantial information on I_{Ca} density in atrial and ventricular myocytes from a number of fish species under different experimental conditions [77, 85]. Again, differences in experimental conditions and species

are abundant and there are substantial differences in the measurements of calcium entry through I_{Ca} among species at different temperatures; Burbot : I_{Ca} , 0.81 ± 0.13 pA pF⁻¹ at 4°C, and 1.35 ± 0.18 pA pF⁻¹ at 11°C [55]; trout: 1, 2 and 4 pA pF⁻¹ at 7, 14 and 21 °C, respectively [77]. Among the experimental conditions that have may have the largest impact on I_{Ca} measurements, ruptured-patch technique with a high (5 mM) EGTA-buffered patch pipette solution has been shown to result in overestimation of I_{Ca} when compared to measurements performed with low EGTA (25 μM) or perforated patch-clamp technique [75]. Therefore, estimations of the contribution of the calcium carried by I_{Ca} to the activation of contraction also varies from as little as 25-30% in trout atrial myocytes using perforated patch technique [75,86], to more than 50% in zebrafish ventricular myocytes subjected to perforated patch-clamp technique [87] and close to 100% using ruptured patch-clamp technique with EGTA buffered patch pipettes in carp ventricular myocytes [37] and in trout atrial myocytes subjected to β-adrenergic stimulation [87]. The latter does not, however, necessarily imply that I_{Ca} is the sole source of calcium delivery in these preparations but merely indicates that it is sufficiently large to activate contraction if no other calcium sources are available. As mentioned above, chamber specific differences in the I_{Ca} density have not been systematically investigated in the teleost heart.

Theoretically, reverse mode NCX could also contribute to the activation of contraction at depolarized membrane potentials and/or when sodium channels produce a large local increase in the subsarcolemmal Na concentration. Several studies have shown that fish cardiomyocytes have a high NCX activity than mammalian cardiomyocytes, and the direct contribution of the NCX to the activation of contraction has been estimated to range from 25-45% under physiological conditions [86-87, 90]. However, the relative contribution of I_{NCX} to total sarcolemmal calcium entry depends critically on the membrane potential and the intracellular Na⁺ concentration [74, 85-86]. Moreover, there is evidence that reverse mode NCX can trigger calcium release from the SR in trout atrial myocytes [90].

Finally, calcium release from the SR has also been reported to contribute to the activation of contraction in fish cardiac myocytes with a contribution that varies between 0 and more than 50% depending on species, tissue and experimental temperature. Some of the first publications to examine the effects of ryanodine, a compound that modulates the opening of the SR calcium release channel, on contraction in multicellular tissue preparations were [91-92] but the reported effects on contraction were only marginal at physiological temperatures and stimulation frequencies. Ryanodine was, however, reported to inhibit post rest potentiation, a phenomenon caused by calcium release from the SR [78], and in 1992 it was reported to have a strong effect on contraction in trout [93] and skipjack tuna [94] at 25 °C, suggesting a temperature- and/or species-dependent component of the effect of ryanodine. In 1998, it was shown that steady-state and maximal SR calcium content in trout ventricular myocytes was about 10 times higher than values reported in human cardiomyocytes and that uptake rates were sufficiently fast for the SR to participate in the regulation of calcium handling on a beat-to-beat basis [75] and in 1999 it was shown that brief membrane depolarizations were capable of eliciting calcium release from the SR in trout atrial myocytes corresponding to a contribution of up to 50% of the total calcium required for the activation of contraction [80].

Subsequently it was shown that this contribution of the SR to the activation of contraction was equally important at 21 and 7°C in trout atrial myocytes [95]. These findings were confirmed in trout using simultaneous recordings of membrane currents and calcium transients [89] and with confocal calcium imaging, which also demonstrated that local spontaneous calcium release from the SR (known as calcium sparks in mammalian cardiomyocytes) are also present in trout atrial and ventricular myocytes as well as in zebrafish ventricular myocytes [96]. This study also showed that the spark frequency in trout atrial myocytes was comparable to that recorded in human atrial myocytes under similar experimental conditions [97], but twice as fast as in trout ventricular myocytes. In line with this, the frequency of spontaneous membrane depolarizations, induced by SR calcium release, were also about 6-fold more frequent in trout atrial than ventricular myocytes, suggesting that SR calcium is more labile in atrial than ventricular myocytes. A higher contribution of the SR Ca^{2+} release has also been reported for the tuna heart [38, 67].

Experiments attempting to identify the triggers of SR calcium release showed that both I_{Ca} and reverse mode NCX are capable of triggering calcium release from the SR [90] and, unexpectedly, this study showed that the gain of I_{Ca} and I_{NCX} -induced calcium release was similar in trout atrial myocytes. Moreover, it was shown that the relative contribution of the two mechanisms depended strongly on the membrane potential and the intracellular Na^+ -concentration. Considering that an intracellular $[\text{Na}^+]_i$ between 13 and 14 mM has been reported in trout cardiomyocytes [98] reverse mode NCX could be expected to contribute significantly to the triggering of calcium release from the SR in trout. While in mammals $[\text{Na}^+]_i$ is higher in atrial than ventricular cells [99], no difference has been seen between chambers in rainbow trout [98]. However, with loss of function of NCX in mutant zebrafish (tre mutant) more cardiac fibrillation is seen in the atrium than the ventricle [100]. This suggests that the atrium is more susceptible to spontaneous Ca^{2+} release from the SR as a result of disruptions of normal calcium transients.

5.5. Relaxation of contraction

Under steady-state conditions, the calcium delivered to the myofilaments during the activation of contraction has to be removed from the cytosol during relaxation. Moreover, if equilibrium is to be maintained, the calcium entering across the sarcolemma must be extruded from the cell and calcium released from intracellular organelles must be re-sequestered by the same organelle. Based on the estimates of sarcolemmal calcium entry in the fish heart, sarcolemmal calcium extrusion would therefore be expected to vary between 50 and 100% in fish cardiomyocytes depending on the species and tissue. In the mammalian heart, forward mode NCX generally accounts for the vast majority of the sarcolemmal calcium extrusion [101] and experiments using rapid caffeine application in $0\text{Na}/0\text{Ca}$ -solution showed that this is also true for trout ventricular myocytes [84]. Assuming that the tail current elicited upon repolarization of the membrane potential is primarily carried by forward mode NCX, the time integral of this current can be used to estimate calcium extrusion by the NCX [75]. With this approach calcium extrusion by the NCX was estimated to account for about 55% of the total increase in calcium during activation of contraction suggesting that intracellular recy-

cling of calcium during contraction must account for about 45% of the total calcium transient [86]. On the other hand, measurements of the NCX activity showed a forward NCX-rate under physiological conditions of $36 \text{ amol pF}^{-1} \text{ s}^{-1}$, which is sufficient to remove the total calcium required for the activation of contraction in a few hundred ms [76].

In agreement with the notion that calcium cycling by the SR takes place on a beat-to-beat basis and could contribute to the activation of contraction, measurements of SR calcium load with rapid caffeine application and specific protocols used to measure steady-state and maximal SR calcium loading, showed that the SR calcium content in trout ventricular and atrial myocytes can be up to 10 times higher than in mammalian myocytes [58,61,77,84], and that the rate of SR calcium uptake under physiological conditions is also sufficient to remove the total calcium required for the activation of contraction between beats at physiological beating rates and temperatures in trout atrial and ventricular myocytes [75,95]. In the presence of β -adrenergic stimulation, the relationship between SR calcium uptake and the cytosolic calcium level was shifted towards lower calcium concentrations, but when normalized to the total calcium transport SR calcium uptake was only slightly increased from 35 to 41% by β -adrenergic stimulation [102].

It is interesting to note that the SR calcium load in fish cardiomyocytes is more than 10-fold higher than the amount of calcium released on each beat [84, 95, 103]. The reason for such an excessive SR calcium load compared to the calcium requirements on a beat-to-beat basis remains elusive, but it definitely provides fish species with an intracellular calcium reservoir that allows the heart to function under adverse conditions where SR calcium refilling is impaired for extended periods of time. This also explains why the inhibition of SR calcium uptake (with cyclopazonic acid) or increasing SR calcium leak (with ryanodine) may have a much slower effect on contraction in fish than in mammalian hearts.

Atrial cardiomyocytes have both greater SR Ca^{2+} loads [105-106] and make greater use of SR Ca^{2+} than ventricular cardiomyocytes [60, 105]. The degree of atrio-ventricular difference in SR Ca^{2+} load and usage is also species specific, being larger in rainbow trout than crucian carp or burbot [105].

The reliance on intracellular Ca^{2+} leads to more rapid contraction kinetics of the fish atrium due to faster calcium release and reuptake rates via the SR relative to ventricular tissue in a variety of teleost species (e.g. rainbow trout - [60]; burbot - [105]). Lowering of cytosolic Ca^{2+} concentration in this manner is via sarco-endoplasmic reticulum Ca^{2+} -AT-Pase (SERCA), and thus the higher SERCA protein expression found in atrial contributes to this shorter duration of isometric contraction relative to ventricular myocardium [107]. In addition, the strength of contraction in teleost atrial muscle has greater ryanodine sensitivity than that in ventricles [60, 62] and is similar in magnitude to what is typically seen in many mammals [82].

Interestingly, the maximal capacity and steady-state SR Ca^{2+} load does not necessarily correlate with SERCA activity or ryanodine sensitivity in all ectotherms. Inverse relationships between ryanodine sensitivity and SR Ca^{2+} load have been found in rainbow trout, burbot and crucian carp [105]. While atrial contraction is more strongly inhibited by ryanodine

than ventricular contraction [60], the increased activity of SR Ca^{2+} ATPase may indicate that atria are able to load the SR with Ca^{2+} in the time between contractions. These factors may contribute to a faster recovery of myocardial contractility or increased mechanical restitution rates in atrial muscle. This may mean increased importance of SR Ca^{2+} for contraction during periods of environmental stress. More studies are necessary to clarify the role of SR Ca^{2+} handling in fish cardiomyocytes, especially with the species-specific and chamber-specific variations.

Differences in chamber-specific expression of the contractile element responsible for calcium sensitivity may also guide differences between contractility of the atrium and ventricle. Myofilament Ca^{2+} sensitivity is a critical factor in regulating cardiac contractility. The cardiac TnT subunit of the zebrafish troponin complex has been shown to be required for the contraction of both the atrium and the ventricle [106] as it is in mammals. However, zebrafish possess two copies of the gene for cardiac troponin C, with one demonstrated to be predominantly expressed in the heart of the zebrafish embryo. Loss of function of this gene results in abnormal atrial and ventricular chamber morphology, but the second cTnC gene enables function to be regained in the atrium [107]. This suggests a possible difference in expression of cTnC between chambers that could potentially lead to variation in calcium sensitivity. Gene duplication has led to multiple copies of sarcomeric proteins, and variation between these fish-specific paralogs may be a method of coping with varying environmental conditions.

6. Environmental influences

The interspecific variation of fish cardiac anatomy and physiology is guided by evolutionary adaptation to different habitats, modes of life and activity levels. Because ectothermic fish are exposed to different temperatures either seasonally or via the vertical thermocline, temperature-dependent regulation of cardiac contractility is crucial. Many fish face unpredictable temperature changes, meaning that fish hearts must have intrinsic mechanisms or rely on post-translational modifications to protect against acute temperature changes when there is not enough time for transcriptional regulation to take effect [108, 34].

Cardiac performance may change through independent changes in stroke volume and heart rate, with relative contributions of each varying substantially among fish. During exercise, more athletic fish modulate cardiac output preferentially by increasing SV, while less active species preferentially modulate HR in different species [54-55]. With environmental hypoxia, reflex bradycardia occurs and increased stroke volume is necessary to maintain cardiac output [109-110]. Higher temperatures have been shown to increase heart rate while colder temperatures decrease stroke volume in crucian carp [10]. For this review, the impact of temperature on cardiac contractility will be focused on. Mechanisms of control of both stroke volume and heart rate must be considered when looking at temperature sensitivity of the fish heart.

There appears to be seasonal variation with the activity of pacemaker cells (marine plaice - [111]). Heart rate is maintained despite the lower temperatures without the need for extrinsic modification. Changes in heart rate seen with temperature are not solely dependent on pacemaker cells. Therefore, changes that are seen may be due to ion channel temperature sensitivity. Chronic temperature stress can also modify K^+ channel conductances and reduce action potential duration to enable higher heart rates (trout - [112]). Thermal acclimation modifies functional properties and subunit composition of K_{IR2} channels [113]. Cold temperatures induce decreased I_{K1} , possibly increasing the excitability of cardiomyocytes but increased I_{Kr} leading to limited AP duration and decreased refractoriness of the heart [114]. These changes in I_K appear when it is the predominant current, namely in ventricular cardiomyocytes [66] while the changes in I_{Kr} appear in both atrial and ventricular cardiomyocytes [114]. The density in particular of ventricular I_{K1} increases in warm-acclimated vs. cold acclimated fish (trout - [113]).

The plasticity of E-C coupling in fish has been suggested to be based, at least in part, on these temperature-induced changes in I_K [114]. These modifications lead to changes in Ca^{2+} influx and hence variation in E-C coupling. Ca^{2+} pumps and ion channels themselves are also known to be sensitive to acute temperature change [103]. The large SR calcium stores and low calcium sensitivity of ryanodine receptors have been proposed to play a critical role in maintaining contraction at lower temperatures than mammals [81]. Higher SR Ca^{2+} content may be necessary to initiate and maintain SR Ca^{2+} release events at lower temperatures [80,115].

Different species may have variations in response to temperature depending on their evolutionary history. Eurythermal fish such as trout must be able to cope with seasonal temperature fluctuations as well as more acute temperatures through the thermal gradient. Other stenothermal species are only able to cope with a more limited temperature range, though this range may be considered on the extreme end of the range for a eurythermal fish (e.g. Antarctic fish). Interspecies variation in excitation-contraction coupling seen with temperature acclimation may be indicative of the capacity for thermal niche expansion. This substantial variation in SR Ca^{2+} release relative to SL Ca^{2+} influx ratio exists between species as well as being dependent on acute and chronic temperature changes. Cold-tolerant active fish have a larger capacity to store Ca^{2+} in the SR than mammals (e.g. active teleosts - [115]; scombroids - [116]). The atrio-ventricular differences in Ca^{2+} loading are also more pronounced in cold-acclimated than warm acclimated fish (trout - [112]).

However, the contribution of the SR to cytosolic Ca^{2+} management varies with temperature in fish hearts. With cold acclimation, SR function is also enhanced more strongly in atrial than ventricular myocytes (trout - [60]). More cold tolerant species may also possess greater SR Ca^{2+} content to begin with (bluefin tuna and mackerel - [112]). The degree of modification of I_{Ca} to offset the negative effects of colder temperatures appears to be species specific, with many teleosts such as trout demonstrating relatively temperature-insensitive Ca^{2+} flux [103] whereas certain scombroids show significant reductions in I_{Ca} with decreasing temperatures [112]. This temperature sensitivity may differ between atrial and

ventricular cardiomyocytes, with ventricular maximum SR Ca^{2+} load varying only in the ventricle of most scombrids [112].

As previously mentioned, isoform compositional changes can lead to variation in contractility. Modulation of MHC isoform expression patterns with temperature also leads to changes in myofibrillar ATPase activity (crucian carp - [10]). This may also be a species-related difference, since not all species display the same changes. The degree to which each chamber varies is still unclear. Differing paralog usage between the chambers may be linked to temperature-induced Ca^{2+} sensitivity differences. Examples of this may exist in important of the contractile element. Troponin I, the inhibitory subunit of troponin, has multiple paralogs expressed in cardiac tissue. The relative expression of these paralogs in the ventricle shifts with temperature, possibly leading to changes in the calcium affinity of the entire troponin complex [117]. However, while paralog profiles vary between tissues (heart, slow skeletal) no information is known about the atrium. TnC isoform differences between chambers [107] may also lend insight into Ca^{2+} sensitivity changes with temperature as the affinity of cTnC in trout is temperature dependent [118]. As previously mentioned, the very presence of multiple fish-specific paralogs of important components of the contractile element may be indicative of how fish have evolved mechanisms to thrive in variable environments. Incorporating the chamber-specific variation with temperature response reveals the complexity of these mechanisms in achieving survival.

7. Summary

The unique structure of the fish heart accommodates the variability in function that have allowed species to exploit many different environments. The increased importance of the atrial contraction in guiding changes in cardiac output indicates that some of this physiological versatility may be due to atrio-ventricular differences. These two main chambers of the fish heart differ in terms of contractile properties from the molecular level of E-C coupling and AP morphology, and the whole heart morphology and function reflect this. The fish heart is representative of early embryological stages of higher vertebrates, these studies on both the development and functioning of chamber-specific properties lends insight into proper functioning of the heart not only in fish. However, the degree of atrio-ventricular differences vary between species making it very difficult to generalize species-specific results across phylogeny. The species-specific differences in these two chambers lend insight into how evolutionary history guides responses to environmental factors such as ambient temperature. Fish-specific genome duplications may have allowed for multiple chamber-specific genes with variable functions that allow for flexibility in function. Further work is required to determine how genome duplication has shaped the structure and function of the fish heart and to clarify how this wide-range of species-specific phenotypes has been maintained.

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Advances and Applications of Tracer Measurements of Carbohydrate Metabolism in Fish

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Relevance of carbohydrate metabolism studies in fish

The zebrafish *Danio rerio* has long been used as an animal model for developmental studies due to a number of desirable characteristics for lab study including its short generation time, large number of offspring, transparent embryos, and *ex utero* development of the embryo. This setting proved to be particularly useful for research on vertebrate development [1] and modeling of human disease [2], namely the hematopoietic [3, 4] and the cardiac systems [5, 6]. However, the use of fish for scientific studies has evolved substantially in the last 30 years. The direction of metabolic fish studies changed drastically with the advent of modern aquaculture and a high demand for models to study nutrition, physiology and metabolism. Capture of fish products in the end of the 1990's represented almost 75% of total production but since 2001 that has leveled at around 90 million tons. Meanwhile, aquaculture production has been increasing at an average annual growth rate of 6.2% from 38.9 million tons in 2003 to 52.5 million tons in 2008 [7]. Aquaculture in 2009 already accounted for 38% of the 145 million tones of total fish products, 81% of which were for human consumption [8]. It is estimated that by 2030, half of the production for human consumption will be derived from aquaculture while the harvest of wild fish will not show any significant growth [9]. Farming of aquatic species has existed for many thousands of years: in ancient Asia, carp were left to grow in ponds and rice paddies and later harvested. Similar practices were thought to take place in ancient Egypt with tilapia and in southern Europe in a polyculture regime, including mullet *Mugil* spp, sole *Solea* spp, seabass *Dicentrarchus labrax* and gilthead seabream *Sparus aurata* [10]. These artisanal methods, with little or no active manipulation of the animals

or diets, are still practiced in some parts of the globe. However, as it has evolved into a highly competitive and commercialized business, the technique of aquaculture has also significantly evolved both in targeted species and in farming methodologies. Aquaculture is highly dependent on capture fisheries to provide fishmeal and fish oil required to produce feeds [11, 12]. Thus, the development of well-suited and cost-effective substitute feeds based on carbohydrates has become a matter of extreme importance to the sustainability and profitability of the sector. Along with it, comes the need to improve our understanding of fish carbohydrate nutrition, its physiology, biochemistry and metabolism.

1.2. Carbohydrate metabolism in carnivorous fish

The central organization of metabolic pathways is highly conserved amongst vertebrates. While the metabolic machinery of fish is much the same as that of mammals, the main differences lie in the nutritional and endocrine control of the pathways via various feedback mechanisms. As a water-living organism, fish have specific adaptations in relation to their terrestrial vertebrate counter-parts. As consequence of living in water, their constant thermal equilibrium with the environment is a principal determinant of overall metabolic rates while excess dietary nitrogen can be cleared as ammonia, a highly toxic but rapidly diffusible molecule that is efficiently transferred from blood to the outside water via the gills [13]. Carbohydrates (CHO) are a basic nutritional source of energy and carbon, but carnivorous fish have a limited capability to digest and metabolize them since they are adapted to a diet high in protein [14]. CHO influence growth, feed utilization and deposition of nutrients according to species, quantity, origin and treatment of dietary CHO used [15, 16, 17]. Fish are considered to be glucose intolerant on the basis of sluggish clearance of a glucose load and their metabolism of glucose has often been compared to that of mammals with insulin-dependent diabetes *mellitus* (IDDM) [18]. However, unlike IDDM, the causes of this intolerance cannot be attributed to a simple deficiency of insulin but is instead a reflection of different enzymatic and hormonal control regimes for glucose regulation in fish compared to mammals. The liver utilizes, produces and stores CHO and is an important component of systemic glycemic control in vertebrates. The rate of nutrient absorption coupled with continuous monitoring of blood glucose levels by various organs including the brain and pancreas mediates a combination of nutritional, endocrine and nerve-mediated regulation of hepatic glucose metabolism. This results in net hepatic glucose uptake when CHO is abundant, such as after feeding and net hepatic glucose output when dietary CHO is unavailable, such as during starvation. Glucose is the major energy source for the central nervous system and the only energy source for erythrocytes and the functions of both are dependent on a threshold concentration of blood glucose. Blood glucose levels are maintained through a balance of several factors, including the rate of consumption and intestinal absorption of dietary CHO, the rate of utilization of glucose by peripheral tissues and the loss of glucose through the kidney and finally the rate of removal or release of glucose by the liver [19].

Figure 1 depicts the pathways by which the liver disposes glucose into the bloodstream during fasting and storage and utilization of imported glucose during feeding. The main enzymes involved in these pathways are also represented as well as possible final fates of

glucose anabolism. As for mammals, many tissues such as skeletal muscle can oxidize alternative substrates to glucose for energy such as triglycerides and can therefore function with variable levels of blood glucose [20] but the brain is highly dependent on glucose as an energy substrate therefore a threshold level of plasma glucose is required for maintaining its function. Not surprisingly, the brain is regarded as key component of endogenous glucose sensing mechanisms that have recently been described in fish models [21]. The plasma glucose concentration also has considerable influence on the selection of myocardial substrates for oxidation in resting and active fish [22] and this adaptation is representative of the changes that occur in whole-body glucose utilization in response to changes in glucose availability [23]. During hypoxia, tissues such as the heart and brain upregulate anaerobic metabolic pathways and become more reliant on glycolysis for ATP production [24]. However under normoxic conditions, glucose uptake by muscle is surpassed by that of amino acids and lipids for growth and energy [25]; this also happens during the recovery of muscle glycogen following exhaustive exercise. Thus in general, the direct contribution of blood glucose to muscle energy metabolism seems to be minor [20, 26, 27, 28, 29]. The generally low reliance of fish muscle on glucose as an energy substrate is compatible with the limited availability of carbohydrate in fish diets [18].

1.3. Hepatic glucose storage and disposal

In mammals, glucose enters the hepatocyte through the GLUT-2 transporter, which is primarily expressed in the liver but is also present in kidney, intestine and β -cells of pancreatic islets [19]. Due to its low affinity and high capacity, GLUT-2 transports hexoses in a large range of physiological concentrations necessary for glucose homeostasis, displaying bidirectional fluxes in and out the cells, namely in hepatocytes [30]. Recently, the presence of GLUT-2 transporter was confirmed at both biochemical and molecular levels in species like rainbow trout *Oncorhynchus mykiss* [31, 32] and its expression in seabass *D. labrax* hepatocytes seems to be regulated by hypoxia.

Blood glucose concentration is the net result of the difference between rates of glucose appearance and disposal. Following uptake into the hepatocyte, the ATP-dependent conversion of glucose to glucose 6-phosphate (G6P) is catalyzed by hexokinase IV or glucokinase (GK). GK is the major hexokinase expressed in liver and due to its relatively high K_m , its role in glucose homeostasis, is to increase the hepatic capacity for glucose metabolism during hyperglycemia. Uniquely, GK is not inhibited by its G6P product so it is able to maintain high rates of glucose phosphorylation while the activities of other hexokinases become limited by increased G6P levels. Moreover, GK activity is hormonally controlled via the glucokinase regulatory protein (GKRP), such that its activity is increased by the high insulin levels of the fed state, but rapidly suppressed during the onset of fasting when both portal vein glucose and insulin levels are waning. As a result, when the liver becomes a net producer of glucose during the fasting state, GK is prevented from converting this back to G6P.

The G6P product can be utilized by the pentose phosphate pathway, be recruited for glycogen synthesis (glycogenesis) or be metabolized by the glycolytic pathway to pyruvate. Pyruvate in turn may be oxidized to acetyl coenzyme A (acetyl-CoA) or carboxylated to

oxaloacetate or malate – a process known as anaplerosis. Acetyl-CoA may be oxidized to CO₂ by the tricarboxylic acid cycle (TCA cycle) or utilized for lipid production (lipogenesis) while anaplerotic products can be utilized for gluconeogenesis. G6P can also be hydrolyzed back to glucose, a reaction catalyzed by glucose 6-phosphatase (G6Pase). In fish, it has been shown that G6Pase is poorly regulated by dietary CHO in comparison to GK [33]. When G6Pase and GK are both active at the same time, glucose and G6P are interconverted in a "futile cycle" that results in the consumption of ATP but no net conversion of glucose to products. This activity has important implications for interpreting the metabolism of certain glucose tracers such as [2-²H]- or [2-³H]glucose since it results in disappearance of the label without any net glucose consumption.

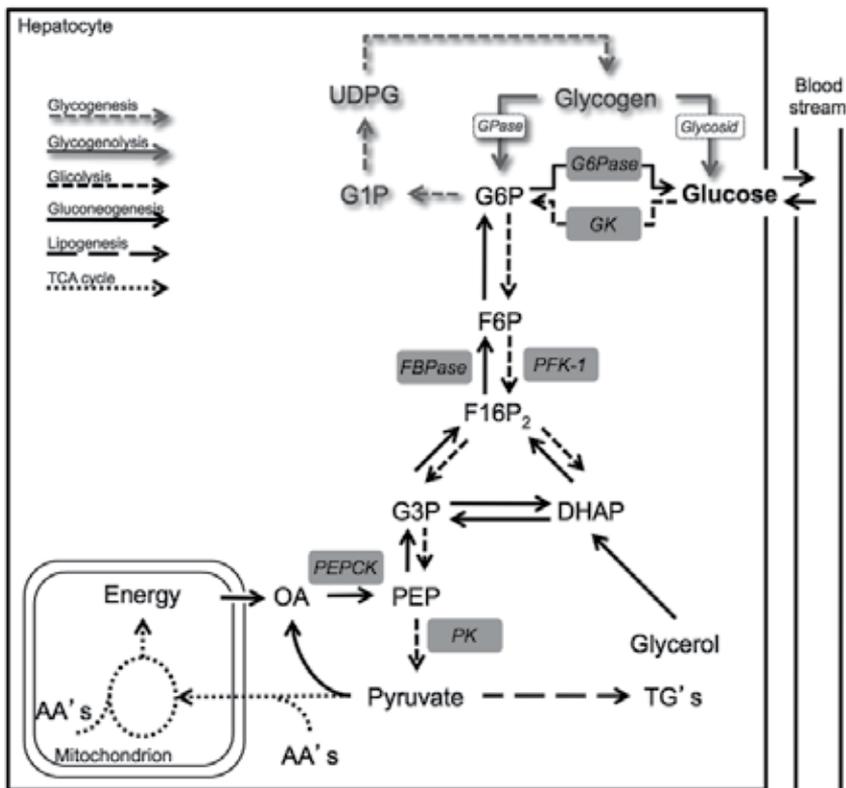


Figure 1. Metabolic model representing main pathways in the liver involving catabolism and anabolism of glucose. Gluconeogenic precursors are represented by pyruvate (and gluconeogenic amino acids, metabolized via the anaplerotic pathways of TCA cycle) as well as glycerol from lipolysis. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P - glucose 6-phosphate; G1P - glucose 1-phosphate; UDPG - uridine diphosphoglucose; F6P - fructose 6-phosphate; F16P₂ - fructose-1,6-bisphosphate; G3P - glyceraldehyde 3-phosphate; DHAP - dihydroxyacetone phosphate; PEP - phosphoenolpyruvate; TG's - triglycerides; OA - oxaloacetate; AA's - amino acids; GK - glucokinase; G6Pase - glucose-6-phosphatase; FBPase - fructose bisphosphatase; PFK-1 - 6-phosphofructo-1-kinase; PEPCK - phosphoenolpyruvate carboxykinase; PK - pyruvate kinase; GPase - glycogen phosphatase; Glycosid - glycosidic enzymes; TCA cycle - tricarboxylic acid cycle.

1.4. Glycogen synthesis and hydrolysis

The liver is the main storage site for glycogen, but tissues such as gills, kidney and brain also sustain relatively high rates of glycogen synthesis from glucose. Net glycogen synthesis results from the glycogen synthase (GSase) and glycogen phosphorylase (GPase) activities that are reciprocally regulated both allosterically and by phosphorylation [34].

GSase exists in a phosphorylated inactive form in the cytosol and it is activated by binding to G6P and this causes a conformational change that makes it a better substrate for protein phosphatases, which then convert the enzyme to the active dephosphorylated isoform. In glycogenesis, G6P is first converted to glucose 1-phosphate (G1P) by phosphoglucomutase and subsequently to uridine diphosphate glucose (UDPG) by UDPG pyrophosphorylase. Glycogen synthase then adds glucose residues from the UDPG donor to the growing glycogen molecule via α -1,4 glycosidic linkages. Hepatic glycogen can be synthesized by the incorporation of glucose into glycogen via a sequence of reactions that was initially characterized in muscle. In this so-called "direct pathway", the glucosyl moiety is transferred into glycogen as an intact entity. It was subsequently discovered that in the liver, glycogen can also be synthesized independently from glucose via gluconeogenic precursors [19]. These contributions are collectively referred to as the "indirect pathway". Since the indirect pathway bypasses GK, and uses precursors that are independent of blood glucose, its contribution to glycogen synthesis may respond to nutritional and hormonal states in a different way to that of the direct pathway. Quantifying the direct and indirect pathway flux components of hepatic glycogen synthesis can therefore further inform adaptations of hepatic carbohydrate metabolism to different diets and hormonal states.

Under fasting conditions, the liver becomes a net producer of glucose and the hydrolysis of glycogen is an important source of glucose production at least in the early stages of fasting. To avoid futile cycling between G6P and glycogen, the activities of GSase and GPase are regulated in a reciprocal manner such that only one or the other is active at a given time. Glycogen may be also hydrolyzed to glucose by the action of glucosidases enzymes that hydrolyze the non-reducing end of polysaccharides directly to glucose. There is evidence of glucosidase activity alongside that of GPase in living fish [35, 36, 37], but the extent of its contribution to glucose synthesis from glycogen and modulation of activity by feeding and fasting are little known.

Most fish species need to periodically cope with starvation as a result of seasonal food limitation or in some cases as natural consequence of their life cycle [38] and the liver plays an important role in the endogenous control of fuel storage and mobilization [39, 40, 41]. Glycogen represents an important source of glucose to be released in the fasted state or when responding to acute stressors. The transition from fasting to feeding states results in a comprehensive realignment of hepatic carbohydrate metabolic fluxes from minimal maintenance of glycemia and peripheral glucose demands by endogenous glucose production during the fasting phase to a high nutrient inflow and replenishment of liver glycogen stores during refeeding [39, 40, 42, 43]. The general consensus is that the liver of fish plays a role in glycemic control through the regulation of hepatic glucose storage and mobilization [33] but its actions are sluggish in comparison to mammalian liver.

1.5. Glycolysis and gluconeogenesis

Glycolysis involves the metabolism of glucose or glycogen to pyruvate. From glucose, the first step in the glycolytic pathway is the phosphorylation of glucose by GK into G6P, as described. From glycogen, G6P is generated following GPase and phosphoglucomutase activities. G6P is isomerized to F6P via G6P isomerase and this is followed by another phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (F16P₂) catalyzed by 6-phosphofructo 1-kinase (PFK-1). These reactions commit the hexose carbon skeletons to pyruvate production. Both phosphorylations are highly regulated and along with conversion of phosphoenolpyruvate (PEP) to pyruvate catalyzed by pyruvate kinase (PK) constitute the irreversible steps of glycolysis. The energy charge of the cell, as well as allosteric and transcriptional processes, contributes to control fluxes through PFK-1 and PK. The free energy released in this process is coupled to substrate-level ATP and NADH formation. Pyruvate is the final product of glycolysis and can undergo further oxidation to acetyl-CoA via pyruvate dehydrogenase (PDH), carboxylation via pyruvate carboxylase (PC) or NADP-malic enzyme or reduction to lactate. In the liver, the relative activities of PDH and PC determine whether pyruvate is oxidized or utilized by anaplerotic pathways such as gluconeogenesis hence the activities of these enzymes are highly regulated depending on the nutritional state. Gluconeogenesis is the principal metabolic pathway that generates glucose in fish. Its main precursors include lactate (converted into pyruvate by lactate dehydrogenase) and gluconeogenic amino acids (those that are metabolized to pyruvate or C4 and C5 TCA cycle intermediates), with minor contributions from glycerol, derived by lipolysis of triglycerides. While conversion of pyruvate to glucose occurs via the same intermediates as glycolysis, different enzymes are used to overcome the unfavorable free energy change of pyruvate to PEP conversion. The conversion of F16P₂ to F6P and G6P to glucose are also mediated by different enzymes to their glycolytic counter-parts (fructose 1,6-bisphosphatase and glucose 6-phosphatase, respectively). Pyruvate is converted to PEP at the expense of two ATP equivalents. First, pyruvate carboxylase generates oxaloacetate (OA) from pyruvate and second, PEP carboxykinase (PEPCK) converts OA to PEP. The free energy change of pyruvate conversion to glucose is positive, hence pyruvate gluconeogenesis requires energy in the form of ATP and reducing equivalents. This is not the case for all gluconeogenic precursors, notably glutamate and glutamine. These are metabolized to α -ketoglutarate, which during its oxidation to OA via the TCA cycle, generates ATP and reducing equivalents in excess of those consumed by PEPCK and glyceraldehyde 3-phosphate dehydrogenase. It is important to note that these amino acids are among the most abundant in dietary protein, therefore from a thermodynamic viewpoint, they facilitate gluconeogenesis from protein.

1.6. Enzymatic and hormonal regulation

Measurement of enzymatic activities involved directly or indirectly in CHO metabolism has been performed in various species and tissues and has proved to be of crucial importance in evaluating adaptation to changes in temperature [44, 45], salinity and osmoregulation [46, 47], feeding status [39, 41, 48, 49], rearing densities [50], diets [51, 52, 53, 54, 55, 56, 57] and to study the effects of hormones [58, 59, 60, 61].

Hormones in vertebrate organisms are a diverse but well conserved group of signaling molecules that regulate and modulate metabolic fluxes. The role of hormones in the regulation of CHO metabolism of fish are far from being completely known due to the variety of responses observed - many of which are dependent on whether studies are performed on intact fish, isolated organs or primary cell cultures. The mammalian paradigm, to which the hormonal regulation in fish is usually compared, has shown to be poorly representative of fish metabolism in a number of cases [62]. Glucose is weakly effective at stimulating insulin secretion in fish compared to mammalian species, although, as expected, an improved response is observed in CHO-tolerant species [63]. On the other hand, amino acids are potent stimulators of insulin secretion even though the intensity of response varies greatly between salmonids and other fish like carps and seabream. This bears some resemblance to mammals, where arginine is also a powerful insulin secretagogue.

In mammals, the actions of insulin are directly opposed by glucagon and adrenal hormones such as adrenaline and these counterregulatory actions are an important component of glucose homeostasis. However, it is unclear in fish to what extent the regulatory and counterregulatory processes are coupled [62]. For example, in response to stress or hypoglycemia, the adrenaline-induced hyperglycemic response of rainbow trout is caused by the stimulation of hepatic glucose production that happens in a dose-dependent fashion [64]. However, the accompanying suppression of glucose clearance described in mammals is not observed in fish. Cortisol is a major regulator of intermediary metabolism and promotes hepatic glucose production and hyperglycemia, primarily as a result of increased hepatic gluconeogenesis fuelled in part by amino acid products of peripheral proteolysis [65].

2. Tracer studies of carbohydrate metabolism in fish

2.1. Overview

A metabolic tracer is, by definition, a substance used to follow the biological transformation of an endogenous substrate (tracee). The tracer must have a unique property that allows its detection but at the same time be chemically identical to the tracee. In most cases, tracers consist of synthetic substrate molecules where one or more atoms in the molecule are substituted for an atom of the same chemical element, but of a different isotope. Isotopic tracers may be identified by radioactivity or differences in mass and/or nuclear spin from the tracee. In order that the tracee pool of interest be not altered by administration of the tracer (thereby potentially perturbing the metabolic activity under observation), its chemical concentration should be insignificant in comparison to that of the tracee. At the same time, this amount of material must possess a high abundance of label relative to the tracee since metabolic flux parameters are typically derived by administering a high density of label and monitoring its dilution by unlabeled tracee molecules. With radioisotopes, the label concentration is measured in terms of specific activity, defined as the number of decays per minute measured per mol of glucose. With stable isotopes, tracer concentration is defined in terms of percent excess enrichment per mol of glucose. Since most stable isotopes are typically

present at background levels (for example, 1.1% of carbon in nature is ^{13}C), this must be accounted for when attributing metabolite enrichment to inflow from a tracer.

Since blood glucose and other carbohydrates can be synthesized endogenously as well as absorbed from the diet, several tracer strategies may be applied to inform carbohydrate metabolism. The first and most easily understood is to incorporate the tracer into dietary carbohydrate and monitor its rate of appearance in endogenous carbohydrate pools (i.e. plasma glucose and liver glycogen) as well as metabolic endproducts (i.e. respired CO_2). This approach provides a direct measure of how a specific dietary carbohydrate source is utilized and disposed into endogenous oxidative and nonoxidative pathways. Oral administration of labeled food [66], either by pellets with ^{13}C -labeled starch and ^{15}N -labeled protein [67] or ^{13}C -labeled rotifers [68], and measurement of isotope appearance in endogenous carbohydrate and protein have also been used to measure substrate utilization in fish. In the case of carbohydrate studies, while the dilution of ^{13}C -enrichment between the feed precursor and sampled product metabolite may qualitatively inform the extent of endogenous glucose production (i.e. high dilution equates to high rates of endogenous glucose production relative to absorption of the ^{13}C -enriched carbohydrate), quantitative data rely on knowing the rate of entry of the ^{13}C -enriched dietary carbohydrate into blood glucose. Since this is a function of absorption and cannot be directly measured, this approach provides only a limited insight into endogenous glucose production. In the second type of measurement, a glucose tracer is directly administered into the blood at a known rate or quantity, and by measuring its dilution by unlabeled endogenous glucose, the rate of appearance (R_a) of blood glucose may be quantitatively defined. Since both absorbed and endogenously-produced glucose contribute to the dilution of the labeled glucose, this measurement does not resolve the contributions of dietary and endogenous sources. Under fasting conditions where there is no input of dietary glucose, glucose R_a equates to endogenous glucose production. In mammals and humans, dietary and endogenous components of glucose R_a may be resolved by combining a feed tracer with a second glucose tracer administered intravenously [69]. The detection method must have the capability of resolving the labeling contribution of blood glucose from each tracer. For example, radioactivity from infused [$3\text{-}^3\text{H}$]glucose and ingested [$\text{U-}^{14}\text{C}$]glucose are resolved by scintillation counters on the basis of their different emission energies. Finally, as previously discussed, endogenous glucose can be derived from multiple sources including gluconeogenesis from a diverse range of precursors as well as glycogen hydrolysis. Neither the labeled dietary carbohydrate nor glucose isotope dilution measurements inform the sources of endogenous glucose production. This represents a significant limitation for understanding carbohydrate metabolism of carnivorous fish since their diet is low in carbohydrate therefore the plasma glucose rate of appearance (R_a) is likely to be dominated by synthesis from endogenous precursors. Moreover in aquaculture, weaning of carnivorous fish from pure fishmeal to diets supplemented with carbohydrates is an important objective for improved sustainability and reduced environmental impact [18]. Hence, there is continuing interest in understanding to what extent their metabolic phenotype can adapt to increased dietary carbohydrate availability and if gluconeogenic utilization of amino acids is spared under these conditions [16]. To effectively address such questions and to im-

prove our overall understanding of piscine glycemic control, endogenous and exogenous contributions to glucose R_a need to be better defined than they are at present.

2.2. Radioactive versus stable isotope carbohydrate tracers

Historically, the use of radioactive isotopes such as ^{14}C and ^3H has provided valuable information characterizing the main pathways of hepatic carbohydrate metabolism. They are easily detected by scintillation counting and their background radioactivity is very low in comparison to metabolite specific activity. Due to the fact that they are weak α - and β -ray emitters, and are therefore easily contained and have relatively high maximum permissible dosages, these tracers are still used in some human and many animal studies. They are more widely used in cell cultures for substrate uptake studies and isotope-dilution measurements where the small scale of the experiments can be accommodated by reasonable radiation containment measures. For this same reason, radioactive isotopes have been particularly useful in the study of larval nutrition. A supply of good quality fish fry is essential for a successful production of juveniles however knowledge of their nutritional requirements is often qualitative rather than quantitative [70]. In this context the innovative methodologies of incorporation of ^{14}C -labeled amino acids [71] or fatty acids [72] in larvae live prey *Artemia* sp. nauplii has improved our understanding of larval nutrition. However, for conventional nutritional studies involving larger fish and tanks, containment of radioactive tracers is often not practically possible therefore in these settings, their stable isotope counterparts (^{13}C and ^2H) are typically deployed. Methods for obtaining glucose rates of appearance (R_a) that were originally developed using ^3H - and ^{14}C -labeled glucose [23, 73, 74, 75, 76] can be easily adapted for stable isotope studies. Moreover, stable isotope tracers can potentially yield much richer metabolic information than their radioactive counterparts due to advances in positional and isotopomer analysis of metabolite enrichment from ^{13}C and ^2H that go above and beyond those which are possible for radiolabeled tracers.

2.3. Tracer administration and measurements in fish compared to mammals

Tracer measurements of whole-body carbohydrate metabolism were originally developed in humans and experimental animal models such as rodents, dogs and primates. Translating these methodologies to fish presents some additional challenges, the most important being the following: firstly, most mammals have accessible peripheral veins that can be catheterized for tracer administration and blood sampling. Catheters may be installed temporarily under anesthesia for the duration of the tracer study, or as indwelling entities for longitudinal studies of conscious free-moving animals. In fish, the dorsal aorta proximal to the gill arches is the main accessible vessel for tracer studies. It has been successfully cannulated for tracer infusion and blood sampling in undisturbed, non-anaesthetized rainbow trout of 800-1200 grams in weight [77, 78, 79]. However, it is unclear if this method can be easily applied to smaller sized fish, or to fish with a different gill-body configuration to rainbow trout, for example flatfish. The second major impediment for fish carbohydrate tracer studies is the fact that glucose appearance and turnover rates are much slower in comparison to mammals, necessitating far longer infusion periods for achieving isotopic steady-state con-

ditions – a critical requirement for investigating precursor-product relationships of biosynthetic pathways. This is particularly problematic for quantitative studies of gluconeogenesis based on the delivery of a labeled precursor substrate such as ^{14}C -lactate or alanine, since both the trace precursor and glucose product pools must be at isotopic equilibrium during the sampling period.

2.4. Measurement of glucose R_a by bolus injection and by primed infusion

There are two approaches for quantifying plasma glucose R_a by isotope dilution. The first involves an intravenous injection of a tracer bolus and monitoring the decrease in specific activity or enrichment of plasma glucose as the tracer is being diluted by unlabeled glucose [75]. The dilution kinetics are best represented by more than one exponential decay function indicating the presence of separate pools of glucose in the body with different clearance characteristics. Because of the relative simplicity of a single injection tracer delivery, this method has been applied in many fish species including kelpbass *Paralabrax clathratus* [73], seabass *D. labrax* [74], *Hoplías malabaricus* [76], common carp *C. carpio* [23]. However, this approach requires frequent blood sampling over a sustained period to adequately describe the complex tracer clearance kinetics. Limited by the number of blood samples that could be drawn from the fish, the study described in [73] extrapolated the clearance kinetics from a smaller set of initial measurements, but the uncertainties of this approach were acknowledged. The alternative primed-infusion method establishes a constant level of tracer in the bloodstream following an appropriate priming dose. Under these conditions, the dilution of the tracer, measured from the ratio of specific activity or enrichment of infused label to that of blood glucose, is equal to the ratio of endogenous glucose appearance and tracer infusion rates. If glucose R_a is variable (for example during meal ingestion) then the rate of infusion needs to be adjusted accordingly in order to maintain a constant ratio of infused glucose to blood glucose specific activity or enrichment. Compared to the bolus injection method, primed infusion measurements require fewer blood samplings; in fact the sole rationale for multiple blood samplings is to verify a constant ratio of infused to blood glucose specific activity or enrichment. Calculation of glucose R_a from steady-state isotope data is more robust compared to single injection since it is independent of the complex and often poorly defined clearance kinetics. However, a primed infusion requires catheterization of a vein to deliver the tracer over an extended period and is technically more difficult than a single bolus injection. While these procedures now allowed glucose R_a to be well determined, and by combination with dietary tracers can determine the contributions of absorbed and endogenous glucose production to glucose R_a , they do not inform the sources of endogenous glucose production. Novel methodologies of resolving the sources of glucose R_a using deuterated water ($^2\text{H}_2\text{O}$) have been developed and can be integrated with primed-infusion glucose R_a measurements [80, 81, 82]. $^2\text{H}_2\text{O}$ is ideally suited for fish metabolic studies since it can be incorporated into aquarium water for an indefinite period and is rapidly incorporated into the fish body water such that the ^2H -enrichment level of the fish tissue water is also fixed for the duration in the ^2H -enriched tank water. As a result of enzyme-catalyzed exchange reactions between bulk water and metabolite hydro-

gens, many important precursor metabolites of glucose and glycogen biosynthesis, such as lactate, pyruvate and alanine also become rapidly enriched to the same level as body water for the study duration. Thus, a constant precursor enrichment that can be indefinitely maintained is possible. $^2\text{H}_2\text{O}$ has recently been used to study protein synthesis kinetics in the catfish *Ictalurus punctatus* [83] and gilthead seabream *S. aurata* [84] as well as blood glucose and hepatic glycogen turnover in seabass *D. labrax* [43, 85]. Because of its enormous potential for informing endogenous carbohydrate metabolism in fish, we will discuss its use in more detail.

3. Deuterated water as a tracer of endogenous glucose and glycogen synthesis

3.1. Overview

Fish and mammals share common pathways for glucose production and consumption [86] hence the underlying principles of plasma glucose ^2H -enrichment from $^2\text{H}_2\text{O}$ that are well described and validated for mammals [80, 87, 88] can be applied to fish. Deuterated water ($^2\text{H}_2\text{O}$) is a relatively inexpensive non-radioactive tracer that can be incorporated in drinking water, or in the case of fish studies, in the tank water. It has been successfully used in humans and other mammals for the study of hepatic intermediary metabolism in both normal and pathological conditions. It rapidly equilibrates with total body water and is distributed evenly into all tissues. It is a practical tracer for both short and long-term metabolic studies. $^2\text{H}_2\text{O}$ is ideally suited for studying fish metabolism since it can be added to the tank water for an indefinite period, during which time it is incorporated into hepatic metabolites such as glycogen and glucose by specific enzymatic reactions in their biosynthetic pathways, as previously described for mammals. Applying these principles to free-swimming fish provides an authentic metabolic profile that is unadulterated by anesthesia or infusion procedures that characterize the administration of classical carbon tracers.

3.2. Basic principles

Deuterium (^2H) is a stable isotope of hydrogen with a nucleus containing one proton and one neutron (the ^1H nucleus contains no neutron). In the NMR experiment, ^2H resonates at a different frequency compared to its ^1H counterpart, allowing tracer levels of ^2H to be observed in the presence of the tracee ^1H . The inherent sensitivity of ^2H (at constant field and with an equivalent number of nuclei) is about 0.9% that of ^1H . Metabolism of ^2H is not exactly equivalent to that of ^1H because of kinetic isotope effects. The strength of a chemical bond between two atoms is dependent in part on their relative masses, hence a C- ^2H bond is stronger than a C- ^1H for any compound. Since metabolite transformation is governed in part by breaking and formation of C-H bonds, the presence of ^2H makes the bonds harder to break thereby potentially slowing the rate of C- ^2H vs. C- ^1H transformation. This can discriminate the transformation of ^2H -enriched metabolites compared to their tracees resulting in apparently slower rates of transformation. Moreover, with bulk levels of ^2H tracers, notably

$^2\text{H}_2\text{O}$, the aggregate isotope effects are toxic and indeed lethal to most living organisms. With tracer studies that utilize $^2\text{H}_2\text{O}$, toxicity from isotope effects is minimized by substituting a relatively low proportion of ^1H by ^2H (<10%). Furthermore, discrimination against ^2H incorporation into metabolites via enzymatic reactions is minimized when the reaction that transfers ^2H from water to the metabolite hydrogen is reversible. Most of the enzymatic steps of intermediary metabolism are reversible with extensive exchange of precursor and product, and under these conditions, discrimination of ^2H incorporation via kinetic isotope effects is not significant. Indeed, when ^2H -discrimination is observed, it informs the unidirectionality of a particular enzymatic step.

These caveats notwithstanding, $^2\text{H}_2\text{O}$ is an inexpensive tracer that is easily delivered into body water by immersion of fish in ^2H -enriched water. It has been successfully used in humans [89, 90, 91] and other mammals [82, 92, 93], for study of hepatic carbohydrate metabolism in physiological and pathophysiological conditions. The methodology was pioneered in humans [80, 87, 94] and was rapidly adopted by others in tissues [81, 88, 95]. As previously discussed, G6P is a common precursor to both glycogen and glucose and in the presence of $^2\text{H}_2\text{O}$, G6P is labeled with ^2H in several positions due to the incorporation of that isotope via exchange with body water. It rapidly equilibrates with total body water and distributes homogeneously within tissues and the body water ^2H -enrichment level can be maintained indefinitely [96]. In fish, incorporation of ^2H from a 5%-enriched saltwater tank into plasma water is rapid, reaching more than 1% in 15 minutes, half of the enrichment of the tank water within 1 hour and approaching that of the tank water after 6h [85].

For studies of carbohydrate metabolism, the ^2H -enrichment distribution of plasma glucose from $^2\text{H}_2\text{O}$ is established according to origin of the G6P precursor (Figure 2.).

If produced from gluconeogenic substrates (gluconeogenic amino acids, pyruvate or glycerol) enrichment in position 2 (H2) is obligatory since conversion of F6P to G6P (facilitated by G6P-isomerase) is part of the gluconeogenic pathway. In mammals, G6P-F6P exchange is extensive and essentially complete hence the hepatic G6P pool is quantitatively enriched in H2 regardless of its origin. This means that newly-synthesized glycogen from G6P is also enriched in this position, regardless of whether the G6P was derived via the direct or indirect pathway [93, 97]. There is some evidence that in fish, hepatic G6P-isomerase activity is sensitive to the nutritional state [43]. In common carp *C. carpio* hepatic G6P-isomerase activity decreased in direct relation to feeding rates [98, 99] but there is no information on how G6P-isomerase activity is modified during the fasting to feeding transition. One possible explanation is that induction of G6P-isomerase activity by feeding is slow compared to activation of glycogen synthesis fluxes. Under these conditions, G6P-isomerase activity could be a rate-limiting step for indirect pathway synthesis of glycogen, at least in the initial stages of refeeding. In principle, sub-maximal G6P-isomerase activity could limit the glycolytic metabolism of G6P derived from glucose and favor its conversion to glycogen via the direct pathway or its utilization by the pentose phosphate pathway.

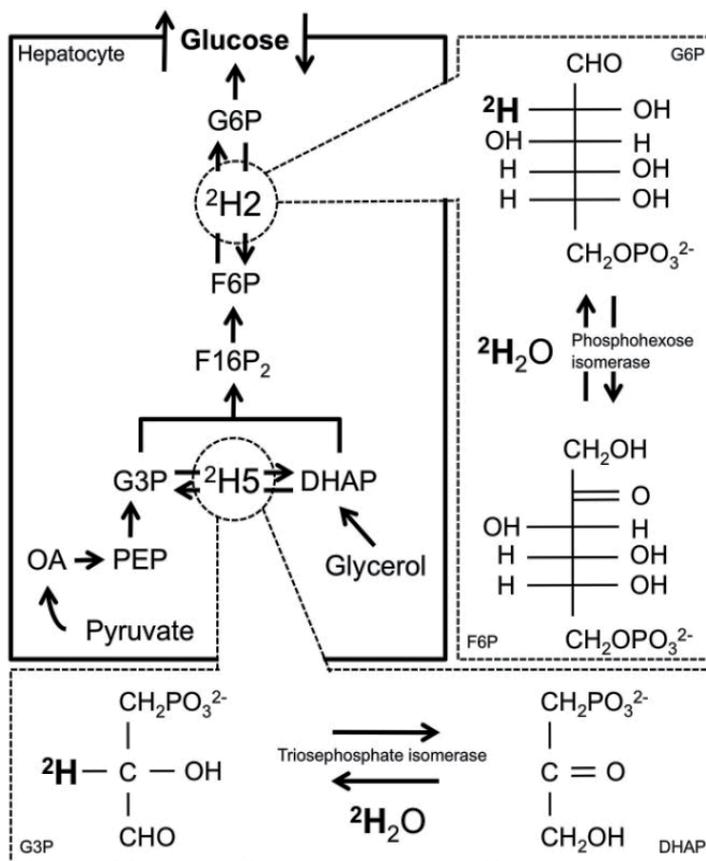


Figure 2. Metabolic model representing gluconeogenesis in the liver with special detail to the labeling with deuterium in position 2 ($^2\text{H}_2$) and 5 ($^2\text{H}_5$) of the glucose molecule. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P - glucose 6-phosphate; F6P - fructose 6-phosphate; F16P₂ - fructose 1,6-bisphosphate; G3P - glyceraldehyde 3-phosphate; DHAP - dihydroxyacetone phosphate; PEP - phosphoenolpyruvate; OA - oxaloacetate; $^2\text{H}_2\text{O}$ - deuterated water.

Besides, ^2H -Enrichment in position 5 of G6P occurs at the level of triose phosphates as shown in Figure 2. Therefore, enrichment of plasma glucose or hepatic glycogen in position 5 reflects the contribution of gluconeogenic fluxes to endogenous glucose production or indirect pathway contributions to hepatic glycogen synthesis.

3.3. NMR and MS methods for ^2H enrichment analysis

Many improvements on the measurement of stable isotope tracer enrichment and analysis of positional labeling information have been largely driven by the development of nuclear magnetic resonance (NMR) and mass spectrometry (MS) technologies. Choosing between these methods depends on the kind of enrichment information that is required from the experiment, the available sample size and access to instrumentation. MS techni-

ques quantify metabolite enrichment by resolving heavier labeled molecules from lighter unlabeled ones. For most MS instruments, the presence of two isotopes with similar increase in the molecular mass, (i.e. ^2H and ^{13}C) cannot be resolved, placing limitations on multiple isotope studies. Positional enrichment can be inferred from fragmentation and analysis of the mass of the daughter fragments (MS-MS). Nevertheless, as fragmentation is dependent on the molecule's chemical structure, the label of interest may or may not be isolated. Chemical derivatization of metabolites is often used to facilitate fragmentation and positional enrichment analysis [87]. MS is highly sensitive and can quantify enrichments from submicromole to picomole amounts of analyte and with appropriate signal calibration and sample purification safeguards, it can be configured for high throughput measurements.

Following a simple method based on a LC-MS/MS procedure for quantifying plasma [6,6- $^2\text{H}_2$]glucose enrichment [100] which does not require glucose derivatization, analysis of glucose can be performed on a few microliters of blood, either whole or as a dried spot on filter paper. This means it can be applied to any size fish and can also be used for repeated sampling of the same fish [85]. This LC-MS/MS measurement provides the mole percent enrichment (MPE) of the glucose molecule, equivalent to the sum of all seven positional enrichments. The principal uncertainties of utilizing plasma MPE levels as a marker of gluconeogenic contribution include the incomplete incorporation of ^2H into sites other than position 5, as seen by the tendency for lower enrichments in positions 1, 3, 4, 6_R and 6_S compared to position 5 by ^2H -NMR analysis [85].

Analysis of ^2H enrichment by ^2H NMR spectroscopy is a method with much lower sensitivity compared to MS, requiring 5-50 μmol of analyte in the typical experimental setting for $^2\text{H}_2\text{O}$ studies (0.5-5.0 % body water enrichment). However, in addition to being nondestructive to the sample, NMR provides a much higher level of positional enrichment information, allows enrichment from multiple stable isotope tracers to be selectively observed, and can provide a global analysis of metabolite enrichments from a complex mixture of metabolites, such as cellular extracts, biological fluids, and intact tissues [101]. This technique relies on the ability of atomic nuclei with odd mass and/or atomic number to align if subjected to an external magnetic field. When irradiated with a certain frequency signal the nuclei in a molecule can change their alignment and the energy frequency at which this occurs can be measured and displayed as an NMR spectrum. Common biologically relevant nuclei that are present at ~100% natural abundance and are observed by NMR include ^1H and ^{31}P and ^{23}Na . Isotopes that are more rare in nature such as ^2H (0.015% of hydrogen) and ^{13}C (1.11% of carbon) can also be observed at natural abundance levels, but molecules that are enriched to higher levels from ^2H - or ^{13}C -enriched precursors can be measured against this background. Since isotopes resonate at a specific frequency, its signals can be uniquely isolated from any other isotope that may be present. Derivatization of the target molecule can be used to provide a more heterogeneous chemical environment therefore improving signal dispersion [102, 103, 104]. This is particularly important for analysis of carbohydrate ^2H enrichment, which feature highly crowded hydrogen signals that are poorly resolved by the inherently small dispersion of ^2H signals (~15% of ^1H signals).

4. Conclusions

There is a compelling need to better understand the metabolism of carbohydrates by fish in general and aquaculture species in particular. Stable-isotope tracer methodologies have evolved such that safe, inexpensive and practical measurements of carbohydrate metabolism may be directly performed on naturally feeding fish in the aquaculture setting. These studies have great potential for informing the efficacy of novel dietary supplements in sparing the conversion of feed protein to carbohydrate as well as improving our general understanding of fish nutritional physiology.

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Ontogenetic Dietary Shifts in a Predatory Freshwater Fish Species: The Brown Trout as an Example of a Dynamic Fish Species

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Additional information is available at the end of the chapter

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1. Introduction

The brown trout (*Salmo trutta*) is a species of Eurasian origin, but it has become naturalized in many other parts of the world. It has an outstanding socio-economic importance, both in commercial and sport fisheries, and it is frequently used as a tourist attraction [1,2]. The case of brown trout is a clear example of a 'dynamic' fish species, as its diet and feeding behaviour can vary greatly among individuals, age classes, seasons and rivers. The composition of brown trout diet is strongly influenced by environmental and biotic factors. For example, water temperature plays an important role, as it influences food intake and the activity of fishes [3], but also the emergence and activity of aquatic insects or other potential prey items. Also water flow rate can be extremely important for drifting feeders such as brown trout, as they regulate food availability. There are many abiotic factors that influence feeding behaviour, but in general, biotic factors such as the locomotor ability of fishes, accessibility, abundance and antipredator behaviour of prey are thought to be the most important factors in the determination of the diet and feeding strategies in fishes. Usually not all the available prey is consumed by the predator, a feature that allows biologists to distinguish between trophic base and trophic niche (Figure 1). The trophic base consists of all potential prey items that the brown trout is able to consume and it is determined by the feeding habits of the fish, the size of the mouth and the anatomical characteristics of its digestive tract. However, the trophic niche is the variety of organisms that are really consumed by the predator, which depends on different factors that play an important role when choosing criteria prey items as, for example, prey abundance, including site-specific

prey accessibility, prey size, energetic selection criteria and prey preference. In this context, the trophic niche for brown trout is very flexible and is usually broader in adults than juveniles.

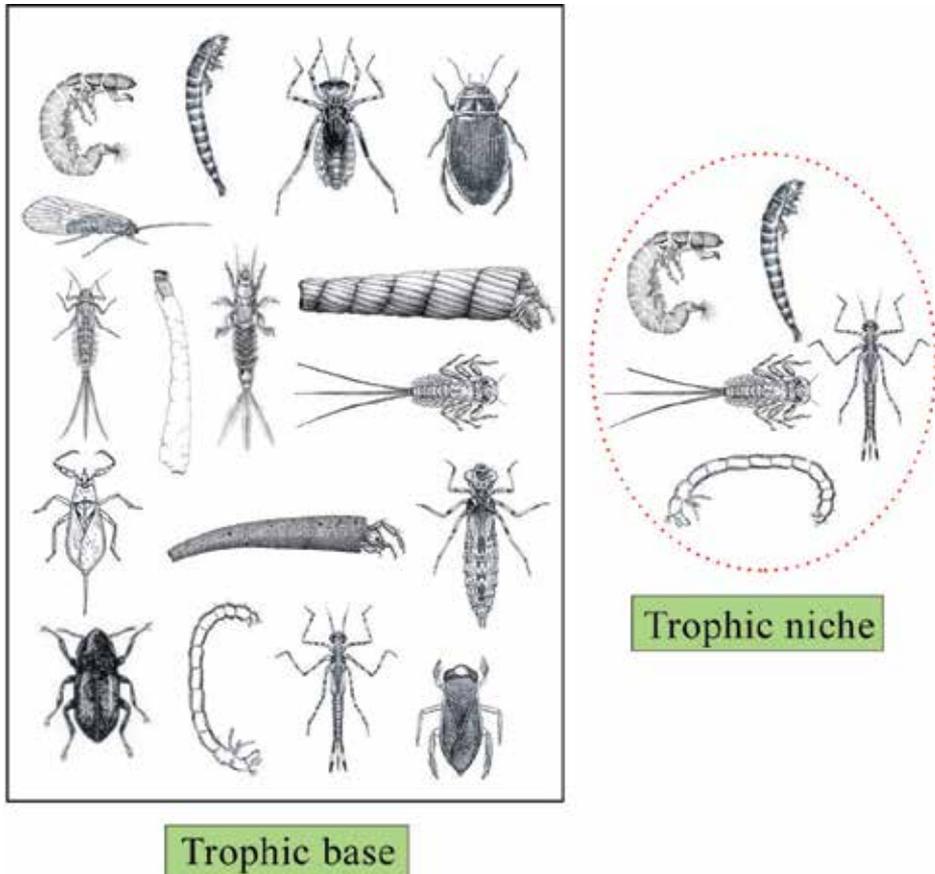


Figure 1. Graphical example of the trophic base and niche for brown trout.

A knowledge of the foraging ecology of fishes is fundamental to understanding the processes that function at the individual, population and community levels since the factors that influence the acquisition and assimilation of food can have significant consequences for the condition, growth, survival and recruitment of fishes [4]. In this context, the development of effective conservation programmes requires a clear understanding of fish ecological requirements, so the knowledge of its feeding habits is essential to achieve this objective. For example, the knowledge on how food is shared among individuals of the same population is critical for understanding its functioning. Hence, conclusions of field studies on feeding could help wildlife managers to take measures to preserve fish populations, especially for threatened and exploited species. In this chapter we will briefly discuss the variables that are involved in the feeding behaviour of brown trout as an example of a predatory freshwater fish species.

2. Methodology and types of analysis employed in feeding and ontogenetic dietary shifts studies

The majority of researchers have conducted feeding studies in feral fish populations based on diet descriptions of the stomach contents, using occurrence, numerical, gravimetric and volumetric methods. The main disadvantage of feeding studies is that fish are systematically killed in order to study their stomach contents. However, due to the decline of many natural fish populations, the studies that use non-lethal methods are now more frequent. Different techniques have been used to collect stomach contents without harming the fish such as gastric lavage, emetics or forceps [5-7]. The effectiveness of the gastric lavage is not related to the size of the trouts, but rather to the prey's own morphological characteristics, the degree of repletion of the stomach and the extent of digestion of the food [8]. The effectiveness of this method is inversely related to the degree of repletion [8].

2.1. Prey selection analysis

Prey selection is an important part of fish feeding ecology. In order to study prey selection of fishes, several indices have been employed, such as the Savage index [9] and Ivlev's selectivity index [10]. The Savage index varies from zero (maximum negative selection) to infinity (maximum positive selection), whereas possible values of Ivlev's selectivity index range from -1 to +1, with negative values indicating rejection or inaccessibility of the prey, zero indicating random feeding, and positive values indicating active selection. Moreover, several researchers have demonstrated that studies based on food selection provide insight into factors involved in prey choice of brown trout [e.g. 11-13].

2.2. Stomach content analysis

In the early 80s Hyslop reviewed the methods used to study the feeding behaviour of fishes and their application to stomach content analyses [14]. Hyslop pointed out the difficulties in the application of these methods and, where appropriate, proposed alternative approaches. Food overlap between age classes can be assessed with Schoener's overlap index [15]. The overlap index has a minimum of 0 (no prey overlap), and a maximum of 1 (all prey items in equal proportions), and diet overlap is usually considered significant when value of the index exceeds 60% [16]. A chi-square (χ^2) test can be used to test for significant differences in the diet composition between age classes [e.g. 17].

2.3. Graphical methods

Graphical methods proposed by Costello [18] and Tokeshi [19] were used to illustrate the relative importance of prey species and to assess the feeding strategy of fish species. Amundsen and collaborators designed an alternative method of Costello graphical method, by plotting prey abundance (A_i) (y - axis) against the frequency of occurrence in diet (F_i) (x - axis) for each prey species. Information on prey importance, feeding strategy and niche breadth can be

obtained by examining the distribution of points along the diagonals and axes of the graph [20] (Figure 5 and Section 4.1).

2.4. Niche breadth indexes

Marshall and Elliott compared univariate and multivariate numerical and graphical techniques for determining inter- and intraspecific feeding relationships in estuarine fish [21] and on the basis of this study, different indices have been employed by ichthyologists to study niche breadth and diet specialisation. Generally, the Shannon diversity index was combined with the Levin's index to assess niche breadth [21] and the evenness index was used to evaluate diet specialisation, these being indices employed to study feeding habits in brown trout populations [22,23]. However, stable isotope analysis is a potentially powerful method of measuring trophic niche width, particularly when combined with conventional approaches [24]. For this reason, over the past two decades this methodology has been employed to study the trophic interactions and dietary niche in different fish species, and it has been recently used to study ontogeny and dietary specialization in brown trout [25,26].

2.5. Multivariate approaches

Recently prey trait analysis has been proposed as a functional approach to understand mechanisms involved in predator-prey relationships [27,28]. Despite the disadvantages of this methodology [29 and references therein], it has been used in order to get a deeper insight into the mechanisms that regulate diet composition and feeding habits of fishes, providing extremely valuable ecological information and complementing traditional diet analysis [23,29,30]. For the application of prey trait analysis, researchers have to use the same trait database and trait analyses as de Crespín de Billy [27]. To evaluate the potential vulnerability of invertebrates to fish predation, de Crespín de Billy and Usseglio-Polatera created a total of 71 different categories for 17 invertebrate traits [(1) macrohabitat, (2) current velocity, (3) substratum, (4) flow exposure, (5) mobility/attachment to substratum, (6) tendency to drift in the water column, (7) tendency to drift at the water surface, (8) trajectory on the bottom substratum or in the drift, (9) movement frequency, (10) diel drift behaviour, (11) agility, (12) aggregation tendency, (13) potential size, (14) concealment, (15) body shape (including cases/tubes), (16) body flexibility (including cases/tubes) and (17) morphological defences] [28]. The information of this trait database is structured using a 'fuzzy coding' procedure; thus, a score is assigned to each taxon describing its affinity for each category of each trait, with '0' indicating 'no affinity' to '5' indicating 'high affinity'. The taxonomic resolution (order, family and genus) use in the classification process corresponds to the lowest possible level of determination of taxa in fish gut contents. When identification to genus is not possible or in the case of missing information for a certain genus, the value assigned for a trait is that of the family level, using the average profile of all other genus of the same family. Additionally, all the taxa and their assigned scores for each category can be found in previous works [27,28]. Prey trait analysis should be carried out with the software R (version 2.11.1), its ADE4 library for the analysis in R is free and downloadable at <http://cran.es.r-projet.org/>. Finally, the analysis of prey traits has provided ichthyologists with important clues for understanding the ontogenetic dietary shifts

in freshwater fish species. As shown in sections 4 and 5, it is an important tool to disentangle the food resource partitioning among both sympatric age classes and fish species.

3. Diet composition of newly emerged brown trout fry

In brown trout populations there is strong evidence for a critical period with high mortality in the first few weeks after fry emergence [3]. Furthermore, the most critical stage for population regulation in the whole life cycle is the density-dependent mortality of young trout in the first few weeks of the life cycle soon after the young fish start to feed [3 and references therein]. Thus, first feeding of newly emerged fry is very important for brown trout survival in this phase of the life cycle, and in newborns of brown trout first feeding can occur even prior to emergence [13,31,32]. In this sense, the feeding behaviour of newly emerged brown trout fry has been studied in both laboratory conditions and in natural spawning areas. Results of those studies show that feeding in recently emerged fry can be initiated before complete yolk exhaustion [13,31,32]. Zimmerman and Mosegaard observed that alevins of brown trout began feeding in experimental conditions when yolk constituted approximately 40% of the total alevin dry weight [31]. Other researchers have indicated that brown trout fry under natural conditions start feeding when having almost 30% of yolk sac remaining compared to the presumed original size of the yolk sac at hatching [32], while in a recent study no food particles have been found in the stomachs of fry having >10% of the yolk sac remaining [13].

The optimal foraging theory (OFT) explains adaptation via natural selection through quantitative models, which led to a better understanding of foraging behaviour. Hence, OFT predicts that predators should select prey that maximise the energetic gains available in relation to the energetic costs of capturing, ingesting and digesting the prey [33,34]. In this context, Many researchers have found that chironomid larvae and baetid nymphs seem to be the most important food items for newborns in different geographical areas [e.g. 13,32,35]. These are probably the most accessible invertebrates living in the gravel interstices on nesting grounds at the moment of emergence, providing over 80% of the energetic input [13]. However, although chironomid larvae and baetid nymphs seem to be the most important food items for newborns, newly emerged brown trout fry can show differences in the selection of these prey items. Although Baetidae is abundant in the benthos, this taxon is negatively selected according to Ivlev's selectivity index, whereas Chironomidae remains positively selected (Figure 2), demonstrating that abundance of prey items in the benthos is not the only factor explaining the complex mechanism that operates in the food selection during this phase of the ontogeny. Thus, prey size may affect the prey ingestion in early fish larvae, and much literature focuses on the relationship between prey size and mouth size as the primary factor of prey selection [e.g. 36]; but in general, other factors apart from size, such as locomotor skills of fish or accessibility and antipredator behaviour of prey items play an important role in feeding behaviour. These hypotheses that could explain the absence of some items in the stomachs in spite of their abundance in the benthos [13].

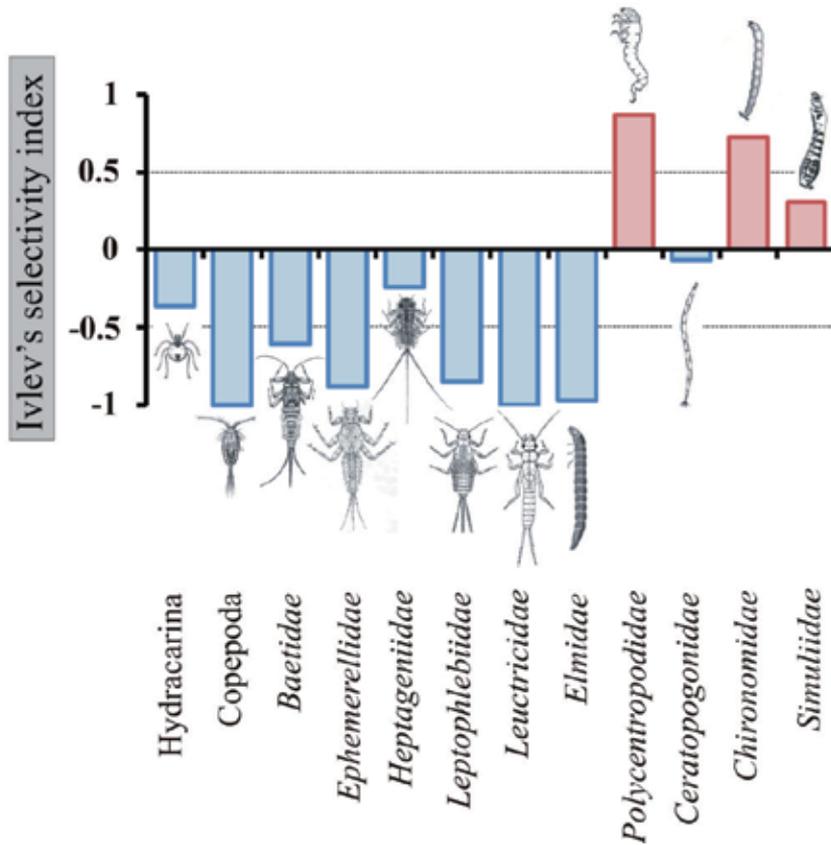


Figure 2. Prey selectivity according to Ivlev's selectivity index of newly emerged brown trout fry in the River Iso (NW Spain) (modified from [13]).

The feeding diversity of juvenile fishes is generally greater than during the larval period, and there is often an increase in the importance of species-specific dietary traits [4]. However, recent studies have demonstrated that at the moment of complete yolk absorption, the fry shows a dramatic shift in niche breadth, which might be related to the improvement of swimming and handling ability of fry for capturing and ingesting both aquatic invertebrates and aerial imagoes [13].

A common practice in many countries associated with river restoration is the rehabilitation of spawning sites with different techniques [37], but recently different authors have emphasized the importance of the complete recolonization of spawning grounds by benthic macroinvertebrates, including first instars, in order to assure the presence of the required amount of prey for the feeding of young fry after restoration works [13]. Hence, at the moment of hatching, a

certain density of small prey should be present in the gravel, as searching for food is limited to the nest area and fry forage on available prey [13].

4. Diet changes with age: Food resource partitioning and change in piscivorous behaviour

In Salmonid populations, dominant fishes may exclude less aggressive individuals, limiting their access to resources within patches. For example, dominant Atlantic salmon *Salmo salar* Linnaeus, 1758 may exclude subordinates from high-quality patches by intimidation or direct aggression [38,39]. However, subordinate fish may gain access to food by using high-quality patches when dominants are absent [40] or may be constrained to foraging in marginal areas [41]. When brown trout and Atlantic salmon co-occurred, trout has been observed to be dominant over salmon, holding feeding stations by swimming actively in the central regions of food patches, whereas salmon occupied the margins, generally remaining stationary on the stream bed [41]. Moreover, in habitats in which food is patchily distributed in time and space, fishes can benefit by moving between patches [42,43], with subordinate animals moving little in comparison with dominant fishes [39].

During their life history brown trout undergo ontogenetic habitat shifts [44 and references therein] due to changes in habitat selection operating at multiple spatial scales [44]. These shifts during fish life stage transitions may be accompanied by a marked reduction in intra-specific competition in the fish population, facilitating the partitioning of resources [e.g. 45,46]. Moreover, dietary analyses usually show high values of diet overlap among age classes, but the differences in the use of feeding habitat and behavioural feeding habits are important adaptive features that may reduce the intra-specific competition in the population [23]. Thus, although the diet comparison among age classes can show a remarkable similarity in their prey utilization patterns, sometimes the high overlap values may not indicate competition, since fishes can adopt different strategies to overcome competence, i.e. resource partitioning among age classes can occur at five different levels: (1) diet composition; (2) prey selection; (3) prey size; (4) habitat utilization for feeding; and (5) niche breadth. Also stomach fullness can vary among age classes as shown in section 4.6.

4.1. Changes in diet composition with age

In brown trout, as in many other fish species, there is normally a change in the diet composition during the life of the fish. Thus, juveniles mainly consume prey items linked to the bottom of the river, many of them interstitial, i.e. living among grains of sand or gravel. Opposite, terrestrial invertebrates and fishes are important resources for large trouts. The contribution of these food items to fish diets increases with predator size or age because larger fish can feed on a wider range of preys as shown in Figures 3 and 4. Within a population, the percentage of the most important prey items change with age. In one study of a river in Italy the percentage of plecopteran nymphs in the diet tended to increase with the individual's age [17]. In another study, *Baetis* spp. dominated in all age classes in different proportions, whereas the percentage

of caddisflies with cases, (*Allogamus* sp.) and mayflies (*Ecdyonurus* spp.) tended to increase with age [23]. Thus, as shown in Table 1, each age class consumes significantly different prey items, Chironomidae being the most frequently consumed prey item in age 0+ and age 3+ (44.41% and 29.84% respectively) and aerial imagoes of Ephemeroptera in age 1+ and age 2+ (77.49% and 49.08% respectively).

Regarding the changes in the proportion of terrestrial invertebrates consumed by trouts during the ontogeny, previous studies have demonstrated that aquatic invertebrates dominated the diet in all age classes [23,47], but it seems clear that terrestrial invertebrates were more frequently consumed by older trout [17,23,48], terrestrial organisms being important prey during warmer seasons in salmonids [49,50].

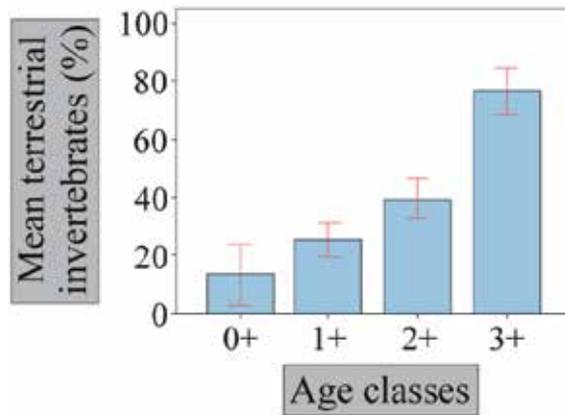


Figure 3. Percentage of terrestrial invertebrates (in terms of relative abundance) consumed by each age class of *Salmo trutta* in the River Anllóns (NW Spain) during summer. Error bars represent 95% confidence intervals.

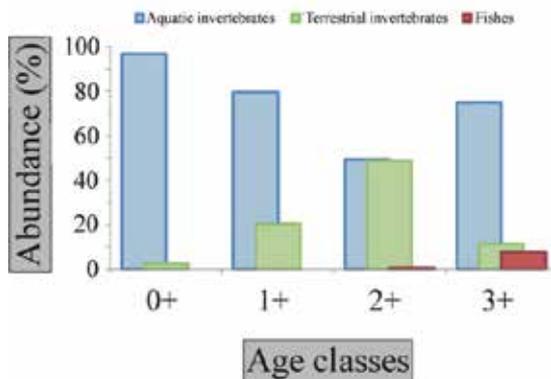


Figure 4. Diet composition consumed by each age class of *Salmo trutta* in the River Lengüelle (NW Spain) during summer.

	Age 0+			Age 1+			Age 2+			Age 3+		
	Diet	Ivlev		Diet	Ivlev		Diet	Ivlev		Diet	Ivlev	
	(%)	Benthos	Drift									
Aquatic invertebrates												
Oligochaeta gen. sp.	0.14	-0.68	1	0	-1	—	0	-1	—	0	-1	—
Ancyliidae	2.07	0.04	1	0.15	-0.85	1	0	-1	—	0	-1	—
Hydrobiidae	2.48	0.90	0.82	2.11	0.88	0.79	0.92	0.74	0.58	4.84	0.95	0.90
Lymnaeidae	1.66	-0.18	-0.01	1.96	-0.10	0.07	20.64	0.79	0.85	29.03	0.85	0.89
Sphaeriidae	0	-1	—	0.53	0.59	1	1.38	0.82	1	0	-1	—
Hydracarina gen. sp.	0.41	0.64	-0.61	0.23	0.43	-0.76	0	-1	-1	0	-1	-1
Ostracoda gen. sp.	0.55	1	1	0.23	1	1	0	—	—	0	—	—
Baetidae	1.93	0.49	-0.06	0.15	-0.63	-0.87	8.72	0.86	0.60	0	-1	-1
Caenidae	5.10	0.94	0.50	0.60	0.59	-0.48	0.46	0.49	-0.57	0	-1	-1
Ephemerellidae	0	-1	-1	0.23	-0.77	-0.86	2.29	0.14	-0.12	0	-1	-1
Ephemeridae	0	—	—	0.08	1	1	0	—	—	0	—	—
Leuctridae	0.55	0.85	1	0.08	0.25	1	0.46	0.82	1	0	-1	—
Aeshnidae	0	—	—	0.08	1	1	0	—	—	0	—	—
Calopterygidae	6.07	1	1	3.78	0.45	0.17	8.72	0.72	0.53	0	-1	-1
Coenagrionidae	0.28	0.61	1	0	-1	—	0	-1	—	0	-1	—
Gomphidae	0.14	-0.13	1	0	-1	—	0	-1	—	0	-1	—
Aphelocheiridae	0.41	-0.27	-0.28	0.08	-0.81	-0.81	0	-1	-1	0.81	0.05	0.05
Gerridae	0.14	1	-0.28	0	—	-1	0	—	-1	0.81	1	0.54
Sialidae	0.14	1	1	0.23	1	1	0	—	—	0	—	—
Dytiscidae	0	—	—	0	—	—	0.46	1	1	0	—	—
Elmidae	0.41	-0.58	-0.56	0.15	-0.83	-0.81	0.46	-0.55	-0.52	0	-1	-1
Brachycentridae	0	-1	—	0.08	-0.64	1	0	-1	—	0	-1	—
Hydropsychidae	2.76	-0.61	0.17	0.45	-0.92	-0.62	0.92	-0.85	-0.36	0.81	-0.87	-0.41
Leptoceridae	0.28	1	1	0	—	—	0	—	—	0	—	—
Limnephilidae	0.55	0.92	1	0.45	0.91	1	0.46	0.91	1	0.81	0.95	1
Philopotamidae	0.14	0.51	1	0	-1	—	0	-1	—	0	-1	—
Polycentropodidae	0	-1	—	0.30	0.25	1	0	-1	—	0	-1	—
Rhyacophilidae	0	-1	—	0	-1	—	0.46	0.49	1	0	-1	—
Sericostomatidae	0.14	1	1	0.15	1	1	0.46	1	1	0	—	—
Chironomidae	44.41	0.31	0.18	4.08	-0.71	-0.76	1.83	-0.86	-0.89	29.84	0.12	-0.01
Simuliidae	15.17	-0.51	-0.18	1.89	-0.92	-0.84	0.92	-0.96	-0.92	12.90	-0.56	-0.26
Tipulidae	0	-1	—	0.08	0.54	1	0	-1	—	0	-1	—
Terrestrial invertebrates												
Ephemeroptera gen. sp.	12.14	—	1	77.49	—	1	49.08	—	1	2.42	—	1
Trichoptera gen. sp.	0.14	—	-0.28	0.45	—	0.30	0	—	-1	0	—	-1
Chironomidae	0.69	—	-0.93	2.04	—	-0.82	0	—	-1	13.71	—	-0.20
Simuliidae	0.69	—	1	0.68	—	1	0	—	—	2.42	—	1

	Age 0+			Age 1+			Age 2+			Age 3+		
	Diet (%)	Ivlev		Diet (%)	Ivlev		Diet (%)	Ivlev		Diet (%)	Ivlev	
		Benthos	Drift									
Arachnida gen. sp.	0	—	—	0.08	—	—	0	—	—	0	—	—
Acanthosomatidae	0	—	—	0.08	—	1	0.46	—	1	0	—	—
Psyllidae	0	—	—	0.15	—	1	0	—	—	0	—	—
Chloropidae	0.14	—	-0.28	0	—	-1	0	—	-1	0	—	-1
Diptera gen. sp.	0	—	-1	0.08	—	-1	0	—	-1	0	—	-1
Syrphidae	0	—	-1	0	—	-1	0	—	-1	0	—	-1
Xylomyidae	0	—	—	0.15	—	1	0	—	—	0	—	—
Cynipidae	0	—	-1	0	—	-1	0	—	-1	0.81	—	0.25
Formicidae	0	—	-1	0	—	-1	0	—	-1	0.81	—	0.54
Carabidae	0	—	—	0.08	—	1	0	—	—	0	—	—
Chrysomelidae	0.28	—	-0.28	0	—	-1	0	—	-1	0	—	-1
Coleoptera gen. sp.	0	—	—	0	—	—	0.46	—	1	0	—	—
Other prey items												
<i>Pseudochondrostoma duriense</i>	0	—	—	0.45	—	—	0.46	—	—	0	—	—
Eggs	0	—	—	0.15	—	1	0	—	—	0	—	—

Table 1. Diet composition and prey selection according to Ivlev's selectivity index in each age class of *Salmo trutta* in the River Furelos (NW Spain) during summer.

The maximum and mean prey size eaten generally increases with size in predatory fish species [51]. Piscivorous behaviour is most frequent in large brown trout, and studies show that it occurs in older individuals with a size of 20–30 cm. Trout in smaller size classes have rarely/never been recorded eating other fish [23,26,52,53]. In contrast, Sánchez-Hernández and collaborators have recorded piscivorous behaviour in an age-0 trout (the individual found was 8.5 cm in fork length) [29]. This behaviour could be related with the hypothesis of Mittelbach and Persson, who stated that fish species that had larger mouth gapes became piscivorous at younger ages and at smaller sizes [51], and demonstrating that mouth gape is not a limitation to use fishes by small trouts [29]. One possible advantage for small trout in diversifying into eating fish is a reduction in competition with other individuals in the same size classes.

The feeding strategy among age classes may be illustrated with graphical methods, such as the modified Costello graphical method [20] based on the relative importance of prey species. Figure 5, shows plots of prey abundance (A_i) against frequency of occurrence of prey in the diet (F_i) for three different age classes. The plots show feeding strategy differs between age classes, varying in degrees of specialization and generalization on different prey types. For all age classes, *Baetis* spp. were the most important prey, always being eaten by more than eighty-five percent of the individuals ($F_i = 87.5\%$ in age-1+ to 100% in age-2+) and represented a high contribution in specific abundance ($A_i = 23.93\%$ in age-1+ to 68.95% in age-0+). However, the majority of the prey items presented low values for both F_i and A_i (lower left quadrant) for all

age classes (Figure 5), displaying evidence of a generalist strategy for these prey (e.g. *Chimarra marginata*, Simuliidae (adult), Chironomidae (adult) and *Ophiogomphus* sp.).

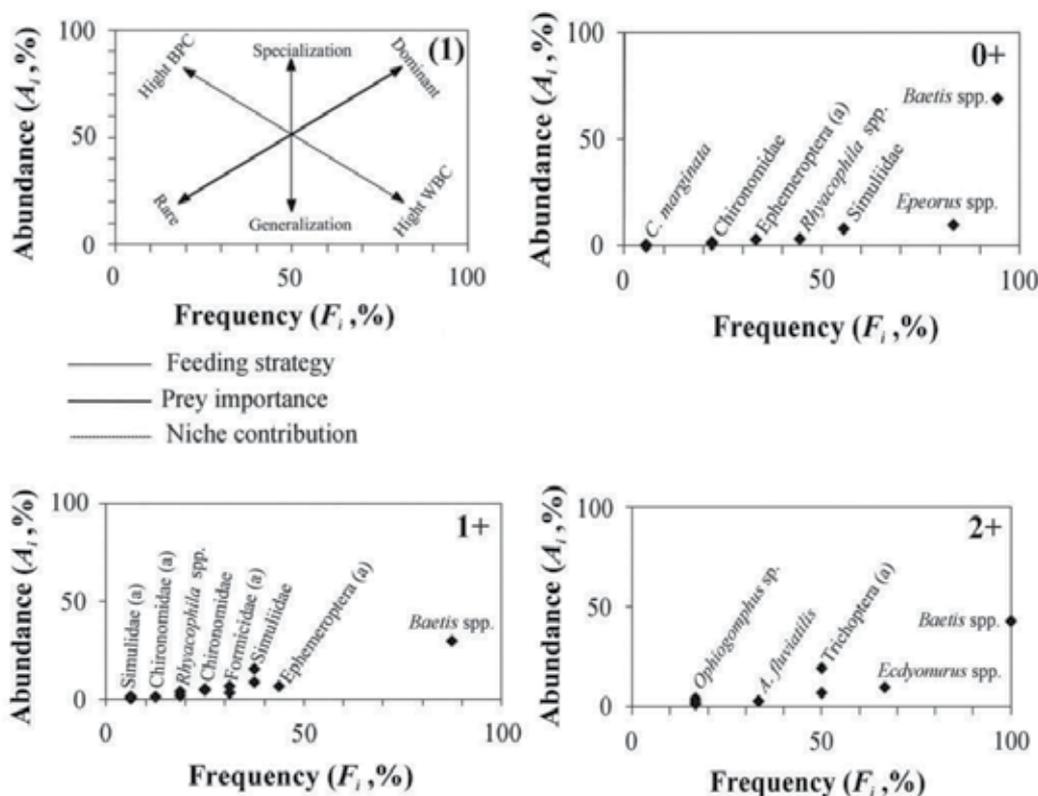


Figure 5. Feeding strategy diagram. (1) Explanatory diagram of the modified Costello method according to Amundsen and collaborators [20]. Data are presented for each age class.

Finally, although in some occasions the diet comparison among age classes can show a remarkable similarity in their prey utilization patterns, sometimes the high overlap values may not indicate competition, since the differences in the behavioural feeding habits are important adaptive features that may reduce the intra-specific competition in the population [23]. Previously, the differences in the behavioural feeding habits among age classes had been studied using prey trait analysis [23], obtaining important advances to disentangle food resource partitioning among cohorts. Details and information needed for the elaboration of prey trait analysis can be found in the introduction section and bibliography [27-29]. Thus, in the reference [23] 'diel drift behaviour' trait showed that age-2+ is clearly separated from the other age classes. Age-2+ tended to feed on prey with no tendency in diel drift behaviour as shown by the presence of *Ancylus fluviatilis*, Gerridae, Coleoptera and Formicidae in their stomachs. On the contrary, age-0+ preferred to feed on prey with nocturnal diel drift behaviour tendency due to the presence of Philopotamidae (*C. marginata*). Age-0+ showed a wider

distribution of values in the fuzzy principal component analysis (FPCA) of the ‘trajectory’ trait; age-0+ tended to feed on prey with oscillatory and by random trajectory (Lumbriculidae, Simuliidae and Chironomidae), whereas age-2+ tended to feed on prey with lineal trajectory due to the presence of Gerridae [23]. On the other hand, no clear differences have been found in the traits ‘tendency to drift in the water column’ and ‘tendency to drift at the water surface’ for prey among age classes [23], however as shown in Figure 6 (fuzzy principal component analysis calculated with the values reported in Table 1), age-3+ is clearly separated from the other age classes, showing that the differences ability to feed at different depths of the water column is possible among cohorts.

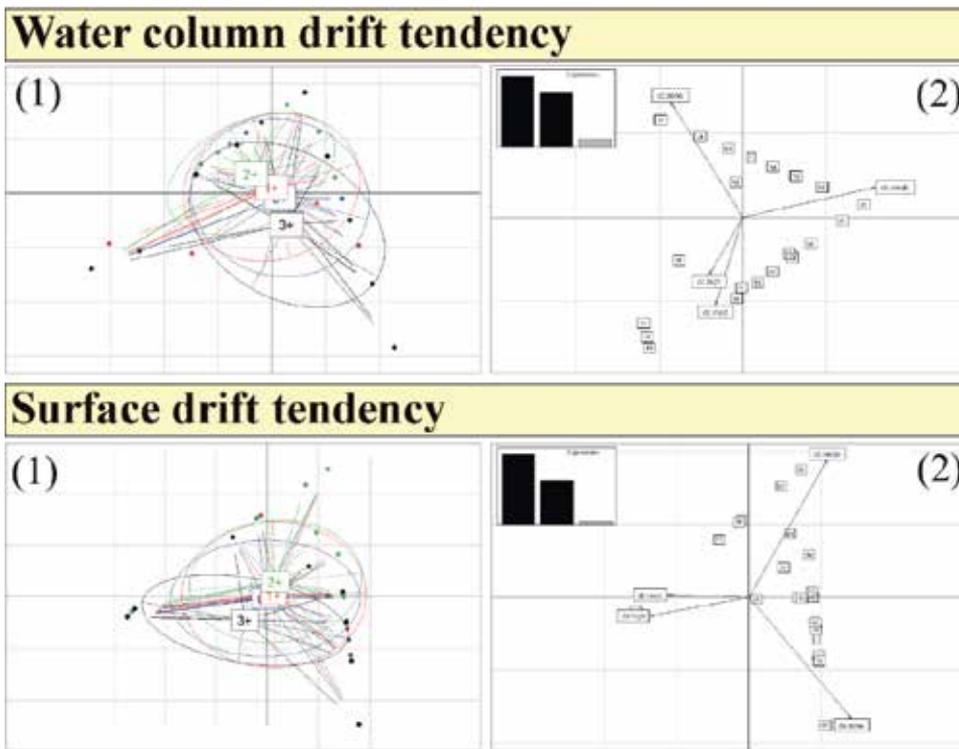


Figure 6. Biplot of gut contents obtained from a fuzzy principal component analysis (FPCA) based on behavioural feeding habits of the four age classes. (1) Similarity results among age classes according to the gut contents of Table 1. Data are presented for each age class. 0+: age-0+, 1+: age-1+, 2+: age-2+ and 3+: age-3+. Ellipses envelop weighted average of prey taxa positions consumed by age classes: Labels (0+, 1+, 2+ and 3+) indicate the gravity centre of the ellipses (2) Factorial correspondence analysis between both traits and prey items and their spatial distribution with histogram of eigenvalues. Details needed for the elaboration of these graphics can be found in the introduction section and bibliography [27-29].

4.2. Changes in prey selection with age

Prey abundance should be an important factor involved in prey choice. However, fishes do not always consume the most abundant taxa available in the environment [13,29,54]. The total

abundance and biomass of invertebrates drifting during the day describe the potential prey available to juvenile brown trout better than abundance and biomass of benthic invertebrates do [55]. Different authors have shown that active choice guided by energetic optimization criteria appeared to be of limited importance in determining the size composition of prey eaten by trouts [11]. These authors also stated that the operating mechanisms of prey-size selection are probably not independent of the characteristics of the size-frequency distribution of the available prey. Moreover, in brown trout spatial and temporal variations in prey selection are possible, due to the preference for the different prey items related to the site-specific prey accessibility [12]. As shown in section 3, the size-frequency distribution of potential prey items in the benthos was different to that of prey in the stomachs of newly emerged brown trout [13], a result that agrees with observations made in other fish species [e.g. 54] and observations made in age-0+ individuals of *S. trutta* during the autumn [29]. In spite of the high abundance of Elmidae in the benthos, this prey item was negatively selected [29]. The rejection of the elmid beetle may be due to their low energetic value, as they have an intense sclerotisation, but it may also be due to their bad taste [56-58]. Hence, other factors, besides prey abundance, including site-specific prey accessibility, prey size, energetic selection criteria and prey preference of fishes, play an important role in the feeding behaviour of freshwater fishes [54].

Fochetti and collaborators found a high preference for species of Trichoptera by trout younger than 3+, a preference for plecopteran species by those older than three years, and a general negative avoidance for species of Ephemeroptera by all age classes [17]. As shown in Table 1, prey selection is clearly different among age classes and important prey items such as Chironomidae in age 0+ and age 3+ and aerial imagoes of Ephemeroptera in age-1+ and age-2+ are positively selected in the benthos and drift respectively. Overall, as different researchers have demonstrated, the mechanisms involved in prey selection among age classes in the same population are complex and may be related at different levels: (1) prey selection in fishes is related to prey characteristics, such as size, locomotor skills, accessibility or anti-predator behaviour, (2) prey selection in fishes is related to fish characteristics, like prior experience, locomotor skills, stomach fullness, mouth gape, sensory capabilities and fish size and (3) prey selection in fishes is related to physical habitat characteristics, as flow patterns and structural complexity of habitat [12,13,34,54].

4.3. Changes in prey size with age

Ontogenetic dietary shifts may also occur at the level of prey size [23,54]. Steingrímsson and Gíslason showed a consistent, but moderate, shift towards larger prey with increased body size in brown trout [59]. Several researchers have found that mean prey size increases as predator size increases [e.g. 23,48,54,60]. The size-frequency distributions of the available terrestrial prey were always greatly dominated (75–90%) by the two smallest size classes (1–2 and 2–3 mm long), prey over 4 mm long being extremely scarce, while size distributions of aquatic prey were less skewed [11]. Thus, in general it is correct to state that trout fed mainly on prey within 1–4 mm size range [11,48], with 2–3 mm prey being the most commonly consumed [48]. On the contrary, Rincón and Lobón-Cerviá showed that organisms 1–2 mm long were generally the most numerous [11]. By age classes the average prey size consumption

is different with higher values in age-2+ ($8.4 \text{ mm} \pm 1.62$) than age-0+ ($4.2 \text{ mm} \pm 0.25$) and age-1+ ($5.9 \text{ mm} \pm 0.51$), but there are no significant differences between age-0+ and age-1+ [23]. Figure 7 illustrates the age-related variation in prey size, showing that mean prey size tends to increase with age.

In conclusion, prey-size selection is probably dependent on the characteristics of the size-frequency distribution of the available prey [11], and the size-related differences in the diet of trout can be related to gape-limitations, increasing mean prey size and maximum prey-size with trout size [48].

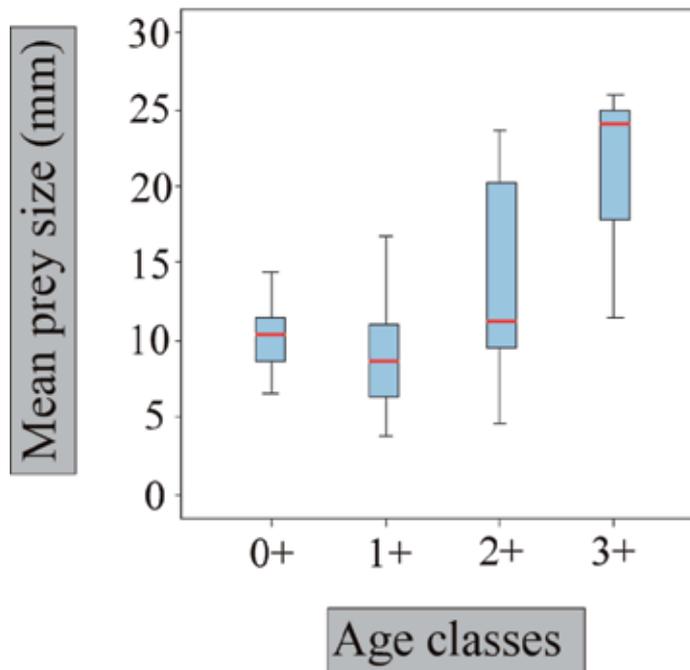


Figure 7. Box plots of the age-related variation in prey size of *Salmo trutta* in the River Furelos (NW Spain) during summer. The solid line within each box represents the median, the bottom and top borders indicate the 25th and 75th percentiles, the notches represent the 95% confidence intervals.

4.4. Changes in the habitat used for feeding with age

In fishes, patches used for feeding and refuges are normally different, as shown by several researchers [61-63], due to brown trout being a habitat generalist. Also, patterns in habitat selection have been shown to be driven by physical and environmental factors operating at multiple spatial scales [44].

Many organisms exhibit ontogenetic shifts in their diet and habitat use, which often exert a large influence on the structure and expected dynamics of food webs and ecological commu-

ities [64]. Special attention has been given to ontogenetic shift in habitat preference in brown trout populations [e.g. 44,65-67]. It is well-known that in brown trout populations habitat use changes during ontogeny, preferring deeper and slower flowing water as they increased in size [e.g. 44,65].

Habitat patches used by brown trout can be monitored by radio telemetry [e.g. 68] and although these studies have shown that brown trout feed on young white suckers *Catostomus commersonii* (Lacepède, 1803) at night in shallow habitats, little information was obtained about the habitat used for feeding. Also, microhabitat use of freshwater fishes has been studied by snorkel observations in previous studies [e.g. 69]. This methodology could be used to study feeding habitat requirements of fish species. However, there are still gaps to be filled before snorkel surveys can be fully adopted in fish diet studies. In fact, one of the main disadvantages of this approach is the need for good visibility. This has been one of the main handicaps because, although brown trout normally yields a bimodal (crepuscular) pattern of activity with a major peak at dawn and a lower one around dusk [70], brown trout can also feed at night [71]. Another limiting factor for the application of snorkel surveys to study feeding habitat requirements is related to the physical characteristic of the river such as current, depth or turbidity. It is well-known that different macroinvertebrates have different preferences for habitats [72] and so prey trait analysis has been proposed as a functional approach to understand mechanisms involved in predator-prey relationships [27-29]. Consequently it may be useful for understanding inter-species interactions and the mechanisms that determine food partitioning between them [29,30]. Nowadays, they have been recently used to provide interesting results about differences in feeding habitat requirements among age classes in brown trout [23], for example, young of the year (0+) tend to capture prey living in moderate current velocities, whereas other age classes (1+ and 2+) tend to feed on prey living in fast current velocities [23].

A previous study on habitat choice in a littoral zone of Lake Tesse (Norway) showed that small trout had a strong association with the bottom and larger trout occurred more frequently higher up in the water column, and suggested that this difference in vertical distribution was also reflected in food choice [73]. In streams, competition among fish species may also be reduced by vertical segregation [e.g. 41,69,74]. In spite that terrestrial invertebrates, as component of the diet, are more important in adults than juveniles of brown trout [23], these same authors found that the ability to feed at different depths of the water is similar among age classes. Nevertheless, it is possible that different age classes of fish may become vertically segregated by concentrating on different prey types living in different parts of the water column as shown in section 4.1. Hence, additional studies are needed in order to clarify whether vertical segregation among cohorts is related to the ability to feed at different depths of the water column.

Finally differences in the use of feeding habitat are important adaptive features that may reduce the intra-specific competition in the population. In this context, fuzzy principal component analysis (FPCA) has shown that age-0+ tended to feed on prey living in moderate current velocities, although overlap was higher between age-1+ and age-2+, preferring to feed on prey living in fast current velocities [23]. Moreover, these researchers have found that age-0+ showed a higher spectrum of prey, which revealed a greater ability to prey on different

macrohabitats, whereas age-1+ and age-2+ preferred to feed on epibenthic prey living in erosional macrohabitats. In our case with the values reported in Table 1 and as shown in Figure 8, 'current velocity' trait shows no clear differences for prey of the four age classes. On the contrary, 'macrohabitat' trait shows that age-1+ has the most ample spectrum for this trait, tending to feed on epibenthic prey living in erosional macrohabitats, whereas age-3+ tends to feed on prey items available in the water column (Figure 8).

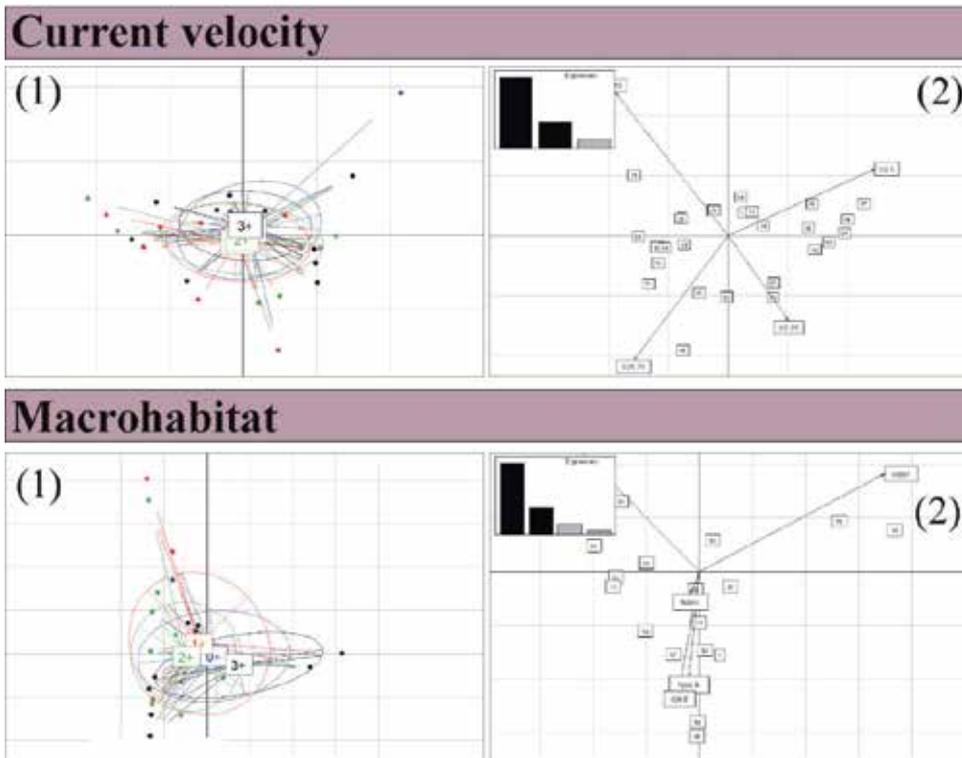


Figure 8. Biplot of gut contents obtained from a fuzzy principal component analysis (FPCA) based on preferential habitat utilization for feeding of the four age classes. (1) Similarity results among age classes according to the gut contents of Table 1. Data are presented for each age class. 0+: age-0+, 1+: age-1+, 2+: age-2+ and 3+: age-3+. Ellipses envelop weighted average of prey taxa positions consumed by age classes: Labels (0+, 1+, 2+ and 3+) indicate the gravity centre of the ellipses. (2) Factorial correspondence analysis between both traits and prey items and their spatial distribution with histogram of eigenvalues. Details needed for the elaboration of these graphics can be found in the introduction section and bibliography [27-29].

4.5. Changes in the niche breadth with age

Deady and Fives showed that niche breadth decreases with fish length in corkwing wrasse, *Symphodus (Crenilabrus) melops* (Linnaeus, 1758), indicating an increase in dietary specialization with increasing length [75]. Magalhães found dietary shifts throughout the ontogeny in an endemic cyprinid of the Iberian Peninsula (*Squalius pyrenaicus* (Günther, 1868)), including

shifts from soft-bodied to hard-shelled prey and decreased animal prey breadth [76]. In contrast, several other researchers have found that niche breadth increases with body size [e.g. 23,77]. Oscoz and collaborators found that larger fish have a higher number of potential prey items available and a wider niche breadth, as indicated by their higher trophic diversity index values [77]. As mentioned in section 3, analysis of diet changes on newly emerged brown trout fry suggests a dramatic shift in niche breadth at the moment of complete yolk absorption, which might be related to the improvement the fry's swimming and handling ability in capturing and ingesting prey [13]. In a recent study on brown trout populations, it has been demonstrated that niche breadth, measured as Levin's index, increases with fish length in *Salmo trutta*. However, no differences were found in the Shannon diversity and evenness indices of prey eaten among age classes [23].

As can be seen in Figure 9; the number of different prey types consumed by *S. trutta* increases during ontogeny. Age-0+ shows the smallest prey spectrum, whereas in age-1+ a significant increase in the prey types consumed by juveniles is observed. However, no significant differences in the dietary niche among ages-1+, 2+ and 3+ are observed in Figure 9. Hence, although no clear results have been observed in the variation of the diversity indices of prey among age classes [23], it could be argued that there is a tendency to increase dietary niche with increasing length or age, at least when the niche breadth of juveniles (0+), subadults (1+) and adults ($\geq 2+$) is compared.

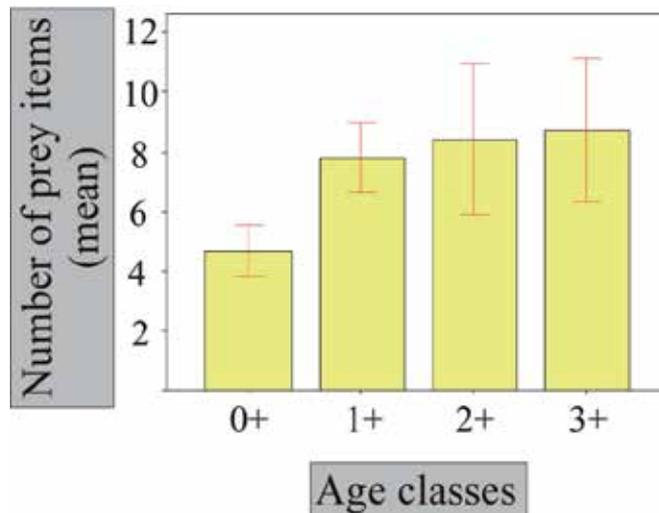


Figure 9. Age-related variation in the number of different prey types consumed by *Salmo trutta* in the River Lengüelle (NW Spain) during summer. Error bars represent the 95% confidence intervals.

4.6. Changes in the fullness index with age

Several researchers have found in different fish species that the stomach fullness index (defined as the weight of the stomach contents (in grams) divided by the weight of the predator (in

grams) and multiplied by 100) varies during ontogeny [78,79]. In salmonids the results are contradictory: in brook charr *Salvelinus fontinalis* (Mitchill, 1814) no differences have been found in the stomach fullness [80], whilst other researchers have demonstrated that the stomach fullness of brown trout varies among size classes [47]. Brown trout between the size of 40 mm and 320 mm fed more intensively, whilst the intensity declined above 320 mm length [47]. In the River Furelos (NW Spain), we have found that stomach fullness during the summer is different among age classes (Kruskal-Wallis test; $p < 0.001$), being higher in age-0+ ($9\% \pm 0.64$) than age-1+ ($1.1\% \pm 0.14$), age-2+ ($1\% \pm 0.25$) and age-3+ ($1.1\% \pm 0.24$) (all Mann-Whitney U test, $p < 0.001$) but no differences have been found between ages-1+, 2+ and 3+ (all Mann-Whitney U test, $p > 0.05$). Moreover, stomach fullness decreases with fish size ($r = -0.72$; $p < 0.001$) (unpublished data). Hence, stomach fullness can vary among age classes; however additional studies are needed in order to clarify whether stomach fullness varies during ontogeny in brown trout.

5. Competition for food between brown trout and other sympatric fish species

Trophic interactions between species are important factors structuring animal communities. Brown trout are top-consumers in freshwater habitats and play an important role as carriers of energy from lower to higher trophic levels (i.e. predators). Many freshwater fish species tend to occupy a specific type of habitat but there are lots of exceptions. For example, spatial niche overlap is considerable where Atlantic salmon and brown trout co-occur, although young Atlantic salmon tend to occupy faster flowing and shallower habitats [e.g. 74]. Moreover, when both fish species co-occur, the habitat used by Atlantic salmon is restricted through interspecific competition by the more aggressive brown trout, indicating an interactive segregation between fish species [e.g. 41,74]. Indeed, it is well known that brown trout is a territorial drift feeder [3,81], and several authors have reported the behavioural dominance of trout over cyprinids in streams [82-84].

The competitive coexistence between species occupying similar niches may be facilitated by a generalisation of niche width as predicted by the optimal foraging theory (OFT), rather than the specialised niche width predicted by the classic niche theory as a response to interspecific competition [85]. However, studies on food partitioning in fish communities have obtained contradictory results. Whereas several authors have found differences in diet composition among sympatric fish species [e.g. 86,87], other researchers concluded that the same food resource can be shared by several species [29,30,85]. In these cases, the differences in behavioural feeding habits, handling efficiency and feeding habitat utilization are important adaptive features that may reduce the inter-specific competition in the fish community and permit the partitioning of food that allows coexistence [29,30]. Thus, sympatric fish species can adopt different strategies to overcome competition and food resource partitioning can occur at different levels.

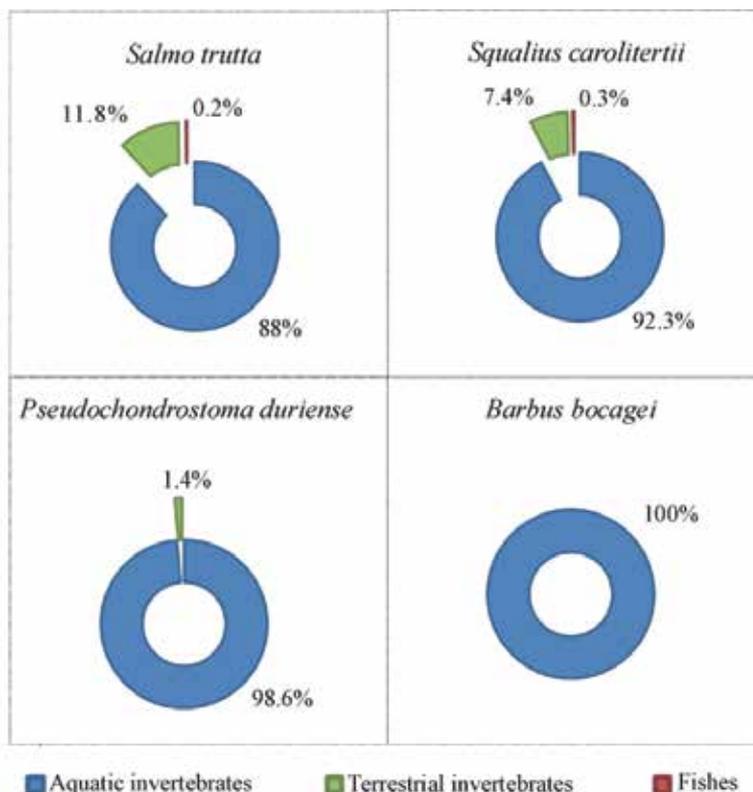


Figure 10. Diet composition consumed by each fish species in the Tormes River (Central Spain) during summer.

Firstly, the use of microhabitats is often different between species, due to segregation of microhabitats, an important factor in reducing the effects of competition for food [69,88,89]. For example, *Barbus bocagei* Steindachner, 1865 occupied deeper habitats and selected lower positions in the water column than *Pseudochondrostoma polylepis* (Steindachner, 1865), and *Squalius pyrenaicus* (Günther, 1868), *P. polylepis* occupied microhabitats with greater velocities than the other two species and *S. pyrenaicus* selected shallower habitats than the other two species [69]. In another study, *S. trutta* showed wider diversity in the habitat used for feeding than *Squalius carolitertii* (Doadrio, 1988), *Pseudochondrostoma duriense* (Coelho, 1985) and *B. bocagei* [30]. Hence, differences were found among species in their ability to feed at different depths of the water column [29,30] as shown in snorkelling studies into microhabitat use in fish [69].

Secondly, different species may specialise in different resources. For example, many cyprinid fish sympatric with trout feed on a significant amount of detritus and plant material not used by trout, leading to reduced inter-specific competition [29,30]. Moreover, resource partitioning may also occur at the level of prey size [29,30,90], although it is not clear whether this size selective strategy is adopted to reduce interspecific competition or it is the result of foraging

behaviour and/or morphological constraints such as gape size [29,91]. Also, terrestrial prey are present primarily on the stream surface and although tend to be absent from the diets of benthic feeders such as *B. bocagei* (Figure 10), terrestrial inputs may constitute an important food resource for freshwater fish species and especially for brown trout. Thus, the utilization of allochthonous food resources such as terrestrial invertebrates by fishes may reduce competition facilitating the partitioning of resources [30].

Thirdly, diel segregation is possible among fish species, and this may also lead to reduced interspecific competition between fish [29,92,93]. According to macroinvertebrate trait analyses, sticklebacks (*Gasterosteus aculeatus* Linnaeus, 1758) and *P. duriense* show a slight preference for prey that drift during the day, whilst age-0 *S. trutta* seem to prefer to feed at dusk, whereas *Achondrostoma arcasii* (Steindachner, 1866) differs from the other three species due to its preference to feed on prey on organisms with weak or no tendency to drift [29]. However, the “diel drift behaviour” of macroinvertebrate prey of brown trout and three sympatric cyprinids is similar [30]. Hence, the differences in the diel feeding behaviour among sympatric fish species might only be adopted in highly competitive communities, where food is a more limiting resource.

6. Conclusion

To summarize, the present study supports the hypothesis differences in the feeding habits and habitat utilization of different age classes of trout could reduce competition for food, by allowing food resource partitioning. Hence, age-related diet shifts occur at five different levels: (1) diet composition changes with fish age; (2) prey selection varies with fish age, probably due to prey-size selection which is in turn dependent on the size-frequency distribution of the available prey; (3) mean prey size increases with fish size and age; (4) habitat utilization for feeding may be different among age classes; (5) niche breadth tends to increase with age and fish size. Finally, also the stomach fullness can vary among age classes. However, additional studies are needed in order to clarify whether stomach fullness varies during the ontogeny in brown trout.

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Edited by Hakan Türker

This book provides an understanding on a large variety of related topics in fish biology.

The further development on molecular and cellular biology and ecology leads to assimilate the newer scientific knowledge in this area. Leading research works from around the world are brought together in this book to produce a valuable source of reference for teachers, researcher, and advanced students of biological science. The first three chapters of this book give a general description of the complex biology of the immune response. Detailed descriptions were also included on understanding of cytokine regulation in teleost immune system. The second three chapters provide information on the environmental stressors on the responses of freshwater fish across molecular to population level. Then, the following two chapters review two special topics; the roles of the atrium and the ventricle across teleost species and the tracer methodologies on the measurements of carbohydrate metabolism. The last chapter discusses the variables that are involved in the feeding behavior of a predatory freshwater fish species.

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