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University Postgraduate Interdisciplinary Doctoral Study of Molecular Biosciences

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Molecular characterization and expression analysis of pro-inflammatory cytokines IL-1β, TNFα1 and TNFα2 in cage-reared Atlantic bluefin tuna *Thunnus thynnus* (L. 1758)

PhD THESIS

Osijek, 2017

BASIC DOCUMENTATION CARD

Josip Juraj Strossmayer University of Osijek **PhD thesis**
University of Dubrovnik
Ruđer Bošković Institute
University Postgraduate Interdisciplinary Doctoral Study of Molecular Biosciences

Scientific Area: Natural Sciences
Scientific Field: Biology

Molecular characterization and expression analysis of three pro-inflammatory cytokines IL-1β, TNFα1 and TNFα2 in cage-reared Atlantic bluefin tuna *Thunnus thynnus* (L. 1758)

Ivana Lepen Pleić

Thesis performed at: Laboratory of Aquaculture, Institute for Oceanography and Fisheries, Croatia; Department of Zoology, University of Aberdeen, Scotland, United Kingdom

Supervisor/s: Ivona Mladineo, PhD

Short abstract:

Atlantic bluefin tuna *Thunnus thynnus* is one of the largest migratory fish species with great economic significance for Croatian industry. The first full-length mRNA and gDNA sequences of *T. thynnus* cytokines IL-1β, TNFα1 and TNFα2 were cloned and characterized within this study. The expression analysis of three cytokines was performed in selected tissues of fish reared over the period of two years; in peripheral blood leukocytes stimulated *in vitro* with LPS, Poly I:C and parasite protein extract; and in gill tissue during natural parasite infection.

Number of pages: 169
Number of figures: 39
Number of tables: 5
Number of references: 385
Original in: English
Key words: Atlantic bluefin tuna; innate immunity; tumor necrosis factor α; interleukin 1β; Digenea; Copepoda; parasite protein extracts

Date of the thesis defense: 03.11.2017.

Reviewers:
1. Đurđica Ugarković, PhD
2. Neda Slade, PhD
3. Željka Trumbić, PhD
4. Jasna Maršić-Lučić, PhD (substitute)

Thesis deposited in: National and University Library in Zagreb, Ul. Hrvatske bratske zajednice 4, Zagreb; City and University Library of Osijek, Europska avenija 24, Osijek; Josip Juraj Strossmayer University of Osijek, Trg sv. Trojstva 3, Osijek

TEMELJN DOKUMENTCIJSKA KARTICA

Sveučilište Josipa Jurja Strossmayera u Osijeku
Sveučilište u Dubrovniku
Institut Ruđer Bošković
Poslijediplomski interdisciplinarni sveučilišni studij Molekularne bioznanosti

Doktorska disertacija

Znanstveno područje: Prirodne znanosti
Znanstveno polje: Biologija

Molekularna karakterizacija i analiza ekspresije triju proinflamatornih citokina IL-1β, TNFα1 i TNFα2 u kavezno uzgojene atlantske plavoperajne tune *Thunnus thynnus* (L. 1758)

Ivana Lepen Pleić

Disertacija izrađena u: Laboratorij za akvakulturu, Institut za oceanografiju i ribarstvo, Hrvatska; Odsjek za zoologiju, Sveučilište u Aberdeen-u, Škotska, Ujedinjeno Kraljevstvo

Mentor/i: prof.dr.sc. Ivona Mladineo

Kratki sažetak doktorske disertacije:

Atlantska plavoperajna tuna *Thunnus thynnus* je jedna od najvećih migratornih vrsta riba s iznimnom važnošću za hrvatsku industriju. U ovoj studiji su klonirane i opisane prve čitave sekvence mRNA i gDNA citokina IL-1β, TNFα1 i TNFα2 u plavoperajne tune. Ekspresija triju citokina analizirana je u odabranim tkivima ribe tijekom dvogodišnjeg uzgoja; u leukocitima krvi stimulirane *in vitro* s LPS-om, Poly I:C-om i proteinskim ekstraktom nametnika; i u škržnom tkivu tijekom prirodne infekcije nametnicima.

Broj stranica: 169
Broj slika: 39
Broj tablica: 5
Broj literaturnih navoda: 385
Jezik izvornika: engleski
Ključne riječi: Atlantska plavoperajna tuna; urođeni imunitet; tumor nekrotični faktor α; interleukin 1β; Digenea; Copepoda; ekstrakt protein parazita

Datum obrane: 03.11.2017.

Stručno povjerenstvo za obarnu:
1. Prof.dr.sc. Đurđica Ugarković
2. Dr.sc. Neda Slade
3. Dr.sc. Željka Trumbić
4. Prof.dr.sc. Jasna Maršić-Lučić, (zamjena)

Disertacija je pohranjena u: Nacionalnoj i sveučilišnoj knjižnici Zagreb, Ul. Hrvatske bratske zajednice 4, Zagreb; Gradskoj i sveučilišnoj knjižnici Osijek, Europska avenija 24, Osijek; Sveučilištu Josipa Jurja Strossmayera u Osijeku, Trg sv. Trojstva 3, Osijek

Research presented in this PhD thesis was performed at the Laboratory of Aquaculture, Institute of Oceanography and Fisheries Split, Croatia, supervised by professor Ivona Mladineo, PhD, and at the Department of Zoology, University of Aberdeen, Aberdeen, Scotland, United Kingdom, under the supervision of professor Christopher J. Secombes, PhD. The supporting grants include the Ministry of Science: Dynamics and Pathology of Parasitofauna in Reared Marine Fish, Unity Through Knowledge Fund (UKF): Development of a Health Genomic Profile for the Captive Atlantic Bluefin Tuna (*Thunnus thynnus*) (23/08) and a personal Fellowship for Doctoral Students from the Croatian Science Foundation to Ivana Lepen Pleić.

Acknowledgments

I would like to thank my supervisor professor Ivona Mladineo for giving me the opportunity to work in this field, for her ideas and her support.

I am very grateful to professor Chris Secombes for welcoming me to his laboratory and introducing me to fish immunology. Without his kind support and advices, a large part of this thesis would not be possible. I am thankful to all the staff, colleagues and friends (Marimar, Heather, Yolanda, Ayham) at the University of Aberdeen for making me feel like home, for their unreserved help and for being a part of what turned out to be a great journey.

I am grateful to Leon Grubišić, PhD and Rino Stanić, colleagues from the Mediterranean Institute for Life Sciences (MedILS) and members of Molecular laboratory at School of Medicine (MEFST) for their assistance and technical support in different aspects of this study.

I would like to thank all my colleagues at the Institute of Oceanography and Fisheries who, at some point, participated in this study, most of all to Ivana Bušelić Garber and Tanja Šegvić Bubić, PhD for all the scientific and less scientific conversations, for their support, but most of all for being a friend when I needed one.

I am forever grateful to my husband Aljoša, for all his love, his patience, for always being the positive one and for teaching me what it means to never give up.

In the end, I want to thank my mother for her endless and unconditional love and support that she has given me through all my life and education.

I dedicate this thesis to her.

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1. Introduction

The immune system is a complex system of cellular and humoral components that protects an organism against other organisms or substances that might cause infection or disease. Its ability to recognize foreign structures and eliminate them via different molecular and cellular mechanisms makes immune system critical for all individuals. Although some important features of immune system have remained the same trough millions of years of evolution and are common for all species, numerous changes have taken place during evolution to enable generation of variability and specialization. Therefore, as new taxonomic categories appeared over time, the immune response experienced recasting. Most notably, while invertebrates relied entirely on nonadaptive innate immune system, vertebrates have developed a new memory-based immune response known as adaptive immunity (McFall-Ngai 2007; Flajnik and Du Pasquier 2008).

Fish, as the first vertebrate group appearing in evolution after adaptive radiation during the Devonian, represent the most successful and diverse group of vertebrates (Tort *et al*. 2003). This heterogeneous group is divided into three superclasses: Agnatha (jawless fish such as the hagfish and lampreys), Chrondrichthyes (cartilaginous fish such as sharks, rays and skates) and Osteichthyes (bony fish) (Zapata *et al*. 1996). Bony fish are further divided into two major classes: Sarcopterygii (lobe-finned fish) and Actinopterygii (ray-finned fish). The teleosts or Teleostei (from Greek: *teleios*; complete, *osteon*; bone) are the larges infraclass in the class Actinopterygii and accounts for 96% of all fish. The members of this group are arranged in about 40 orders and 448 families with over 26,000 species described (Benton 2005; Neara *et al.* 2012). The main reason why teleost fish are by far the most species-rich vertebrate clade is the *whole-genome duplication (WGD)* event that happened early in the life of ray-finned fish, about 350 to 450 million year ago (Volff 2005; Glasauer and Neuhauss 2014). The teleost-specific (TS) WGD resulted in discovery of numerous novel or semi-novel genes and functions in fish, known as “more genes in fish than mammals” concept (Ohno 1970). Large scale teleost genome analysis has revealed numerous gene duplications that are considered to originate from the WGD (Stein 2007). After WGD, duplicated genes go through different scenarios. First scenario is

non-functionalization of one gene duplicate due to the lack of selective restriction on preserving both duplicates. Second scenario is preservation of duplicates due to subfunctionalization (partitioning of ancestral gene functions between duplicates), neofunctionalization (assigning a novel function to one of the duplicates) and dosage selection (preserving genes to maintain dosage balance between interconnected components) (Glasauer and Neuhauss 2014). As genetic diversity translates to protein diversity, it is very likely that among all conserved functions in teleost fish there are still a lot of hidden, unique functionalities, many of which are now starting to be unraveled (Aoki *et al*. 2008). Ultimately, TS-WGD has endowed teleosts with diversification potential that can become beneficial long after WGD, e.g. during phases of environmental change (Glasauer and Neuhauss 2014).

The last decade has yielded significant advances in the understanding of inflammatory responses of, most of all, bony fish. In a number of fish species, the genes encoding hallmark immune molecules have been identified and characterized revealing high similarity to their higher vertebrate counterparts, which places this group in apparent crossroads between the innate and the appearance of the adaptive immune responses (Tort *et al*. 2003; Grayfer and Belosevic 2012).

This thesis represents the first insight into the structure and expression profiles of key innate immune genes in Atlantic bluefin tuna *Thunnus thynnus*, as one of the largest, long-lived, predatory fish species with tremendous value for Mediterranean aquaculture.

1.1. The immune system of teleost fish

Fish immune system, as in other vertebrates, can be divided into *innate (non-specific) immunity* and *adaptive or acquired (specific) immunity*. Both innate and adaptive immune responsee can be divided into *cell mediated response* and *humoral (soluble) factors*. As an initial line of defense against infection, innate immune response precedes adaptive immune response, activating it and modulating its nature. Innate and adaptive immune responses collaborate in the maintenance of homeostasis and are now considered combinational systems (Magnadóttir 2006). Innate immune system responds in general fashion to broad array of foreign stimuli and is activated immediately or within hours of the pathogen's appearance inside the host body. Therefore, it is a crucial factor in disease resistance (Secombes and Wang 2012). The adaptive response is commonly delayed but is crucial for long-

lasting immunity due its ability to produce immunological memory, therefore is a key factor in the vaccine development and preventive function of vaccines (McHeyzer-Williams and McHeyzer-Williams 2005; Sallusto and Lanzavecchia 2009; Secombes and Wang 2012). Immune response is mediated by the complex network of immune cells and soluble mediators. In order to function in the most effective manner, cells of the immune system are organized in tissues and organs collectively referred to as the lymphoid system.

1.1.1. Immune organs in fish

In higher vertebrates, organs and tissues of the immune system are categorized according to the role they play in the immune response. In mammals, the thymus, foetal liver and bone marrow are primary (generative or central) lymphoid organs which support the development of immune cells by producing hematopoietic cells involved in host defense (Secombes and Wang 2012). These cells originate from single undifferentiated type of cells called *stem cells*, which are able to develop into any other hematopoietic cell. Through the process of hematopoiesis, hematopoietic cells give rise to all other blood cells, and are classified into three lineages: *myeloid lineage* (monocytes/macrophages, neutrophilic, basophilic and eosinophilic granulocytes, thrombocytes, mast cells, dendritic cells (DC)), *lymphoid lineage* (B lymphocytes, T lymphocytes, natural killer (NK) cells) and *erythroid lineage* (erythrocytes and platelets) (Yates and Lyszak 2004). In mammals, secondary (peripheral) lymphoid organs comprise encapsulated systemic organs such as the spleen and lymph nodes, as well as non-encapsulated lymphoid tissues associated with mucosal surfaces called mucosal associated lymphoid tissue (MALT). MALT includes GALT (gut-associated lymphoid tissue) in the intestinal tract, BALT (bronchus-associated lymphoid tissue) in the respiratory tract, and lymphoid tissue in the genitourinary tract (Randall 2010; Suzuki *et al*. 2010). Secondary lymphoid organs represent the site of lymphocyte activation by antigen, which is basically any substance derived form foreign microorganisms or host itself that causes an immune system to produce antibodies against it.

Fish possess most generative and secondary lymphoid organs found in mammals, except the bone marrow and lymph nodes. The lymphoid organs of fish comprise (Figure 1.1) (Secombes and Wang 2012; Biller-Takahashi and Urbinati 2014):

- thymus
- head kidney
- spleen
- MALT (gut, gills and skin)

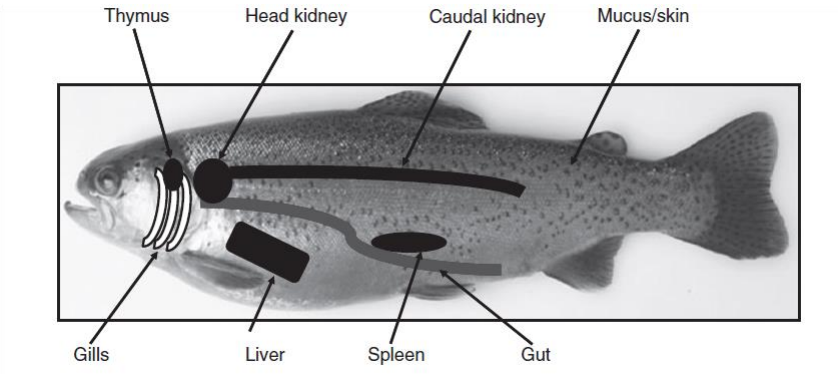


Figure 1.1. Immune organs and their anatomical localization in teleost fish. The approximate site of immune organs is superimposed onto a rainbow trout (*Oncorhynchus mykiss*). Reused from Secombes and Wang (2012).

The order in which different lymphoid organs develop as well as their development timeframe differs among fish species. In marine fish, the first lymphoid organ that develops is head kidney, followed by spleen and then the thymus. In contrary, in freshwater fish, the first organ to become lymphoid is the thymus, although the kidney can contain hematopoietic precursors prior to that event, but not lymphocytes (Zapata *et al*. 2006; Uribe *et al*. 2011).

1.1.1.1. The thymus

The thymus is a double organ located behind the operculum in the dorsolateral position of the gill and is lined by mucous tissue of the pharyngeal epithelium (Ellis 2001). Although it is usually found as a paired organ, in some teleost species the thymus can appear in more than one pair, like cing-fish (*Sicyases sanguineus*) which possess a pair of thymus glands in each gill chamber (Gorgollon 1983). Thymus plays a major role in the development of adaptive immune response of teleost fish, as it provides an inductive environment for the development of T cells from hematopoietic progenitor cells

(thymocytes) and their subsequent maturation (Bowden 2005; Biller-Takahashi and Urbinati 2014). In mammals, the thymus is organized into the outer lymphocyte-rich region (*the cortex*), and an inner, less cellular region (*the medulla*). T-cell progenitors enter the thymus through cortico-medullary blood vessel and can differentiate into natural NK cells, DC and T cell lineage (Secombes and Wang 2012). When maturation is finished, T lymphocytes exit thymic cortex, enter the bloodstream and seed peripheral lymphoid organs. The structure of fish thymus is highly variable between different species but also between developmental stages of one species. For example, while in many fish species, such as salmonids, there is no clear differentiation between cortex and medulla as in higher vertebrates, zonation of turbot (*Scophthalmus maximus* L.) and Atlantic halibut (*Hippoglossus hyppoglossus* L.) thymus is very well observed (Tatner and Manning 1982; Fournier-Betz *et al*. 2000). On the other hand, in carp (*Cyprinus carpio* L.) zonation of the thymus occurs only later, during forth week post-fertilization (Romano *et al*. 1999).

1.1.1.2. The head kidney

The head kidney in teleost fish can be considered as equivalent of a bone marrow in mammals as it is a major site of hematopoiesis (Zapata *et al*. 2006). The bone marrow in mammals represent the site where B cells originate and develop from hemtopoietic stem cells (HSCs). Development of B cells starts with differentiation of HSCs into multipotent progenitor cells, then common lymphoid progenitor cells, B-cell progenitor intermediates and finally naive B cells expressing surface antibodies (Ab), also known as immunoglobulins (Ig) (Santos *et al*. 2011). Since bone marrow is absent in fish, head kidney (*pronephros*) has taken over a role of central hematopoiseis and immune site. Hematopoiesis also occurs in trunk kidney (*mesonephros*), but to a lesser extent due its renal function. The fish kidney is shaped in a form of letter ‘Y’ and placed along the body above swimming bladder. Anterior part, head kidney, penetrates under the gills, while posterior part is extended along the vertebral column. The hematopoiesis that occurs in fish head kidney includes:

- erythropoiesis
- granulopoiesis
- thrombopoiesis

- monopoiesis
- lymphoplasmopoiesis

As a result of different hematopoietic processes, fish head kidney is rich in numerous developmental stages of blood cells, including erythropoietic series (from proerythroblasts to young and mature erythrocytes), granulopoietic series (from myeloblasts to mature granulocytes), monopoietic series (from monoblasts to monocyte), lymphopoietic series (from lymphoblasts to large lymphocytes and small lymphocytes, and active and inactive plasma cells) and thrombopoietic series (from thromboblasts to thrombocytes) (Secombes and Wang 2012).

Melano-machrophage centres (MMCs), often referred to as macrophage aggregates, are also present in fish head kidney. They represent distinctive groupings of pigment-containing cells normally located in the stroma of the hemopoietic tissue of the spleen and the kidney, although in some fish species, they can also be found in the liver (Agius and Roberts 2003). There are also reports of their occasional occurrence in gills, brain and gonads (Macchi *et al*. 1992). The key function of fish MMCs is that of 'metabolic dump' for debris of effete or damaged cells, including red blood cells. No less important is its role in recognition and digestion of antigens (Agius and Roberts 2003). Fish MMCs can also be considered as primordial germinal centers (GC) found within secondary lymphoide organs of mammals and birds (Saunders *et al*. 2010). GCs represent specialized microenvironments where high-affinity antibody-secreting plasma cells and memory B cells are produced as a result of interaction between antigen-specific B cells, follicular B helper T cells (Tfh) and specialized follicular dendritic cells (FDC) (Gatto and Brink 2010). Furthermore, GCs are an important site for affinity maturation, a process in which Tfh-activated B cells produce antibodies with increased affinity for antigens. The key initiator of antibody affinity maturation is activation-induced cytidine deaminase (AID), mutagenic enzyme responsible for modifying the specificity of B cells by producing point mutations at the antibody gene locus. These mutations ideally result in an increased affinity to exogenous antigens, but in some cases these mutations can produce or enhance a B cell's ability to target the host. Thus, B cells producing high-affinity antibodies must be selected over all other B-cells. This selection process occurs in the GCs. Two main evidence that fish MMCs and mammalian GCs are evolutionary related

are: successful labelling fish MMCs with antibody used for labelling mammalian FDCs (CNA-42) and expression of AID in fish cells that co-locate with MMCs (Vigliano *et al*. 2006; Saunders *et al*. 2010).

Besides being hematopoietic-lymphoid organ, fish head-kidney represents an important endocrine organ, homologous to the mammalian adrenal gland. It contains interrenal endocrine cells that secrete cortisol, catecholamines (such as adrenalin and noradrenalin) and thyroid hormones important for modulation of osmoregulation, stress and immune response (Geven and Klaren 2017).

1.1.1.3. The spleen

In mammals, the spleen is the largest secondary lymphoide organ. It consists of blood vessels, ellipsoids, red pulp and white pulp. Ellipsoids are thick-walled capillaries derived from the splenic arterioles which than open in the pulp (Uribe *et al*. 2011). The first main function of the spleen is filtration of blood in search for pathogens and old or damaged blood cells, which occurs primarily in red pulp. Second function is initiation of immune response to blood-borne pathogens which is carried out in lymphocytes-rich white pulp (Bogen 2004). In fish, the spleen also represents important secondary lymphoid organ with the same components as in higher vertebrates, but with less defined red and white pulp. The red pulp is bigger and better developed then the white pulp, containing, within its sinusoids, diverse cell population such as macrophages and lymphocytes. The white pulp basically consists of MMCs and ellipsoids. Blood-borne pathogens are retained within ellipsoidal wall during plasma filtration and subsequently taken over by macrophages and transferred to MMCs for further elimination (Secombes and Wang 2012).

1.1.1.4. MALT

The aquatic ecosystem (freshwater or sea water) represents microbial-rich environment in constant contact with every mucosal epithelial surface of the fish body. Being exposed to a wide range of microbial populations, fish mucosal barriers have developed both innate and adaptive immune responses. However, not all microorganisms are threatening. Nevertheless, majority of them have positive effect on fish physiology. Thus, in order to maintain homeostasis and avoid immune response

against harmless antigens, the mucosal immune system favors tolerance rather than responsiveness (Salinas 2015; Salinas and Parra 2015).

Every mucosal surface contains associated lymphoid tissues (MALT). So far, four different MALTs have been described in teleost fish: gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Salinas *et al*. 2011; Gómez *et al*. 2013; Tacchi *et al*. 2014; Salinas and Parra 2015) (Figure 1.2)

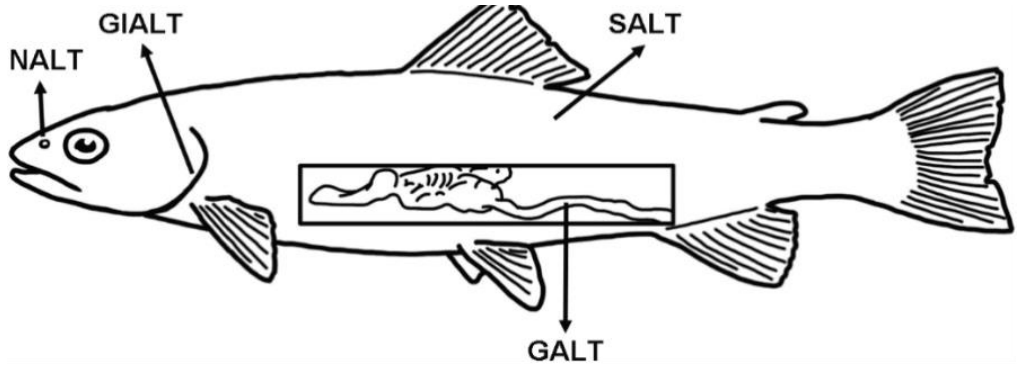


Figure 1.2. Schematic representation and anatomical localization of four main mucosa-associated lymphoide tissues (MALT) in teleost fish; NALT (nasopharynx-associated lymphoid tissue), GIALT (gill-associated lymphoid tissue), SALT (skin-associated lymphoid tissue) and GALT (gut-associated lymphoid tissue). Reused from Salinas (2015).

In higher vertebrates, mucosal surfaces consist of inductive sites, involved in antigen sampling and stimulation of naive T and B lymphocytes, and effective sites where effector cells contribute to the antibody production (Brandtzaeg *et al*. 2008). The effective sites are present within MALT of all vertebrates and comprise unorganized, diffuse leukocytes disseminated along the mucosal surfaces, also known as diffuse MALT. The inductive sites comprise organized lymphoid structures, so-called organized MALT, which can only be found within the mucosal epithelia of endothermic vertebrates (Salinas 2015). Since teleost do not possess organized lymphoid tissue it is proposed that diffused MALT perform both inductive and effector functions (Parra *et al*. 2016). As an exemple, GALT will be described in more detail.

1.1.1.4.1. Gut-associated lymphoid tissue (GALT)

The gastrointestinal tract (the gut) has a dual role: a digestion of nutrients and maintenance of the immune homeostasis. In mammals, GALT is composed of organized MALT, such as Payer's patches or mesenteric lymph nodes and diffused MALT composed of dispersed effector cells at sites such as *lamina propria* or intraepithelial lymphocytes. The teleost GALT ultrastructure is less complex then in mammals, lacking Payer's patches or mesenteric lymph nodes. The immune cells that can be found within *lamina propria* comprise macrophages, eosinophilic and neutrophilic granulocytes, T and B lymphocytes and plasma cells. On the other hand, the intraepithelial compartment contains mostly T cells and small number of B lymphocytes (Rombout *et al.* 2011; Salinas and Parra 2015; Salinas *et al*. 2011).

Teleost intestine can also be divided into three segments, based on the microscopial anatomy of its mucosa and the local absorptive cells, called enterocytes: the first segment with enterocytes considered as absorptive cells; the second segment with enterocytes characterized by large supranuclear vacuoles, irregular microvilli zone and high pinocytotic activity and strong take up of macromolecules, and the third segment with enterocytes with osmoregulatory function (Rombout *et al*. 2011). It is suggested that the second segment of teleost intestine plays a principal role in the uptake and processing of antigens (Rombout and van den Berg 1985). Furthermore, teleosts possess a type of cells that share morphological similarities with mammalian microfold cells (M cell) found mainly above the Peyer's patches (Fuglem *et al*. 2010). In mammals, M cells are specialized in the uptake of antigens from intestine lumen and their transport to lymphoid tissues. In addition to teleost enterocytes and M cell-like cells, large intraepithelial macrophages are also suggested to participate in antigen uptake from the lumen of the intestine (Chen *et al*. 2015).

1.1.1.5. Liver as immune organ

Beside its critical role in metabolism of proteins, carbohydrates and lipids, liver also represents an important immune organ. In mammals, the liver is a primary hematopoietic organ during the prenatal development. The immune cell population retained in postnatal liver comprise myeloid cell lineage and intrahepatic lymphocytes including B cells and T cells, NK cells and NK cells expressing T cell

receptor (NKT cells) (Nemeth *et al*. 2009). Although the immunological roles of fish liver are still understudied, emerging evidence suggests its active engagement in immune response. Fish liver is involved in immune regulation and gene expression following viral (Castro *et al*. 2014) and bacterial infection (Martin *et al*, 2010; Millán *et al*. 2011) with similar resident immune cell population as mammalian liver, i.e. myeloid cells, B and T lymphocytes (Moller *et al*. 2014).

1.1.2. Innate immune system of fish

The innate immune response can briefly be described as quick, relatively temperature-independent, non-specific protection that does not depend upon pathogen recognition (Ellis 2001) The innate immune system is unique for its specific receptors called pathogen recognition receptors or pattern recognition receptors (PRRs) which can distinguish 'infectious non-self' from the ‘non-infectious self'. This recognition system of innate immunity has its roots in ancient evolutionary history, dating from the time of Metazoa (Porifera) which evolved around a billion years ago. Activation of innate immune response is triggered by detection of exogenous or endogenous infectious agents by PPRs. Exogenous molecules that originate from pathogens are called pathogen associated molecular patterns (PAMPs) and can be divided to: bacterial PAMPs (which include bacterial cell wall components such as lipopolysaccharide (LPS) and peptidoglycan, flagellin, and bacterial DNA or RNA), viral PAMPs (which include the cytosolic compartment for viral genome amplification, mRNA metabolism and viral protein expression, as well as double-stranded and single-stranded non-capped RNA), and fungal PAMPs (which are associated with early germ tube formation and hyphal forms that express high levels of zymosan) (Wilkins and Gale 2010; Boltaña *et al*. 2011). Endogenous molecules released by damaged or stressed host cells are called danger-associated molecular patterns (DAMPs) and include proteins such as heat shock proteins, calgranulins, serum amyloid A and non-protein molecules such as uric acid, ATP, potassium efflux, ROS, and heparin sulphate (Paccinini and Midwood 2010).

PRR families that have been identified so far include the C-type lectins (CLRs), the Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and absent in melanoma (AIM)-like receptors (ALRs) (Hansen *et al*. 2011). Basic structure of all PRRs is the same and consists of a protein domain

for recognizing PAMPs or DAMPs connected to a protein domain that interacts with downstream signaling molecules (Secombes and Wang 2012). Activated PRRs can trigger various innate immune responses including the complement cascade, apoptosis, leukocyte activation and migration, and production of inflammatory cytokines and interferons (Lee and Kim 2007).

The innate immune system is commonly divided into three main components: physical barriers, cellular components and humoral factors.

1.1.2.1. Physical barriers

Fish scales and mucus of epidermal layer of skin, gills and gastrointestinal tract represent the first barrier against infectious microorganisms. Fish mucus is secreted by epidermal goblet cells whose main property is production of gel-forming glycoproteins called mucins. Main components of mucus are water and glycoproteins associated with wide range of functions such as excretion, ionic and osmotic regulation, respiration, reproduction and communication, but also disease resistance. Mucus has two important protection roles as a part of innate immune response. Beside the efficient prevention of pathogen adherence by being constantly produced and sloughed off, fish mucus also contains numerous innate immune factors including proteolytic enzymes (such as bacteriolytic enzyme lysozyme), complement proteins, lectins, antibacterial peptides and immunoglobulin IgM (Magnadóttir 2006; Subramanian *et al*. 2007).

1.1.2.2. Cellular components of fish innate immune system

A variety of cell types are actively involved in fish innate immune response including macrophages, granulocytes (e.g. neutrophils), non-specific cytotoxic cells (NCC) and natural killer (NK)-like cells. Mast cells and rodlet cells also play an important role in innate immune response (Secobes and Wang 2012; Firdaus-Nawi and Zamri-Saad 2016).

1.1.2.2.1. Macrophages

Macrophages originate from hematopoietic progenitors by direct differentiation or via circulating blood monocytes (Hodgkinson *et al*. 2015). They are of great importance for immune response to pathogens and they contribute to the maintenance of homeostasis. Together with scavenger endothelial

cells, macrophages constitute mononuclear phagocytic system which eliminates both physiologic and foreign waste products from the circulation via endocytosis and phagocytosis (Whyte 2007; Secombes and Wang 2012). Furthermore, machrophages participate in three different homeostatic activities, i.e. host defense, wound healing and immune regulation, thus can be classified as classically activated, wound healing and regulatory macrophages, respectively (Mosser and Edwards 2008). Macrophages express various receptors on their cell surface, including PRRs such as TLRs and CLRs, scavenger receptors and complement receptors. They are also an important source of cytokines and chemokines, a potent signaling proteins which mediate effective immune response, link innate and adaptive immunity and in turn influence the macrophage's microenvironment (Secombes and Wang 2012; Dauque and Descoteaux 2014) (more details about cytokines are given in *Section 1.1.5*). Macrophages are also essential for antigen presentation to cells of adaptive immune system, i.e. T cells, as they are one of accessory cells or antigen presenting cells (APCs) (Dauque and Descoteaux 2014) (more details about antigen presentation are give in *Section 1.1.3.1*).

1.1.2.2.2. Neutrophils

Neutrophils or neutrophilic granulocytes play a pivotal role in inflammatory immune response against a variety of bacterial, viral, protozoan and fungal pathogens, in most cases successfully removing them from the host organism (Secombes and Wang 2012). Guided by chemotactic factors released by injured tissue, neutrophils are the first granulocytes to appear at the injured site, followed by macrophages. At the site of injury, neutrophils destroy microorganisms through phagocytosis with proteolytic enzymes, antimicrobial peptides and cell damaging reactive oxygen species (ROS) (Biller-Takahashi and Urbinati 2014). In addition, fish neutrophils can release extracellular fibers, called neutrophil extracellular traps (NET), composed of DNA, histones, and proteins which can bind and destroy bacteria, fungi and parasites, and inactivate viruses (Palić *et al*. 2007; Toledo-Ibarra *et al*. 2013).

1.1.2.2.3. Non-specific cytotoxic cells (NCC) and NK-like cells

Cell-mediated cytotoxicity (CMC) represents one of the major protective immune defense mechanisms against viral diseases. Virus-infected cells can be recognized and processed by cells of

innate or adaptive immune system, thus CMC is also referred to as non-specific and specific, respectively (Utke *et al*. 2007). In mammals, non-specific CMC reactions are carried out mainly by natural killer (NK) cells. Fish also possess non-specific CMC mechanisms, performed by two types of NK cell homologues: non-specific cytotoxic cells (NCC) and NK-like cells. NK-like cells in fish are isolated from blood leukocytes and are shown to spontaneously kill allogeneic (cell type that is from the same species but genetically distinct), xenogeneic (cell types of different species and different genotypes) and virus-infected target cells (Fischer *et al*. 2006). On the other hand, NCCs are the most active in head kidney and spleen where they tend to target various cells including tumor cells, virus-transformed cells and some protozoa. NCCs are able to spontaneously kill the affected cells through apoptotic and necrotic mechanisms (Firdaus-Nawi and Zamri-Saad 2016).

1.1.2.2.4. Mast cells

In mammals, mast cells play a central role in wound healing and angiogenesis, as well as in defense mechanisms, participating in both innate and adaptive immunity (Weller *et al*. 2011). Activated *in vivo* mast cells respond directly to pathogens by phagocytosis and ROS production, but also send signals to other tissues to modulate both innate and adaptive immune responses. Within seconds of stimulation, mast cells can undergo degranulation followed by production of pro- and anti-inflammatory cytokines, chemokines and heparin (Marshall 2004). Mast cells are a component of most teleost species innate immunity and are localized in the vicinity of blood vessels in the intestine, gills and skin (Sfacteria *et al*. 2015). Increased number of mast cells in tissues and organs of teleosts is associated with a variety of stress conditions including exposure to heavy metals and herbicides, parasitic infections and chronic inflammation (Lauriano *et al*. 2011). Mast cells degranulation, release of cytokines and subsequent inflammatory reaction, has also been shown in teleost fish following bacterial infection (Reite and Evensen 2006).

1.1.2.2.5. Rodlet cells

Rodlet cells are unique type of cells in fish, characteristic for their cytoplasmic inclusions, so-called rodlets, and a thickened capsule-like cell border (Siderits and Bielek 2009). Although there are some suggestions that rodlet cells only have secretory function (Mendonca *et al*. 2005), the close

relationship between the presence of infectious agents and the presence of rodlet cells indicates that they also have a defensive role in teleost fish, especially against parasitic helminths (Secombes and Wang 2012).

1.1.2.3. Humoral factors of fish innate immunity

Humoral factors comprise cell receptors or other molecules that are soluble in body fluids, such as plasma. Innate humoral defense of teleost fish includes various inhibitors of bacterial growth, such as: transferrin, lysozyme, C-reactive protein, alkaline phosphatase, antimicrobial peptides, complement, lectins.

1.1.2.3.1. Transferrin (Tf)

Transferrin (Tf) is a multifunctional protein involved in the regulation of iron metabolism, crucial for growth and survival. Tf binds and transports iron around the body via bloodstream, maintaining optimal levels of free iron and in that way controlling the balance between its beneficial and toxic effects (Kohgo *et al*. 2008; Garcia-Fernandez *et al*. 2011). As the the effector in innate immune response, Tf provides low free iron environment which limits survival of microorganisms and controls their infectiousness (Firdaus-Nawi and Zamri-Saad 2016).

1.1.2.3.2. Lysozyme

Fish lysozyme, as in higher vertebrates, is an important part of innate immune defense due to its significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Being a glycoside hydrolase, lysozyme catalyzes the hydrolysis of glycosidic bonds of bacterial cell wall peptidoglycans which ultimately leads to its lysis. Lysozyme is produced by leukocytes (mainly monocytes and neutrophils) and can be found in mucus, lymphoid tissue, plasma and other body fluids of most fish species (Magnadóttir 2006; Firdaus-Nawi and Zamri-Saad 2016). In addition to its bactericidal function, fish lysozyme can also act as opsonin that within the process of opsonization chemically modifies microbes or apoptotic cells in order to provoke stronger immune response (i.e. enhances phagocytosis or activates the complement system) (Grinde 1989).

1.1.2.3.3. C-reactive protein

C-reactive protein (CRP) was first found in the serum of patients with acute inflammation as a substance that reacted with the 'C' carbohydrate antigen of *Pneumococcus* sp. bacterium, thus named 'C-reactive' protein (Firdaus-Nawi and Zamri-Saad 2016). In humans, CRP is a prime blood test marker for inflammation in the body. High concentrations of CRP can also be found in fish blood, eggs and mucus. As inflammation acute phase protein, CRP is able to bind to the pathogen and promote its opsonization and consequently complement activation and phagocytosis (Biller-Takahashi and Urbinati 2014).

1.1.2.3.4. Alkaline phosphatase

Alkaline phosphatase (AP) is a lysosomal enzyme and found in fish skin and intestinal mucus and blood serum (Nigam *et al*. 2012). High activity of fish AP is associated with stress conditions (Ross *et al*. 2000), parasitic infections (Fast *et al*. 2002) as well as wound healing (Rai and Mittal 1983).

1.1.2.3.5. Antimicrobial peptides

Antimicrobial peptides (AMP) are low molecular wight proteins that play a major role in innate immune defense against wide variety of bacteria, viruses and fungi by lytic or ionophoric (pore-forming) functions (Smith *et al*. 2010). AMPs can be found in all areas of the body exposed to pathogens including mucus, circulatory system, gill tissue, liver. Beside their antimicrobial functions, AMPs also participate in inflammatory responses such as recruitment of neutrophils, promotion of mast cell degranulation and enhancement of phagocytosis (Plouffe *et al*. 2005).

1.1.2.3.6. Complement

The complement system is an important part of innate immune system and is responsible for promotion of inflammatory responses, pathogen elimination, clearance of apoptotic and necrotic cells, as well as for modulation of the adaptive immune responses, performed by more then 30 distinct plasma and membrane-associated proteins (Nakao *et al*. 2011; Secombes and Wang 2012). Mammalian and teleost complement systems are considered both structural and functional equivalents, except for the significantly wider distribution of complement components in teleost fish. While in

mammals complement components are mainly produced in the liver, in teleosts they can be found in head and renal kidney, intestine, gill, skin, brain and gonads (Løvoll *et al*. 2007). The complement system has three activation pathways: classical pathway (an antibody-dependent activation triggered by antigen-antibody complex), alternative pathway (activated by direct binding to cell surface molecules of microorganisms, not involving specific recognition) and lectin pathway (triggered by recognition of microbial surface carbohydrate) (Secombes and Wang 2012). Central components of complement system are C5a and C3b, able to trigger biological processes of opsonization resulting in recruitment neutrophils and macrophages, phagocytosis chemotaxis of leukocytes and inactivation of the released bacteria toxin (Biller-Takahashi and Urbinati 2014).

1.1.2.3.7. Lectins

Lectins are a group of proteins characterized by their ability to bind carbohydrates with high specificity (Nilsson 2007). In fish, lectins can be found in skin, gills, eggs, electric organ, stomach, intestine, liver and plasma (Ng *et al*. 2015). Lectins mediate pathogen recognition in the immune system leading to either establishment of mutually favorable interactions with harmless microbes, or activation of innate and adaptive responses against potentially pathogenic ones (Vasta *et al*. 2011). Fish egg lectins were also suggested to provide some protection to developing egg and preventing the transmission of pathogenic organisms from mothers to their offspring (Firdaus-Nawi and Zamri-Saad 2016). They also participate in process of opsonisation as well as in other functions such as agglutination, fertilization, embryogenesis and morphogenesis (da Silva Lino *et al*. 2013).

1.1.2.4. Inflammation

The inflammation is a highly regulated protective response of body tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is considered an innate immune mechanism that involves immune cells, blood vessels, and molecular mediators. The primary role of inflammation is the resolution of tissue damage, which includes the elimination of initial cause of cell injury, removal of necrotic cells and tissues damaged during original insult and/or the inflammatory process and lastly, initiation of tissue repair and recovery of homeostasis (Grayfer and Belosevic 2012). The classical signs of inflammation are (Xiao 2017):

- redness - due to dilatation of small blood vessels within the damaged area
- swelling - due to accumulation of fluid in the extravascular space and to some extent due to the accumulation of inflammatory cells
- heat - due to increased blood flow resulting in vascular dilation and the delivery of warm blood to the area
- pain - partially due to the stretching and distortion of swollen tissues and partially due to chemical mediators of acute inflammation such as bradykinin
- loss of function - partly caused by conscious or reflective restraint of movement due to pain, and partly by physical immobilization due to severe swelling

The general steps of inflammation are:

a) vasodilatation and increased vascular permeability
b) leucocyte migration and removal of debris
c) resolution and healing

The initial recognition of tissue damage and/or pathogens is mediated by resident immune cells such as macrophages and mast cells which express various PRRs on their surface. Upon activation, these cells release mediator factors, such as vasoactive amines, histamine and serotonin, in order to extend and make blood capillaries more permeable, allowing the migration of leukocytes. Granulocytes (mainly neutrophils, but also basophils and eosinophils) are the first cell type to arrive at the inflammation focus, being responsible for the removal of irritants, bacteria, or damaged cells and tissues through phagocytosis. The remaining pathogenic cells and cellular debris are phagocytosed by macrophages (Magnadóttir 2006).

1.1.3. Adaptive immune system of fish

The origin of the mammalian type of adaptive immune system appeared simultaneously with the appearance of jawed vertebrates (i.e. Gnathostomata), about 450 million years ago. The key events in the evolution of vertebrate immune system that enabled the development of adaptive immune system were the appearance of the thymus, B and T lymphocytes, RAG (recombination activation gene)

enzymes (Secombes and Wang 2012), and especially the development of unique mechanism of genetic recombination called V(D)J recombination (Firdaus-Nawi and Zamri-Saad 2016). Namely, in order to respond to great diversity of antigens, adaptive immune system must be capable of producing a large number of different immunoglobulins from a small number of coding genes. This is accomplished by V(D)J recombination, a process of antigen receptor random rearrangement of V(variable), D (diversity), J (joining) gene segments. V(D)J recombination occurs in the bone marrow (for B cells) and thymus (for T cells) during early stage of lymphocytes maturation. RAG1 and RAG2 are key components of the V(D)J recombination machinery. They initiate the recombination by cleaving DNA at the recombination signal sequence (RSS) site, and are subsequently involved in recombination of VDJ genes and final rejoining of DNA (Market and Papavasiliou 2003). V(D)J recombination, therefore, enables the generation of numerous and diverse immunoglobulin superfamily (IgSF) antigen receptors on cell membrane T lymphocytes (TCR - T cell receptor) and B lymphocytes (BCR – B cell receptor) (Hirano *et al*. 2011). Hence, the adaptive immune response is mediated by two major groups of lymphocytes; B lymphocytes that mediate humoral (antibody) responses and T lymphocytes that mediate cell-mediated immune responses.

Adaptive immunity is triggered after the pathogen has evaded the innate immune system and generated an antigen threshold level leading to the activation of antigen presenting cells (APCs) (Janeway *et al*. 2001a). Adaptive immune system defense mechanisms comprise three major milestones:

- recognition of specific antigen during the process of antigen presentation
- generation of responses that will efficiently eliminate pathogen or pathogen-infected cells
- development of immunological memory through generation of memory B and T cells

1.1.3.1. Antigen presentation

Antigen presentation is an immune process essential for T cell recognition of foreign antigens and activation of T cell-mediated immune response. Namely, while BCR recognizes antigens in their native form (Harwood and Batista 2010), TCR can recognize antigen only after it has been processed and bound as linear peptide fragments to a set of cell surface proteins called major histocompatibility

complex (MHC) on antigen presenting cells (APCs) (Janeway *et al*. 2001a). There are two classes of MHC molecules, termed MHC class I and MHC class II. MHC class I molecules possess β2 subunits and can only be recognized by CD8 (cluster of differentiation 8) co-receptors. CD8 is a transmembrane glycoprotein that serves as a co-receptor for the TCR of cytotoxic T cells (but can also be found on natural killer cells and dendritic cells (DCs)) (Gao and Jakobsen 2000). On the other hand, MHC class II molecules comprise β1 and β2 subunits and can only be recognized by CD4 co-receptors on T helper cells, monocytes, macrophages, and DCs. Thus, class of MHC molecules defines which type of lymphocytes may bind with high affinity to the presented antigen (Kambayashi and Laufer 2014; den Haan *et al*. 2014).

APCs can also be divided into two categories: *professional* and *non-prefessional*. Professional APCs include macrophages, DCs and B cells. These cells digest pathogen either by phagocytosis (macrophages and DCs) or by receptor-mediated endocytosis (B cells), process the pathogen-derived antigen into peptide fragments and send them to cell surface where they are bound to MHC class II molecules. $CD4^+$ T helper cells recognize and interact with the antigen-MHC class II complex on the membrane of the APCs leading to their activation and further differentiation (Kambayashi and Laufer 2014). Exogenous antigens derived from exogenous pathogens, such as bacteria, parasites or toxins are usually processed by professional APCs (Janeway *et al*. 2001b; Kambayashi and Laufer 2014).

Non-professional APCs include all cell types, with the exception of non-nucleated cells such as erythrocytes. They are specialized for processing endogenous antigens derived from intracellular bacteria and viruses and presenting them via MHC class I molecules to $CD8^+$ cytotoxic T-cells (den Haan *et al*. 2014).

1.1.3.2. The humoral adaptive immune response in fish

1.1.3.2.1. B cells

Humoral immunity is mediated by B lymphocytes whose main functions include: antigen presentation as one of professional antigen presenting cells (APCs), production of antibodies against antigens and development of immunological memory by differentiation into memory B cells (Firdaus-Nawi and Zamri-Saad 2016). Fish head kidney is a major site of B cell production and maturation into the

mature naive B cells. Germinal centers (GC) found in fish head kidney and spleen are an important site for activation and subsequent differentiation of B cells into antibody producing plasma cells or memory B cells. B cells activation is triggered by their encounter with antigens and can be T cell-dependent (for protein-derived antigens) or T cell-independent (for polysaccharides- and unmethylated CpG DNA-derived antigens) (Nutt *et al*. 2015). T cell-dependent B cell activation requires activation of T helper cells by activated APCs, in this case B cell. Thus, once BCR on antigen presenting B cell binds an antigen, the antigen is processed and presented to T helper cells through MHC class II on surface of B cells. This leads to activation and differentiation of T helper cells, which then express surface protein cluster of differentiation 40 ligand (CD40L). CD40L binds to B cell surface protein CD40 which induce activation and differentiation of naive B cells (Janeway *et al*. 2001b; Kurosaki *et al*. 2015). On the other hand, T cell-independent B cell activation is triggered by direct binding of an antigen to BCR and doest not require T cell activation (Janeway *et al*. 2001b).

Activated B cells can differentiate either into: (i) the plasmablasts, a short-lived, proliferating, low affinity antibody-producing cells which then further differentiate into the plasma cells, a long-lived, not-proliferating, high affinity antibody-producing cells (Nutt *et al*. 2015); or (ii) memory B cells, which circulate through the body and are responsible for secondary antibody response. Secondary response is triggered upon memory B cell encounter with the same antigen which activated their parent B cells, since these two share the same surface BCR (Kurosaki *et al*. 2015).

1.1.3.2.2. Antibodies

Antibodies, or immunoglobulins, are multimeric Y-shape glycoproteins that belong to the immunoglobulin superfamily (IgSF), a large family of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells (Maverakis *et al*. 2015). A typical Ig molecule comprises two heavy (H) and two light (L) chains, each containing one amino-terminal variable (V) domain and one (in the L chain) or more (in the H chain) carboxyl-terminal constant (C) domains (Secombes and Wang 2012). The arms of the Y contain the sites that can recognize and bind antigens, called Fab (fragment, antigen-binding) region. Fab region is composed of one C and one V domain from each H and L chain of the antibody. The base of Y plays a role in modulation of immune

cell activity. This region is called Fc (fragment, crystallizable) region, and is composed of two H chains. Fc region interacts with receptors on the surface of the immune cells (e.g. B cells, macrophages, granulocytes, mast cells), called Fc receptors, enabling antibodies to activate these cells and induce immune response (Corley 2004). Within the antigen-binding site (or paratope) of Fab there is small, extremely variable region called hypervariable region, which enables the existence of millions of antibodies with slightly different structures of antigen-binding site. This wide variety of antibody paratopes is a result of random recombination of V, D and J gene segments during V(D)J recombination (Market and Papavasiliou 2003; Diaz and Casali 2002). Enormous diversity of antibody paratopes allows the immune system to recognize an equally wide variety of antigens (Rhoades and Pflanzer 2002).

Antibodies are secreted mostly by plasma cells, and used by immune system for identification and neutralization of pathogens. Antibodies are expressed in two physical forms: a membrane-bound and a soluble. Membrane-bound antibodies are attached to the surface of B cells and serve as the antigen-specific component of BCR complex. They have essential role in the activation of B cells during immune response and their subsequent differentiation. Membrane-bond antibodies are also important for internalization of antigen and for its presentation to T cells. Soluble antibodies are found in blood and secretions where they serve for recognition and clearance of foreign antigens (Corley 2004). Soluble antibodies circulate the bloodstream and other body fluids, where they recognize and bind to the specific antigen that initiated their production. Upon binding, antibodies inactivate pathogens by blocking their ability to bind to receptors on host cells. Binding of antibody also marks a certain pathogen for destruction and elimination by immune cells with the surface receptors for Ig molecules such as phagocytes, e.g. neutrophils, macrophages, mast cells (Schroeder and Cavacini, 2010).

In mammals, there are five types of antibody isotypes based on five different types of Fc regions:

- *IgM* is expressed on the surface of B cells as monomer and in a secreted form as pentamer. It is the first response Ig which eliminates pathogens during early stage of B cell-mediated immunity, before there is sufficient IgG (Corley 2004, Geisberger *et al*. 2006).

- *IgA* is the most produced Ig. It predominately exists in the form of dimers with small amount of trimers and tetramers. IgA is found in mucosal areas of gut, respiratory and urogenital tract, but also saliva, tears and breast milk. It prevents colonization of pathogens and can form complexes that bind to multiple antigen molecules (Underdown and Schiff 1986).
- *IgG* is monomeric Ig which provides majority of antibody-based immunity against invading pathogens. It is the only antibody capable of crossing the placenta to give passive immunity to the foetus (Corley 2004).
- *IgD* functions mainly as an antigen receptor on B cells in a form of monomer (Geisberger *et al*. 2006), but can also stimulate basophils and mast cells to produce antimicrobial factors (Chen *et al*. 2009).
- *IgE* is a monomeric Ig which binds to allergens and triggers histamine release from mast cells and basophils. It also has a role in protection against parasitic worms (Corley 2004).

Teleost fish possess three major types of Ig: IgM, IgD and IgT (named as IgZ in zebrafish) which are unique for teleost fish (Parra *et al*. 2016). All three isotypes have been identified in all teleost fish species examined so far, with the exception of IgT which has still not been found in channel catfish, *Ictalurus punctatus*, and medaka, *Oryzias latipes* (Secombes and Wang 2012; Parra *et al*. 2016).

IgM is predominant Ig isotype in teleost blood/serum, while IgD and IgT are found in lesser amounts (Secombes and Wang 2012). Teleost IgM is generally present as a tetramer in both serum and mucus (Salinas *et al*. 2011). Similar to mammalian IgM, teleost IgM plays a key role in systemic immune responses with effector functions such as complement activation, opsonization, neutralization and immune exclusion (Ye *et al*. 2013). Teleost IgD protein exists in two monomer forms (long and short), but its role in pathogen infections is yet to be determined (Parra *et al*. 2016). The teleost IgT is a mucosal-epithelial Ig preferentially expressed in the gut where it can be induced specifically by a mucosal pathogen (Zhang *et al*. 2010). However, its effector functions are still not completely clarified, although it is suggested that it is involved in immune exclusion (Parra *et al*. 2016). Although both IgT and IgM can be found in very early developmental stages (4 days post-fertilization) in teleost

fish, the expression of IgT increases more rapidly suggesting that IgT may have protection role for fish larvae (Zhang *et al*. 2011, Secombes and Wang 2012).

1.1.3.3. The cellular adaptive immune response in fish

Cell-mediated immunity involves activation of T lymphocytes which can be categorized according to their function into two general populations: cytotoxic T cells (CTLs) and helper T (Th) cells. In addition, after the encounter with the antigen, naive T cells can also differentiate into the memory T cells responsible for secondary cell-mediated reaction (Firdaus-Nawi and Zamri-Saad 2016).

CTLs are also known as $CD8^+$ T cells due to their expression of CD8 molecules involved in recognition of an antigen via their interaction with MHC class I molecules on surface of all nucleated cells (Nakanishi *et al*. 2015). CTLs can directly kill infected cells by inducing apoptosis, a programmed cell death, in two different ways: (i) by releasing cytotoxins into the target cell, where they trigger caspase (cysteine-aspartic proteases) cascade leading to apoptosis, or by (ii) expression of the surface protein FAS ligand (FasL) which binds to Fas molecules expressed on the target cell enabling the formation of the death-induced signaling complex (DISC). DISC again triggers caspase cascade ultimately leading to apoptosis of cell expressing surface Fas (Janeway *et al*. 2001c).

Th cells are also known as $CD4^+$ T cells due to their expression of CD4 molecules that interact with MHC class II molecules on APCs (Nakanishi *et al*. 2015). Upon activation, naive Th cells may differentiate into at least five major subtypes: Th1, Th2, Th17, inducible T-regulatory (iTreg) and follicular B helper T (Tfh) cells, that play a critical role in orchestrating immune responses through production of different cytokines (Laing and Hansen 2011). Teleost Th1 cells are involved in immune responses to intracellular viral and bacterial infections by producing IFN-γ. Th2 cells produce IL-4/13, and IL-6 which contribute to their role in expelling extracellular parasites (e.g. helminths). Th17 cells control extracellular bacteria and fungi by producing IL-17, IL-21 and IL-22. Tfh induces activation and differentiation of B cells by expressing CD40L, IL4/13 and IL 21. Inducible T-regulatory (iTreg) cells produce TGFβ1 and are important for maintenance of lymphocyte homeostasis and immune tolerance (Laing and Hansen 2011; Secombes and Wang 2012). Th signature cytokines are also

important for activation of Th cells, thus represent their positive feedback (Secombes and Wang 2012).

1.1.4. Overall mechanisms of fish immune response

The immune system can be described as a set of cellular and humoral components which defend the body against foreign harmful substances or infectious microorganisms. In order to distinguish 'infectious non-self' from the ‘non-infectious self', innate immunity system employs its recognition receptors PRRs which recognize exogenous PAMPs or endogenous DAMPs. The first obstacle in invading pathogen needs to pass is a physical barrier in the form of fish scales and mucous surfaces of skin, gills and gastrointestinal tract. If the pathogen successfully breaks through, it is recognized by PRRs which then trigger various cellular and humoral components of innate and subsequently adaptive immunity system. The innate immunity involves cellular components such as macrophages, granulocytes (e.g. neutrophils) and non-specific cytotoxic cells which have been employed by inflammatory cytokines to kill and digest the invading pathogens through a phagocytosis. Simultaneously, the innate humoral components (e.g. lysozyme, C-reactive protein, antibacterial peptides, complement) alone or in collaboration with cellular components destroy invading microbes or inhibit their growth (Aoki *et al*. 2008). These processes, especially phagocytosis by local macrophages, may result in complete elimination of pathogen. However, if an infection progresses despite the inflammation, cytotoxic cell and phagocyte activity of the innate immune system, a more specific and coordinated response is required to destroy the pathogen. Thus, the adaptive immunity system is triggered. When a pathogen evades the innate immune system for long enough, it generates a threshold level of a pathogen-derived antigen. Professional APCs, i.e. macrophages, dendritic cells or B cells, recognize and engulf the antigen. After the degradation process, selected small linear peptides fragments from the pathogen are sent to the surface of APCs and bound to surface (MHC) class II molecules. $CD4^+$ T cells or Th cells recognize presented antigen and bind to MHC class II via T cell receptor, TCR. Formation of APC-Th cell complex triggers activation and differentiation of Th cells. Th cells differentiate into Th1, Th2, Th17, iTreg or Tfh cells, depending on type of infection, which then secrete different cytokines to facilitate different types of immune responses. Tfh cells thus induce

activation and differentiation of B cells by expressing surface protein CD40L and cytokines IL4/13 and IL 21. Activated B cells differentiate into either plasma cells that produce specific antibody or memory B cells that create immunological memory of specific antigen (Figure 1.3). The released soluble antibodies bind specifically to the pathogen blocking its binding to the host cells. Disabled pathogen is recognized and eliminated by phagocytic cells such as macrophages, neutrophils or mast cells via phagocytosis.

Intracellular pathogens such as viruses undergo another effective mechanism of elimination. They are recognized, phagocytized and processed by non-professional APCs, which can basically be any nucleated cell, and displayed on MHC class I molecules on their surface. CD8 + T cells or CTLs recognize and bind to MHC class I which triggers its activation. Activated CTLs can directly kill infected cells by inducing cell apoptosis (Figure 1.4).

Upon the repeated exposure to the same antigen, circulating memory B cells located in the mucosal layer of the exposed fish stimulate production and release of specific antibodies against the pathogen and/or cytotoxic cells to prevent adhesion and subsequent invasion of the pathogen. If the pathogen passes mucosal barrier and invade the host body, it again triggers components of innate immune system but also induces a secondary immune reaction of adaptive immune system. Secondary reaction is faster and stronger then the primary due to the activated memory B and memory T cells which enhance production and release of specific antibodies and enhance the activity of CTLs, respectively.

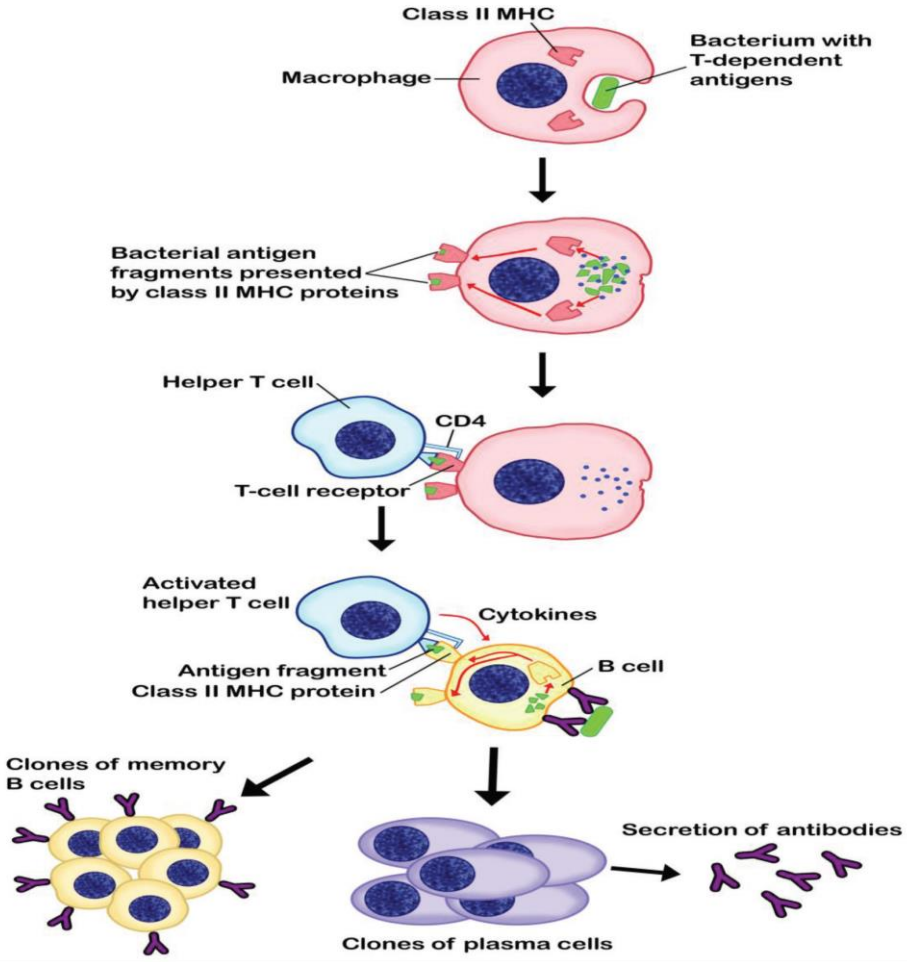


Figure 1.3. T-dependent activation of B cells. Antigen derived from exogenous pathogen (i.e. bacterium) is processed by antigen presenting macrophage (professional APC) and displayed on surface MHC class II proteins. CD4+ T helper cell recognizes and binds to MHC class II which triggers T helper cell activation and secretion of appropriate cytokines. Naive B cell recognize and binds to activated T helper cells which then triggers activation and differentiation of B cells into antigen producing plasma cells and memory B cells. Reused from Firdaus-Nawi and Zamri-Saad (2016).

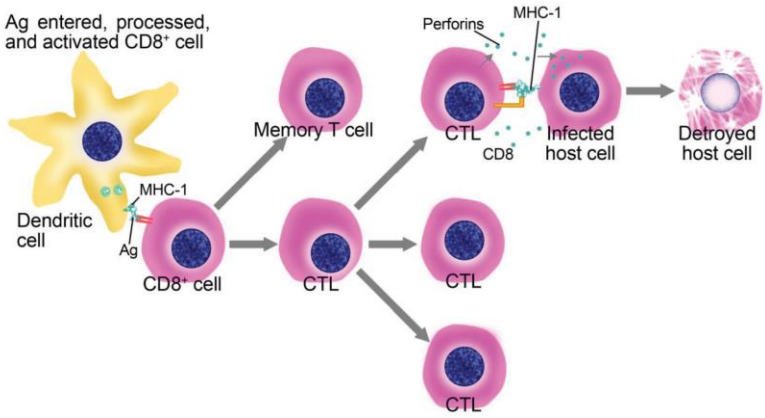


Figure 1.4. Elimination of endogenous antigen (Ag) by cytotoxic T cell (CTL). Ag is processed by antigen presenting dendritic cell (professional APC) and displayed on surface MHC class I (MHC-1) proteins. CD8+ T cell recognizes and binds to MHC-1 which triggers T cell activation and differentiation into CTLs and memory T cell. CTL recognizes and binds to infected host cell expressing MHC-1 on its surface (non-professional APC). CTL releases pore forming cytolytic protein which forms pores on the target cell allowing cytotoxins also released by CTL to enter and destroy infected cell by inducing apoptosis. Reused from Firdaus-Nawi and Zamri-Saad (2016).

1.1.5. Fish cytokines – mediators of immune response

Every aspect of immune response requires precise regulation to ensure that it occurs only under appropriate conditions, lasts for appropriate period of time and has appropriate magnitude. That kind of regulation is, in most cases, regulated by cytokines, simple polypeptides or glycoproteins of less than 30 kDa, (Thomson 1994; Callard and Gearing 1994). These molecules regulate communication between different cells of immune system and have a number of different functions in both humoral and cell-mediated immune response (Tzianabos and Wetzler 2004). Cytokines are able to modulate both innate and adaptive immune response. For example, upon their production at the site of infection, cytokines regulate the capacity of local and newly arrived phagocytes to eliminate invading pathogens by modulation of inflammation signals. Furthermore, cytokines also regulate antigen presenting cells (APCs) and their migration to lymph nodes to initiate the adaptive immune response leading to generation of cytotoxic T cells and the production of antibodies (Wang *et al*. 2011; Wang and Secombes 2013). Cytokines can be produced by a variety of cells, both immune (e.g. monokines, macrophages, B lymphocytes, T lymphocytes, mast cells) and nonimmune (e.g. endothelial cells, fibroblasts, stromal cells). However, the two principal producers of cytokines are the Th cell and the macrophages. Initially, cytokines produced mainly by leukocytes were designated as *lymphokines* or as *monokines* if they were produced mainly by monocytes and macrophages. However, later studies have shown that the majority of cytokines can be produced by more then one type of cells, thus these terms have fallen out of usage as a part of official classification of cytokines. Cytokines generally function as intercellular messenger molecules that evoke particular biological activities after binding to a receptor on a responsive target cell. The cell that is affected by the cytokine carries cell surface receptor that binds specifically to that one cytokine. The main biological activities of a number of immune-related cytokines include (Tzianabos and Wetzler 2004):

- modulation of both cellular and humoral immune responses
- induction of inflammatory responses
- regulation of hematopoiesis
- control of cellular proliferation and differentiation

- induction of wound healing

All cytokines possess several common properties (Hamblin 1993; Vilcek and Le 1994; Tzianabos and Wetzler 2004):

1. Their constitutive production is usually low as they are not stored in their active state but are synthesized and released as a result of new gene transcription following cell stimulation. Since transcription is typically transient and mRNA short-lived, cytokine secretion is a brief self-limiting event.

2. The cellular response of cells to cytokines usually occurs in matter of hours requiring production of mRNA and proteins de novo.

3. Cytokines can induce or inhibit the production of other cytokines creating regulatory networks that modulate cytokines' biologic effect.

4. Cytokines may act on the cell from which they were secreted having ***autocrine*** activity or on proximal cell type having ***paracrine*** activity. In general, cytokines perform in the vicinity in which they are secreted, but in few cases (such as IL-1 and TNFα) they can also act in an ***endocrine*** manner, and be carried via the blood stream to target cells.

5. Cytokines can regulate cellular activity in several different interactive ways:

    - ***Pleiotrophy*** - cytokines can induce different responses in different targets, despite the fact that they act on target cells via high affinity specific receptors.

    - ***Redundancy*** - different cytokines can have comparable effects on same target cells.

    - ***Synergism*** - occurs when two signals are necessary for stimulation of target cells or when contact with the first signal (cytokine) is crucial for induction of receptor expression for the second signal. Cytokines often act in synergy with other cytokines but also with pathogen-derived molecules. Because of that characteristic it is unlikely that *in vivo* a cell will ever encounter a single cytokine.

- ***Antagonism*** - one cytokine can inhibit the action of another cytokine (less well documented).

Considering their ability to stimulate or inhibit immune response, cytokines can also be divided to:

1. ***Pro-inflammatory*** cytokines, such as TNFα, IFNγ and IL-1β, up-regulate inflammatory processes by enhancing antimicrobial functions of immune cells and in that way facilitate the pathogen clearance. Although they are primarily responsible for initiation of effective defense against pathogens, their overproduction can be harmful and may ultimately lead to shock, multiple organ failure, and death (Pinsky *et al.* 1993; Marty *et al.* 1994).

2. ***Anti-inflammatory*** cytokines, such as TGFβ and IL-10, down-regulate inflammatory processes and direct cell functions towards tissue repair mechanisms. These cytokines are crucial for silencing aggravated inflammatory process and maintenance of homoeostasis for proper organ function (Gerard *et al.* 1993; Howard *et al.* 1993). On the other hand, excessive anti-inflammatory response may also result in the suppression of body immune function (Bone 1996; Fisher *et al.* 1996).

The production of pro-inflammatory and anti-inflammatory cytokines is therefore strictly controlled by complex feedback mechanisms (Zimmer *et al*. 1996; Kasai *et al*. 1997; van Dissel *et al*. 1998).

1.1.5.1. Classification of cytokines

Fish possess a repertoire of cytokines similar to those of mammals, many of which have been cloned in different teleost species (Whyte 2007; Secombes et al. 2011). Current knowledge of fish cytokines is based on experimentally obtained and described models and signalling networks of their mammalian homologues.

According to conventional classification cytokines have been divided into several families including (Secombes *et al*. 1996):

- *Interferons (IFN),* a family of cytokines originally identified for their ability to "interfere" with viral replication. IFNs can also activate inflammatory cells and are of central importance

in host defense against intracellular and extracellular pathogens (Tzianabos and Wetzler 2004).

- *Chemokines* or chemoattractant cytokines, a family of small (8-10 kDa) structurally related proteins that regulate immune cell migration under both inflammatory conditions and homeostasis. Immune cells that are attracted by chemokines include monocytes/macrophages, granulocytes and T lymphocytes (Bird and Tafalla 2015). Once they are released by infected or damaged cells, chemokines attract immune cells by forming a concentration gradient which allows cells to move towards the higher local concentration of chemikines, i.e. source of infection (Callewaere *et al*. 2007). Chemokines are also known as "second-order" cytokines, being induced by "first-order" cytokines with pro-inflammatory roles, such as interleukins, tumor necrosis factors or interferons (Peatman and Liu 2007).

- *Colony stimulating factors (CSF),* a family of secreted glycoproteins that drive the proliferation and differentiation of blood cells from hematopoietic stem cells (Zou and Secombes 2016). They also participate in replenishing leukocytes populations during immune reactions (Tzianabos and Wetzler 2004).

- *Interleukins (IL),* a large group of pluripotent cytokines produced by a variety of lymphoid and non-lymphoid cells which are involved, at least partly, in all immune reactions. Although the term 'interleukin' originally describes a specific function (interaction between leukocytes), cytokines which are now designated as interleukins are in fact multifunctional. Interleukins represent one of the critical early inducted cytokines that orchestrate expression of other cytokines during infection or inflammation. They can also induce migration of leukocytes and enhance proliferation of macrophages and phagocitosys (Huising *et al.* 2004).

- *Tumor necrosis factors (TNF),* a family of structurally related proteins, either soluble or membrane-bound, that are produced during lymphoid organ development and inflammation. TNFs are involved mainly in cellular regulation such as cell killing/survival during immune responses and inflammatory reactions (Gruss 1996).

However, with increasing knowledge about molecular structure of cytokines this classification had to be revised. Classification of cytokines is now based on their molecular structure which further dictates their receptors, the down-stream signalling pathways that are activated and their function (Zou and Secombes 2016):

1. β-Trefoil cytokines

The β-Trefoil cytokine family, also referred to as **Interleukin-1 (IL-1) family**, is named by protein fold (i.e. secondary structure) in which the protein backbone is twisted into a trefoil knot shape. It consists of 11 members that can functionally be classified into (Zou and Secombes 2016):

- pro-inflammatory group (IL-1α, IL-1β, IL-18, IL-33 and IL-36α, IL-36β and IL-36γ)
- anti-inflammatory group (IL-1Ra - receptor antagonist for IL-1α and IL-1β; IL-36Ra - receptor antagonist for IL-36α, β and γ; IL-37 and IL-38)

Fish Novel IL-1 Family Members (nIL-1Fm) identified in fish and characterized as teleost specific are:

- IL-1β3 gene and IL-1β4 pseudogene in salmonids (Husain *et al*. 2012)
- gcIL-1R1 - IL-1β receptor antagonist in grass carp (Yao *et al*. 2015)

2. β-Jellyroll cytokine

β-Jellyroll cytokine family, also termed **Tumor necrosis factor (TNF) superfamily**, is named by protein fold composed of eight beta strands arranged in two four-stranded sheets. It consists of 19 members in human, mainly membrane bond proteins. There are three major members of this family in fish with a critical role in regulation of inflammatory response, cell survival/apoptosis, proliferation and differentiation (Zou and Secombes 2016):

- TNF-α
- lymphotoxin (LT) α (also called TNF-β)
- LT-β

3. Cysteine Knot Cytokines

Cysteine knot cytokines are named by protein fold which is composed of three disulphide bridges formed from pairs of cysteine residues. They consist of two major families:

- *IL-17 family cytokines*

  Members of this family mainly have pro-inflammatory functions. There are six members identified in mammals: IL-17A to IL-17F. In fish only two clear homologues are identified: IL-17B and IL-17D but with multiple isoforms termed IL-17A/F (Wang *et al*. 2015).

- *Transforming growth factor-β (TGF-β) family cytokines*

  Members of TGF-β family regulate cell development, proliferation, differentiation, migration, and survival of lymphocytes, DC, NK cells, macrophages and granulocytes. Three isoforms are identified in mammals: TGF-β1 to TGF-β3. Fish possess specific TGF-β isoform (TGF-β6) whose role is still undetermined (Funkenstein *et al*. 2010).

4. Type I α Helical Cytokines:

Helical cytokines are characterized by four-helix bundle fold in which four helices are coiled together like strands of a rope with hydrophobic core in the center. This large family can be divided into several subfamilies:

- *IL-2 Subfamily*

  This subfamily includes IL-2, IL-4/13, IL-7, IL-15 and IL-21 whose central action is mainly directed to lymphocytes, their survival, proliferation and differentiation. Interestingly, fish IL-4/13 share similarities with both IL4 and IL13 in mammals. Also, they are present in two copies, IL4/13A and IL4/13B as a result of WGW event in fish (Wang and Secombes 2015).

- *Beta Chain Cytokines*

  In mammals, this subfamily includes IL-3, IL-5 and granulocyte-macrophage CSF (GM-CSF). Fish possess recently discovered IL-3/IL-5/GM-CSF related genes (Yamaguchi *et al*. 2015), whose nature and functions are yet to be discovered.

- *IL-6 Subfamily*

Cytokines belonging to IL-6 subfamily play a pivotal role in hematopoiesis and act as both pro- and anti-inflammatory. In fish, this subfamily includes IL-6, IL-11 and molecules termed CNTF-like and M17 (Zou and Secombes 2016).

- *IL-12 Subfamily*

IL-12 subfamily of cytokines includes IL-12 IL-23, IL-27 and IL-35, with only IL-12 functionally studied in fish. IL-12 mediates stimulation of interferon γ (IFNγ) secretion from resting lymphocytes and NK cells. Some fish species appear to possess two functional IL-12 isoforms, due to the existence of IL-12 subunit paralogues. It is also suggested that two isoforms signal via different receptors and are differentially expressed during bacterial, viral and parasitic infection (Wang *et al*. 2014).

- *Colony Stimulating Factors (CSF)*

CSF subfamily can be divided in respect to their target cell into: CSF-1 or macrophage colony stimulating factor (M-CSF), CSF-2 or granulocyte-macrophage CSF (GM-CSF) and CSF-3 or granulocyte-CSF (G-CSF). There are two major isoforms of M-CSF in fish, M-CSF1 and M-CSF2, which are probably the result of teleost WGD event (Wang *et al*. 2008).

5. **Type II α Helical Cytokines**

Type II α Helical Cytokines comprise three major families:

- *IL-10 subfamily*

This subfamily in fish includes IL-10, IL-22, IL-26 and IL-20 like (IL-20L) which is homologue to the mammalian IL-19/IL-20/IL-24. Only IL-10 and IL-22 are functionally characterized in fish. IL-10 inhibits inflammation, promote T cell proliferation, memory B cells, and IgM production (Piazzon *et al*. 2015a; Piazzon *et al*. 2015b), while IL-22 activates antimicrobial peptides and antibacterial immunity (Qi *et al*. 2015).

- *Type I IFN*

This family can be divided into two groups: group I IFNs (IFN a, d, e) and group II IFNs (IFN b, c, f), which both induce expression of the antiviral effectors, promote apoptosis and regulate inflammation (Zou and Secombes 2016).

- *Type II IFNs*

  In teleosts two members of this family have been identified and are termed IFN-γ and IFN-γ related or IFN-γrel. IFNγ can activate phagocytes and enhance antigen presentation, while IFN-γrel is involved in the regulation of anti-bacterial and antiviral immunity (Zou and Secombes 2016).

6. **Open face β sandwich**

Secondary structure of open face β sandwich family, also referred to as chemokines, is characterized by two opposing antiparallel β-sheets. Chemokines are defined by the presence of four conserved cysteine residues and are divided into four distinct subfamilies based on the arrangement of the first two cysteine residues within their peptide structure. In mammals, chemokine subfamilies are: *CXC (α*), *CC (β), C* and *CX3C* classes. In fish, no CX3C class chemokines have ever been reported (Bird and Tafalla 2015), whilst C class chemokines have been reported only in zebrafish, *Danio rerio*. However, a new fish-specific chemokine subfamily has been identified in zebrafish and designated as CX class (Nomiyama *et al*. 2008).

In response to pathogens, chemokines mount initial steps of both innate and adaptive immune response by promoting immune cells migration to the site of infection, but also by regulating their subsequent differentiation (Bird and Tafalla 2015).

1.1.5.2. Cytokine receptors

Cytokines activity is mediated through binding to high-affinity cell surface receptors on target cells. All cytokine receptors contain three functional domains necessary for their biological activity:

- *recognition domain* - protruding from the cell surface, involved in specific binding of the cytokine

- *hydrophobic transmembrane domain* - attaches the receptor to the cell

- *intracellular signaling domain* - binds enzymes called kinases or couples with G-protein, which are both involved in signal transduction by activation of downstream proteins

Cytokine receptors are expressed on different cell types allowing cytokines to affect a diverse array of cells. On the other hand, these receptors also share structural homologies and can bind different cytokines, though with different affinity, enabling cytokines their redundancy (Tzianabos and Wetzler 2004).

Cytokine receptors can be classified into several receptor families (Tzianabos and Wetzler 2004; Wang *et al*. 2009):

- **Type I cytokine receptors,** which share conserved extracellular domain with approximately 200 amino acids. They bind cytokines such as IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL11, IL12, GM-CSF and G-CSF.
- **Type II cytokine receptors,** which share structural similarities in their ligand-binding domain and are receptors mainly for interferons, but also bind IL10 and IL22.
- **Chemokine receptors,** which contain 7 transmembrane domains and couple to G-protein for signal transduction.
- **Tumor necrosis factor receptor (TNFR) family** shares a cysteine-rich common extracellular binding domain.
- **TGF-beta receptors,** which have cytoplasmic serine/threonine rich domain, thus represent serine/threonine kinase receptors.
- **Immunoglobulin (Ig) superfamily receptors** shares structural homology with immunoglobulins and bind cytokines such as IL1α and β, IL-18, IL-33, CSF-1R.

Cytokine-mediated signal transduction is associated with several signaling pathways, depending on the type of cytokine receptor, ultimately leading to transcription of cytokine responsive genes. All cytokine receptors, especially Type I and II cytokine receptors are associated with so-called JAK/STAT signaling pathway. Janus kinase (JAK) is a family of tyrosine kinase which phosphorylates tyrosine residues of certain proteins. The binding of cytokine to its receptor activates associated JAK which then phosphorylates itself and the receptor, creating a binding site for associated signal transducers and activators of transcription (STAT). JAK again phosphorylates and

activates STAT, which then translocates to nucleus and induces transcription of cytokine responsive genes (Tzianabos and Wetzler 2004; Haan *et al*. 2006; Murray 2007).

Chemokine receptors associate with G-proteins to transmit cell signals following ligand binding. G proteins are characterized by their ability to hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and thus activate (when bond to GTP) and deactivate (when bond to GDP) themselves. Activated G protein activates different signaling cascades including the activation of so called 'second messengers', intracellular signaling molecules which can couple with downstream kinase cascades, such as mitogen activated protein kinase (MAPK) cascade, amplifying the strength of the initial chemokine-derived first signal (Patel *et al*. 2013).

Immunoglobulin superfamily and TNF receptors can transduce signal via specific interleukin-1 receptor activated protein kinase (IRAK) 4 (Weber *et al*. 2010) and death inducing signaling complex (DISC) (Rahman and McFadden 2006), respectively, both leading to activation of transcription factor - nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB), which ultimately induces transcription of certain cytokine responsive genes. Later signal transduction pathways are discussed in detailed in *Sections 1.1.6.1.* and *1.1.7.1*.

1.1.6. Tumor necrosis factor alpha (TNFα) in teleost fish

Tumor necrosis factor alpha (TNFα) belongs to a large family of structurally related cytokines called Tumor necrosis factor superfamily TNFSF, an ancient family of structurally related cytokines whose orthologs can be traced back to protostomian invertebrates (Secombes *et al*. 2016). In mammals, TNFα exists in two biologically active forms: a 26 kDa membrane-bound protein and a 17 kDa secreted form, generated by proteolytic cleavage of the 26 kDa protein at its C terminus with TNFα converting enzyme (TACE) (Gearing *et al*. 1994; McGeehan *et al*. 1994; Moss *et al*. 1997). The cleaved mature peptide forms a trimer and binds to its receptor eliciting a response. The 17 kDa TNFα has a structure typical of TNF family members, composed of eight anti-parallel β-strands, forming a "jely-roll" β-structure (Figure 1.5) (Ware *et al*. 1998). A predicted TACE cut site is present in all fish

TNFα sequences, suggesting a conserved mechanism for TNFα processing and release in all vertebrates (Secombes *et al*. 2016).

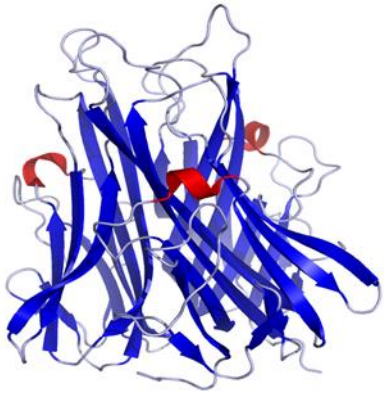


Figure 1.5. Crystal structure of TNFα as published in the Protein Data Bank (PDB: 1TNF).

1.1.6.1. Functional characteristics of TNFα

TNFα is a central inflammatory mediator initially identified as a serum component capable of eliciting "hemorrhagic necrosis" of certain tumors (Carswell *et al*. 1975). Fish TNFα has been found to be a pluripotent immune gene expressed at early stage of infection with a key role in orchestration of cytokine production and inflammation, as well as in regulation of lymphoid organ development and migration and proliferation of leukocytes (Zou and Secombes 2016). Studies have also shown that the main target of fish TNFα are endothelial cells, suggesting that TNFα is mainly involved in the recruitment of leukocytes to the inflammatory foci rather than in their activation (Roca *et al*. 2008). Furthermore, TNFα can induce its own production and that of other cytokines such as IL-1, IL-6 and IL-8 (Hong *et al*. 2013). TNFα is produced by macrophages in response to immunological challenges such as bacteria (lipopolysaccharides), viruses, parasites, mitogens, and other cytokines (Frederick *et al*. 2004). Following its production, TNFα mediates cellular response by binding to one of two receptors on cell membrane: TNFα receptor 1 (TNFR1) or TNFα receptor 2 (TNFR2). It can induce either NF-κB-mediated survival or caspases-mediated apoptosis, depending on the cellular context (Rahman and McFadden 2006). The apoptotic pathway is activated upon TNFα binding to death

domain (DD) on TNFR1, which can activate caspase cascades via DD containing signaling intermediates, ultimately leading to apoptosis. Namely, activated TNFR1 induces the formation of death inducing signaling complex (DISC), a protein complex formed by members of so-called death receptors such as RIP (receptor interacting protein), TRAD (TNFR1- associated death domain) and FADD (Fas-associated death domain). DISC activates caspase 8 or 10 which then induce activation of pro-apoptotic proteins of Bcl-family leading to cell death (Micheau and Tschopp 2003; Muppidi *et al*. 2004; Rahman and McFadden 2006). On the other hand, the NF-κB mediated survival pathway is activated following TNFα interaction with either TNFR1 or TNFR2. Activated receptors attract and bind TNF receptor-associated factor (TRAF). This complex activates signaling proteins such as NF-κB inhibitor kinase (NIK) and mitogen activated protein kinase kinase (MAPKK) 1 (MEKK1), which then activates the inhibitor of NF-κB (IκB) kinase (IKK). Activated IKK phosphorylates IκB, leading to its dissociation from NF-κB and subsequent degradation by proteasome. Free NF-κB is translocated into the nucleus where it, as the transcriptional factor, induces the expression of anti-apoptotic and inflammatory genes, resulting in cell survival and initiation of immune response (Wang *et al*. 1998; Rahman and McFadden 2006). Through adaptation processes during evolution, many viruses have developed different strategies to neutralize TNFα by direct binding and inhibition of the ligand or its receptor, or by modulation of various downstream signalling events, e.g. downregulating the cellular death receptors, interacting with TRAF, blocking caspase activation, blocking or activating NF-κB (Benedict and Banks 2003; Rahman and McFadden 2006).

In addition to its roles in acute infection, TNFα can be associated with oocyte maturation (Crespo *et al*. 2010) and liver development in fish (Qi *et al*. 2010), as well as with pathogenesis of several chronic fish diseases (Xu *et al*. 2012; Ronza *et al*. 2015). TNFα also represents one of the major cytokines secreted by adipose tissue, i.e. adipokin in fish (Liu *et al*. 2015). It has been designated as limiting factor of lipid deposition, inhibitor of preadipocytes differentiation and stimulator of mature adipocyte lipolysis, and it is suggested to be an important regulatior of lipid metabolism in fish (Albalat *et al*. 2005; Wang *et al*. 2012; Liu *et al*. 2015).

1.1.6.2. Identification of TNFα in fish

First fish TNFα was identified and characterized by Hirono *et al*. (2000) in Japanese flounder *Paralychthys olivaceus*. It had only 20-30% amino acid identity with mammalian TNFs, but had very similar intron/exon organization. The expression of flounder TNFα gene was elicited in peripheral blood leukocytes (PBL) by stimulation with different pathogen-associated molecular patterns (PAMP) suggesting that fish share conserved role of this cytokine in inflammatory responses with higher vertebrates. TNFα is currently one of the most well-studied fish cytokines, being described in several teleost fish, including: brook trout, *Salvelinus fontinalis* (Bobe and Goetz 2001), rainbow trout, *Oncorhynchus mykiss* (Laing *et al*. 2001), gilt-head sea bream, *Sparus aurata* (Garcia-Castillo *et al*. 2002), red sea bream, *Pagrus major* (Cai *et al*. 2003), common carp, *Cyprinus carpio* (Saeij *et al*. 2003), channel catfish, *Ictalurus punctatus* (Zou *et al*. 2003b), zebrafish, *Danio rerio* (Savan *et al*. 2005), Nile tilapia, *Oreochromis niloticus* (Praveen *et al*. 2006), turbot, *Psetta maxima* (Ordas *et al*. 2007), goldfish, *Carassius auratus* (Grayfer *et al*. 2008), ayu fish, *Plecoglossus altivelis* (Uenobe *et al*. 2007), sea bass, *Dicentrarchus labrax* (Nascimento *et al*. 2007), Atlantic salmon, *Salmo salar* (Morrison *et al*. 2007), Pacific bluefin tuna, *Thunnus orientalis* (Kadowaki *et al*. 2009), orange-spotted grouper, *Epinephelus coioides* (Lam *et al*. 2011), southern bluefin tuna, *Thunnus maccoyii* (Polinski *et al*. 2013). Notably, all fish TNFα proteins possess the TNF family signature, [LV]- x-[LIVM]-x3-G-[LIVMF]-Y-[LIMVMFY]2-x2-[QEKHL] (Laing *et al*. 2001), which underlines the evolutionary conservation of this cytokine. Also, the gene organization of 4 exons and 3 introns is found in all known vertebrate TNFα molecules determined so far.

1.1.6.3. Isoforms of TNFα in fish

The first functional characterization of a fish TNFα was reported by Zou *et al*. (2003), who suggested that pro-inflammatory roles of TNFα are conserved among all teleosts. This and many later studies also revealed the existence of some obvious differences between teleost TNFα and their mammalian counterparts, such as the presence of multiple TNFα isoforms in some species. Thus, Pacific bluefin tuna (Kadowaki *et al*. 2009), salmon (Haugland *et al*. 2007) and orange-spotted grouper (Lam *et al*. 2011) express two TNFα genes (TNFα1 and TNFα2), whereas common carp (Savan and Sakai 2004)

and rainbow trout (Hong *et al*. 2013) possess a third TNFα (TNFα3). On the other hand, Japanese flounder (Hirono *et al*. 2000) possesses only one TNFα copy. Furthermore, TNFα1 has shown rather strong constitutive expression in different tissues of healthy fish but relatively poor up-regulation following immune challenge *in vitro* and *in vivo* (Reyes-Cerpa *et al*. 2012), in contrast to TNFα2 with low constitutive expression but fast and strong post-stimulation induction (Zou *et al*. 2002; Kadowaki *et al*. 2009). TNFα3, on the other hand, exhibited generally lower constitutive expression than the other two genes, but was most responsive to early post-stimulation time point (Hong *et al*. 2013). The differential expression and modulation of the TNFα paralogues could be the results of subfunctionalization or even neo-functionalization, enabling fine regulation of TNFα-mediated cellular responses (He and Zhang 2005; Hong *et al*. 2013).

1.1.7. Interleukin-1 beta (IL-1β) in teleost fish

The interleukin-1 (IL-1) family of cytokines, is another major mediator of inflammation which can induce the expression of numerous non-structural, function-associated genes during infection (Bird *et al*. 2002a). Its members tend to be either pro-inflammatory, or act as antagonists to inhibit the activities of other family members (Palomo *et al*. 2015). IL-1β is the best characterized and most studied cytokine amongst the 11 mammalian IL-1 family members. In mammals, IL-1β is expressed as a 30 kDa inactive precursor, pro-IL-1β, that lacks a signal peptide and does not follow the conventional endoplasmic-reticulum (ER)/Golgi route of secretion. IL-1β is fully activated and able to elicit immune functions only after it is proteolytically cleaved into a 17.3 kDa mature peptide (Figure 1.6) by caspase 1. This is a cysteine protease also called IL-1β converting enzyme (ICE), although several other proteases can also cleave IL-1β but at different cleavage site (Bird *et al*. 2002a; Ogryzko *et al*. 2014). Caspase 1 cuts pro-IL-1β at a conserved aspartic acid residue (D) typically located between residues 113 and 117 releasing mature peptide (Zou *et al*. 1999b). However, in order to process IL-1β, caspase 1 itself must first be activated. Activation of caspase 1 takes place in the inflammasome, a multiprotein complex expressed in myeloid cells (i.e. monocytes, macrophages, granulocytes, mast cells, DCs) in response to danger or pathogen-associated signals (Vojtech *et al*. 2012). The inflammasome generally includes one or more pathogen recognition receptors PRRs, the

adaptor protein ASC, and caspase 1 (Broz and Monach 2011). Therefore, the release of mature IL-1β is a two-step process which requires two separate inflammatory signals. The first step is the transcriptional upregulation (e.g. via transcriptional factor NF-κB) and production of both pro-IL-1β and pro-caspase 1, as a result of cytokine signaling or myeloid cells encounter with pathogen-associated molecular pattern PAMPs. The second step is the assembly and activation of the inflammasome whose function is to produce biologically active mature IL-1β via activation of caspase 1. This step requires secondary inflammatory signal, usually in the form of DAMPs or cytokine signals in the cytosol (van de Veerdonk *et al*. 2011; Ogryzko *et al*. 2014). Caspase 1 is also associated with the transport of mature peptide out of the cell (Carretti *et al*. 1992; Thornberry *et al*. 1992; Tocci 1997).

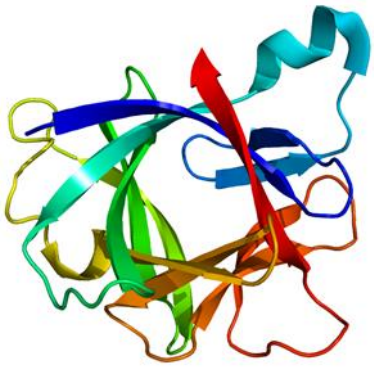


Figure 1.6. Crystal structure of IL-1β. Adapted from the Protein Data Bank (PDB: 31BI).

Fish IL-1β orthologs lack an identifiable D residue, i.e. caspase 1 cut site. However, processing of teleost pro-IL-1β into an active form involves inflammatory caspases (Zou and Secombes 2016). Teleost fish inflammatory caspases have been primarily sequenced in zebrafish, as a generally accepted model organism for studying host-pathogen interactions and immunity (Vojtech *et al*. 2012). Zebrafish orthologues of human inflammatory caspases 1 and 5, named caspase A and caspase B, are both able to cleave pro-IL-1β, although human caspase 5 does not cleave pro-IL-1β itself, but potentiates IL-1β processing mediated by caspase 1 (Vojtech *et al*. 2012; Martinon *et al*. 2002).

Interestingly, zebrafish caspase A and B require two cut sites for successful processing of IL-1β: D122 plus D104 for caspase A, and D122 plus D104 or D88 for caspase B (Vojtech *et al*. 2012). Therefore, zebrafish IL-1β is cleaved at two different sites into two mature peptides of 22- and 18-kDa.

1.1.7.1. Functional characteristics of IL-1β

IL-1β is one of the earliest expressed pro-inflammatory cytokines with overlapping functions with TNFα, enabling organism to respond promptly to infection (Zou and Secombes 2016). IL-1β is produced by a variety of cells, but mainly blood monocytes and tissue macrophages. It affects almost every cell type playing a central role in the initiation of systemic and local responses to infection or injury by activating macrophages, T and B lymphocytes and NK cells (Netea *et al*. 2010; Dinarello 2011). IL-1β exerts its activity by binding to IL-1 type I receptor (IL-1R1), which then recruits and binds IL-1 receptor accessory protein (IL-1RAP), which serves as a co-receptor for signal transduction of IL-1/IL-1RI complexes. Activated trimeric complex assembles two signaling proteins: myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor activated protein kinase (IRAK) 4. This triggers autophosphorylation of IRAK4, which then phosphorylates and activates IRAK1 and IRAK2. IRAK1 and IRAK2 form a complex with TNF receptor-associated factor 6 (TRAF6) causing its oligomerization and activation, and subsequent stimulation of signaling pathways leading to activation of transcription factors NF-kB and activator protein 1 (AP-1) (Cao *et al*. 1996a; Weber *et al*. 2010). Namely, TRAF 6 is ubiquitin ligase involved in ubiquitination of other proteins and itself. Ubiquitinated TRAF6 forms complex with transforming growth factor-β (TGF-β)-activated protein kinase-binding protein 2 and 3 (TAB2 and TAB3) and TGF-β–activated protein kinase (TAK1). Ubiquitination of TAK1 promotes its association with mitogen activated protein kinase (MAPKK) 3 (MEKK3). TAK1 and MEKK3 represent the main linkage between IL-1R-triggered immune response and final gene activation (Cao *et al*. 1996b; Weber *et al*. 2010). TAK1 can activate inhibitor of NF-κB (IκB) kinase (IKK), which then phosphorylates IκB leading to its degradation. Once NF-κB is dissociated from IκB, it can translocate into the nucleus and induce transcription of numerous IL-1β responsive genes (Weber *et al*. 2010). TAK1 and MEKK3 can also activate MAPKKs such as MKK4, MKK7, MKK3 and MKK6, ultimately leading to activation of AP-1. Both pathways

result in the induction of numerous IL-1β responsive genes including chemokines (IL-8), pro-inflammatory cytokines (TNFα, IL-6, IL-34), anti-inflammatory cytokines (IL-10 and TGF- β), cyclooxygenase 2 (COX2), acute-phase proteins (e.g. C-reactive protein, transferrin, complement factors) and cell adhesion molecules (Burns *et al*. 2003; Reis *et al*. 2012). IL-1β can also bind to IL-1 type II receptor (IL-1R2). However, since IL-1R2 contains only short cytoplasmic terminus and lacks signaling-competent part of intracellular domain specific for IL1R1, it is incapable of intracellular signaling and serves as decoy receptor that blocks ligand action. Together with IL-1Ra, IL-1R2 serves as a negative regulator of IL-1β signaling (Weber *et al*. 2010; Palomo *et al*. 2015; Zou and Secombes 2016).

In addition to its role in regulation of immune response, fish IL-1β is also involved in regulation of other physiological processes particularly muscle metabolism. Recent studies have shown that IL-1β is able to induce dilation of coronary microvessels (Costa *et al*. 2015), as well as trigger expression of genes controlling muscle mass (Heidari *et al*. 2015), growth and metabolism (Pooley *et al*. 2013).

1.1.7.2. Identification of IL-1β in fish

The first fish IL-1β cDNA sequence was identified by Secombes *et al*. (1998) in trout and exhibited 49-56% amino acid identity to the mammalian IL-1β. Interestingly, the trout IL-1β did not possess a putative ICE cleavage site required for the maturation-cleavage of the mammalian IL-1β. Nevertheless, the expression of trout cytokine could be induced in head kidney tissue and leukocytes following PAMPs (in this case LPS, lipopolysaccharid) stimulation (Secombes *et al*. 1998; Zou *et al*. 1999a), suggesting its pro-inflammatory nature. In teleosts, IL-1β is not constitutively expressed but activated only after *in vitro* stimulation with different PAMPs, or as the result of various infections *in vivo* (Reyes-Cerpa *et al*. 2012). So far IL-1β has been characterized in various fish species including: rainbow trout, *Oncorhynchus mykiss* (Zou *et al*. 1999a), common carp, C*yprinus carpio* (Fujiki *et al*. 2000), sea bass, *Dicentrarchus labrax* (Scapigliati *et al*. 2001), gilthead seabream, *Sparus aurata* (Pelegrin *et al*. 2001), small spotted catshark, *Scyliorhinus canicula* (Bird *et al*. 2002b), channel catfish, *Ictalurus punctatus* (Wang *et al*. 2006), Nile tilapia, *Oreochromis niloticus* (Lee *et al*. 2006), haddock, *Melanogrammus aeglefinus* (Corripio-Miyar *et al*. 2007), yellowfin sea bream,

Acanthopagrus latus (Jiang *et al*. 2008), orange-spotted grouper, *Epinephelus coioides* (Lu *et al*. 2008), southern bluefin tuna, *Thunnus maccoyii* (Polinski *et al*. 2013). All known fish IL-1β are characterized by conserved family signature [FCL]-x-S-[ASLV]-xx-[PRS]-xx-[FYLIV]-[LI]-[SCAT]-T- xxxxxxx-[LIVMK].

1.1.7.3. Isoforms of IL-1β in fish

Additional isoforms of IL-1β are quite often in teleost fish, due to the fact that many fish species are tetraploid and have undergone the WGD. Second IL-1β gene (IL-1β2) was first described in rainbow trout (Pleguezuelos *et al*. 2000). Comparison of the two trout genes revealed that IL-1β2 has 82% amino acid identity to the IL-1β1, and as other non-mammalian IL-1β genes, no putative ICE cleavage site. Induction of trout IL-1β2, as it was already shown with IL-1β1, can be elicited by *in vitro* cultured trout leukocytes (Pleguezuelos *et al*. 2000). Shortly after this discovery, two highly similar IL-1β genes have also been described in catfish, sharing 94.3% amino acid sequence identity. In this case, expression analysis indicated that both IL-1β genes are expressed in normal catfish tissues as well as after bacterial infection, but exhibited distinct expression profiles (Wang *et al*. 2006). Carp also contains two IL-1β isoforms, where IL-1β2 shares 74% identity with the IL-1β1 and 95-99% identity with other known IL-1β2 transcripts (Farrar *et al*. 1991). The post-stimulation expression of the two carp IL-1β isoforms differed in a way that the expression of IL-1β1 gene was on average at least ten-fold greater than that of IL-1β2. In light of all the above, and facts that the transcripts of IL-1β2 had shown to have high substitution numbers in the coding regions and that predicted IL-1β2 proteins are truncated compared to the IL-1β1, it was suggested that the IL-1β2 may be in fact a pseudogene (Farrar *et al*. 1991). Recently, two more IL-1β genes, IL-1β3 and IL-1β4, were cloned and characterized in rainbow trout (Husain *et al*. 2012). Interestingly, the IL-1β4 sequence has two stop codons, a deletion and an insertion, implying that salmon IL-1β4 is a transcribed pseudogene. It is also striking that IL-1β3 has only low identities to salmonid IL-1β1 (31.4–32.2%). A relatively high constitutive expression of trout IL-1β3 in gills, spleen and kidney and the up-regulation by PAMPs, pro-inflammatory cytokines and viral infection, suggests its role in inflammation and host defense (Husain *et al*. 2012). In addition, high expression of IL-1β3 in trout ovary suggests its possible role in

reproduction system (Husain *et al*. 2012). This is supported by IL-1β expression in seabream gonads, where it is associated with tissue reorganization during the adult reproductive cycle (Chaves-Pozo *et al*. 2009).

Ultimately, these findings indicate the existence of two types of teleost IL-1β originating from the WGD. Type I teleost IL-1β genes, including IL-1β3, share a similar 6 coding exon structure as in tetrapods. Type II teleost IL-1β genes, including IL-1β1 and IL-1β2, lack one or two coding exons at their 5'-end, and share higher identities within this subgroup than within the type I subgroup (Husain *et al*. 2012).

1.2. Atlantic bluefin tuna

Atlantic (or Northern) bluefin tuna (ABFT), *Thunnus thynnus*, is a seawater ray-finned bony fish belonging to the Scombridae family. Genus *Thunnus*, also referred to as the true tunas, consists of eight species of tuna and is divided into two subgenera: *Thunnus* (*Thunnus*) or the bluefin group, and *Thunnus* (*Neothunnus*) or the yellowfin group. Bluefin tunas are the largest of the *Thunnus* species (Scombridae) and are characterized by a long lifetime, wide geographic distribution and endothermy (Block and Stevens 2001). Today tuna aquaculture comprises only three *Thunnus* species all belonging to the bluefin group: ABFT which is cultured in Croatia and several other Mediterranean countries, Southern bluefin tuna *Thunnus maccoyii* (SBFT) which is cultured in Australia, and Pacific bluefin tuna *Thunnus orientalis* (PBFT) cultured in Mexico and Japan (Benetti *et al*. 2016).

1.6.1. ABFT biological properties

Adult ABFT (Figure 1.7) is characterized by its impressive biological properties such as size (up to 3 m in length and 700 kg of weight), swimming speed (up to 90 km/h) and enormous muscular strength which allows extensive transoceanic migrations (Benetti *et al*. 2016). Furthermore, since ABFT is a pelagic fish that never stops swimming to ventilate (like other tunas and mackerel sharks), it continuously generates heat which is used to elevate and maintain the temperature of highly-aerobic tissues of the skeletal muscle, eye, brain and viscera above water temperatures. The ABFT, as all tunas, achieves this kind of endothermy by conserving nearly all the metabolic heat and the heat

generated by centrally located swimming muscle. It possesses special complex of arteries and veins lying very close to each other in the periphery of the body, called *rete mirabile* ("wonderful net"), through which generated heat is being transferred from venous blood to the cold arterial blood coming from the gills. Also, a counter-current exchange system of blood flowing in opposite directions facilitates this heat transfer (Cech *et al*. 1984) (Figure 1.8). This way the effects of surface cooling are mitigated enabling ABFT to sustain up to 98% of its body temperature. This property of endothermy is highly developed in ABFT and contributes to its extensive capacity for migration (Graham and Dickson 2001) during which ABFT can withstand cold (down to 3°C) as well as warm (up to 30°C) temperatures while maintaining stable internal body temperature (Block *et al.* 2001).

Figure 1.7. Atlantic bluefin tuna, *Thunnus thynnus*. Taken from MarineBio.org.

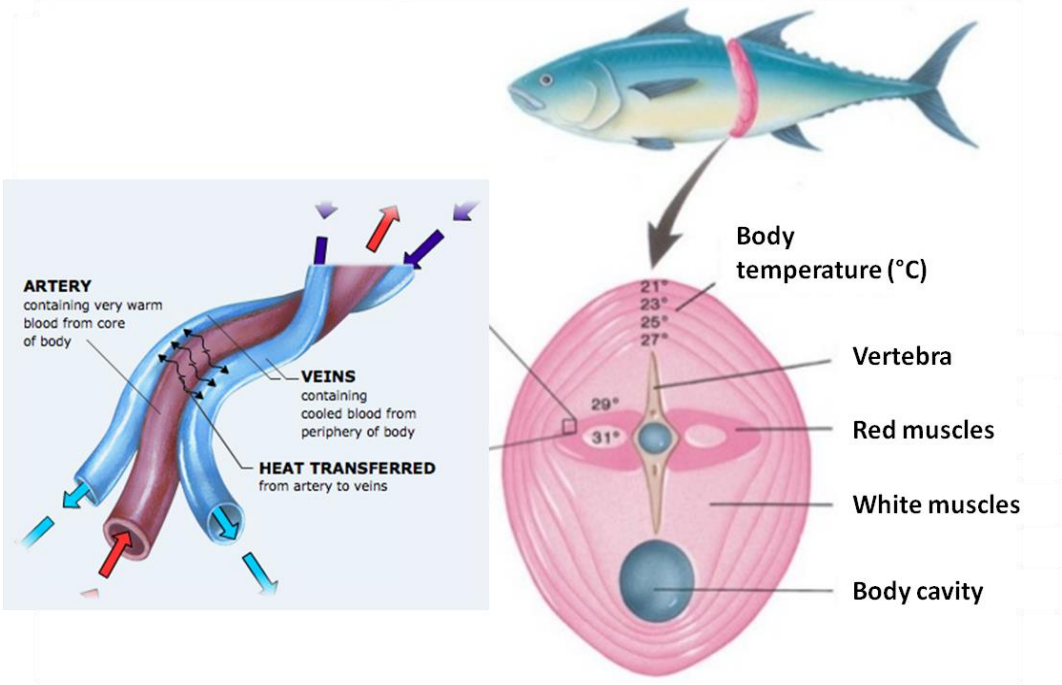


Figure 1.8. Scheme of a counter-current heat exchange system between veins and artery in tuna.

1.6.1. ABFT habitat

Among the tuna, ABFT has the widest geographical distribution and it is the only large pelagic fish living permanently in temperate Atlantic waters (Bard *et al.* 1998; Fromentin and Powers 2005). It inhabits the pelagic ecosystem of the entire North Atlantic and its adjacent seas, from the equator to the North of Norway and from the Gulf of Mexico through the Mediterranean Sea to Black Sea (Mather *et al.* 1995). ABFT migrates seasonally from the Atlantic Ocean where it feeds, to the Gulf of Mexico or the Mediterranean Sea where it spawns (ICCAT, 2008) (Figure 1.9). Based on those two spawning grounds International Commission for the Conservation of Atlantic Tunas (ICCAT) has determined two ABFT populations: (i) Western population, which spawns in the Gulf of Mexico with spawning peak in May and (ii) Eastern population which spawns in the Mediterranean Sea with spawning peak in June (Zohar *et al*. 2016). Although ABFT preferentially occupies the surface and subsurface waters, both juveniles and adults frequently dive to depths of 500 m to 1000 m (Lutcavage *et al.* 2000; Block *et al.* 2001; Brill *et al.* 2001) in search for food and/or to cool the body temperature (Carey and Robinson 1981; Holland *et al.* 1992; Bard *et al.* 1998; Gunn and Block 2001; Musyl *et al.* 2003).

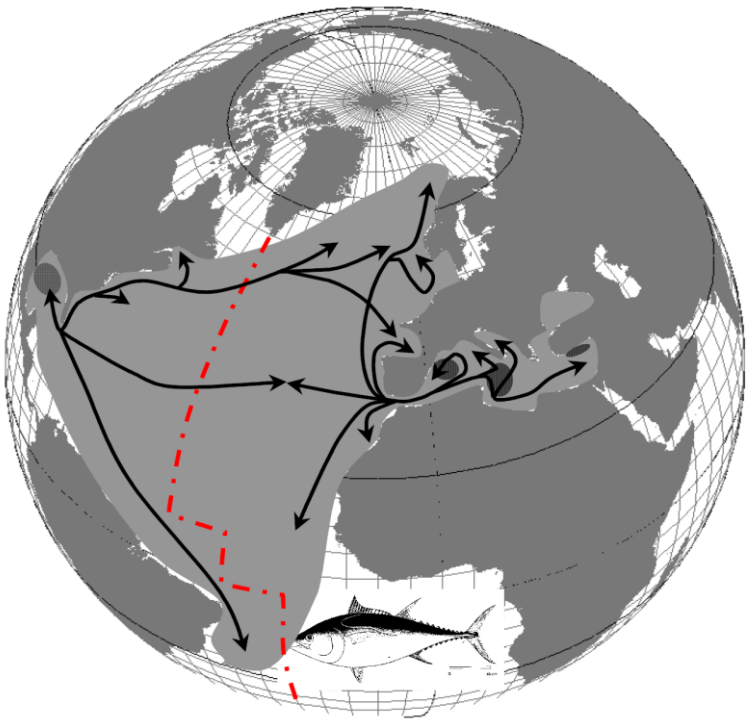


Figure 1.9. Spatial distribution of Atlantic bluefin tuna, *Thunnus thynnus* (grey shading). The main migration routes (black arrows), main spawning grounds (dark grey areas) and the stock delimitation border (vertical red dashed line) are depicts. Reused from Fromentin and Powers (2005).

1.6.3. Capture-based aquaculture of ABFT in Croatia

ABFT represents one of the economically most important species for fisheries industry reaching extremely high commercial value. High market demand for ABFT dictated by constantly growing sushi-sashimi market in Japan resulted in overfishing and drastic ABFT population reductions (Benetti *et al*. 2016). ABFT farming was introduced into Mediterranean aquaculture in the early nineties aiming at the production of tuna with the optimal protein and fat content especially for sushi and sashimi market (Karakulak *et al*. 2016). Today, ABFT represents the most valuable finfish aquaculture product recognized, with more than half of the world's total production concentrated in the Mediterranean Sea (Ottolenghi 2008).

In Croatia, the first ABFT culture began two decades ago. Presently, tuna exported to the Japanese market accounts for 50% of the total national fisheries export, highlighting the importance of ensuring

optimal and sustainable conditions during its farming cycle. ABFT for aquaculture are targeted by purse-seiners and traditional trap fishermen during their spawning migrations (South Adriatic in Croatia) and transported to farming cages (Figure 1.10.A). Typical size range in the Mediterranean is from 40 to 400 kg, while in Croatia it is much lower, from 8 to 30 kg. Thus, while fattening period in the Mediterranean ranges from 3 to 7 months, farming period in Croatia is prolonged and lasts up to 2 years until required product quality and commercial size are reached (Mylonas *et al*. 2010), which makes tuna farming in Croatia unique in the world. Consequently, ABFT aquaculture in Croatia is characterized as farming rather than fattening (Miyake *et al*. 2003). During farming period and depending on the baitfish availability, ABFT are fed with variety of fresh or defrosted, previously frozen baitfish, such as sardinella (*Sardinella aurita*), pilchard (*Sardina pilchardus*), herring (*Clupea harengus*), mackerel (*Scomber scombrus*), bogue (*Boops boops*), and some cephalopods (Vita *et al*. 2004) (Figure 1.10.B).

In order to allow recovery of wild population stocks and at the same time enable constant and sustainable supply, since the early 2000s the European Union has turned its focus to closing the life cycle of ABFT. Thus, research and development consortiums such as the REPRODOTT (Reproduction of the Bluefin Tuna in Captivity - feasibility study for the domestication of *Thunnus thynnus*) and SELFDOTT (Self-sustained Aquaculture and Domestication of Bluefin Tuna *Thunnus thynnus*) have been funded by the European Commission, European governments and companies (Karakulak *et al*. 2016; de la Gándara *et al*. 2016). The world's largest land-based facility for ABFT has been completed in Spain in April 2015, allowing spawning of ABFT under controlled conditions and for prolonged period. The aim of such facility is to provide a sufficient amount and quantity of fertilized ABFT eggs and ABFT juveniles for both aquaculture industry and scientific research. Cyprus and Turkey have also reported significant progress regarding production of ABFT juveniles from their own broodstock, suggesting that the Mediterranean tuna aquaculture is one step closer to commercialization of closed-cycle production of ABFT (de la Gándara *et al*. 2016).

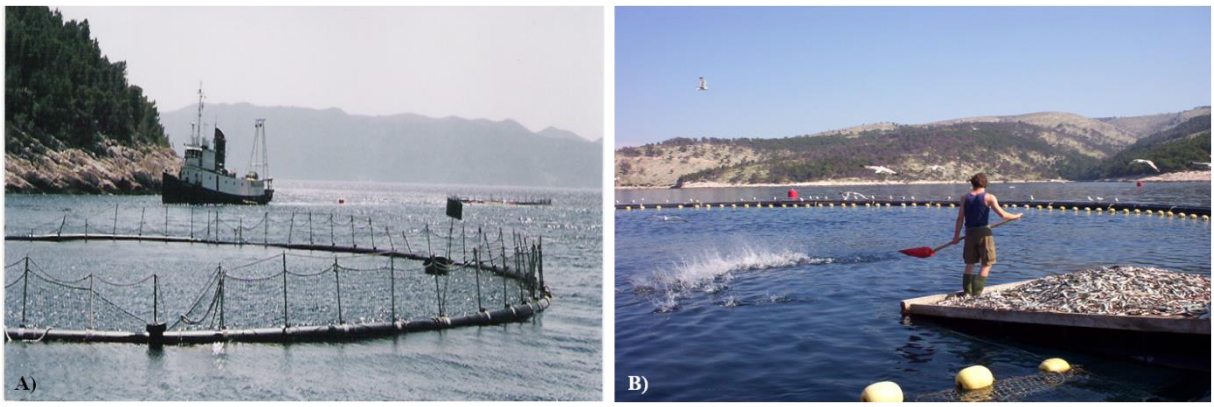


Figure 1.10. Capture-based Atlantic bluefin tuna farming in Croatia. A) Farming cage in tuna farm Sardina d.o.o., Brač; B) Manually feeding tuna with defrosted baitfish.

1.6.4. Health status of farmed ABFT

Sustainable aquaculture relies on successful health management of farmed fish. In case of cage culture, it is nearly impossible to have control over waterborne pathogens. Therefore, this kind of aquaculture often contributes to their outbreaks (Nowak 2007). Furthermore, stress due to limited space, presence of microorganisms, unpredictable environmental factors and unbalanced diet, is also a frequently present factor in cage culture. All the above can have a negative effect on host immunocompetence and ultimately lead to immunosuppression and disease outbreak. Due to its long-distance migration, ABFT is exposed to numerous types of pathogens, e.g. parasites, viruses, bacteria (Munday *et al*. 2003; Nowak *et al*. 2006). However, pathogen-associated mass mortalities have been rarey reported, especially in the Mediterranean farming systems.

Feeding tuna with imported frozen baitfish was a subject of polemics due to the potential risk of dissemination of viral agents, such as viral hemorrhagic septicaemia virus (VHSV) by the frozen herring (Jones *et al*. 1997; Marty *et al*. 1998) to tuna or to the wild fish populations. However, in almost 25 years of ABFT aquaculture in the Mediterranean, there was no evidence of presence of pathogenic viruses or clinical signs related to them. In Japanese facilities, viral diseases such as red seabream iridoviral desease (RSIVD) and viral nervous necrosis (NNV) were shown to cause mortalities of larval and juvenile PBFT (Balli *et al*. 2016).

Although bacterial diseases are rare in farmed ABFT, there are few reports of bacterial agents causing mortalities without obvious clinical signs. For example, asymptomatic pasteurellosis which led to granulomatous changes in liver and spleen was unexpectedly discovered during harvest of seemingly

healthy ABFT (Perić 2002). On the other hand, *Photobacterium damsela* subsp. *piscicida* outbreak (Mladineo *et al*. 2006), provoked by increased water temperature and excess of volatile amines content in frozen baitfish (Šimat *et al*. 2009), led to mass mortalities of ABFT in 2003 and 2004 (Mladineo *et al*. 2006; Šimat *et al*. 2009). Furthermore, *Photobacterium damsela* subsp. *damselae*, *Vibrio* sp., and *Tenacibaculum* sp. caused septicemia-related lesions in ABFT larvae (Gustinelli *et al*. 2011).

No economically threatening parasitoses have ever been reported in adult reared ABFT in the Adriatic or Mediterranean farming system. Nevertheless, parasitoses and mortalities are more common in other bluefin tuna species. For example, in SBFT occurrence of mortalities have been associated with encephalitis caused by opportunistic scuticociliate *Uronema nigricans* (Munday et al. 1997) or epizootic hyperinfections of sea lice *Caligus chiastos* and sanguinicolid blood fluke *Cardicola forsteri* (Hayward *et al*. 2010). Blood flukes appear to cause the most harmful parasitic diseases with potential deadly outcome in both SBFT and PBFT (Balli *et al*. 2016) (Table 1.1). However, although sanguinicolid blood flukes have been identified in farmed ABFT (Nowak *et al*. 2006, Aiken *et al*. 2007; Palacios-Abella *et al*. 2015), they apparently do not cause ABFT mortalities (Table 1.1).

Cardicola Species	Definitive Host	Effect on Industry
C. forsteri	SBFT	Mortality
	ABFT	None
C. orientalis	SBFT	Mortality
	PBFT	Mortality
	ABFT	None
C. opisthorchis	PBFT	Mortality
	ABFT	None

Table 1.1. *Cardicola* species, their definitive hosts and effect on the industry. Mortality occurs in untreated SBFT and PBFT. Adapted from Balli *et al*. (2016).

Metazoan parasite communities found in ABFT comprise trematodes, cestodes, nematodes, crustaceans, myxozoans and microsporidians (Mladineo *et al*. 2008). Digenean trematodes, with *Didymosulcus katsuwonicola* and *Koellikerioides intestinalis* as core species, are the most frequent and abundant parasite group found in various ABFT organs (Mladineo and Tudor 2004; Mladineo *et*

al. 2011). Interestingly, parasite assemblages according to host size differ between wild and captive ABFT; while the most diverse parasitofauna is usually found in the largest specimens of wild ABFT (Culurgioni *et al*. 2014), the opposite was observed in captivity. Mladineo *et al*. (2011) have reported a disappearance of three didymozoid species and decreasing trend of other parasite populations towards the end of the rearing cycle in ABFT from Croatian tuna farms. Such decreasing trend in parasite populations has rarely been observed in other intensive aquaculture productions (Yamaguti 1970; Karlsbakk 2001). It is still unclear whether environmental factors or host immunity resistance and genetic predispositions, or combination of all mentioned, influence this phenomenon (Mladineo *et al*. 2011), but it certainly underlines the complexity of host-parasite interactions in which the behaviour of both the host and the parasite depends on the common interaction.

Intensification of tuna aquaculture industry, especially closed-cycle production may contribute to the appearance of new tuna health issues. Understanding the immune response of the farmed ABFT will provide a better insight into potential health risks which can arise during farming process and perhaps enable their mitigation. Studies of immune response in tunas have predominately been focused on the PBFT and SBFT. In case of PBFT, studies encompass ontogeny of the immune response (Watts *et al*. 2003), cytokines expression (Kadowaki *et al*. 2009; Mladineo and Block 2010) as well as IgM and IgT expression in lymphoid organs (Mashoof *et al*. 2014) and *Cardicola*-infected organs (Polinski *et al*. 2014a). Studies of SBFT immune response encompass analysis of serum Ig, lysozyme and complement activity (Watts *et al*. 2002), measurement of *Cardicola*-specific antibody presence (Watts *et al*. 2001; Aiken *et al*. 2008; Kirchhoff *et al*. 2012), lysozyme and complement activity (Kirchhoff *et al*. 2011, 2012) and cytokines expression (Polinski *et al*. 2013, 2014b). In contrary, ABFT immune system has not been widely studied and prior to this thesis, researchs referring to cloning and expression analysis of any important immune genes in this species were not undertaken.

1.7. Research objectives

The main research objectives of this thesis were:

1. Identification and characterization of the pro-inflammatory cytokines in reared Atlantic bluefin tuna (ABFT) *Thunnus thynnus* trough:

    - Cloning of the full-length complementary DNA (cDNA) and genomic DNA (gDNA) sequences of ABFT pro-inflammatory cytokines TNFα1, TNFα2 and IL-1β

    - Comparison of ABFT TNFα1, TNFα2 and IL-1β molecules with known sequences in other vertebrates, especially teleost fish
    - Designing of the 3D models of ABFT TNFα1, TNFα2 and IL-1β using homology modeling method

2. Analysis of ABFT TNFα1, TNFα2 and IL-1β expression profiles *in vitro*:

    - in peripheral blood leukocytes (PBLs) after stimulation with different PAMPs, lipopolysaccharid (LPS) and polyinosinic-polycytidylic acid (Poly I:C)
    - in PBLs after stimulation with *Pseudocycnus appendiculatus* (Copepoda) and *Didymosulcus katsuwonicola* (Digenea) protein extracts

3. Analysis of ABFT TNFα1, TNFα2 and IL-1β expression profiles *in vivo*:

    - in selected tissues of ABFT over the duration of the farming process (at three time points)
    - at the *D. katsuwonicola* and *P. appendiculatus* parasitation site

4. Pathohistological analysis of gill filaments infected with *D. katsuwonicola* and *P. appendiculatus*

2. Materials and methods

2.1. Atlantic bluefin tuna sampling

Atlantic bluefin tuna (ABFT) *Thunnus thynnus* sampling (Table 2.1) was done on several occasions at tuna facility Sardina d.o.o., Brač. All fish handling procedures followed established standards for the care and use of animals, which were previously approved by the Ethical Committee for Animal Welfare at the Institute of Oceanography and Fisheries, Croatia. All sampling was performed using sterilized surgical kits.

Table 2.1. Atlantic bluefin tuna ABFT sampling schedule with description of sampled fish, extracted tissue samples and purpose of sampling. Newly caught group comprised healthy juvenile fish that were caught in the central part of the South Adriatic Sea, transferred in a towing cage tugged to the farming site and left for acclimation for two weeks. Damaged group comprises reared juvenile fish with wounds and lesions on the skin that led to mortalities in some instances during the acclimation period. Fish necropsy included assessment of gross pathology and histopathology, bacteriology and parasitology as previously described (Mladineo *et al*. 2006: Mladineo *et al*. 2011). Damaged fish showed signs of septicemia. Farm-acclimated group comprised healthy fish reared for 18 months.

YEAR	NUMBER AND DESCRIPTION OF SAMPLED FISH	TISSUE SAMPLED	PURPOSE OF SAMPLING
Summer 2010. Transfer and acclimatization period	**14 juvenile tuna** 8-10 kg Designation: newly caught	5 tail and dorsal fin clip	DNA extraction for full-length sequencing
		5 head kidney and liver samples	RNA extraction for full-length sequencing
		9 head kidney and liver samples	RNA extraction for expression analysis
Summer 2011. Transfer and acclimatization period	**10 juvenile tuna** 8-10 kg Designation: damaged	head kidney and liver samples	RNA extraction for expression analysis
Winter 2011. Commercial harvest after 18 months of farming	**10 adult tuna** 65-75 kg Designation: farm-acclimated	head kidney and liver samples	RNA extraction for expression analysis
Winter 2012. Commercial harvest after 18 months of farming	**14 adult tuna** 85-115 kg	peripheral blood	Isolation od peripheral blood leukocytes (PBLs)
Winter 2013. Commercial harvest after 18 months of farming	**7 adult tuna** 85-115 kg Designation: uninfected	gill filaments	RNA extraction for expression analysis
	7 adult tuna 85-115- kg Designation: *Didymosulcus katsuwonicola*-infected	gill filaments	RNA extraction for expression pathohistological analysis
		20 *D. katsuwonicola* cysts	Preparation of parasite protein extracts (PE)
	7 adult tuna 85-115- kg Designation: *Pseudocycnus appendiculatus* -infected	gill filaments	RNA extraction for expression pathohistological analysis
		7 adult *P. appendiculatus*	Preparation of parasite protein extracts (PE)

2.2. Genomic DNA extraction

Collected tail or dorsal fin clips were preserved in absolute ethanol until used. Genomic DNA (gDNA) was isolated from ABFT tail or dorsal fin clips, previously preserved in absolute ethanol, following a simplified DNA isolation procedure (Laird *et al*. 1991). Briefly, pieces of fin or liver/head kidney tissue were cut into smaller fragments (cca 5 mm), washed in distilled water and transferred into 1,5 ml tube containing 200 µl of lysing buffer (0,01M Tris-HCl, 0,01M EDTA, 0,15M NaCl, 2% (w/v) SDS). Tissues were digested by adding 8 µl of Proteinase K (1mg/ml) to each tube and incubating in shaker set on 700 rpm for 3-4 h (or until tissue was digested) on 55°C. Samples were then centrifuged at 13,000 rpm (~17,900 x g) for 10 min at room temperature. Supernatant containing gDNA was transferred into clean 1.5 ml tube and gDNA was precipitated by adding 1 volume of isopropanol (e.g. 200 µl of lysing buffer : 200 µl of isopropanol) and centrifuging at 3.000 rpm for 10 min. Supernatant was discarded and the pellet containing gDNA was washed with 800 µl of 70% ethanol and centrifuged for 10 min. The pellet was air-dried for 15-20 min and dissolved in 20-40 µl of TE buffer (0.05M Tris-HCl, 1mM EDTA). DNA quantity and quality were assessed using a spectrophotometer (Eppendorf, Qiagen, Ilden, Germany) at 260 and 280 nm. Samples with a 260/280 ratio between 1.7 and 1.9 were used as templates in PCR reactions. After isolation, the gDNA was kept at -20 °C for long-term storage.

2.3. RNA isolation

Collected ABFT head kidney, liver or gill tissue samples were stored in RNAlater (Qiagen) at –20 °C until RNA extraction. Total RNA was isolated from liver, head kidney and gill tissues, and peripheral blood leukocytes (PBL). Total RNA was extracted from 50 to 100 mg of liver, head kidney or gill tissue and 5-10 x 10^6 cells using Tri Reagent (Sigma Aldrich, USA) following the manufacturer's instructions. Briefly, after samples were manually homogenized in 1 mL of TRI Reagent, homogenates were incubated at room temperature (rt) for 5 minutes (min) and 200 ml of chloroform was added. Samples were shaken vigorously for 15 seconds (sec), incubated at rt for 15 min and centrifuged at 12,000 x g for 15 min at 4 ºC. Aqueous (upper) phase was transferred into a new tube and RNA was precipitated by adding equal volumes of isopropanol (Sigma Aldrich). Samples were

then vortexed for 5-10 sec, incubated at rt for 5 min and centrifuged at 12,000 x g for 10 min at 4 ºC. Supernatant was removed and the RNA precipitate (gelatinous whitish pellet on the bottom of the tube) was subsequently washed with 1 ml of 75 % ethanol and centrifuged at 12,000 × g for 5 min at rt. After supernatant was removed, the RNA pellet was air dried for 3-5 min and dissolved in 20-40 µl RNase/ DNase free water (Sigma Aldrich). RNA was quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies) and stored at -80 °C if not used immediately.

2.4. Synthesis of cDNA

Prior to cDNA synthesis, total RNA was treated with 1 unit/µl of RNase free DNase I (Thermo Scientific) following the manufacturer's instructions. Briefly, the reaction mix was prepared by adding 1 µg of RNA sample, 1 µl of 10x reaction buffer with $MgCl_2$ (supplied with the kit), 1 µl of RNase free DNase I and DEPC-treated Water to 10 µl. The mix was then incubated at 37 °C for 30 min. To inactivate Dnase I without degradation of RNA, 1 µL 50 mM EDTA was added to reaction mix and incubated at 65 °C for 10 min.

cDNA was synthesised from 1 µg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) following manufacturer's instructions. Reverse transcription (RT) master mix was prepared on ice from 2 µl of 10x RT Buffer, 0,8 µl of 25x dNTP Mix (100 mM), 2 µl of 10x RT Random Primers, 1 µl of RNase Inhibitor, 1 µl of MultiScribe™ Reverse Transcriptase and 3.2 µl of Nuclease-free H_2O per reaction. Subsequently, 10 µl of RT master mix was combined with 10 µl of RNA and incubated at 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. cDNA prepared as described was used as a template for PCR and real-time PCR.

2.5. Cloning and sequencing

For cloning purposes, PCR products were first ligated into pGEM-T Easy Vector (Promega, Madison, WI) by mixing 3 µl of PCR product with 5 µl of 2X Rapid Ligation Buffer, 1 µl of T4 DNA Ligase (3 Weiss units/µl) and 1 µl of pGEM®-T Easy Vector (50 ng) and incubated at 4 °C over night (ON). Ligation reactions were then transfected into RapidTrans TAM1 competent *E. coli* (Active Motif, US) by adding 2 µl of each legation reaction into separate tubes containing competent cells and placed on ice for 20 minutes. The cells were then heat-shocked for 45 seconds in a water bath at exactly 42 °C

and immediately returned to ice for another 2 minutes. Then, 250 µl of Super Optimal broth with Catabolite repression (SOC) medium (Invitrogen) was added to every tube and incubated for 1.5 hours at 37 °C with shaking (~150 rpm). Subsequently, 100 µl of each transformation culture was plated onto lysogeny broth (LB) agar (Invitrogen) containing 100 µg/ml of ampicillin, and left to grow at 37 °C ON. Clones were than screened for PCR insert using T7 and SP6 primers (Table 2.2). Colonies containing the correct size insert were grown overnight in 5 ml LB medium (Invitrogen) containing 5 µl of ampicillin (100 µg/ml), in shaking incubator at 200 rpm and 37 °C. Plasmid DNA from at least 5 independent colonies was purified using a QIAprep Spin Miniprep kit (Qiagen) following the manufacturer's instructions. All the buffers and columns were supplied with the kit. Briefly, 1-5 ml bacterial overnight culture was pelleted by centrifugation at >8000 rpm (6800 x g) for 3 min on rt. Pelleted bacterial cells were then resuspended in 250 µl Buffer P1 and transferred to a new microcentrifuge tube. To lyse bacterial cells, 250 µl of Buffer P2 was added and the reaction was mixed thoroughly by inverting the tube 4–6 times until solution became clear and blue. The reaction was neutralized by adding 350 µl of Buffer N3 and mixing (by inverting the tube 4-6 times) until the solution became colorless. The reaction mix was then centrifuged for 10 min at 13,000 rpm (~17,900 x g) and approximately 800 µl of supernatant was transferred to the QIAprep 2.0 spin column, and centrifuged for 60 s at 13,000 rpm. The QIAprep 2.0 spin column was washed by adding 0.75 ml of Buffer PE and centrifuged for 30-60 sec at 13,000 rpm. The QIAprep 2.0 spin column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 35 µl of Buffer EB (10 mM TrisCl, pH 8.5) was added to the center of the QIAprep 2.0 spin column, incubated for 1 min, and centrifuged for 1 min at 13,000 rpm.

The purified plasmids were sent to Macrogen (the Netherlands) for sequencing with vector specific primers T7 forward and SP6 reverse.

2.6. Rapid Amplification of cDNA Ends (RACE)

The GeneRacer™ Kit (Thermo Fisher Scientific, USA) provides a method to obtain full-length 5′ and 3′ ends of cDNA using known cDNA sequence, ensuring the amplification of only full-length

transcripts via elimination of truncated messages from the amplification process. All reagents were supplied with the kit except ethanol.

2.6.1. Production of complementary DNA

Production of cDNA for 3' and 5' RACE was performed from 2 μg of total RNA, using a GeneRacer™ Kit following the manufacturer's instructions (Figure 2.1). Main steps of this method were:

1) **Dephosphorylation of total RNA or mRNA** (removal of 5' end phosphates from RNA species that lack protective 5' cap structure). This step eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo (Figure 2.1.A).

    - In order to remove the 5′ phosphates, total RNA was treated with calf intestinal phosphatase (CIP) by mixing 2 μl of total RNA (5 μg), 1 μl of 10X CIP Buffer, 1 μl of RNaseOut™ (40 U/μl), 1 μl of CIP (10 U/μl) and 5 μl of diethyl pyrocarbonate (DEPC) treated water. Reaction was gently mixed by pipetting, briefly vortexed, centrifuged to collect fluid and incubated at 50 °C for 1 hour. After incubation, reaction was once more centrifuged briefly and placed on ice. RNA was precipitated by adding 90 μl of DEPC treated water and 100 μl of phenol:chloroform:isoamyl alcohol (25:24:1). Reaction was then vortexed vigorously for 30 sec and centrifuged at 13,000 rpm for 5 min at rt. Top, aqueous phase (~100 μl) was transferred to a new tube and 2 μl 10 mg/ml mussel glycogen and 10 μl 3 M sodium acetate (pH 5.2) were added. The reaction was subsequently washed with 220 μl of 95% ethanol and frozen on dry ice for 10 minutes. RNA was pelleted by centrifuging at 13,000 rpm for 20 min at 4 °C. Supernatant was removed and 500 μl of 70% ethanol was added. Reaction was then inverted several times, vortexed briefly and centrifuged at 13,000 rpm for 2 min at 4 °C. Remaining ethanol was removed by a pipet and the pellet was air-dried for 1-2 minutes at rt. The RNA pellet was resuspended in 7 μl of DEPC water.

2) **Removal of the 5′ cap structure** from intact, full-length mRNA. This treatment leaves a 5′ phosphate required for ligation to the GeneRacer™ RNA Oligo (Figure 2.1.B).

- In order to remove the 5′ cap structure from intact, previously obtained full-length mRNA, RNA from the previous step was treated with tobacco acid pyrophosphatase (TAP) by mixing 7 µl of RNA, 1 µl of 10 x TAP Buffer, 1 µl of RNaseOut™ (40 U/µl) and 1 µl of TAP (0.5 U/µl). Reaction was gently mixed by pipetting, briefly vortexed, centrifuged to collect fluid and incubated at 37 °C for 1 h. After incubation, reaction was once more centrifuged briefly and placed on ice. RNA was precipitated and pelleted as described under 1).

3) **Ligation of the GeneRacer™ RNA Oligo** to the 5′ end of the mRNA using T4 RNA ligase. The GeneRacer™ RNA Oligo provides a known priming site for GeneRacer™ PCR primers for amplification of 5' end (Figure 2.1.C).

- The GeneRacer™ RNA Oligo was ligated to the 5′ end of the mRNA using T4 RNA ligase by adding 7 µl of decapped RNA to the tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 µg) and incubating at 65 °C for 5 min to relax the RNA secondary structure. Reaction was then placed on ice to chill for 2 min and briefly centrifuged. Subsequently, the following reagents, 1 µl of 10x Ligase Buffer, 1 µl of 10 mM ATP, 1 µl of RNaseOut™ (40 U/µl) and 1 µl of T4 RNA ligase (5 U/µl) were add to the tube and incubated at 37 °C for 1 h. After incubation, reaction was once more centrifuged briefly and placed on ice. RNA was precipitated and pelleted as described under 1).

4) **Reverse transcription of the ligated mRNA** using chosen reverse transcriptase and the GeneRacer™ Oligo dT Primer to create RACE ready first-strand cDNA with known priming sites at the 5′ and 3′ ends (Figure 2.1.D).

- Ligated mRNA was reverse transcribed using SuperScript™ III RT and the GeneRacer™ Oligo dT Primer to create RACE ready first-strand cDNA with known priming sites at the 5′ and 3′ ends. The following reagents were added to the 10 µl of previously obtained ligated RNA: 1 µl of GeneRacer™ Oligo dT Primer, 1 µl of dNTP Mix and 1 µl of distilled water. The reaction was then incubated at 65 °C for 5 min (to remove any RNA secondary structure), chilled on ice for at least 1 min and briefly centrifuged. The following reagents were

subsequently added to 13 µl of ligated RNA and primer mixture: 4 µl of 5x First Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOut™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl). Reaction mix was then incubated at 25 °C for 5 min, briefly centrifuged and again incubate at 50 °C for 60 min. RT reaction was inactivated by heating at 70 °C for 15 min and chilling on ice for 2 min. Subsequently, 1 µl of RNase H (2 U) was added to the reaction mix and incubated at 37 °C for 20 min and stored at -20 °C until used.

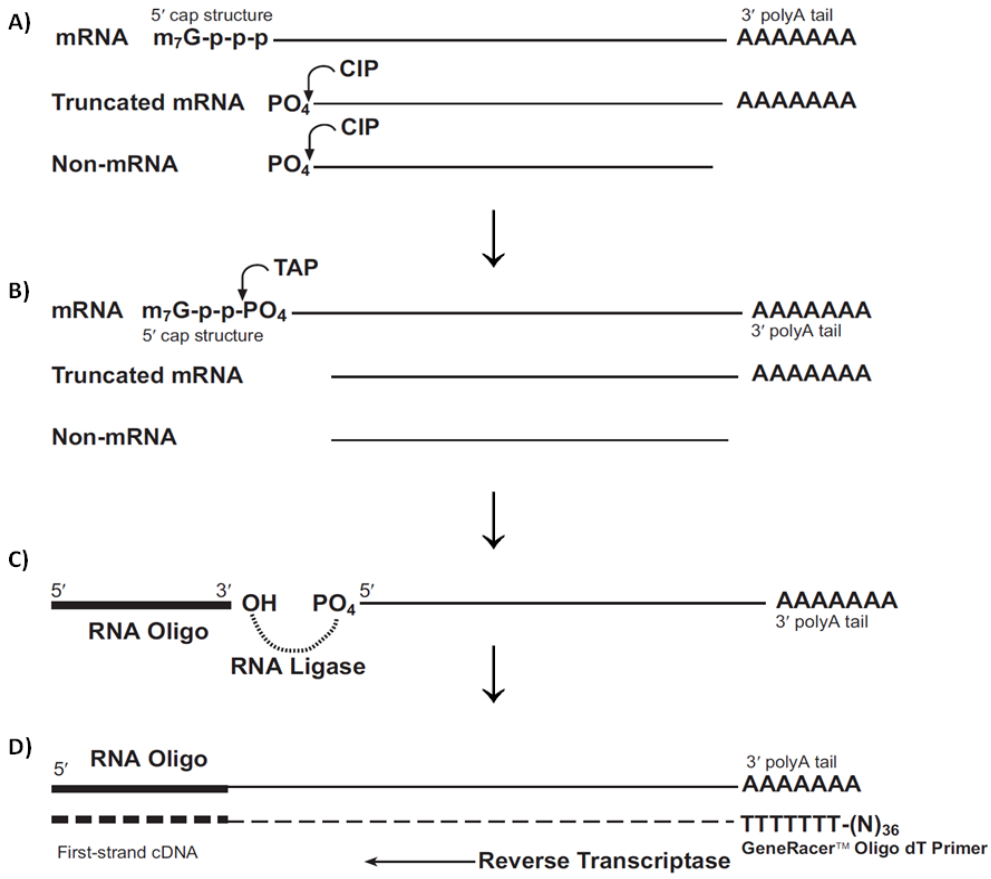


Figure 2.1. Overview of the production of cDNA for 3' and 5' RACE using GeneRacer™ Kit, as described in the user manual. A) Dephosphorylation of total RNA or mRNA. To remove 5' end phosphates, RNA was treated with calf intestinal phosphatase (CIP). This step eliminates uncapped mRNA and non-mRNA from subsequent ligation; B) Removal of the 5′ cap structure from full-length mRNA using tobacco acid pyrophosphatase (TAP). This step leaves free 5′ phosphate required for ligation; C) Ligation of the GeneRacer™ RNA Oligo to the 5′ end of the mRNA using T4 RNA ligase. This step provides a known priming site for GeneRacer™ PCR primers after transcription of mRNA; D) Reverse transcription of the ligated mRNA using SuperScript™ III reverse transcriptase and the GeneRacer™ Oligo dT Primer. This step provides RACE ready first-strand cDNA with known priming sites at the 5′ and 3′ ends.

2.6.2. Rapid Amplification of cDNA Ends PCR (RACE-PCR)

Amplification of 5' and 3' cDNA ends was performed using GeneRacer™ Kit and following the manufacturer's instructions. Main steps were:

1. In order to obtain 5′ ends, the first-strand cDNA (obtained as described in *Section 2.6.1.*), was amplified using a reverse gene specific primer (see *Section 2.7.*) (Table 2.2) and the GeneRacer™ 5′ Primer (homologous to the GeneRacer™ RNA Oligo) (Figure 2.2). Only mRNA that has the GeneRacer™ RNA Oligo ligated to the 5′ end and that is completely reversely transcribed, will be amplified using PCR. If needed, additional PCR with nested or semi-nested primers was performed.

2. In order to obtain 3′ ends, the first-strand cDNA (obtained as described in *Section 2.6.1.*), was amplified using forward gene specific primer (see *Section 2.7.*) (Table 2.2) and the GeneRacer™ 3′ Primer (homologous to the GeneRacer™ Oligo dT Primer) (Figure 2.3). Only mRNA that has a polyA tail and is reversely transcribed, will be amplified using PCR. If needed, additional PCR with nested or semi-nested primers was performed.

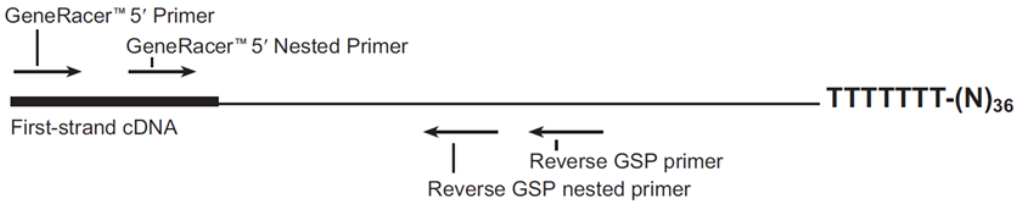


Figure 2.2. Overview of the amplification of 5' cDNA end using reverse gene primers and the GeneRacer™ 5′ Primers.

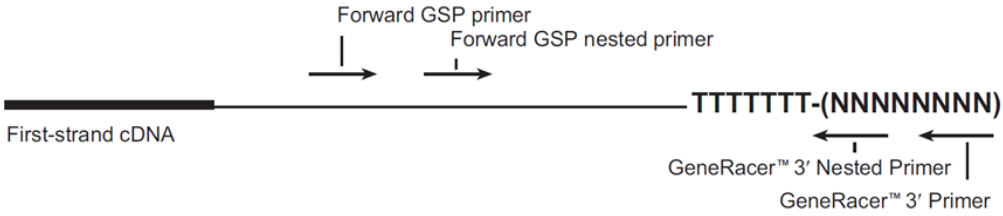


Figure 2.3. Overview of the amplification of 3' cDNA end using forward gene specific primers and the GeneRacer™ 5′ Primers.

2.7. Cloning and sequencing of ABFT TNFα1, TNFα2 and IL1β cDNA and gDNA using RACE method

The initial ABFT TNFα1, TNFα2 and IL1β fragments were isolated using cDNA derived from liver tissue RNA and the primers bftTNF1-F/bftTNF1-R (Table 2.2; Fig 2.4.A), bftTNF2-F/bftTNF2-R (Table 2.2; Fig 2.4.B) and bftIL1-F/bftIL1-R (Table 2.2; Fig 2.4.C) respectively, designed to areas of the highest homology with aligned known teleost TNF sequences. PCR was run in 25 µl reactions combining 1 µl of each primer (10 µM), 1 µl of cDNA, along with 2.5 µl of 10x PCR buffer, 1 µl of $MgCl_2$ (50 mM), 0.5 µl of dNTP (0.25 mM each), 0.1 µl of BIOTAQ™ DNA Polymerase (5 u/µl; Bioline, UK) and DNase/RNase free PCR water, to a volume of 25 µl. PCR conditions for TNFα were as follows: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 58 °C (for TNF1)/65 °C (for TNF2) for 30 sec and 72 °C for 1 min, followed by 1 cycle of 72 °C for 10 min. In order to obtain the initial sequence of IL1β, touchdown PCR was performed. PCR products were visualized on a 1.5 % agarose gel containing ethidium bromide (100 ng/ml). Products of adequate size were cloned and sequenced as described in *Section 2.5*. The initial IL1β, TNFα1 and TNFα2 ABFT fragments were then used to design ABFT specific primers (see Table 2.2).

The 3' and 5' ends of ABFT TNFα1, TNFα2 and IL1β mRNA were obtained using RACE-PCR with cDNA prepared for 3' and 5' RACE as described in *Section 2.6*. PCR was run in 25 µl reactions combining 0.5 µl of each primer (10 µM), 1 µl of cDNA, along with 2.5 µl of 10x PCR buffer, 1 µl of $MgSO_4$ (50 mM), 0.5 µl of dNTP (10 mM), 0.1 µl of Platinum Taq DNA Polymerase (5 u/µl; Invitrogen) and DNase/RNase free PCR water, to a volume of 25 µl. PCR conditions for both runs of nested PCR were as follows: 1 cycle of 94 °C for 2 min; 35 cycles 94 °C for 30 sec, 50 °C (first run) and primer specific temperature (second run), 72 °C for 1 min; followed by 1 cycle of 7 °C for 10 min.

The 3' end of the TNFα1 cDNA was obtained in two parts. PCR was first carried out with primer pair bftTNF1-3'F1/GeneRacer™ 3' Primer, with the second semi-nested PCR carried out with bftTNF1-3'F2/GeneRacer™ 3' Primer. This led to the amplification of an incomplete 3' untranslated region (UTR). The complete 3' UTR was obtained by PCR carried out with primer pair bftTNF1-

3'F3/GeneRacer™ 3' Primer followed by a nested PCR carried out with bftTNF1-3'F4/GeneRacer™ 3' Nested Primer (Figure 2.4.A). The 5' end of TNFα1 was obtained using PCR carried out with primer pair bftTNF1-5'R1/GeneRacer™ 5' Primer, followed by the second nested PCR carried out with bftTNF1-5'R2/GeneRacer™ 5' Nested Primer (Figure 2.4.A).

The 3' end of TNFα2 cDNA sequence was obtained using primer pair bftTNF2-3'F1/ GeneRacer™ 3' Primer, with the second nested PCR carried out with bftTNF2-3'F2/ GeneRacer™ 3' Nested Primer (Figure 2.4.B). The complete 5' UTR of the TNFα2 sequence was obtained using nested PCR carried out first with primer pair bftTNF2-5'R1/GeneRacer™ 5' Primer and then bftTNF2-5'R2/GeneRacer™ 5' Nested Primer.

The IL-1β 3' end was obtained using primer pair bftIL1-3'F1/ GeneRacer™ 3' Primer, with the second nested PCR carried out with bftIL1-3'F2/ GeneRacer™ 3' Nested Primer (Figure 2.4.C). The 5' end was obtained in two parts. PCR was first carried out with primer pair bftIL1-5'R1/GeneRacer™ 5' Primer, with the second nested PCR carried out with bftIL1-5'R2/GeneRacer™ 5' Nested Primer, but gave an incomplete 5' UTR. The complete IL-1β 5' UTR was obtained using PCR carried out with primer pair bftIL1-5'R3/GeneRacer™ 5' Primer followed by a nested PCR carried out with bftIL1-5'R4/GeneRacer™ 5' Nested Primer (Figure 2.4.C).

The IL1β, TNFα1 and TNFα2 gene organization was obtained by PCR amplification of genomic DNA, using primer combinations bftTNF1-gF/bftTNF1-gR, bftTNF2-gF/bftTNF2-gR and bftIL1-gF/bftIL1-5'R1 (Table 2.2; Figure 2.4) designed within the 5' and 3' UTR's of the generated full-length cDNA sequences. PCR conditions were as follows: 1 cycle of 94 °C for 2 min; 35 cycles of 94 °C for 30 sec, annealing set at primer specific temperature, 72 °C for 2 min; followed by 1 cycle of 72 °C for 10 min.

All PCR products were cloned and sequenced as described in *Section 2.5.*

Table 2.2. Oligonucleotide primers used to clone and/or amplify the ABFT TNFα1, TNFα2, IL-1β and β-actin genes.

Name	Nucleotide sequence (5'→3')	Use
bftTNF1-F	CCAGGCRGCCATCCATTTAGAAG	
bftTNF1-R	CGCTGACCTCACCGCGCTCATCAG	
mβactin-F	ATCGTGGGGCGCCCCAGGCACA	
mβactin-R	CTCCTTAATGTCACGCACGAT TTC	
bftTNF2-F	TGAATGCAAGGTAGCGCTGGATG	Primers used to obtain
bftTNF2-R	TGGTCTGGTCGGAACTTGTGGCG	initial fragments
bftIL1-F	GTGGCTCTGGGCATCAAG	
bftIL1-R	GGTGCTGATGTACCAGTTGG	
Universal T7	GTAATACGACTCACTATAGGG	Universal primers
Universal SP6	ATTTAGGTGACACTATAG	
bftTNF1-3'F1	GGATTTGCGACGACTGTG	
bftTNF1-3'F2	GCTGGAGTGGAGAGTTGAT	
bftTNF1-3'F3	TCTTGGTGCCGTGTTTCAG	
bftTNF1-3'F4	ACGGAAACCAATCAGCAAT	
bftTNF2-3'F1	CCCTCAATCCGGCCTCTACTTTG	Primers for 3' RACE
bftTNF2-3'F2	CCATCTGAGCCATACTGTGAAGCG	
bftIL1-3'F1	AGTGGACGACAAAAACAGCC	
bftIL1-3'F2	GAGCGACAAGGTACGGTTTC	
GeneRacer™ 3' Primer	GCTGTCAACGATACGCTACGTAACG	
GeneRacer™ 3' Nested Primer	CGCTACGTAACGGCATGACAGTG	
bftTNF1-5'R1	TTTCCCGCTCCCTGCTCGTCG	
bftTNF1-5'R2	TGGCTGTAGACGAAGTAGAGGC	
bftTNF2-5'R1	CATTGTCCTCTCCTTGTCCTGTCC	
bftTNF2-5'R2	CAACAAGGAGAGCAGTAGCAGCCG	
bftIL1-5'R1	AAGGTTCGGTAGCGGTTGGCGG	Primers for 5' RACE
bftIL1-5'R2	GGTGCTAATATTCTTCCCAGTGTCC	
bftIL1-5'R3	CTCACTCTCTAACACACTTTGCTCC	
bftIL1-5'R4	CCAGCAAGATGTTGAGCAGG	
GeneRacer™ 5' Primer	CGACTGGAGCACGAGGACACTGA	
GeneRacer™ 5' Nested Primer	GGACACTGACATGGACTGAAGGAGTA	
bftTNF1-gF	GAGAGAAGTATCACCACAGAGCG	
bftTNF1-gR	CTTCGTATCCTCTCAATTAGTATCACAGC	Primers used to obtain
bftTNF2-gF	AGGAAACACACAACGCAGAG	genomic DNA
bftTNF2-gR	AGGCAAACACACCAAAGAAGG	
bftIL1-gF	GGGATAACCAACCAAACTAACAGAAC	
bftβactin-rtF	CAGGGAGTGATGGTGGGTATGG	
bftβactin-rtR	GAAGGTCTCGAACATGATCTGGGTC	
bftTNF1-rtF	GAAAACGTCTCACACCTCTCAGCC	Primers for expression
bftTNF1-rtR	CAGCTGAAACACGGCACCAA	studies
bftTNF2-rtF	CAGTGGAATGGAAAAATCAGG	
bftTNF2-rtR	CTTCACAGTATGGCTCAGATGG	
bftIL1-rtF	GAAATGAGATGCAACGTGAGCG	
bftIL1-rtR	CACTTTGCTCCTCTAAAATGCTGTCC	

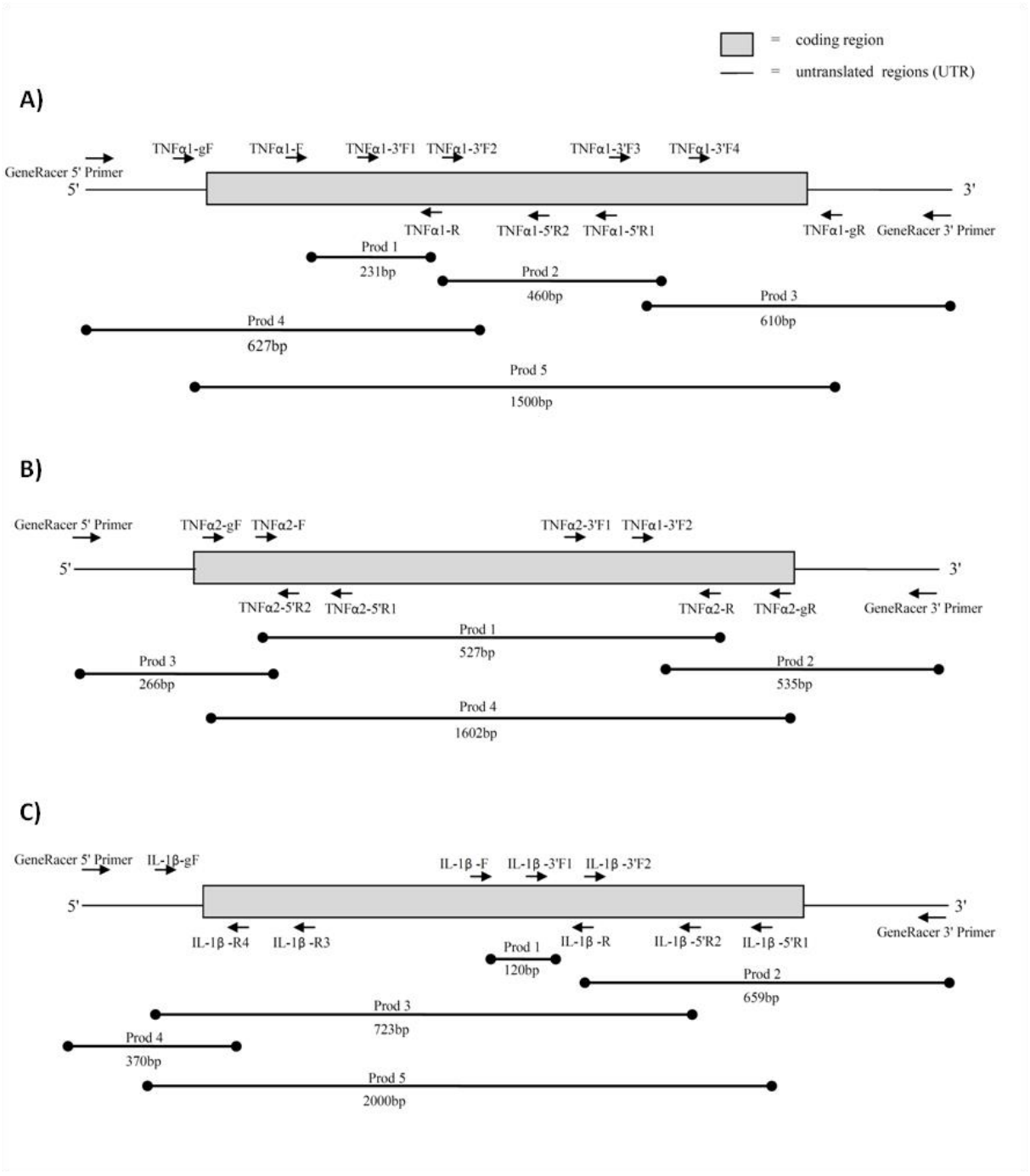


Figure 2.4. Positions of primers used to clone the ABFT TNFα1 (A), TNFα2 (B) and IL-1β (C) cDNA and gDNA sequences. Primers are illustrated with arrows and lines represent relative size and positions of the resulting products (Prod).

2.8. ABFT TNFα1, TNFα2 and IL1β sequence characteristics and phylogenetic analysis

Sequences were analyzed for similarity with other known vertebrate sequences using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990). Comparison between more than two sequences was performed using the CLUSTAL W (v1.60) multiple sequence alignment package (Thomson *et al.* 1994). Calculation of amino acid and nucleotide homology between sequences was performed using MatGat (Matrix Global Alignment Tool) (Campanella *et al*. 2003). The transmembrane region predictions were made using TMpred (Hofmann and Stoffel 1993). Phylogenetic trees were generated with MEGA 5 (Tamura *et al*. 2011) using the neighbour-joining method, p-distance and complete deletion of gaps. The branches were validated by bootstrap analysis from 10 000 repetitions, which are represented by numbers at the branch nodes. Predicted amino acid sequences were analyzed by SignalP version 3.0 (Dirløv Bendtsen *et al*. 2004) and the hydrophobicity profile was determined using Kyte and Doolittle plots (Kyte and Doolittle 1982). Protein family signatures were predicted using the PROSITE database (Hulo *et al*. 2007) and glycosylation sites were determined using NetNGlyc 1.0. Server (Gupta *et al*. 2004).

2.9. ABFT TNFα1, TNFα2 and IL-1β protein modeling

The three-dimensional models of ABFT TNFα1, TNFα2 and IL-1β were predicted by homology modeling using the SWISS-MODEL Protein Modeling Server (Arnold *et al*. 2006). Prediction tools PsiPred (Jones 1999), DISOPRED (Jones and Ward 2003) and MEMSAT (Jones *et al*. 1994) within SWISS-MODEL Workspace were used to predict the secondary structure elements, occurrence of disordered regions and putative transmembrane regions, respectively, in order to optimize selection of possible modeling templates. Identification of a suitable template for the ABFT protein structures prediction was performed using BLAST (Altschul *et al.* 1997) search implemented in SWISS-MODEL Workspace (Arnold *et al*. 2006). As the sequence identities between ABFT proteins and potential homologous templates with known three-dimensional structure were, in all three cases, less than 50%, alternative sequence alignment methods were used to improve quality of the modeling results. Alignments of the protein sequences were made with the ClustalW program in MEGA5 (Tamura *et al*. 2011) and some manual refinements were carried out to account for the positions of

critical structural features. Several templates and associated alignments were tested and stereo chemical plausibility, packaging quality and global structure quality of the resulting models were evaluated using the PROCHECK (Laskowski *et al*. 1993), ANOLEA (Melo and Feytmans 1998) and QMEAN (Benkert *et al*. 2008) programs. Finally, on the basis of the best results, human TNFα (PDB id: 1tnf), human TNFα (PDB id: 2zjc, chain B) and human IL-1β (PDB id: 1iob, chain A) were identified as the most suitable structural templates for model prediction of the ABFT TNFα1, TNFα2 and IL-1β, respectively. The resulting theoretical models were displayed as protein monomers and analyzed with SWISS-PDB viewer DeepView (Guex and Peitsch 1997). While the IL-1β molecule seemed to exist as a monomer, the trimeric models of ABFT TNFα1 and TNFα2 were generated by superposing the homology model with each of the template's chains (A, B and C).

2.10. Preparation of parasite protein extracts (PE)

Adult digenean *Didymosulcus katsuwonicola* (N=30) (Figure 2.5) were collected from fish gills and kept on ice in phosphate buffer saline (PBS, pH 7.4) during transportation to laboratory. Didymozoids were first extracted from connective-tissue cysts, washed and then frozen in PBS (pH 7.4) at -20 °C. After removal from gills, adult copepod *Pseudocycnus appendiculatus* (N=6) (Figure 2.6) were immediately frozen in PBS at -20 °C. Once thawed, PBS buffer was decanted and the remaining parasites were weighted. Upon adjusting final concentration of samples with PBS (0.25 g of original wet weight per ml), parasites were frozen in liquid nitrogen and manually homogenised by sterile pestle. Samples were intermittently sonicated (Sonoplus 2200, Bandelin, Germany) on ice for 60 sec using following instrument set up: duty cycle 10%, power 20%. After centrifugation at 4 °C, 600 x g for 10 sec, supernatants containing total protein samples were collected and filtered using 0.45 µm syringe filters. Protein concentration of each sample was determined using Bradford assay or NanoDrop 1000 and adjusted to 1 mg/ml with Leibovitz's L-15 medium (Thermo Fisher Scientific, USA).

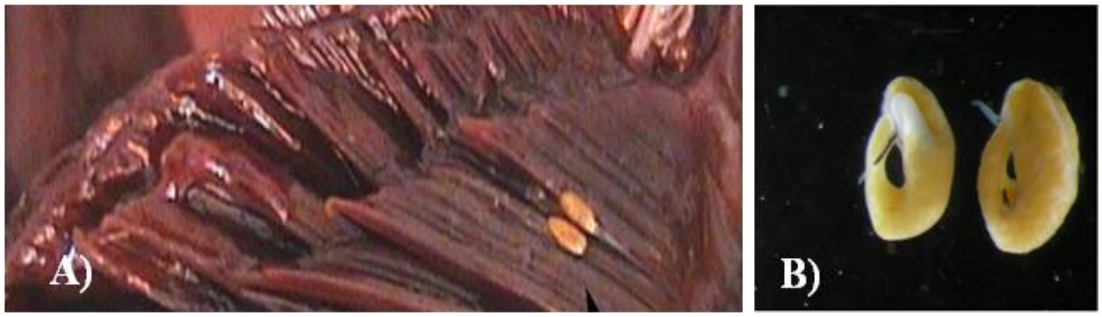


Figure 2.5. ABFT gills infected with didymozoid trematode *Didymosulcus katsuwonicola*. A) Two didymozoid connective-tissue cysts attached to gill filaments; B) Each cyst contains two hermaphroditic individuals.

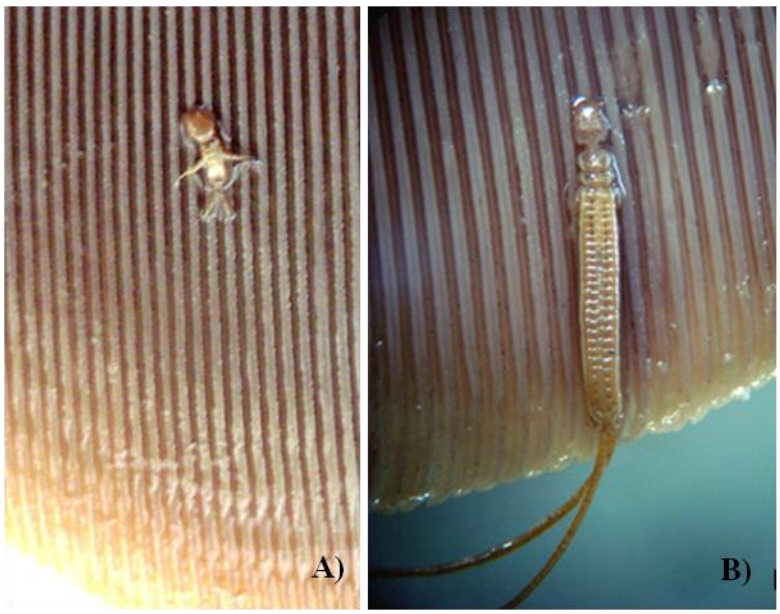


Figure 2.6. Atlantic bluefin tuna gills infected with siphonostomatoid copepod *Pseudocycnus appendiculatus*. A) Male; B) Female.

2.11. Establishment and stimulation of ABFT peripheral blood leukocytes

Blood was obtained from the severed lateral artery in the pectoral dent of healthy ABFT, collected into EDTA treated collection tubes and held on ice during transportation to the laboratory settings. ABFT peripheral blood was diluted 1:5 with Leibovitz's L-15 medium, supplemented with 10 u/ml of heparin, 2% foetal calf serum (FCS) (Thermo Fisher Scientific, USA), 1% penicillin/streptomycin P/S (10000 u/ml Penicillin G sodium; 10000 µg/ml Streptomycin sulphate). Diluted blood was then layered onto 51% iso-osmotic Percoll (Thermo Fisher Scientific, USA) solution (1:3) and peripheral blood leukocytes (PBLs) were separated from the blood by centrifugation at 2000 x g for 20 min. The PBLs were removed from the interface and washed twice in L-15 medium now supplemented with 15% FCS and 1% P/S. To ensure viability greater than 95%, isolated cells were visualised by hemocytometer following trypan blue staining. Cells were then re-suspended in L-15 (1% P/S, 15% FCS) to a final concentration of 10^7 cells mL^{-1}, seeded onto 6 well plates and left for acclimatisation for approximately 3 h at 23 °C. Afterwards, cultures were stimulated with 50 µg/ml LPS (*Escherichia coli* 0111:B4 lipopolysaccharides); 50 µg/ml Poly I:C (polyinosinic-polycytidylic acid sodium salt, Sigma); 1 µg/ml and 10 µg/ml of *D. katsuwonicula* PE; or 1 µg/ml and 10 µg/ml of *P. appendiculatus* PE for 1, 3, 5 and 12 h in triplicates. The treatments were terminated by re-suspending cells in 1 ml of TriReagent (Thermo Fisher Scientific, USA).

2.12. Expression studies using real-time PCR

For expression analysis of the three ABFT cytokines, ABFT β-actin was used as the reference gene. The initial ABFT β-actin fragment was amplified using mβactin-F and mβactin-R primers, designed initially to the mouse β-actin sequence (Table 2.2). PCR conditions for amplification of β-actin were 1 cycle of 94 °C for 5 min, 20 cycles of 94 °C for 30 sec, 58 °C for 30 s and 72 °C for 1 min, followed by 1 cycle of 72 °C for 10 min. PCR products were cloned and sequenced as described in *Section 2.5.* and the partial sequence of ABFT β-actin was used to create specific primers for real-time PCR.

The expression of IL-1β, TNFα1, TNFα2 and β-actin was measured using real-time PCR target-specific primers bftIL1-rtF/rtR, bftTNF1-rtF/rtR, bftTNF2-rtF/rtR and bftβactin-rtF/rtR (Table 2.2). The suitability of each primer pair in real-time PCR assays was tested using conventional PCR with

cDNA and genomic DNA as templates. Samples loaded onto an agarose gel stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA) confirmed that primer pairs did amplify a product of the correct size from the cDNA and that there was no genomic DNA contamination. A negative control (no template) reaction was also performed for each primer pair tested.

Real-time PCR was carried out using SYBR green I (Thermo Fisher Scientific, USA) in a LightCycler 480 System (Roche Applied Science, UK). Template cDNA (prepared as described previously) was diluted with 200 µl of TE buffer (pH 8.0) and each sample was run in duplicate. The cycling protocols were as follows: an initial denaturation of 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 sec, annealing at primer specific temperature (58-64 °C) for 30 sec and 72 °C for 30 sec, acquiring the melting curve from 75 to 98 °C. Fluorescence outputs were measured and recorded at 80 °C after each cycle for 40 cycles and quantified by comparison with a serial 10-fold dilution of pooled reference samples for each primer pair used. Transcript levels of both genes were calculated using the LightCycler 480 System integrated software. Expression levels of ABFT IL-1β, TNFα1 and TNFα2 cDNAs were normalized to the reference gene, ABFT β-actin, which had an average (± SEM) ct value of 16.70 ± 0.26 in all samples. The relative expression (presented as arbitrary units) was calculated as the expression of the target gene divided by that of β-actin times 100,000. The results represent the average + SEM of each group of fish.

One-way PERMANOVA based on Euclidean distance was used to test the null hypothesis of no differences in expression level of all three cytokines. Significance was set at p=0.05, with p-values being obtained using 999 permutations of unrestricted permutation of raw data with Monte-Carlo simulation included. PERMANOVA is a flexible and robust test that can be used with any distance similarity matrix and it constructs an F-ratio from sums of squared distances within and between groups that is analogous to Fisher's F-ratio (Anderson 2001).

2.13. Microscopy of semithin sections

For patohistological analyses, small fragments of parasite-infected gills were collected and fixed in 3.5% paraformaldehyde and 3% glutaraldehyde in 0.1 M PBS (phosphate buffer solution). Tissue was postfixed in 1% osmiumtetroxide for 1 h, then dehydrated in an ascending series of acetone and embedded in Durcopan resin. Semithin sections (0.5 µm) were stained with 1% toluidine and examined under an Olympus BX 40 light microscope.

3. Results

3.1. Identification and characterization of ABFT TNFα1, TNFα2 and IL-1β

3.1.1. Identification of ABFT TNFα1 and TNFα2 complete cDNA and gDNA sequences

The ABFT TNFα1 cDNA sequence is 1386 bp long (GenBank ID: JQ807663; Figure 3.1) and consists of a 147 bp 5' UTR, a 744 bp open reading frame (ORF) encoding a protein of 247 amino acids and a 495 bp 3' UTR containing AU-rich elements (ARE), including seven instability motifs (ATTTA), two endotoxin-responsive motifs (ATATTTAT and TTATTTA) and one polyadenylation signal (ATTAAA) located 17 bp upstream of the polyA tail.

The ABFT TNFα2 cDNA sequence is 999 bp (GenBank ID: KF134538; Figure 3.2) long with a 738 bp ORF encoding a protein of 245 amino acids. The 5' UTR consists of 63 bp and the 3' UTR of 197 bp, containing two alternative non-canonical polyadenylation signals (ACTAAA), one located 150 bp and the other 16 bp upstream of the polyA tail. No ARE or endotoxin-responsive elements were present.

The ABFT TNFα1 gene sequence (GenBank ID: JQ807664; Figure 3.1) measures 1889 bp and contains four exons interrupted with three short introns. The first exon includes 5' UTR and the first 186 bp of TNFα1 ORF. Exon two contains 52 bp and exon three contains 54 bp of the ORF. Finally, exon four includes 452 bp of the ORF and the entire 3' UTR region. The three introns contain 121, 111 and 271 nucleotides, respectively (Figure 3.3; Figure 3.4). Intron three is 1 bp smaller than in PBFT.

The ABFT TNFα2 gene sequence (GenBank ID: KF134537; Figure 3.2) is 1908 bp long and also contains four exons and three introns. The first exon includes 5' UTR and the first 174 bp of the ORF. Exon two contains 28 bp and exon three contains 57 bp of the ORF, while exon four includes 479 bp of the ORF and the entire 3' UTR region. The three TNFα2 introns contain 127, 372 and 411 nucleotides, respectively (Figure 3.3; Figure 3.4), while introns one and two are 2 and 1 bp smaller than in PBFT.

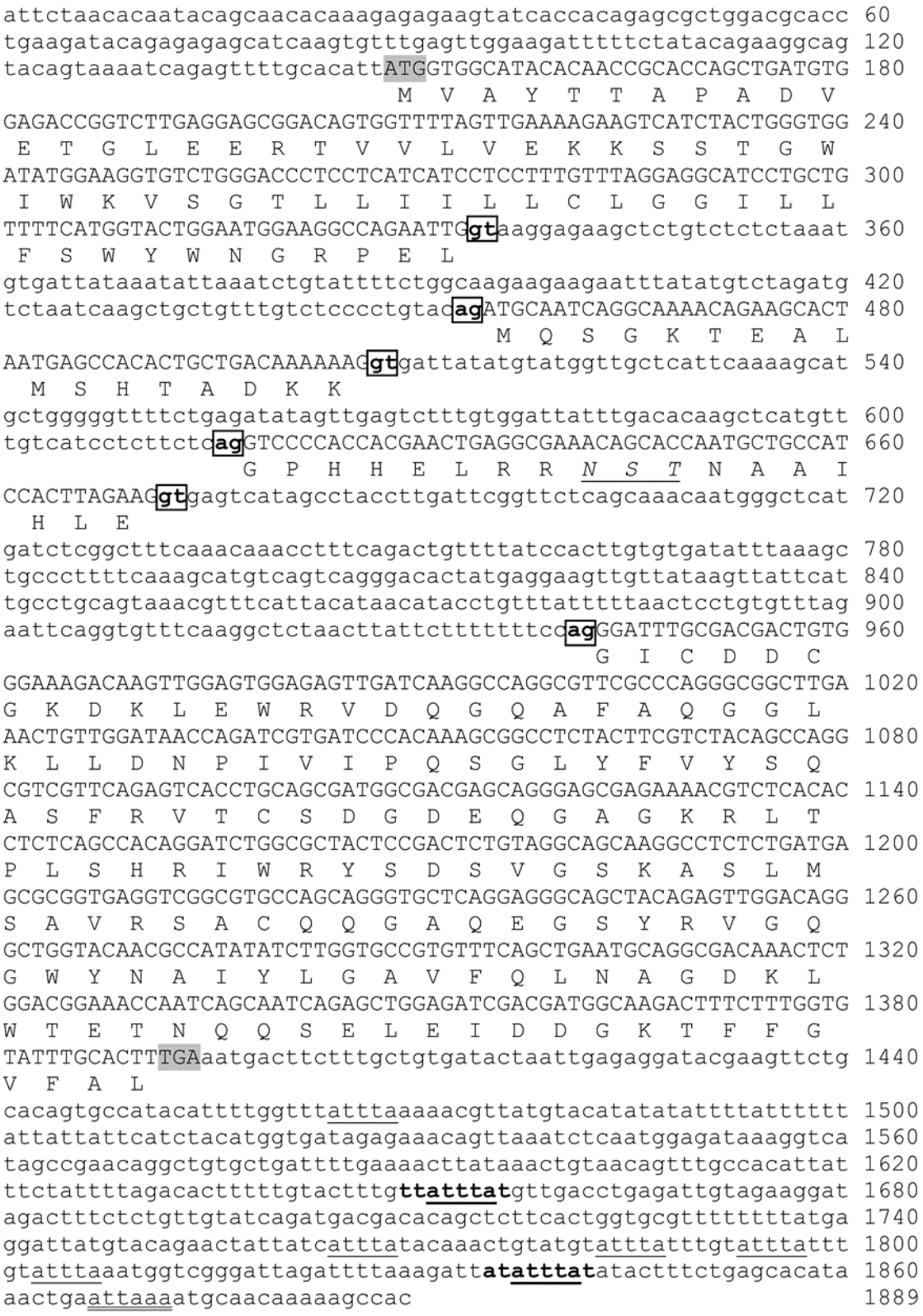


Figure 3.1. Full-length nucleotide (cDNA/gDNA) and deduced amino acid sequence of the ABFT TNFα1. The. ORF sequence is shown in upper case and UTR's and intron sequences are shown in lower case. Features shaded include the start and stop codon. In the 3′UTR the 7 RNA instability motifs (ATTTA) are underlined, and a polyadenylation signal (ATTAAA) is indicated by double underlining. A potential N-glycosylation site (position 94–96) is italicized and underlined, and potential endotoxin-responsive motifs (T/ATATTTAT) are shown in bold type. Intron splice sites are in bold and boxed.

```
aaaaccagagtgacagtagaagaagaaacaaagctctcaataaggctgctaagagtctga 60
acaATGGAAGGTGAATGCAAGGTAGCGCTGGATGCTGCTGTCCACATAGGAGCTAGGAAA 120
    M  E  G  E  C  K  V  A  L  D  A  A  V  H  I  G  A  R  K
CACACAACGCAGAGTGTTAAACCCAGCTCAAAGCTAACCACAGCCGTGTTGGCGTTCACA 180
 H  T  T  Q  S  V  K  P  S  S  K  L  T  T  A  V  L  A  F  T
TTCTGTTTTGCTGCTGCGGCTGCTACTGCTCTCCTTGTTGTCAACCAGCATACCAAGgtg 240
 F  C  F  A  A  A  A  A  T  A  L  L  V  V  N  Q  H  T  K
ggaacatacgttctggaatagtctctgaattattgaagggcctggatgcctttcagagtg 300
agtggcctttcttgcctgaatccagtgttctaacagtctctttaccattctcttcttgaa 360
acagGGGACAGGACAAGGAGAGGACAATGATGgtgagaactcttcccttaattctcccaa 420
     G  T  G  Q  G  E  D  N  D
aagttcaagactgtacttttatattaatggctttgtagtatctaataactgatcatgtg 480
ctgaaatgtaataatattaatatattaaatgcattagcttatatcagttattgaactta 540
aaacatagagcaagtgaatgaatacttcaaatttgcaaagaaaagaacatctcagtagac 600
atacctgactgtttttctgcagctaatgttgaaccaatagtgaaaactatcttactctac 660
tctgattctgatactaattgcaccagagagtcacacagggaaatgtcaagctgcagcagg 720
agactagctaattttctaaacctctatcttctctccttttacagATCTTCGTCACACATT 780
                                                D  L  R  H  T  L
GAGGCAAATTTCCAACATAAGAGCTGCCATTCATCTAGAAGgtaaagacacacaatatac 840
  R  Q  I  S  N  I  R  A  A  I  H  L  E
ctacacttatacttggggctagggatctatgtggttcatacttagactcatcaaaaaagg 900
aagcactatgttgtcttcctctggttcattgagatcccatttaatagtccagtggttct 960
caaatcccagttcatcaaggcagttgtcttggatatttagtagggttagggttagtggtc 1020
cttgaggaagttctagggcttcctgaagaggctctatgggttctcaaggctgtcacaggg 1080
cttcttgaggaagttcttggggtttttgaagaagttccagggattttgtagaacatttca 1140
agactcactgaggtaattctcagggtcccttaactcgaactgtcagaaattcaagataat 1200
ctccatcaccatgtctctctactcttcaacagGTGAATACAACCCTGACTACAAATCTGA 1260
                                    G  E  Y  N  P  D  Y  K  S  D
TGTGAAGACATCAGTGGAATGGAAAAATCAGGTGGACCAGTCCCACTCTCAAGGAGGGCT 1320
  V  K  T  S  V  E  W  K  N  Q  V  D  Q  S  H  S  Q  G  G  L
GAAACTTGAAGAAAATGAGATTGTGATCCCTCAATCCGGCCTCTACTTTGTGTACAGCCA 1380
  K  L  E  E  N  E  I  V  I  P  Q  S  G  L  Y  F  V  Y  S  Q
GGCATCTTTCCGGGTCAGCTGCAGCAGCAGTGACTCCACCTCAAAGTCCATGGTCCATCT 1440
  A  S  F  R  V  S  C  S  S  S  D  S  T  S  K  S  M  V  H  L
GAGCCATACTGTGAAGCGTTGGTCCAACTCATATGGGAACGGTGACGCCACAAGTTCCTA 1500
  S  H  T  V  K  R  W  S  N  S  Y  G  N  G  D  A  T  S  S  Y
CCAGACCATCCTGCACTCTGTCCGCACCGCCTGCCAAAAGACGGTCAGCCGTGATCCGGA 1560
  Q  T  I  L  H  S  V  R  T  A  C  Q  K  T  V  S  R  D  P  D
TGAGGATGGGAGCTGGTACTCCACTGTGTACATGGGCGCTGTGTTCAGCCTGAATAAAGG 1620
  E  D  G  S  W  Y  S  T  V  Y  M  G  A  V  F  S  L  N  K  G
CGACAAGCTGAAGACAGTGACAGAGGAGAAGATCTTGCCCAAGCTGGAGGATGAGCCAGG 1680
  D  K  L  K  T  V  T  E  E  K  I  L  P  K  L  E  D  E  P  G
GAAGACCTTCTTTGGTGTGTTTGCCTTGTAAggacaggtgactgagggcaaccgccgcaa 1740
  K  T  F  F  G  V  F  A  L
atatgaatatacactaaacagtcatgcaatgttgactttacacccaaaccgtaaagactg 1800
catcagaaggtcattacacaaggctgtgtaaaccaagaaaatagcgccatgttaagtgta 1860
gaaattgagaaaacagttcacagtaaactaaaagtaatgtgctaccac              1908
```

Figure 3.2. Full-length nucleotide (cDNA/gDNA) and deduced amino acid sequence of the ABFT TNFα2. The ORF sequence is shown in upper case and UTR's and intron sequences are shown in lower case. Features shaded include the start and stop codon. In the 3′UTR, two potential non-canonical polyadenylation signals (ACTAAA) are indicated by double underlining. Intron splice sites are in bold and boxed and parts of intron sequence that differ from those in PBFT TNFα2 are in bold and underlined.

	exon1/utr	exon1	intron1	exon2	intron2	exon3	intron3
Atlantic Tuna TNFα1	147	186	121	52	111	54	271
Pacific Tuna TNFα1	148	186	121	52	111	54	272
Atlantic Tuna TNFα2	63	174	127	28	372	57	411
Pacific Tuna TNFα2	63	174	129	28	373	57	411
Yellow Croaker TNFα1	168	189	101	55	103	60	291
Trout TNFα1	140	192	89	49	137	60	397
Carp TNFα1	149	180	83	28	109	72	149
Mouse TNFα	167	186	516	55	178	48	294
Human TNFα	175	186	600	46	187	48	301

Figure 3.3. Exon/intron size of ABFT TNFα1 and TNFα2 vs other known TNFs. The exon/intron sizes of the human, mouse and selected fish species TNFα were obtained from NCBI Human Genome Resources (http://www.ncbi.nlm.nih.gov/genome/).

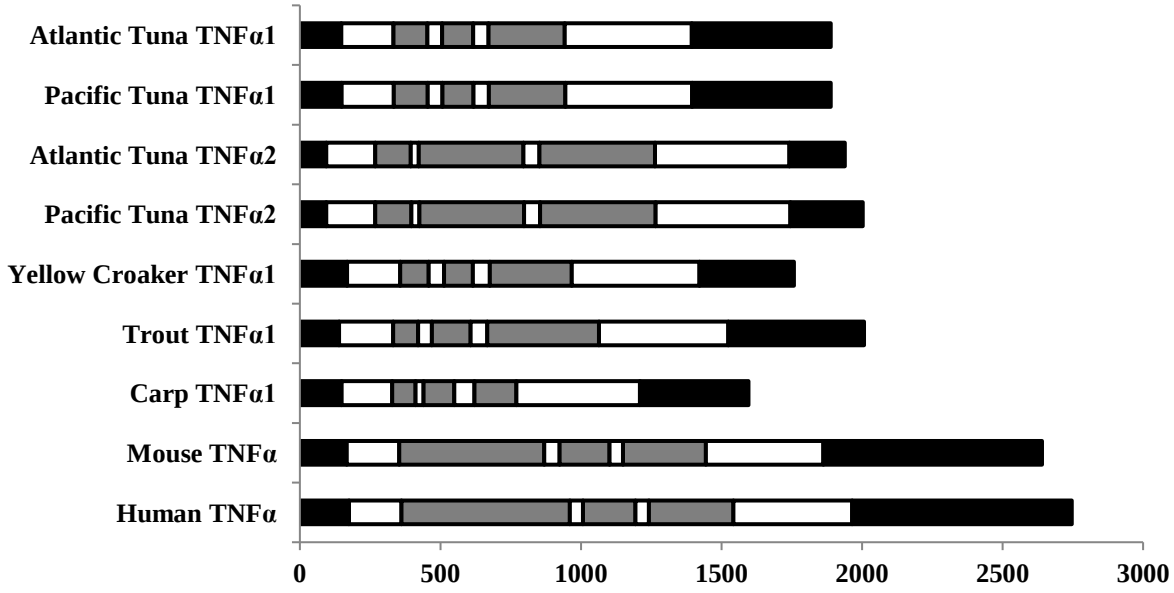


Figure 3.4. Gene organisation and exon/intron size of ABFT TNFα1 and TNFα2 vs other known TNFα genes. Introns are indicated by grey boxes, UTR's by black boxes and ORF's by white boxes. The intron sizes of the human and mouse TNFα and selected fish species were obtained from NCBI Human Genome Resources (http://www.ncbi.nlm.nih.gov/genome/).

3.1.2. Analysis of ABFT TNFα1 and TNFα2 protein sequences

Analysis of both ABFT TNFα predicted proteins (Figure 3.5) revealed a sequence IVIPQSGLYFVYSQA with excellent homology to the TNFα family signature [LV]-x-[LIVM]-x_3-G-[LIVMF]-Y-[LIVMFY]$_2$-x_2-[QEKHL]. The putative TNF-alpha converting enzyme (TACE) cut site is in position E^{84} - L^{85} of the ABFT TNFα1 amino acid sequence and in position T^{72} - L^{73} of the TNFα2 sequence, resulting in mature peptides of 163 and 173 amino acids as in PBFT. A potential transmembrane domain located at position 35-55 (VSGTLLIILLCLGGILLFSWY) of TNFα1 sequence and at position 31-53 (LTTAVLAFTFCFAAAAATALLVV) of TNFα2 sequence, were identified using the TMpred software (Hofmann and Stoffel 1993) indicating that both ABFT TNFα proteins can be membrane-bound. In comparison with other known vertebrate TNFα amino acid sequences it was revealed that two cysteine residues crucial for correct folding of the mature TNFα are conserved in both ABFT TNFα1 (C^{150} and C^{190}) and TNFα2 ($C^{143}$and C^{187}). While the TNFα1 molecule contained one potential N-glycosylation site at position 94–96, none were found in TNFα2.

ABFT TNFα1 and TNFα2 shared the highest amino acid and nucleotide identity with the PBFT TNFα homologues (Table 3.1). ABFT TNFα1 also has relatively high identity to other perciformes TNFα molecules but has only 39% amino acid identity to TNFα2. This homology was reflected in the phylogenetic tree analysis (Figure 3.6), constructed using the NJ method, that grouped ABFT TNFα1 with TNFα1 from the PBFT and the other *Perciformes*, branching away from *Cypriniformes* and *Siluriformes*. ABFT TNFα2, however, grouped with its homologue in PBFT and appeared closer to the *Cyprinid* and *Silurid* molecules.

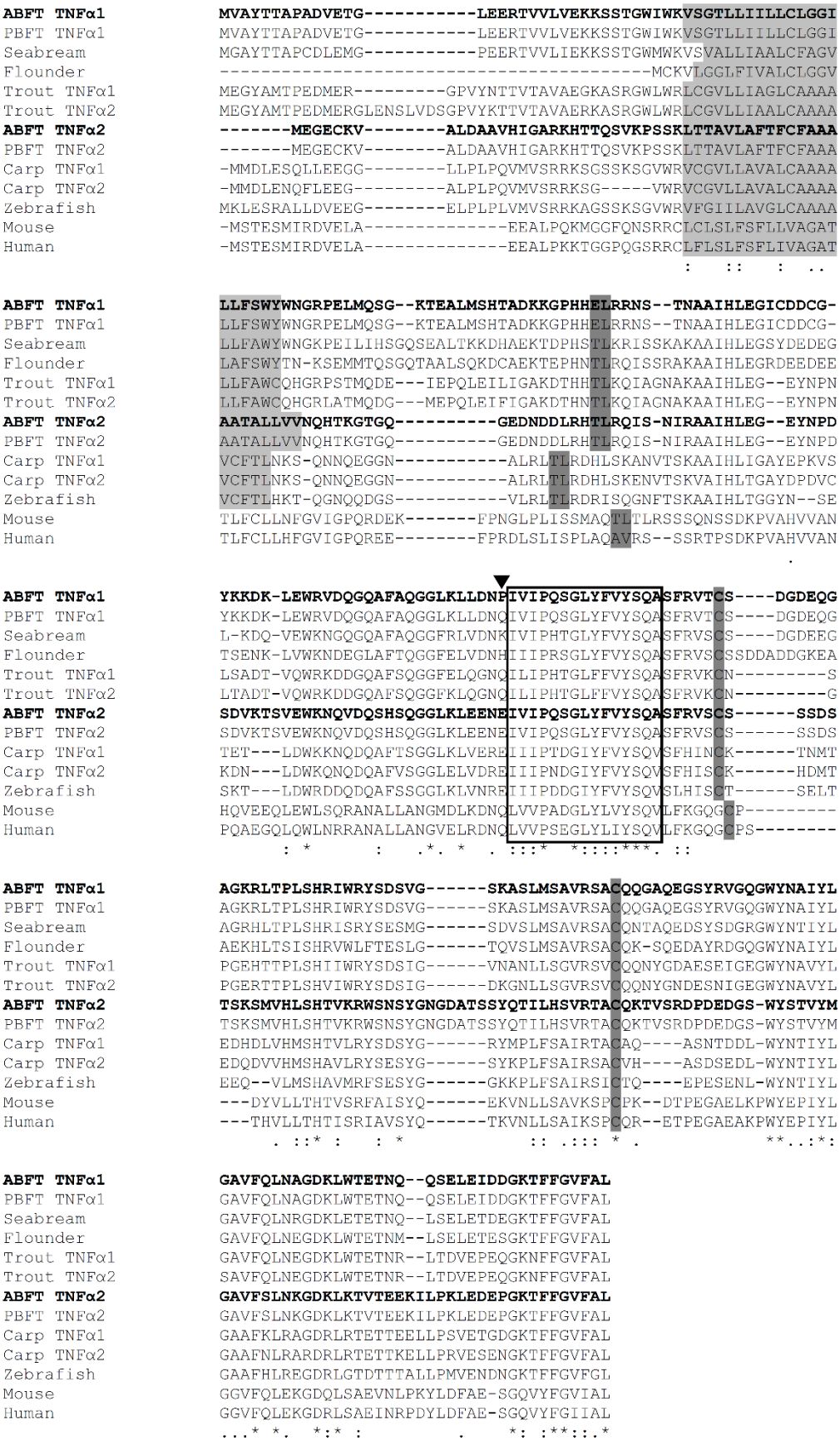


Figure 3.5. Multiple alignment of the predicted ABFT TNFα1 and TNFα2 (shown in bold type) with selected known vertebrate TNFα molecules. Identical (*) and similar (: or .) residues identified using CLUSTAL W (v1.60) are indicated. The TNF family signature is boxed. The transmembrane domain is highlighted in light

grey, while a potential cleavage site that generates the mature peptide and two cysteine residues crucial for correct folding of the mature TNFα are in dark grey. The only aa difference to the PBFT TNFα1 sequence is indicated with (▼). No differences are seen between ABFT and PBFT TNFα2 sequences. The EMBL accession numbers of the TNFα genes are: PBFT TNFα1, BAG72141.1; Seabream, CAC88353.1; Flounder, BAA94969.1; Trout TNFα1, CAB92316.1; Trout TNFα2, CAC16408.1; PBFT TNFα2, BAG72142.1; Carp TNFα1, CAC84641.2; Carp TNFα2, CAC84642.2; Zebrafish, NP_998024.2; Mouse, BAA19513.1; Human, NP_000585.2.

Table 3.1. Amino acid and nucleotide homology of ABFT TNFα1 and TNFα2 with human and selected fish sequences.

	TNFα1		TNFα2	
Species	Amino acid identity (%)	Nucleotide identity (%)	Amino acid identity (%)	Nucleotide identity (%)
Pacific BFT_TNFα1	99.6	98.0	38.2	45.8
Striped Beakfish	75.1	52.4	40.8	38.9
Seabass	73.4	69.2	38.4	41.3
Yellow Croaker	72.6	75.6	38.9	45.3
Orange-spotted Grouper	72.3	49.8	40.0	53.3
Seabream	70.8	55.2	40.0	37.5
Sea perch	69.7	54.9	40.4	52.4
Turbot	68.3	70.4	41.2	46.3
Pufferfish	60.0	60.5	39.2	45.9
Flounder	52.5	62.9	39.2	49.6
Trout_TNFα1	50.8	44.0	40.3	35.8
Trout_TNFα2	49.8	55.2	39.6	44.1
Pacific BFT_TNFα2	39.0	46.2	100.0	95.6
Carp_TNFα1	39.0	53.7	37.5	46.6
Carp_TNFα3	39.0	44.6	38.3	42.6
Channel catfish	37.8	46.1	42.0	51.6
Zebrafish	37.7	34.5	37.0	43.4
Ayu	35.2	46.8	44.0	38,5
Carp_TNFα2	34.8	31.9	36.0	51.4
Human	29.8	45.7	25.5	39.5

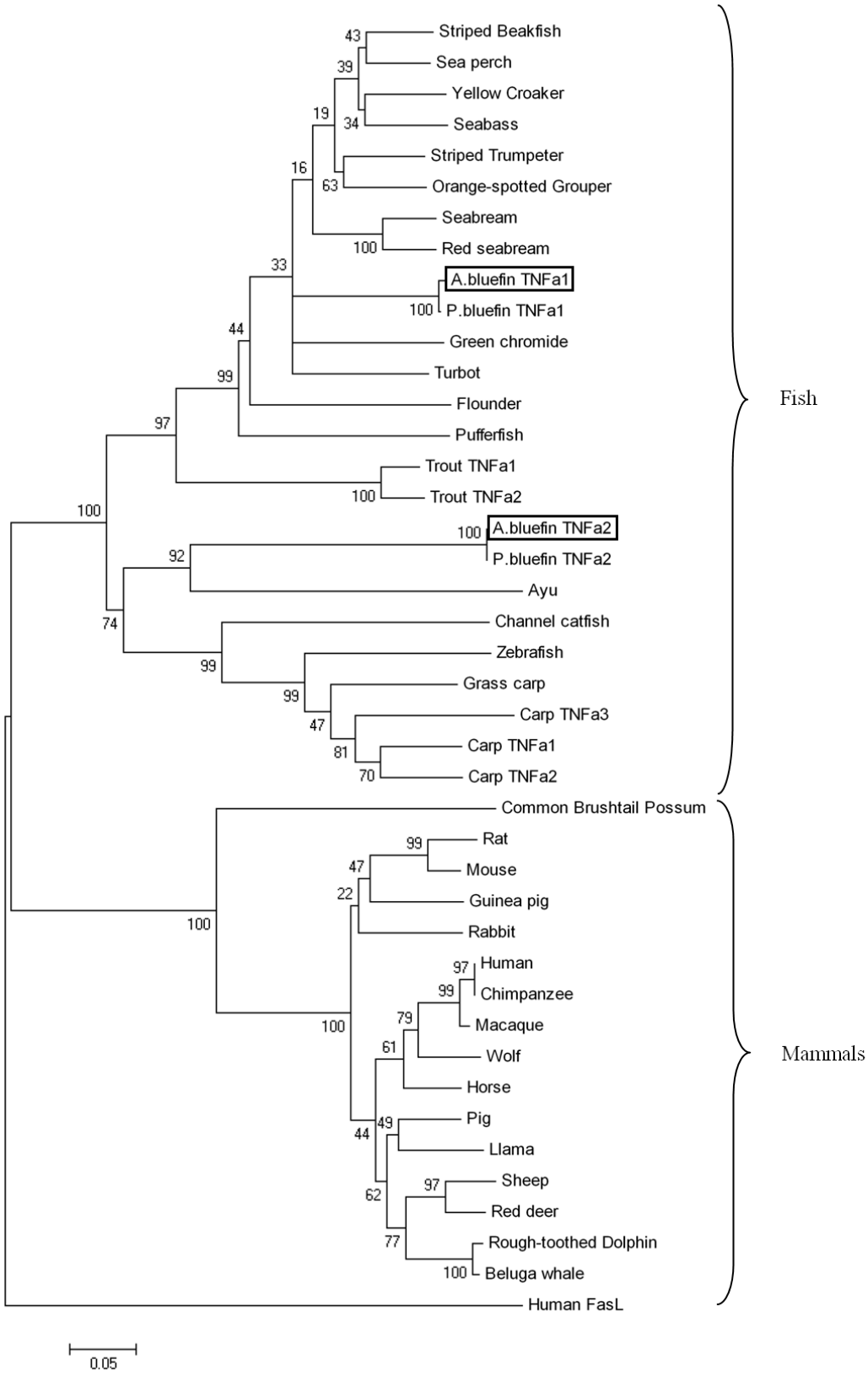


Figure 3.6. Phylogenetic analysis of the ABFT TNFα1and TNFα2 with other known vertebrate TNFα molecules. The sequences were aligned using CLUSTALW and the tree was generated with MEGA 4 using the neighbour-joining method. The branches were validated by bootstrap analysis from 2000 repetitions and are represented by numbers at the branch nodes. Human Fas ligand was used as outgroup. The GenBank accession

numbers of the TNFα sequences used in this study are as follows: Striped beakfish TNFα, ACM69339.1; Sea perch TNFα, AAR02413.2; Yellow croaker TNFα, ABK62876.1; Sea bass TNFα, AAZ20770.1; Striped trumpeter TNFα, ACQ98509; Orange-spotted grouper TNFα, AEH59794.1; Seabream TNFα, CAC88353.1; Red seabream TNFα, AAP76392.1; P. bluefin TNFα1, BAG72141.1; Green chromide TNFα, AEM59514.1; Turbot TNFα, ACN41911; Flounder TNFα, BAA94969.1; Pufferfish TNFα, NP_001033074.1; Trout TNFα1, CAB92316.1; Trout TNFα2, CAC16408.1; PBFT TNFα2, BAG72142.1; Ayu TNFα, DD019003; Channel catfish TNFα, NP_001187101.1; Zebrafish TNFα, NP_998024.2; Grass carp TNFα, ADY80577.1; Carp TNFα3, BAC77690.1; Carp TNFα1, CAC84641.2; Carp TNFα2, CAC84642.2; Common brushtail possum TNFα, AAB49506.1; Rat TNFα, AAR91624.1; Mouse TNFα, BAA19513.1; Guinea pig TNFα, AAB06492.1; Rabbit TNFα, NP_001075732.1; Human TNFα, NP_000585.2; Chimpanzee TNFα, BAE92774.1; Macaque TNFα, BAD69724.1; Wolf TNFα, AAB32391.1; Horse TNFα, AAA30959.1; Pig TNFα, NP_999187.1; Llama TNFα, BAC75383.1; Sheep TNFα, CAA39437.1; Red deer TNFα, AAA50759.1; Rough-toothed dolphin TNFα, ABC68490.1; Beluga whale TNFα, AAL56946.1; Human FasL, AAH17502.1.

3.1.3. Identification of ABFT IL-1β complete cDNA and gDNA sequence

The ABFT IL-1β cDNA consists of 1294 bp (GenBank ID: KF134540; Figure 3.7) containing a 177 bp 5’ UTR, 459 bp 3’ UTR and a 724 bp ORF encoding a protein of 246 amino acids. The IL-1β 3’UTR contains nine instability motifs (ATTTA), three endotoxin-responsive motifs (TTATTTAT) and one polyadenylation signal (AATAAA) 16 bp upstream of the polyA tail. Five instability motifs are also found within the introns. The ABFT IL-1β translation contains three potential N-glycosylation sites at positions 8-10, 132-134 and 203-205, respectively.

The ABFT IL-1β gDNA sequence (GenBank ID: KF134539; Figure 3.7) is 2443 bp long and comprises four exons and four introns. The first exon contains 97 bp of 5’ UTR and the first 206 bp of the ORF, exons three and four contain 165 and 134 bp, respectively, of the ORF. Finally, exon five includes 219 bp of the ORF and the entire 3’ UTR region. The four introns contain 543, 225, 157 and 224 nucleotides, respectively (Figure 3.8; Figure 3.9). After PCR amplification with primer pair bftIL1-gF/bftIL1-gR using liver and head kidney cDNA of “damaged” ABFT as template, two transcripts were revealed: a fully spliced RNA transcript (exons 1-4) and a transcript containing exons 1-4 plus intron 1.

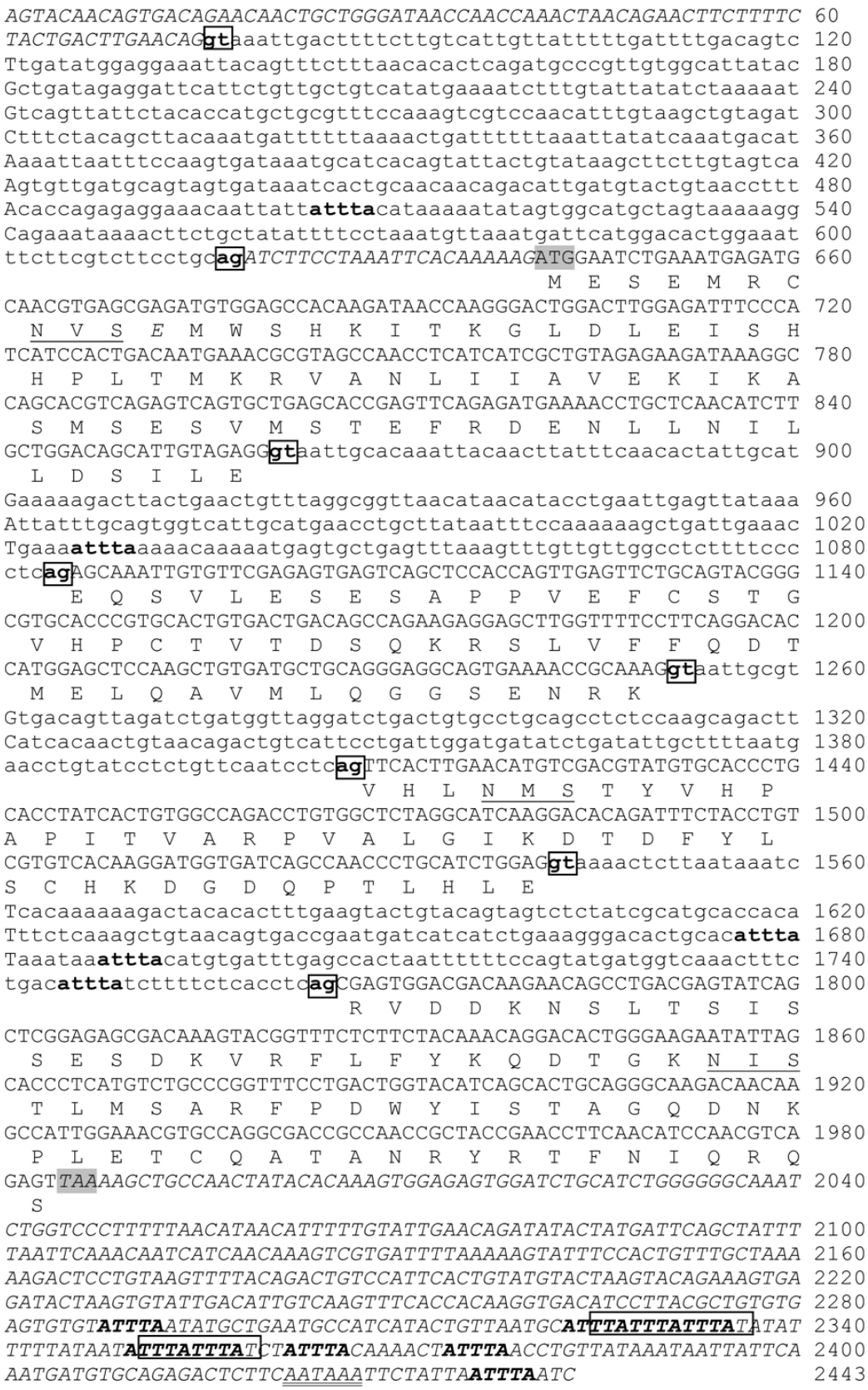


Figure 3.7. Full-length nucleotide (cDNA/gDNA) and deduced amino acid sequence of the ABFT IL-1β. Exon sequences are shown in upper case with 5' and 3' UTRs italicized and intron sequences are shown in lower case. Features shaded include the start and stop codons. RNA instability motifs (ATTTA) are shown in bold type, potential endotoxin-responsive motifs (TTATTTAT) are boxed and the polyadenylation signal (AATAAA) is indicated by double underlining. Potential N-glycosylation sites (positions 8-10, 132-134 and 203-205) are underlined. Intron splice sites are in bold and boxed.

	exon1/utr	intron1	exon1/utr	exon1	intron2	exon2	intron3	exon3	intron4	exon4	intron5	exon5	intron6	exon6/utr	utr
Atlantic Tuna IL-1β	75	543	22	206	225	165	157	134	224	219					459
Seabream IL-1β	76	406	26	125	495	160	679	134	149	252					403
Seabass IL-1β	76	423	4	220	558	165	382	134	108	297					396
Trout IL-1β1	101	288	16	33	155	202	623	180	236	137	447	231			466
Trout IL-1β2	102	280	19	33	187	193	289	171	339	137	549	231			518
Trout IL-1β3	151	132	4	32	611	52	255	223	371	186	157	140	122	213	1024
Carp IL-1β	49	102	4	41	168	79	90	178	699	174	94	137	89	219	332
Xenopus IL-1β	54	110	11	47	90	46	993	211	988	180	1384	146	953	219	502
Mouse IL-1β	72	721	15	47	533	49	1540	202	547	171	1150	131	720	207	434
Human IL-1β	72	460	15	47	564	52	1981	202	547	165	1236	131	721	210	594

Figure 3.8. Exon/intron size of ABFT IL-1β vs other known IL-1βs. The exon/intron sizes of the human and mouse IL-1β and selected fish species were obtained from NCBI Human Genome Resources (http://www.ncbi.nlm.nih.gov/genome/).

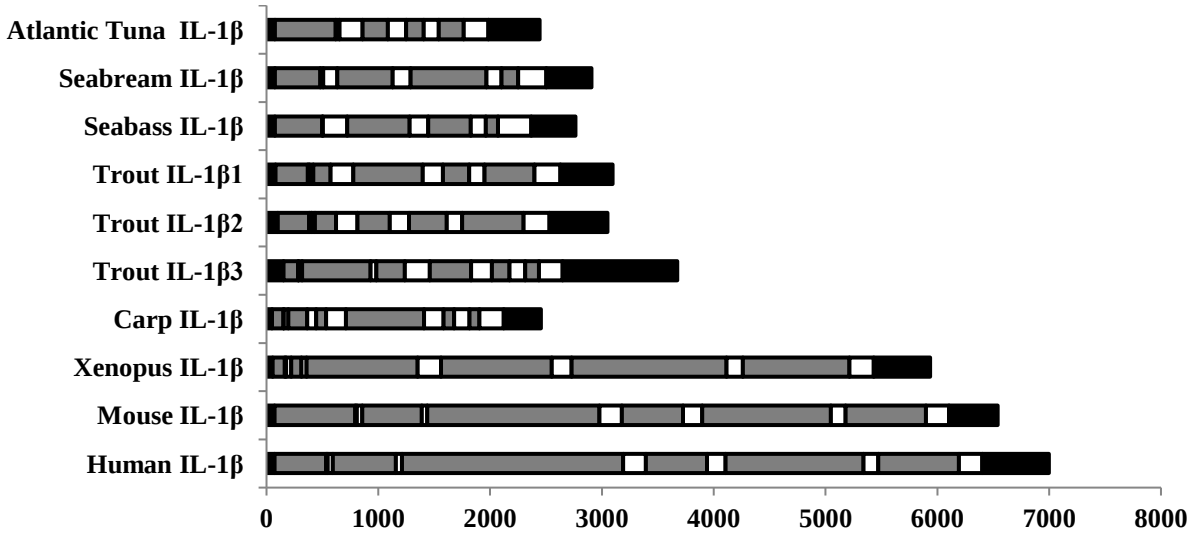


Figure 3.9. Gene organisation and exon/intron size of ABFT IL-1β vs other known IL-1β genes. Introns are indicated by the grey boxes, UTR's by black boxes and ORF's by white boxes. The intron sizes of the human and mouse IL-1β and selected fish species were obtained from NCBI Human Genome Resources (http://www.ncbi.nlm.nih.gov/genome/).

3.1.4. Analysis of ABFT IL-1β protein sequence

Analysis of the ABFT IL-1β amino acid sequence (Figure 3.10) showed a good level of conservation in the predicted 12 β-sheets but the absence of an aspartic acid responsible for the cleavage of mammalian IL-1β, typical of non-mammalian IL-1β's. The predicted protein revealed a sequence LMSARFPDWYISTAGQDNKPL with homology to the modified IL-1β family signature [FCL]-x-S-[ASLV]-xx-[PRS]-xx-[FYLIV]-[LI]-[SCAT]-T-xxxxxxx-[LIVMK]. ABFT IL-1β shared the highest amino acid identity (96.3%) with its homologue in Southern bluefin tuna SBFT (*Thunnus maccoyii*), followed by lemonfish, trumpeter, mandarin fish, turbot, halibut and olive flounder IL-1β amino acid sequences, all with more than 60% identity (Table 3.2). Phylogenetic tree analysis reflected the amino acid homology (Figure 3.11) showing that ABFT IL-1β branches together with SBFT IL-1β inside a larger group consisting of other *Perciformes* and members of the *Pleuronectiformes*, separate from the *Cypriniformes*, but clearly a member of the fish type II IL-1β's (Husain *et al*. 2012).

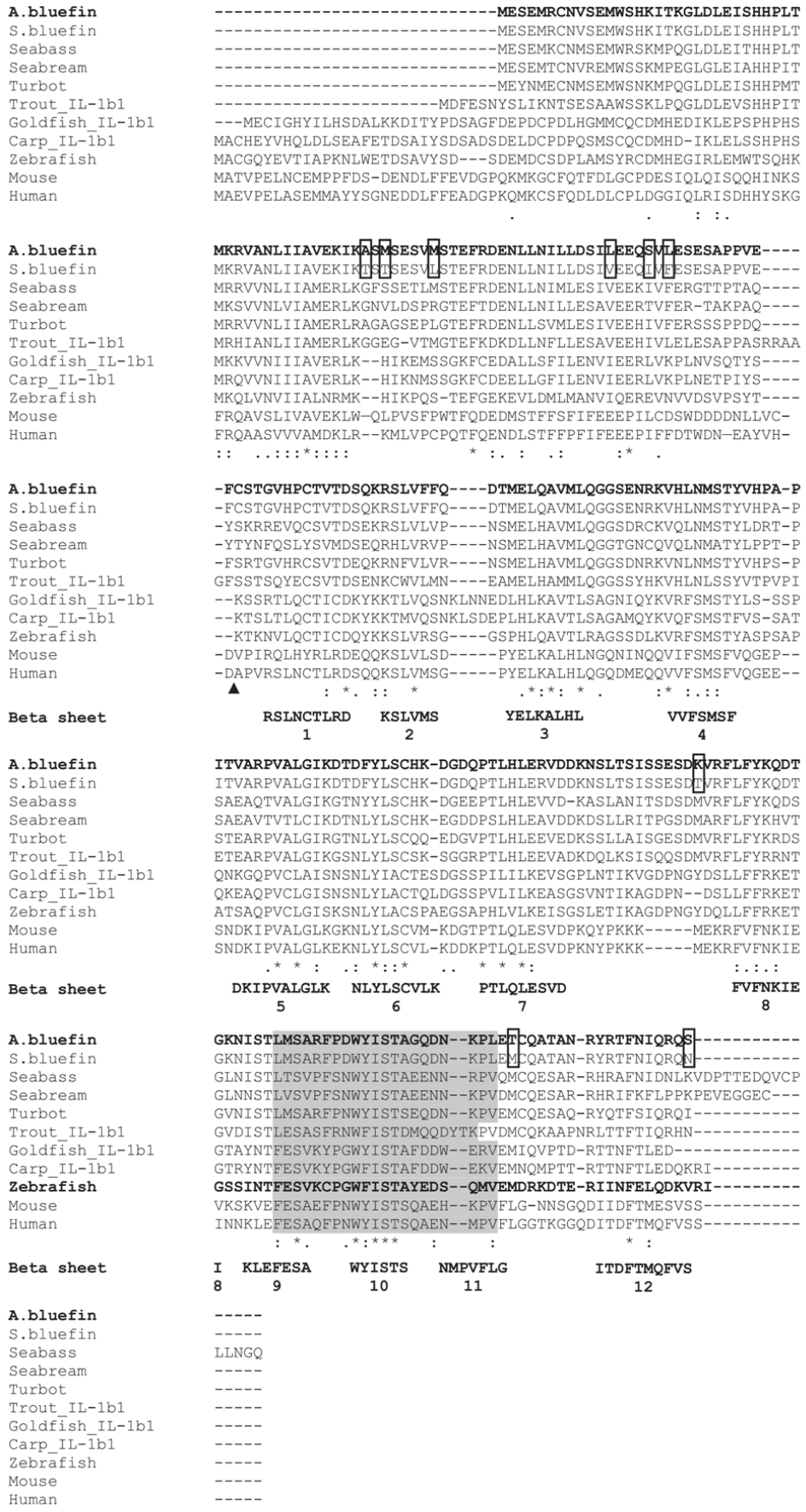


Figure 3.10. Multiple alignment of the predicted ABFT IL-1β amino acid sequence (shown in bold letters) with selected known vertebrate IL-1β molecules. Identical (*) and similar (: or .) residues identified using CLUSTAL

W (v1.60) are indicated. Areas of high conservation within the 12 β –sheets are indicated in bold, below the alignment. The IL-1β family signature is shaded. The mammalian ICE cut site crucial for full activation of the mature IL-1β peptide is indicated with (▲). Nine aa differences between Atlantic and Southern BFT IL-1β sequence are boxed. The EMBL accession numbers of the IL-1β genes are: SBFT AGH24759.1; Sea bass, CAC80553.1; Seabream, CAC81783.2; Turbot, CAC33867.2; Trout IL-1β1, CAA11684.1; Goldfish IL-1β1, CAC80551.1; Carp IL-1β1, BAA24538.1; Zebrafish, AAH98597.1; Mouse, AAA39276.1; Human, AAA59135.1.

Table 3.2 Amino acid and nucleotide homology of ABFT IL-1β with human and selected fish IL-1β sequences.

Species	Amino acid identity (%)	Nucleotide identity (%)
Souther BFT	96.3	98.2
Lemonfish	67.5	61.4
Trumpeter	67.0	56.6
Halibut	65.4	65.6
Turbot	65.4	67.8
Mandarin fish	64.8	71.5
Olive flounder	63.6	61.1
Sea perch	59.8	69.2
Striped beakfish	58.2	64.8
European seabass	57.3	66.4
Orange-spotted grouper	56.5	65.8
Trout IL-1β1	54.6	57.4
Gilthead seabream	52.8	65.1
Trout IL-1β2	51.6	31.6
Atlantic cod	50.0	52.5
Haddock	50.0	21.2
Goldfish IL-1β2	31.3	48.3
Common carp IL-1 β2-1	29.5	39.7
Common carp IL-1β2-2	28.5	42.7
Zebrafish	29.3	46.9
Common carp IL-1β1	28.1	49.5
Goldfish IL-1β1	27.8	51.0
Human	27.2	52.5
Leopard shark	24.1	50.6
Smaill Spotted Catshark	23.5	48.5

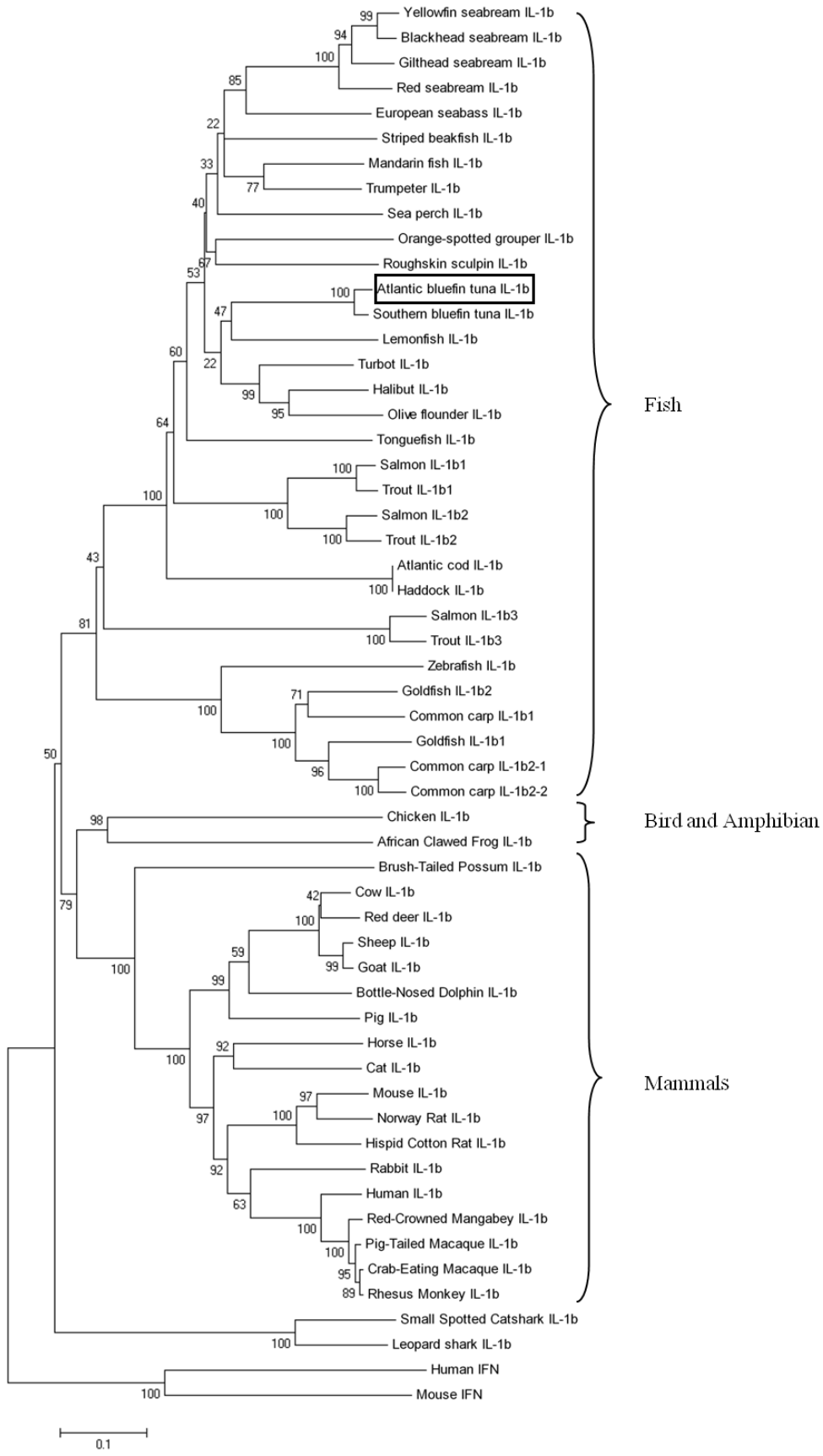


Figure 3.11. Phylogenetic analysis of the ABFT IL-1β with other known vertebrate IL-1β molecules. The sequences were aligned using CLUSTALW and the tree was generated with MEGA 4 using the neighbour-joining method. The branches were validated by bootstrap analysis from 2000 repetitions and are represented by numbers at the branch nodes. Human and Mouse IFN-© were used as outgroups. The GenBank accession

numbers of the IL-1β sequences used in this study are as follows: Yellowfin seabream, AAV74185.1; Blackhead seabream, AFM93777.1; Gilthead seabream, CAC81783.2; Red seabream, AAP33156.1; European sea bass, CAC80553.1; Striped beakfish, ACH87392.1; Mandarin fish, AAV65041.1; Trumpeter, ACQ99510.1; Sea perch, ABP38359.1; Orange-spotted grouper, ABV02594.1; Roughskin sculpin, AFH88676.1; Southern BFT, AGH24759.1; Lemonfish, AAT65502.1; Turbot, CAC33867.2; Halibut, ACY54774.1; Olive flounder, BAB86882.1; Tonguefish, ACU55137.1; CCH6376.1; Salmon IL-1β1, NP_001117054.1; Trout IL-1β1, CAA11684.1; Salmon IL-1β2, AGKD01067865; Trout IL-1β2, CAB53541.3; Atlantic cod, CAD79352.2; Haddock, AJ550166.2; Salmon IL-1β3, CCH6376.1; Trout IL-1β3, AJ557021; Zebrafish, AAH98597.1; Goldfish IL-1β2, CAC80552; Common carp IL-1β1, BAA24538.1; Goldfish IL-1β1, CAC80551.1 Common carp IL-1β2-1, CAC19887.1; Common carp IL-1β2-2, CAC19888.1; Chicken, CAA75239.1; African clawed Frog, CAB53499; Common brushtail possum, AAD21871.1; Cow, AAA30585.1; Red deer, AAA62234.1; Sheep, CAA38566.1; Goat, BAA09675.1; Bottle-nosed dolphin, BAA87947.1; Pig, AAA02584.1; Horse, BAA07718.1; Cat, AAA30814.1; Mouse, AAA39276.1; Norway rat, AAA41426.1; Hispid cotton rat, AAL18817.1; Rabbit, BAA04863.1; Human, AAA59135.1; Red-crowned mangabey, AAA86704.1; Pig-tailed macaque, AAA86715.1; Crab-eating macaque, BAA09677; Rhesus monkey, AAA86709.1; Small spotted catshark, CAC80866.1; Leopard shark, AB074142.1; Human IFN-γ, 56786138; Mouse IFN-γ, 33468859.

3.1.5. Homology modeling of ABFT TNFα1, TNFα2 and IL-1β

In order to find a suitable template among different sequences in the protein databases, for structure prediction of the first 3D models of immune proteins in tuna, the ABFT TNFα1, TNFα2 and IL-1β amino acid sequences were analyzed using BLAST (Altschul *et al.* 1997) search implemented within the SWISS-MODEL Workspace (Arnold *et al*. 2006). The search identified 31 homologous sequences with significant similarity (E-value set at $1x10^{7}$ and using the blosum62 matrix) with the ABFT TNFα1, 23 with TNFα2 and 20 with IL-1β. Sequences with more than 30% identity with ABFT sequences were considered as possible templates. All alignments were performed using ClustalW implemented in MEGA 5 (Tamura *et al*. 2011).

Three-dimensional models were constructed using human templates identified as the most suitable: TNFα (PDB id: 1tnf, chain B) as template for ABFT TNFα1, TNFα (PDB id: 2zjc, chain B) as template for ABFT TNFα2, and IL-1β (PDB id: 1iob, chain A) as template for ABFT IL-1β. Structure assessments of all three models showed that more than 90% of residues, according to PROCHECK (Laskowski *et al*. 1993), lie within the allowed regions, with the majority of model parts built correctly, therefore with the overall model showing good structural quality.

3.1.5.1. 3D models of ABFT TNFα1 and TNFα2

All eight amino acids crucial for maintenance of the human TNFα conformation and two of eleven amino acids involved in receptor binding in human TNFα (Van Ostade *et al*. 1991; Zhang *et al*. 1992)

were conserved in both ABFT TNFα molecules (Figure 3.12). The predicted model of the ABFT TNFα1 and TNFα2 monomers form a 'jelly roll' sandwich composed mainly of β-strands, with an intra molecular disulphide bridge C^{150}- C^{190} (TNFα1) and C^{143}- C^{187} (TNFα2) stabilizing each monomer, and showing excellent compatibility with the human TNFα tertiary structures (Figure 3.13A and C). The quaternary structure of ABFT TNFα1 and TNFα2 trimers were obtained by superposing the predicted ABFT TNFα1/TNFα2 monomers onto each of the human trimer chains (Figure 3.13B and D).

```
ABFT TNFα1          -PHHELRRNSTNAAIHLEGICDDCGK----DKLEWRVDQGQAFAQGGLKLLDNPIVIPQS
ABFT TNFα2          LRHTLRQISNIRAAIHLEGEYNPDYKSDVKTSVEWKNQVDQSHSQGGLKLEENEIVIPQS
Human TNFα          -VRSSSRTPSDKPVAHVVANPQAEGQ------LQWLNRRANALLANGVELRDNQLVVPSE
                      :   :  . ... *: .  :   :      ::*     ::   .*::* :* :*:*..

ABFT TNFα1          GLYFVYSQASFRVTCSDGDEQGAGKRLTPLSHRIWRYSDSVG------SKASLMSAVRSA
ABFT TNFα2          GLYFVYSQASFRVSCSSSDS--TSKSMVHLSHTVKRWSNSYGNGDATSSYQTILHSVRTA
Human TNFα          GLYLIYSQVLFKGQG-------CPSTHVLLTHTISRIAVSYQ------TKVNLLSAIKSP
                    ***::***. *:           .  . *:* : * : *        :  .:: ::::.

ABFT TNFα1          CQQGAQEGSYRVGQGWYNAIYLGAVFQLNAGDKLWTETNQQSELEIDD--GKTFFGVFAL
ABFT TNFα2          CQKTVSRDPDEDGS-WYSTVYMGAVFSLNKGDKLRTVTEEKILPKLEDEPGKTFFGVFAL
Human TNFα          CQRETPEG--AEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAES-GQVYFGIIAL
                    **: . ..    .. **..:*:*.**.*: **:* :  :.    .: :  *:.:**::**
```

Figure 3.12. Alignment of ABFT TNFα1, TNFα2 and human TNFα protein sequences. Identical (*) and similar (: or .) residues identified using CLUSTAL W (v1.60) are indicated. Dashes represent gaps added to optimize alignment of the sequences. Amino acids important for maintenance of the human TNFα conformation and amino acids involved in receptor binding are in bold and colored in red and green, respectively. ABFT amino acids that correspond to those in the human are equally colored.

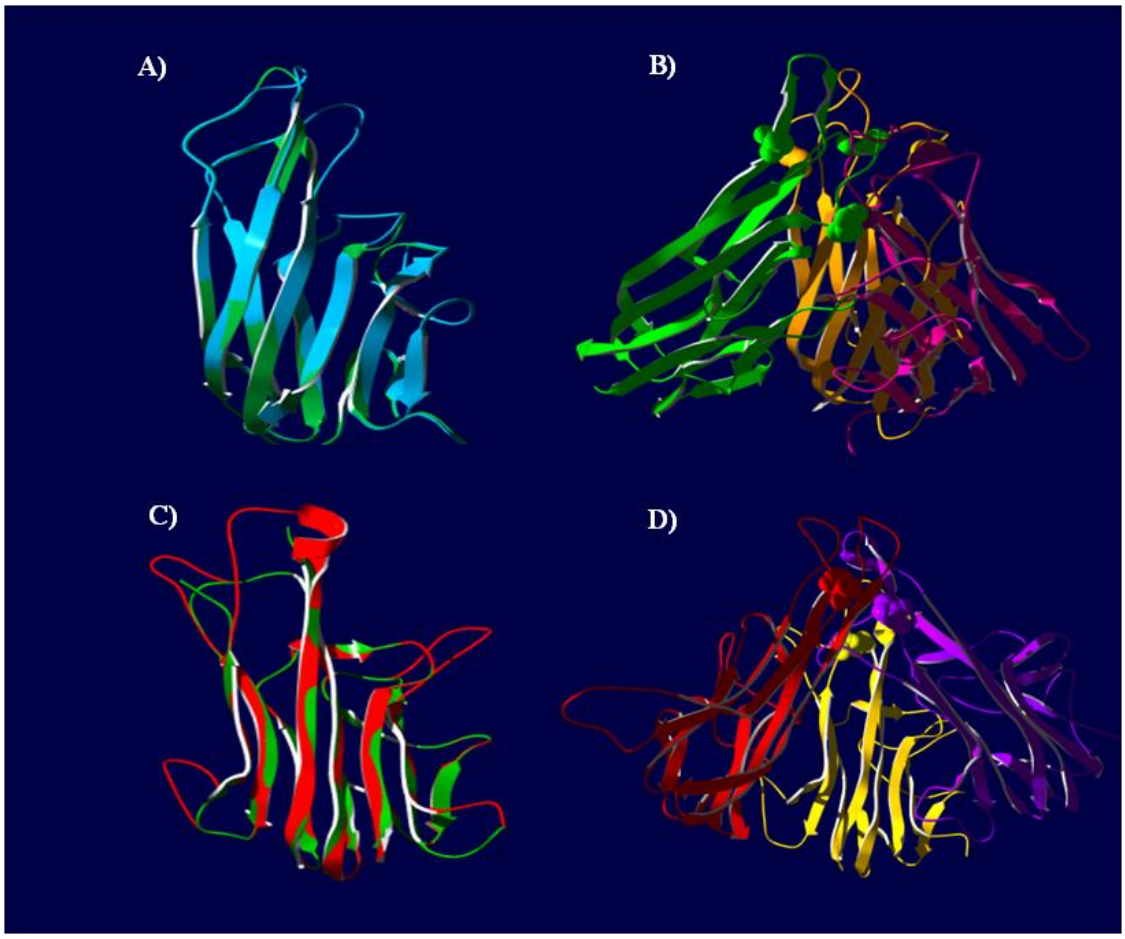


Figure 3.13. Homology modeling of ABFT TNFα1 and TNFα2. Modeling results represented as ribbon diagrams showing: (A, C) superposition of the predicted ABFT TNFα1 and TNFα2 monomers (shown in blue and red, respectively) with the human TNFα counterpart (shown in green), and arrows representing beta-strands; (B, D) predicted quaternary structure of ABFT TNFα1 and TNFα2 trimers, each chain represented by a different color and disulphide bridges shown as spheres.

3.1.5.2. 3D model of ABFT IL-1β

ABFT IL-1β showed 40% overall sequence identity with its human homologue (1iobA) but within the 12 β-strands, identity was even higher (45%) (Figure 3.14) suggesting similar folding patterns and therefore similar tertiary structure (Figure 3.15). The predicted ABFT IL-1β, as the human IL-1β protein, exists as a monomer and forms a so-called β-trefoil structure. However, only one of seven residues important for binding of human IL-1β to its receptor (IL-1RI) (Labriola-Tompkins *et al*. 1991) was conserved in ABFT IL-1β suggesting a diverse receptor-ligand binding pattern.

```
ABFT IL-1β          PPVEFCSTGVHPCTVTDSQKRSLVFFQDTMELQAVMLQGGSENRKVHLNMSTYVHPAPIT
Human IL-1β         APVRSLN-----CTLRDSQQKSLVMSG-PYELKALHLQGQDMEQQVVFSMS-FVQGEESN
                    .**.  .      **: ***::***:    . **:*: *** . :::* :.** :*:     .
                       RSLNCTLRD          KSLVMS    YELKALHL          VVFSMSF
                            1               2         3                4

ABFT IL-1β          VARPVALGIKDTDFYLSCHKDGDQPTLHLERVDDKNSLTSISSESDKVRFLFYKQDTGKN
Human IL-1β         DKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDPKN----YPKKKMEKRFVFNKIEIN-N
                       *****:*:.::****  ..*:***:** ** **     ..:. : **:* * : . *
                    DKIPVALGLK   NLYLSCVLK     PTLQLESVD                   FVFNKIEI
                         5           6            7                          8

ABFT IL-1β          ISTLMSARFPDWYISTAGQDNKPLETCQATANR-YRTFNIQRQS-
Human IL-1β         KLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFVSS
                       : **:**:*****:  :* *:    :...:    *.:*  *
                    KLEFESA      WYISTS     NMPVFLG         ITDFTMQFVS
                       9           10         11               12
```

Figure 3.14. Alignment of ABFT and human IL-1β protein sequences. Identical (*) and similar (: or .) residues identified using CLUSTAL W (v1.60) are indicated. Dashes represent gaps added to optimize the alignment of the two sequences. Residues involved in human receptor binding are shown in yellow and the 12 β-sheets are indicated below the sequence. ABFT amino acids that correspond to those involved in human receptor binding are equally colored.

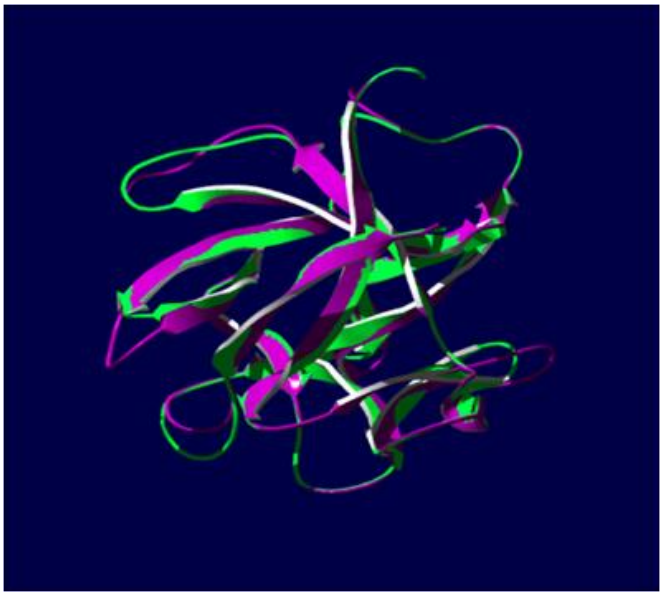


Figure 3.15. Homology modeling of ABFT IL-1β. Modeling results represented as ribbon diagrams showing superposition of the predicted ABFT IL-1β monomer (shown in purple) with the human IL-1β counterpart (shown in green), with arrows representing beta-strands.

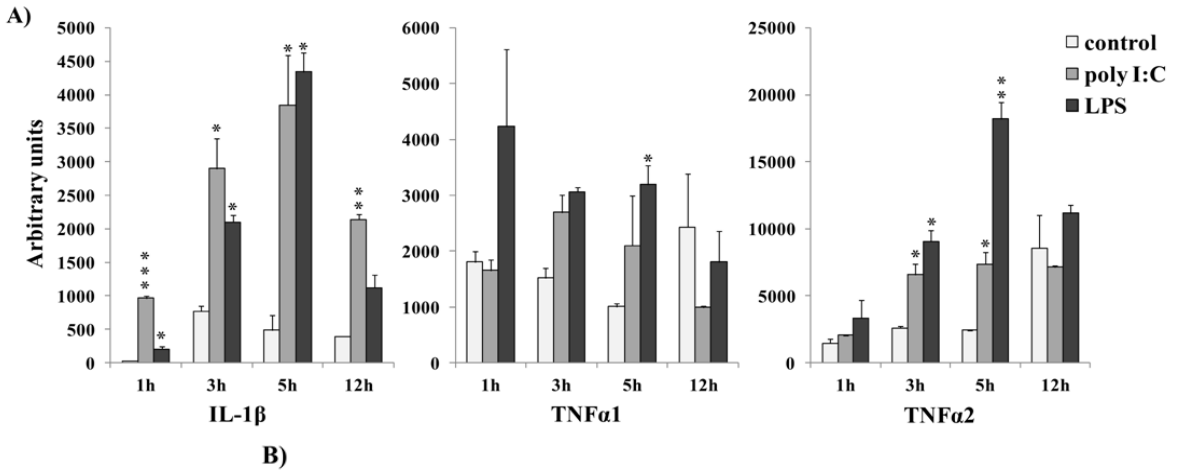
3.2. Analysis of ABFT TNFα1, TNFα2 and IL-1β expression profiles *in vitro*

The expression of ABFT IL-1β, TNFα1, TNFα2 and reference gene β-actin was measured using real-time PCR and target-specific primers bftIL1-rtF/rtR, bftTNF1-rtF/rtR, bftTNF2-rtF/rtR and bftβactin-rtF/rtR (Table 2.2). Real-time PCR was carried out using SYBR green I (Thermo Fisher Scientific, USA) in a LightCycler 480 System (Roche Applied Science, UK).

3.2.1. Expression of ABFT TNFα1, TNFα2 and IL-1β in PBL after stimulation with PAMPs

Expressions of ABFT IL-1β, TNFα1 and TNFα2 were examined in PBL cells after Poly I:C and LPS stimulation for 1, 3, 5 and 12 h. Stimulation with Poly I:C did not significantly up-regulate TNFα1, exhibiting change under or equal 2-fold in all samples examined. The only significant difference in TNFα1 expression between stimulated and control samples, was registered after 5 h of LPS stimulation, showing a 3.4-fold induction. On the other hand, TNFα2 showed significant difference in expression between control samples and both Poly I:C and LPS-stimulated samples after 3 h and 5 h of incubation, having the highest fold induction (7.6-fold) after 5 h of LPS stimulation (Figure 3.16A and B).

IL-1β was significantly up-regulated after Poly I:C stimulation from 1 h to 12 h as well as from 1 h to 5 h after LPS stimulation (Figure 3.16A), exhibiting up to 32.4-fold induction at first hour of stimulation with Poly I:C. The highest fold induction after LPS stimulation (11.7-fold) was measured after 5 h of incubation. Induction of IL-1β was biologically significant in all samples examined (Figure 3.16B).



B)

Fold Change	Poly I:C				LPS			
	1h	3h	5h	12h	1h	3h	5h	12h
IL-1β	**32.4**	**3.9**	**13.4**	**5.4**	**6.6**	**2.8**	**11.7**	**2.9**
TNF1α	0.9	1.8	**2.0**	0.5	**2.4**	**2.1**	**3.4**	0.9
TNF2α	1.0	**2.9**	**3.7**	0.9	1.6	**3.5**	**7.6**	1.4

Figure 3.16. Expression of ABFT IL-1β, TNFα1 and TNFα2 expression in PBL after stimulation with PAMPs. The expression of ABFT IL-1β, TNFα1, TNFα2 and reference gene β-actin was measured using real-time PCR and target-specific primers bftIL1-rtF/rtR, bftTNF1-rtF/rtR, bftTNF2-rtF/rtR and bftβactin-rtF/rtR. A) Relative expression of ABFT IL-1β, TNFα1 and TNFα2 in PBLs after stimulation with 50 µg/ml of LPS and Poly I:C for 1, 3, 5 and 12 h. The relative expression (arbitrary units) was calculated as the expression of the target gene divided by that of β-actin times 100,000. The results represent the average +SEM of three replicates. Significant change in expression between stimulated and time-matched control samples is shown above the bars as *p ≤0.05; **p ≤ 0.01 and ***p ≤ 0.001; B) Fold change of transcript expression after stimulation of PBLs for 1, 3, 5 and 12 h. The fold change was calculated as the average expression level of stimulated samples divided by that of the time-matched controls.

3.2.2. Expression of ABFT TNFα1, TNFα2 and IL-1β in PBL after stimulation with parasite protein extracts (PE)

Expression of ABFT cytokines was examined in PBL cells after stimulation with two different concentrations (1 µg/ml and 10 µg/ml) of *Didymosulcus katsuwonicola* and *Pseudocycnus appendiculatus* PE for 1, 3, 5 and 12 h, and both statistical and biological significances of cytokine induction were observed.

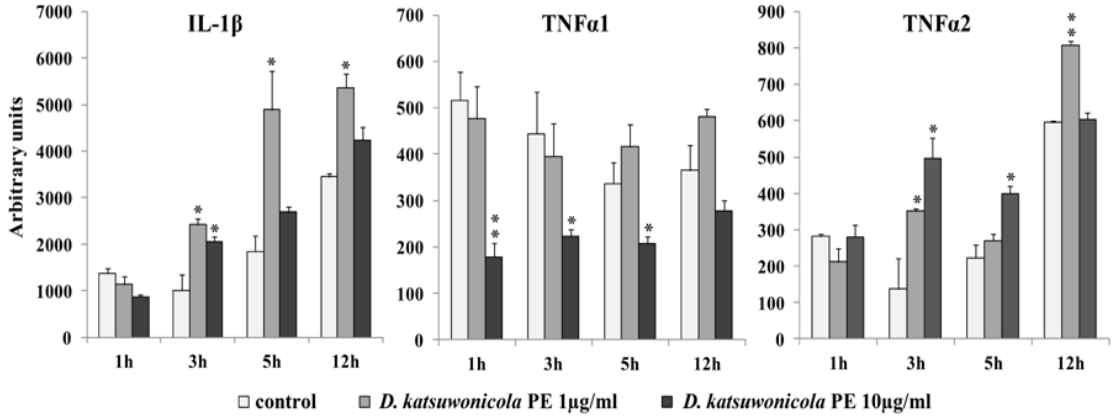
TNFα1 was significantly down-regulated after treatment of PBL with 10 µg/ml of *D. katsuwonicola* PE from 1 h to 5 h (Figure 3.17A), while stimulation with 10 µg/ml of *P. appendiculatus* PE caused its significant up-regulation after 1 h and 12 h of incubation (Figure 3.18A). TNFα2 was up-regulated

from 3 h to 12 h of incubation with *D. katsuwonicola* PE at both concentrations (Figure 3.17A). Incubation with *P. appendiculatus* PE caused up-regulation of TNFα2 after 1 h, 5 h and 12 h but only when 10 µg/ml of PE was added (Figure 3.18A).

IL-1β was significantly up-regulated compared to non-stimulated time-matched controls after treatment with 1 µg/ml of *D. katsuwonicola* PE from 3 h to 12 h (Figure 3.17A), while treatment with 10 µg/ml of the same PE up-regulated IL-1β only after 3 h of incubation. Stimulation with *P. appendiculatus* PE caused significant up-regulation of IL-1β only after 12 h and when 10 µg/ml of PE was used (Figure 3.18A).

After calculating fold changes, biologically significant induction of IL-1β was recorded after treatment with 1 µg/ml of *D. katsuwonicola* PE between 3 h and 5 h (Figure 3.17B) and after 12 h of incubation with 10 µg/ml of *P. appendiculatus* PE (Figure 3.18B). TNFα1 was significantly induced only after 1 h of incubation with 10 µg/ml of *P. appendiculatus* PE (Figure 3.18B). TNFα2 was significantly induced after 3 h of incubation with 1 µg/ml and 10 µg/ml of *D. katsuwonicola* PE (Figure 3.17B) and 1 h and 12 h after treatment with 10 µg/ml of *P. appendiculatus* PE (Figure 3.18B).

A)



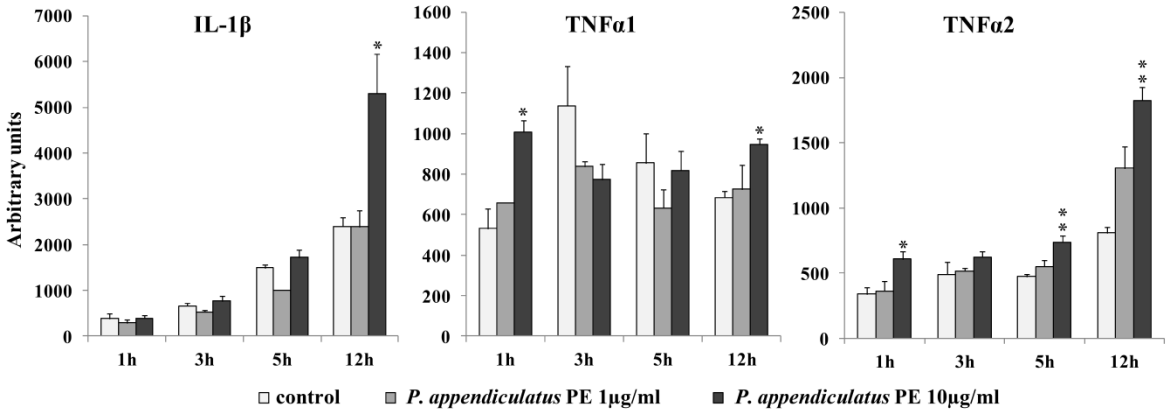
B)

Fold Change	*D. katsuwonicola* PE 1µg/ml				*D. katsuwonicola* PE 10µg/ml			
	1h	3h	5h	12h	1h	3h	5h	12h
IL-1β	0.8	**2.1**	**2.2**	1.4	0.7	1.7	1.3	1.1
TNF1α	0.9	0.9	1.3	1.3	0.3	0.5	0.6	0.8
TNF2α	0.7	**2.4**	1.0	1.3	0.9	**5.8**	1.6	1.0

Figure 3.17. Modulation of ABFT IL-1β, TNFα1 and TNFα2 expression in PBL after stimulation with *D. katsuwonicola* PE. The expression of ABFT IL-1β, TNFα1, TNFα2 and reference gene β-actin was measured

using real-time PCR and target-specific primers bftIL1-rtF/rtR, bftTNF1-rtF/rtR, bftTNF2-rtF/rtR and bftβactin-rtF/rtR. A) Relative expression of ABFT IL-1β, TNFα1 and TNFα2 in PBLs after stimulation with 1 µg/ml and 10 µg/ml of *D. katsuwonicola* PE for 1, 3, 5 and 12 h. The relative expression (arbitrary units) was calculated as the expression of the target gene divided by that of β-actin times 100,000. The results represent the average +SEM of three replicates. Significant change in expression between stimulated and time-matched control samples is shown above the bars as *p ≤0.05 and **p ≤ 0.01; B) Fold change of transcript expression after stimulation of PBLs for 1, 3, 5 and 12 h. The fold change was calculated as the average expression level of stimulated samples divided by that of the time-matched controls.

A)



B)

Fold Change	*P. appendiculatus* PE 1µg/ml				*P. appendiculatus* PE 10µg/ml			
	1h	3h	5h	12h	1h	3h	5h	12h
IL-1β	0.9	0.9	0.6	1.0	1.2	1.2	1.2	**2.7**
TNF1α	1.5	0.9	0.8	1.1	**2.0**	0.8	1.0	1.3
TNF2α	1.1	1.1	1.2	1.5	**2.3**	1.3	1.6	**2.1**

Figure 3.18. Modulation of ABFT IL-1β, TNFα1 and TNFα2 expression in PBL after stimulation with *P. appendiculatus* PE. The expression of ABFT IL-1β, TNFα1, TNFα2 and reference gene β-actin was measured using real-time PCR and target-specific primers bftIL1-rtF/rtR, bftTNF1-rtF/rtR, bftTNF2-rtF/rtR and bftβactin-rtF/rtR. A) Relative expression of ABFT IL-1β, TNFα1 and TNFα2 in PBLs after stimulation with 1 µg/ml and 10 µg/ml of *P. appendiculatus* PE for 1, 3, 5 and 12 h. The relative expression (arbitrary units) was calculated as the expression of the target gene divided by that of β-actin times 100,000. The results represent the average +SEM of three replicates. Significant change in expression between stimulated and time-matched control samples is shown above the bars as *p ≤0.05 and **p ≤ 0.01; B) Fold change of transcript expression after stimulation of PBLs for 1, 3, 5 and 12 h. The fold change was calculated as the average expression level of stimulated samples divided by that of the time-matched controls.

3.3. Analysis of ABFT TNFα1, TNFα2 and IL-1β expression profiles *in vivo*

3.3.1. ABFT cytokine expression in newly caught, damaged and farm-acclimated fish

Initially, a partial ABFT β-actin sequence was determined (GenBank ID: JF271923) to allow the design of primers to measure real-time expression of this housekeeping gene in this species. The expression levels of ABFT β-actin, TNFα1, TNFα2 and IL-1β were measured using real-time PCR in head kidney and liver tissue taken from newly caught ABFT, damaged juvenile ABFT and farm-acclimated ABFT at harvest. Using the β-actin gene as a reference, the results showed that all three cytokines were constitutively expressed to some degree in all samples examined, with no significant differences in expression levels of TNFα1 and TNFα2 between the liver and head kidney ($p = 0.308$ and $p = 0.15$, respectively) (Figure 3.19A and B). However, expression of IL-1β was found to be significantly higher in liver tissue compared to head kidney ($p = 0.029$) (Figure 3.19C). The putative influence of ABFT health condition on the expression level of three cytokines was further investigated in both liver and head kidney tissue. Damaged fish with wounds showed significantly higher levels of TNFα1 expression in liver tissue compared to newly caught fish (approximately 6 times greater; $p = 0.007$) and farm-acclimated fish at harvest (approximately 8 times greater; $p = 0.037$) (Figure 3.20A). TNFα2 and IL-1β expression was also significantly higher in liver tissue of damaged fish compared to newly caught fish, where the expression of TNFα2 was approximately 2.5 greater ($p = 0.034$) and the expression of IL-1β was approximately 10 greater ($p = 0.003$). However, when compared to expression levels of farm acclimated fish, no significant differences were found (Figure 3.20B and C). In head kidney tissue no significant differences in the expression of all three cytokines were found between all three groups of tuna.

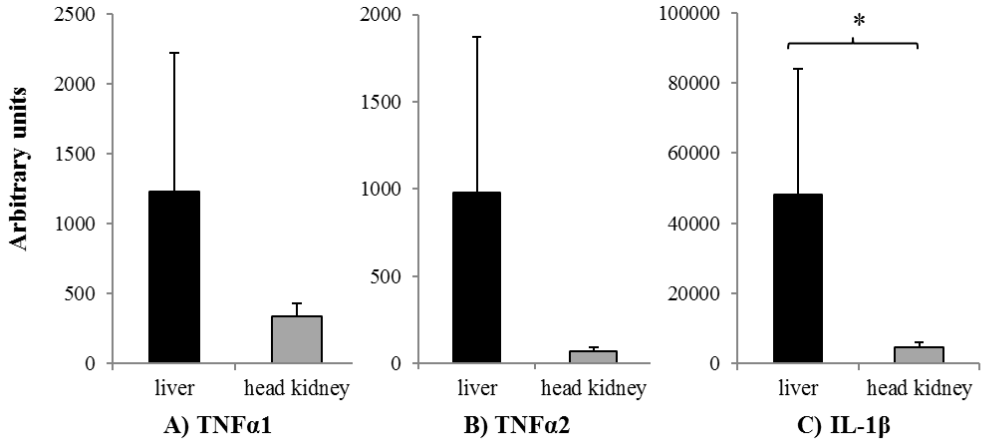


Figure 3.19. ABFT TNFα1 (A), TNFα2 (B) and IL-1β (C) expression in liver and head kidney. Gene expression was measured using real-time PCR and normalized against ABFT β-actin. Target-specific primers used are bftIL1-rtF/rtR, bftTNF1-rtF/rtR, bftTNF2-rtF/rtR and bftβactin-rtF/rtR. Data are presented as means±SD of 5-8 fish per group. The asterisk indicates a significant difference ($p<0.05$) in expression of IL-1β between the two tissues.

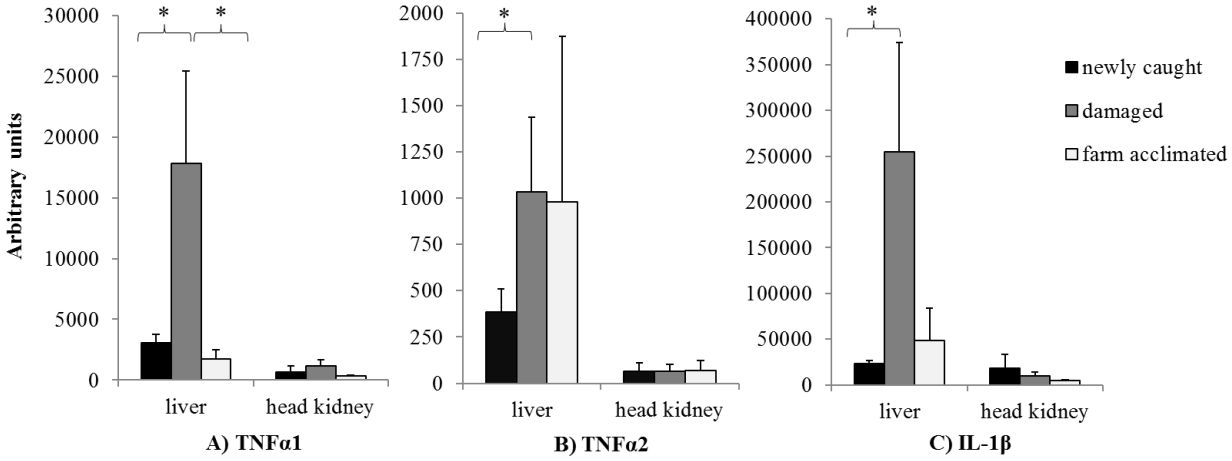


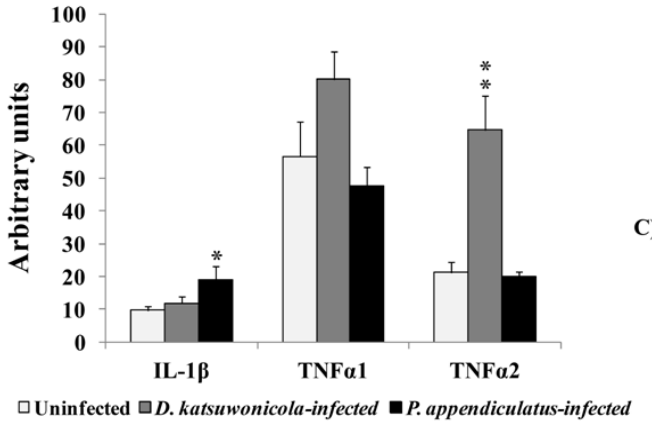
Figure 3.20. Expression analysis of TNFα1, TNFα2 and IL-1β in different groups of cage-reared ABFT. TNFα1 (A), TNFα2 (B) and IL-1β (C) expression in liver and head kidney of a newly caught, damaged and farm acclimated tuna, normalized against ABFT β-actin. Data are presented as means ± SD of 5-10 fish per group. Asterisks indicate significant differences ($p<0.05$) in expression relative to damaged fish.

3.3.2. ABFT TNFα1, TNFα2 and IL-1β expression in infected and uninfected gills

P. appendiculatus-infected gills showed significantly higher expression of IL-1β compared to uninfected gills, while *D. katsuwonicola*-infected gills showed significantly higher expression of TNFα2. TNFα1 showed no significant difference in expression between gills infected either with *P. appendiculatus* or *D. katsuwonicola* and uninfected gills (Figure 3.21A and B). TNFα1 and TNFα2

showed significant difference in expression between two groups of infected gills; in *P. appendiculatus*-infected gills (Figure 3.21A) TNFα1 and TNFα2 expression was 59% and 31%, respectively, of that in gills parasitized by *D. katsuwonicola* (Figure 3.21C). In contrast, no significant difference was found in IL-1β expression between two groups of infected gills (Figure 3.21C).

A)



B)

Fold change	*D. katsuwonicola*-infected gills	*P. appendiculatus*-infected gills
IL-1β	1.2	**2.0**
TNFα1	1.4	0.8
TNFα2	**3.0**	0.3

C)

	P.appendiculatus/D. katsuwonicola
IL-1β	0.72
TNFα1	**0.59****
TNFα2	**0.31****

Figure 3.21. Induction of cytokines in the course of parasite gill infection of ABFT. A) Relative expression of ABFT IL-1β, TNFα1 and TNFα2 in gill infected with *Didymosulcus katsuwonicola* (Didymozoidae, Digenea) and *Pseudocycnus appendiculatus* (Pseudocycnidae, Copepoda). The relative expression (arbitrary units) was calculated as the expression of the target gene divided by that of β-actin times 100,000. Data are presented as average +SEM of 7 fish per group. Significant induction relative to non-infected individuals is shown above the bars as *p ≤0.05 and **p ≤ 0.01; B) Fold change of transcript expression between parasite-infected and uninfected gills calculated as the average expression level of infected samples divided by that of uninfected samples; C) Ratio of expression levels of ABFT IL-1β, TNFα1 and TNFα2 between *D. katsuwonicola*- and *P. appendiculatus*-infected gills. **Indicates that difference in expression levels of selected genes between gills of two parasitized groups is significantly different with **p ≤ 0.01.

3.4. Pathohistology of parasite-infected gill filaments of ABFT

3.4.1. Pathohistology of gills infected with digenean *Didymosulcus katsuwonicola*

Didymozoid cysts, overlaid by gill epithelial layer (Figure 3.22A), clustered two individuals just above a large afferent filament artery (Figure 3.22A, upper insert). Its *tunica adventitia* composed mainly of collagen fibres merged with connective capsule surrounding parasites, while *tunica intima* had granulated cells on its surface (Figure 3.22A, upper insert). In vicinity of cysts, small afferent lamellar arterioles and a peripheral nerve with myelinated and non-myelinated axons were observed

(Figure 3.22A, lower insert). Didymozoid cysts consisted of a connective tissue capsule of collagen fibres of varying thickness, being more pronounced proximal to the attachment site compared to the periphery. Connective tissue capsule encapsulated both the afferent filament artery and lateral afferent lamellar arterioles. Bundles of loose collagen fibres were interspersed by fibroblasts and fibrocytes, and encompassed numerous small anastomosing capillaries (Figure 3.22B). Didymozoid hind body's cuticle was in direct contact with connective tissue capsule, leaving no intercystic space between digenean and the host's capsule. A thin basement membrane distally overlaid connective tissue capsule and supported stratified squamous epithelium (Figure 3.22B). This epithelium was approximately 10 strata thick in the part proximal to the tuna filament and abundant with mast cells and eosinophils that migrated also into the host's connective capsule (Figure 3.22C). Several rodlet cells appeared in subepithelial area as well. On the contrary, peripheral part of the didymozoid cyst was overlaid with approximately 5 strata of epithelial cells, abundant in mucous goblet cells (Figure 3.22D).

3.4.2. Pathohistology of gills infected with copepod *Pseudocycnus appendiculatus*

Copepod attached proximally at the filament base by a sclerotized claw of second antennae, deeply embedded into gills epithelium (Figure 3.23A). Tissues adjacent to the parasitation site were fragmented, showing haemorrhages and strong proliferation of mucous and rodlet cells (Figure 3.23B). In contrast to didymozoid tissue reaction, only few mast cells and eosinophils were observed (Figure 3.23C). Tissue mass proximal and in between the two antennae claws consisted of multi-layered squamous epithelium, lacking the above mentioned cell types (Figure 3.23D). In some instances, basement membrane was thick and disrupted. Distally from the attachment site, and medially to the claws' grip, cells embedded in connective tissue underwent necrotic/apoptotic changes. A demarcation between apparently unchanged epithelial cells and cell undergoing death process was observed as an accumulation of dark proteinaceous matrix (Figure 3.23E). Necrotic/apoptotic cells were dark-stained, with nuclei undergoing karyolysis, karyopyknosis and karyorrhexis (Figure 3.23F). No particular parasite encapsulation or lymphocytic infiltration was observed.

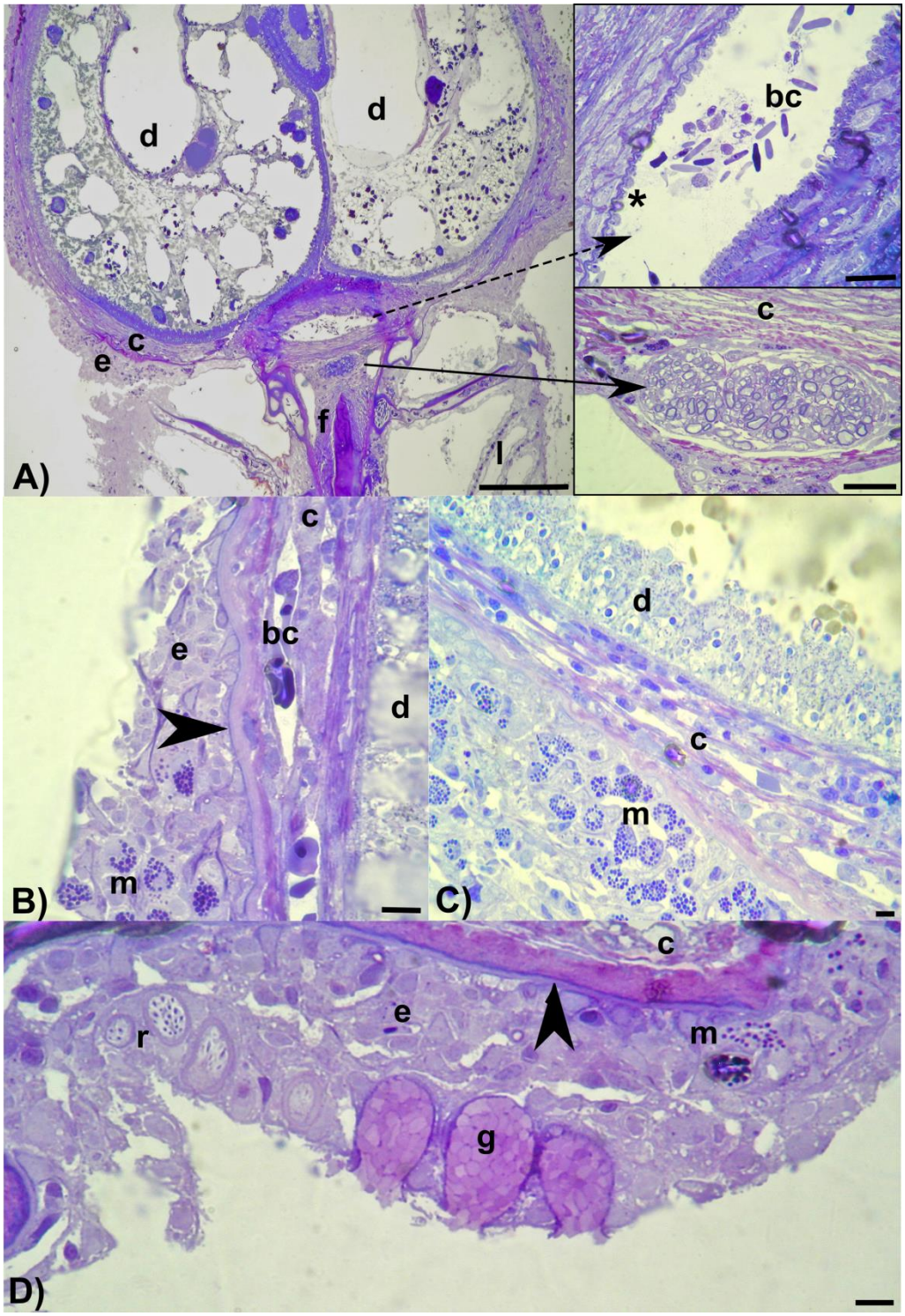


Figure 3.22. Pathohistological semithin section (0.5 µm) of ABFT gill filament infected with *Didymosulcus katsuwonicola* (Didymozoidae, Digenea) (1% toluidine blue). A) Didymozoid cyst with two individuals (d), situated proximally under the squamous epithelium (e) of gill filament (f) and close to lamellae (l), is surrounded by a connective tissue capsule (c) (scale bar=1 mm). Dashed arrow points to the upper insert where afferent filament artery, situated below the cysts, shows different blood cells (bc) in the lumen, and small granulated cells (*) attached to its *tunica intima* (scale bar=25 µm). Full-line arrow points to the lower insert, showing a peripheral filament nerve abundant with (non)myelinated axons, situated below the afferent filament artery and connective tissue capsule (c) (scale bar= 100 µm); B) Connective tissue capsule (c) lays in close contact to the didymozoid hind body (d) and is interspersed by anastomosing capillaries filled with blood cells (bc). Note a thin

basement membrane (arrowhead) supporting filament squamous epithelium (e) abundant with mast cells and eosinophils (m) (scale bar=10 µm); C) Strong epithelial infiltration of mast cells and eosinophils (m) is depicted above connective tissue capsule (c) overlaying didymozoid hind body (d). Note loose appearance of the capsule (c) abundant with cellular elements and relatively thin connective fibres (reddish) (scale bar=10 µm); D) Distal part of the connective tissue capsule (c) shows a thin basement membrane (arrowhead), overlaid by squamous epithelium (e) where rodlet cells (r) and large, active mucous goblet cells (g) are present. On right, note mast cells in different stages of degranulation (m) (scale bar=10 µm).

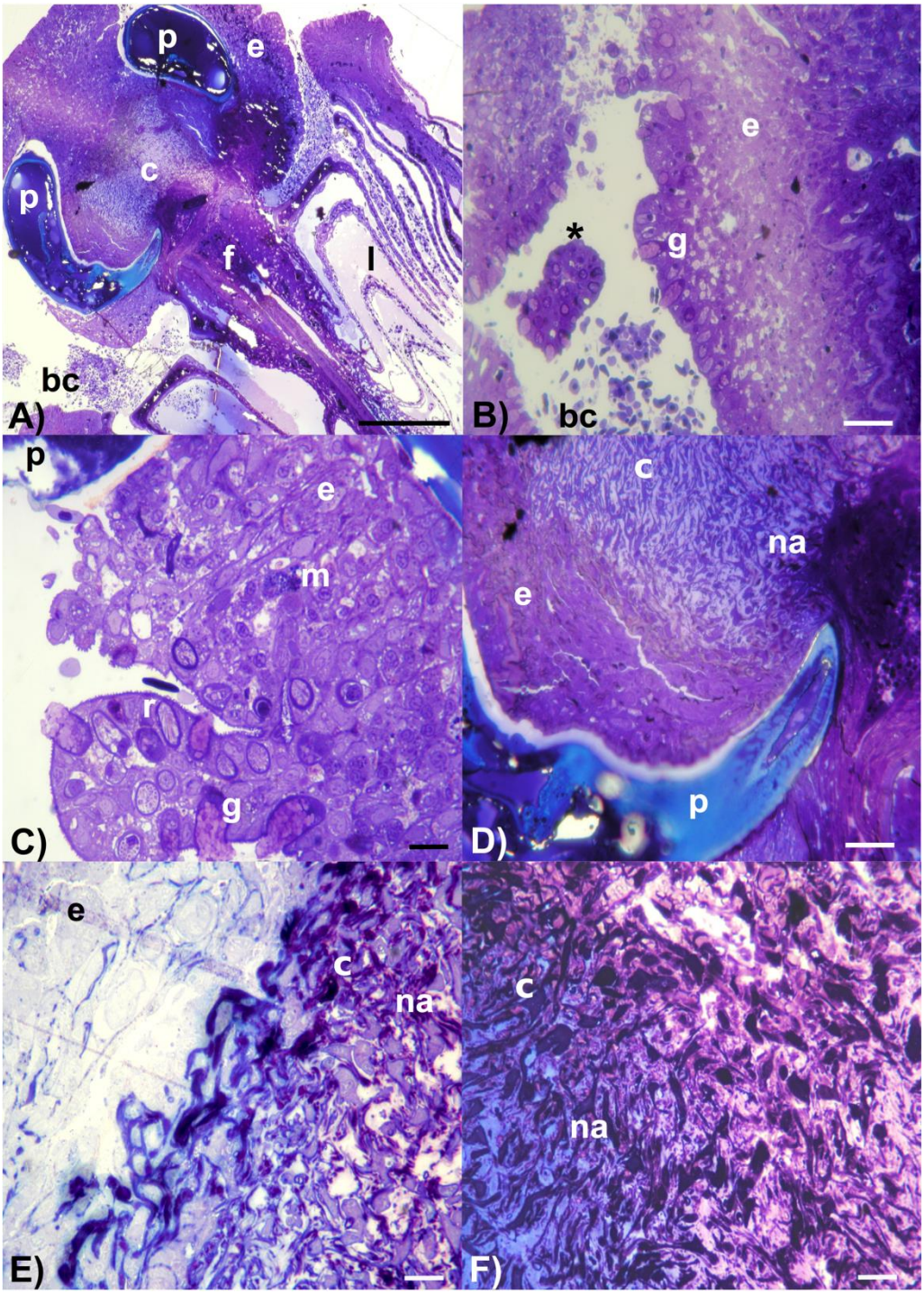


Figure 3.23. Pathohistological semithin section (0.5 µm) of ABFT gill filament infected with *Pseudocycnus appendiculatus* (Pseudocycnidae, Copepoda) (1% toluidine blue). A) Claws of copepod second antennae (p) are attached to the gill squamous epithelium (e) and connective tissue (c) of the filament (f), distally of lamellae (l). Note blood cells (bc) proximal to the attachment site (scale bar=1 mm); B) Gill epithelium (e) proximal to

copepod attachment site shows fragmentation of the tissue (*), secretion of mucous goblet cells (g) and haemorrhaging (bc) (scale bar=100 µm); C) A detail of the infected epithelium (e) evidencing considerable number of mucous goblet (g) and rodlet cells (r), with few mast cells and eosinophils (m). Note in the left and right upper corner, sclerotized copepod claw (p) (scale bar=10 µm); D) At the parasitation site (p) beneath gill epithelium (e), connective tissue (c) was interspersed with high number of necrotic/apoptotic cells (na) (scale bar=100 µm); E) Demarcation area between apparently unchanged epithelial cells (e) and cells undergoing necrosis/ apoptosis (na) in the connective tissue (c) was observed (scale bar=10 µm); F) Area of connective tissue (c) was marked by extensive number of necrotic/ apoptotic cells (na) (scale bar=10 µm).

4. Discussion

4.1. Characterization of Atlantic bluefin tuna pro-inflammatory cytokines

4.1.1. Atlantic bluefin tuna TNFα1 and TNFα2 sequences

The size of ABFT TNFα1 (247 bp) and TNFα2 (245 bp) amino acid sequences is congruent with the average size of known fish TNFα's, which is 242 amino acids (Frederick *et al*. 2004). It also showed relatively high homology with other known TNFα's with the highest sequence identity with PBFT TNFα (Table 3.1) being expected since these are closely related species belonging to a single subgenus, although geographically separated in the North Pacific and the North Atlantic (Block and Stevens 2001). Thus, ABFT TNFα2 was identical to its homologue in PBFT (Kadowaki *et al*. 2009), whereas TNFα1 differed in a single amino acid (Proline P^{29} instead of Alanine).

Interestingly, while the ABFT TNFα1 mRNA sequence was of the same length as its homologue in PBFT (Kadowaki *et al*. 2009), TNFα2 mRNA was 60 bases shorter than PBFT TNFα2 transcript due to the employment of an alternative polyadenylation signal resulting in shortening of the 3'UTR. Cleavage in the 3'UTR at alternative polyadenylation site can give raise to transcripts with the same coding region and thus production of the same protein, but also various 3'UTR lengths. The resulting variation of regulatory element composition or in some cases, their removal in the 3'UTR can change downstream transcript regulation, stability, and even gene expression patterns (Di Giammartino *et al*. 2011; Gupta *et al*. 2014; Dickson and Wilusz 2010). Interestingly, ABFT TNFα2 3'UTR does not contain AU-rich elements or endotoxin-responsive elements otherwise typical for genes with transient expression, like cytokines. These elements represent major determinants of RNA stability and are involved in the regulation of transcription during cell growth and differentiation as well as during immune response (Caput *et al*. 1986; Sachs 1993; Roca *et al*. 2007). The same was noticed in PBFT TNFα2, implying rather unique situation when compared to other teleost TNFα2 mRNAs, like salmon with 6 (Haugland *et al*. 2007), trout with 8 (Zou *et al*. 2002) and carp with 3 (Saeij *et al*. 2003) instability motifs within their TNFα2 3'UTR. Instability motifs are conserved in both mammalian and fish TNFα (Hel *et al*. 1998; Hirono *et al*. 2000; Laing *et al*. 2001; Garcia-Castillo *et al*. 2002; Cai *et al*. 2003; Saeij *et al*. 2003; Zou *et al*. 2003a) emphasizing the possibility of similar translation

regulation within all vertebrates. Both TNFα genes have the intron splicing consensus (GT/AG) conserved at the 5′ and 3′ ends of the introns. The gene organization of 4 exons and 3 introns is found in all known vertebrate TNFα molecules determined so far (Figure 3.3; Figure 3.3).

Alignment with selected vertebrate TNFα sequences (Figure 3.5) showed that two ABFT TNFα molecules contained a TNF family signature composed of 14 amino acids highly conserved in all known TNF family members that is located within β-strands in the central part of the protein (Ware *et al*. 1998). This indicates that both mature peptides have a structure adequate to initiate receptor-mediated apoptosis signalling (Lam *et al*. 2001). Substitution of Leucine (L) at position 1 with Isoleucine (I), Valine (V) at position 3 with Isoleucine (I) and Leucine (L) at position 10 with Phenylalanine (F), seen within the both ABFT TNFα1 and TNFα2 is consistent with amino acid differences found so far between fish and mammalian TNFα sequences (Frederick *et al*. 2004). Furthermore, two cysteine residues responsible for correct folding of the mature TNFα in mammals (Rink and Kirchner 1996) are also conserved in ABFT TNFα1 (C^{150} and C^{190}), TNFα2 (C^{143} and C^{187}) and other vertebrate TNFα sequences implying that the formation of a disulphide bond within the TNFα monomer is of great importance. A putative TACE restriction site ([TS]/[LV][KR]) crucial for the release of the mature peptide of both mammals and fish (Hirono *et al*. 2000; Laing *et al*. 2001; Garcia-Castillo *et al*. 2002; McGeehan *et al*. 1994) was also conserved in both ABFT TNFα genes.

ABFT TNFα1 and TNFα2 shared only 39% of amino acid identity, similar to that reported in PBFT, but much lower when compared to two TNFα species in trout (Laing *et al*. 2001) and carp (Saeij *et al*. 2003) that share 88.2% and 81% amino acid identity, respectively. It suggests that these ABFT TNFα1 and TNFα2 have distinct phylogenetic separation and physiological roles. This was well reflected on phylogenetic tree where ABFT TNFα1 and TNFα2 did not branch together in contrast to those in trout and carp. Furthermore, ABFT TNFα1 clustered in the same clade as other members of *Perciformes* away from younger *Cypriniformes* and *Siluriformes* clades, while ABFT TNFα2 was closer to both *Cypriniformes* and *Siluriformes* (Figure 3.6).

4.1.2. Atlantic bluefin tuna IL-1β sequence

The predicted ABFT IL-1β amino acid sequence showed the highest identity with SBFT IL-1β (Table 3.2) with the total of nine different amino acids (Figure 3.10). Interestingly, instability motifs (ATTTA) were found in the ABFT 3'UTR and in all introns, except intron 3. Since the same pattern was seen in trout IL-1β, there is a possibility that these intron instability motifs influence or have a role in regulating splicing of pre-RNA as well as mRNA expression (Zou *et al*. 1999a). Furthermore, gene organization analysis revealed that the ABFT IL-1β gene contains only four introns, in contrast to trout with five introns, and carp and mammals with six introns (Figure 3.9). As reported in tilapia (Lee *et al*. 2006), sea bream (Pelegrin *et al*. 2001), rainbow trout (Zou *et al*. 1999a), sea bass (Buonocore *et al*. 2003), common carp (Engelsma *et al*. 2001) and human (Dinarello 2003), ABFT IL-1β has an intron within the 5' UTR and therefore exon 1 is untranslated, unlike in chicken and catshark with fully translated exon 1 (Dinarello 1997). The size of ABFT IL-1β intron 1 was larger than in other fish and human genes, while the remaining introns were typically much shorter than in mammals, resulting in a smaller gene. Within mRNA derived from damaged ABFT liver and head kidney, a second transcript was detected containing exons 1-4 plus intron 1. Incomplete splicing is often reported in IL-1β genes, as seen in trout where two variants occur: one that retains intron 5 and another with introns 4 and 5 (Zou *et al*. 1999a). Retention of introns in IL-1β transcripts is also reported in carp (Engelsma *et al*. 2001) and sea bass (Buonocore *et al*. 2003). However, it is rather unlikely that these transcript variants have any biological activity (Scapigliati *et al*. 2004; Peddie *et al*. 2001).

When aligned with other selected vertebrate IL-1β mRNAs, ABFT sequence showed the highest homology to others along the predicted secondary structured regions consisting of 12 β-sheets (Figure 3.10). IL-1β family signature, which was previously modified from conventional family signature in order to include haddock (Corripio-Miyar *et al*. 2007), sea bass (Buonocore *et al*. 2003) and trout (Zou *et al*. 1999a), was also found within β-sheets (9-11). After sequence analysis by SignalP version 3.0 and using Kyte and Doolittle plots, it was clear that ABFT IL-1β does not have a signal peptide. In mammalian species proteolytical cleavage of pro-IL-1β by IL-1β converting enzyme (ICE) is

necessary for full activation of mature IL-1β peptide. However, ABFT like other non-mammalian species (Carretti *et al*. 1992; Weining *et al*. 1998; Zou *et al*. 1999b; Fujiki *et al*. 2000; Hong *et al*. 2001; Pelegrin *et al*. 2001; Scapigliati *et al*. 2001; Bird et al. 2002b; Wang *et al*. 2004; Lee *et al*. 2006; Wang *et al*. 2006; Corripio-Miyar *et al*. 2007; Jiang *et al*. 2008; Lu *et al*. 2008), has no aspartic acid residue, crucial for recognition of ICE. With no ICE cut site present, the cleavage of pro-IL-1β in non-mammalian vertebrates occurs through specific enzyme actions (Irmler *et al*. 1995). Possible cut site has been predicted in chicken (Kaiser *et al*. 2001), carp (Hong *et al*. 2001), trout (Carretti *et al*. 1992; Zou *et al*. 1999b) and sea bass (Scapigliati et al. 2001), where recombinant protein was synthesized and active, although the mechanism of how this pro-peptide is processed remained unclear. Recently, teleost fish inflammatory caspases were identified in zebrafish and shown to cleave pro-IL-1β (Vojtech *et al*. 2012).

ABFT IL-1β showed the highest identity with lemonfish (67%), closely followed by trumpeter, mandarin fish and flounder (Table 3.2). These relationships were evident within the phylogenetic tree analysis where ABFT IL-1β formed a group with the rest of *Perciformes* and *Pleuronectiformes* farther away from the evolutionary younger *Cypriniformes* clade (Figure 3.11).

4.1.3. Homology modeling of Atlantic bluefin tuna TNFα1, TNFα2 and IL-1β

ABFT TNFα1 and TNFα2 showed good compatibility with the human TNFα tertiary structure due to the conservation of residues and motifs crucial for secondary and tertiary structures (Figure 3.12). These conserved residues included all eight amino acids important for the maintenance of the TNFα conformation, as it was observed within human TNFα (Van Ostade *et al*. 1991; Zhang *et al*. 1992) and more recently within sea bass TNFα (Nascimento *et al*. 2007). On the other hand, the amino acids which are important for receptor-ligand binding in human TNFα (Van Ostade *et al*. 1991; Zhang *et al*. 1992) are poorly conserved within ABFT TNFα molecules, indicating that this protein may share the same tertiary structure as the mammalian protein but that it has different receptor-ligand binding mechanisms.

Analysis of the human IL-1 (PDB id: 1iobA) protein model did not reveal any specific interactions that could result in the formation of stable quaternary structures (Shaanan *et al*. 1992). Likewise, the predicted model of ABFT IL-1β suggests it does not form a complex in solution. Like other members of the IL-family, it forms a β−trefoil fold characterized by 12 β-strands; six strands forming a tapered β-barrel, which is closed at the wide end by another six strands (Murzin *et al*. 1992). Although ABFT IL-1β has similar tertiary structure with its human homologue, it probably binds differently to its receptor (IL-1R1), sharing only one of seven residues essential for that interaction (Figure 3.14).

4.2. Expression of Atlantic bluefin tuna IL-1β, TNFα1 and TNFα2 *in vitro*

It is known that the expression of many teleost TNFα and IL-1β can be induced in PBLs by PAMPs LPS and Poly I:C (Zou *et al*. 2000; Engelsma *et al*. 2001; Scapigliati *et al*. 2001; Bird *et al*. 2002b; Zou *et al*. 2002; Saeij *et al*. 2003; Lee *et al*. 2006; Haugland *et al*. 2007; Lu *et al*. 2008; Kadowaki *et al*. 2009; Polinski *et al*. 2013). Therefore, the expression of ABFT IL-1β, TNFα1 and TNFα2 was examined in PBLs using the same stimulants. In addition, expression levels of three cytokines were also quantified during *in vitro* stimulation of PBLs with *Pseudocycnus appendiculatus* and *Didymosulcus katsuwonicola* protein extracts.

4.2.1. Expression of ABFT TNFα1 and TNFα2 in LPS and Poly I:C-stimulated PBL

Both TNFα1 and TNFα2 were constitutively expressed in control PBL cells reaching the highest induction 5 h after stimulation. However, two TNFα genes exhibited differences in their respective expression patterns (Figure 3.16). Whereas TNFα1 showed no significant differences between control cells and LPS/Poly I:C stimulated cells, except after LPS stimulation after 5 h, ABFT TNFα2 showed significant up-regulation after 3 and 5 h post-LPS and Poly I:C stimulation. Majority of studies evidenced different expression of those two TNFα genes indicating that TNFα2 had stronger post-stimulation expression than TNFα1, as in the case of LPS stimulation of salmon head kidney leukocyte cells (Haugland *et al*. 2007), trout macrophages (Zou *et al*. 2002), carp head kidney phagocytes (Engelsma *et al*. 2001) or PBFT PBL (Kadowaki *et al*. 2009). Authors speculated that different expression of two TNFα genes is a result of different number of instability motifs, like in trout (Zou *et al*. 2002), or polymorphisms in 3' UTR, like in carp (Engelsma *et al*. 2001), influencing

TNFα mRNA half-life and translation efficiency. This could explain different expression of ABFT TNFα2 with no instability motifs and TNFα1 with seven. On the other hand, differential expression between TNFα1 and TNFα2 in salmon is probably caused by other mechanisms considering that two genes do not differ in number of instability motifs. These diverse findings are probably a consequence of different experimental designs, tissues or cells examined, duration of stimulation or infection, type of expression analysis as well as mechanisms influencing mRNA.

4.2.2. Expression of ABFT IL-1β in LPS and Poly I:C-stimulated PBL

No constitutive expression of IL-1β was observed 1 h after LPS and Poly I:C stimulation, as previously reported (Corripio-Miyar *et al*. 2007), confirming its stimulant dependency in tuna PBL. Low up-regulation, however, was observed after 3 h in control non-stimulated cells that increased and maintained constant level untill 12 h. The similar pattern, potentially related to isolation procedure and cell adaptation to culture plates, was already reported in non-stimulated rainbow trout (Brubacher *et al*. 2000), tilapia (Lee *et al*. 2006) and mammalian (Dinarello *et al*. 2003) cells. In both LPS and Poly I:C-stimulated cells, statistically significant up-regulation of IL-1β was observed during the whole experimental period, except for LPS-stimulated cells at 12 h (Figure 3.16A). Such pattern was previously confirmed in LPS and Poly I:C-stimulated orange-spotted grouper PBL (Lu *et al*. 2008), LPS-stimulated carp head kidney phagocytes (Engelsma *et al*. 2001), trout head kidney leukocytes (Zou *et al*. 2000), sea bass blood, head-kidney, spleen, gills and liver leukocytes (Scapigliati *et al*. 2001), Nile tilapia head kidney cells (Lee *et al*. 2006), SBFT kidney homogenates and PBL (Polinski *et al*. 2013) and LPS-stimulated Atlantic cod adherent head kidney cells. The latter in contrast failed to respond to Poly I:C stimulation (Seppola *et al*. 2008).

The highest induction of IL-1β occurred at 5 h post-LPS stimulation, while stimulation by Poly I:C inducted IL-1β already at 1 h, although the magnitude of expression was the highest at 5 h post-stimulation (Figure 3.16B). The reason for the latter lower fold change during incubation could be attributed to the increased basal expression of IL-1β in control cells after 3 h.

4.2.3. Expression of ABFT IL-1β, TNFα1 and TNFα2 in parasite PE-stimulated PBL

There are only few *in vitro* studies of influence of parasite antigens on immunity-related genes in teleost fish. Franke *et al.* (2014) examined leukocyte response of three-spined sticklebacks (*Gasterosteus aculeatus*) to helminth parasite antigens by analysing a number of viable cells and granulocyte to lymphocyte ratio and by comparing different parasite species across different host populations. Additionally, other authors (Hoole and Arme 1988; Taylor and Hoole 1993; Jones *et al*. 1995) performed *in vitro* leukocytes assays with parasite-derived substances demonstrating immunosuppressive influence of different endoparasites belonging to classes Cestoda, Trematoda and Kinetoplastida, respectively. However, those studies did not assess possible effects of parasitic substances on induction or suppression of genes related to innate immune defense mechanisms. Expression analysis of pro-inflammatory cytokines in response to *in vitro* stimulation of PBLs with parasite protein extracts presented in this study, are therefore the first in teleost fish. Treatment with adult *D. katsuwonicola* PE induced dose-depended up-regulation of IL-1β (after 3 h to 5 h) and TNFα2 (after 3 h) (Figure 3.17), while adult *P. appendiculatus* PE induced all three cytokines; IL-1β (after 12 h), TNFα1 (after 1 h) and TNFα2 (after 1 h and 12 h) (Figure 3.18). While 1 μg/ml of *P. appendiculatus* PE failed to elicit biologically significant induction, 10 μg/ml of the same PE resulted in significant induction of all three cytokines. Treatments with 1 μg/ml and 10 μg/ml of *D. katsuwonicola* PE both led to significant induction of TNFα2, but higher fold change was recorded when using higher concentration. In contrast, significant induction of IL-1β was present only when 1 μg/ml of *D. katsuwonicola* PE was added, congruent to *in vivo* expression pattern in *Didymosulcus*-infected gills, where TNFα2 was dominant. Noteworthy is that during all treatments fold change in parasite PE-stimulated cells was relatively low (2.7 approximately) compared to LPS or Poly I:C treatments, especially in the case of IL-1β. If we consider such immune response as being relatively mild, our findings support the assumption that parasite adaptive mechanisms efficiently enable its survival within the host. Similar was recently observed by Reyes-Becerril *et al*. (2017) in yellowtail amberjack, *Seriola lalandi*. Study showed that expression of *S. lalandi* pro-inflammatory cytokines IL-1β and TNFα is induced by antigens derived from adult monogenean parasite *Neobenedenia melleni*. Contrary, antigens from larval stage of *N. melleni* failed to evoke cytokine response. Authors

suggested that different level of antigenicity between two developmental stages of *N. melleni* arise from parasites ability to adjust antigenic activity as a part of their adaptation strategy. Thus, *N. melleni* larvae modify their surface antigens to avoid host immune response, whereas adults benefit from it. Namely, adult *N. melleni* reproductive activities require large amount of food derived from host skin mucus and plasma protein leakage (Reyes-Becerril *et al*. 2017) which is, in turn, induced by pro-inflamatory cytokines such as IL-1 and TNFα (Buchmann 1999).

4.3. Expression of Atlantic bluefin tuna TNFα1, TNFα2 and IL-1β *in vivo*

Expression of TNFα1, TNFα2 and IL-1β and their importance as potential biomarkers for cage-reared ABFT was evaluated among three groups of ABFT: 1) newly caught fish, 2) farm-acclimated fish and 3) damaged fish. The first two groups were apparently healthy tuna, while the latter group exhibited wounds and lesions in different parts of their body, suggesting entrapment in the cage net or abrasions from fast swimming in high density. They also showed behavioral changes such as slower and unbalanced swimming, disorientation and lack of appetite, suggesting their shift in health status towards a disease. Expression of three cytokines was also evaluated during natural gill infection with *Pseudocycnus appendiculatus* and *Didymosulcus katsuwonicola*.

4.3.1. Expression of ABFT TNFα1 and TNFα2 during critical points of the farming process

This study has confirmed that ABFT, like PBFT (Kadowaki *et al*. 2009), carp (Saeij *et al*. 2003), trout (Zou *et al*. 2002), and salmon (Haugland *et al*. 2007) expresses at least two TNFα genes. In contrast, Southern blot analysis revealed the presence of only one TNFα copy in Japanese flounder (Hirono *et al*. 2000). ABFT TNFα1 and TNFα2 mRNAs are both constitutively expressed in liver and head kidney tissue of healthy tuna. No significant difference in expression of two TNFα genes was found, although a trend for a higher expression in liver tissue was noticeable for both (Figure 3.19A and B). Also, expression levels of both genes had approximately the same magnitude in liver and head kidney tissue, indicating equivalent expression patterns of TNFα1 and TNFα2 in ABFT, as it was reported in sea bream (Garcia-Castillo *et al*. 2002). On the other hand, Kadowaki *et al*. (2009) suggested that in PBFT, TNFα1 and TNFα2 are regulated independently, although such up-regulation of TNFα2 was

observed in blood, while liver and head kidney did not show different expression levels of the TNFα genes.

ABFT TNFα1 showed a significantly higher expression level in liver of damaged tuna compared to both newly caught and farm-acclimated fish, whereas TNFα2 expression in damaged fish was significantly higher only in comparison to newly caught fish. However, it must be emphasized that expression of TNFα2 in farm-acclimated group showed large individual differences which could have been the reason for no significant difference of TNFα2 expression levels between this group and the damaged one (Figure 3.20A and B). On the other hand, TNFα genes were not induced in head kidney tissue of any group of fish. Previous studies have also reported different expression patterns between different TNFα isoforms, like in trout (Zou *et al*. 2002; Sigh *et al*. 2004) and the Atlantic salmon (Zou *et al*. 2007). Furthermore, the magnitude of TNFα1 expression was higher than that of TNFα2 in all three groups of ABFT. This finding contrasts principal expression profile of two TNFα genes. Namely, TNFα1 is shown to have rather strong constitutive expression in different tissues of healthy fish but relatively poor up-regulation by immune challenge (Reyes-Cerpa *et al*. 2012), unlike the TNFα2 whose post-stimulation induction is usually fast and strong (Zou *et al*. 2002; Kadowaki *et al*. 2009). However, there are few studies *in vivo* that have shown interesting reports of shift in expression levels between TNFα2 and TNFα1, from early to late phase of infection. For example, in salmon vaccinated with oil-based adjuvant, TNFα2 is more dominant for the first few days after the vaccination (with a peak in day 2), but its expression levels decline to day 8 followed by an increase of TNFα1 expression after day 10, leading to total domination of TNFα1 over TNFα2 (Haugland *et al*. 2007). Similarly, in trout *Ichtyophthirius multifilis*-infected head kidney TNFα1 showed increasing transcriptional level from 48 h to 26 days post-infection, while TNFα2 were lower or equal to non-infected fish levels (Sigh *et al*. 2004). Authors speculated that transcriptional level shift from TNFα2 to TNFα1 was a consequence of organ-specific up-regulation and later activation of numerous immune cells. Likewise, damaged ABFT suffered infections and/or injuries for an extended period of time, which could have been the reason for stronger expression of TNFα1 compared to that of TNFα2. Therefore, the abundance of melano-macrophage centers (MMC), natural killer (NK) cells and natural

killer T (NKT) cells in liver could be an explanation for the increased cytokine gene expression, since liver can produce acute phase proteins and pro-inflammatory cytokines, as recognized in mammals (González-Amaro *et al*. 1994; Li and Diehl 2003; Gao *et al*. 2007).

4.3.2. Expression of ABFT IL-1β during critical points of the farming process

ABFT IL-1β was constitutively expressed in all samples examined with a significantly higher expression in the liver compared to the head kidney (Figure 3.19C). In contrast, *in vivo* studies in haddock (Fujiki *et al*. 2000), tilapia (Pelegrin *et al*. 2001), sea bass (Wang *et al*. 2004) and carp (Engelsma *et al*. 2001)) observed IL-1β expression only after stimulation. It is possible though that the examined fish from the newly caught and farm-acclimated group, although not infected or injured, were still showing some stress-related stimulation triggering IL-1β expression.

IL-1β had the highest expression level of all three cytokines examined and it was significantly increased in damaged tuna compared to the newly caught fish (but not farm-acclimated fish) (Figure 3.20C). Induction of IL-1β is usually a transient phenomenon, as seen in tilapia stimulated with LPS (Lee *et al*. 2006) where expression of IL-1β was undetectable 7 days post-infection. On the other hand, this study has shown that ABFT IL-1β can be up-regulated for at least one week after possible injury and/or infection. Precedents for a more chronic impact on IL-1β expression are seen in fish with ectoparasite infections, in the Atlantic salmon (Fast *et al*. 2006) where author reported up-regulation of IL-1β 40 days after infection with parasitic copepod *Lepeophtheirus salmonis*. More severe case was reported in striped trumpeter (Cavello *et al*. 2009) where IL-1β up-regulation lasted for more than 10 months after infection with copepod *Chondracanthus goldsmidi*. Thus, chronic injuries, similar to chronic parasite infections, may lead to the prolonged responses as seen in this study.

4.3.3. Expression of IL-1β, TNFα1 and TNFα2 in infected and uninfected ABFT gills

Importance of cytokines during host response to parasite infection has already been proven through their up-regulation in protozoan (Morrison *et al*. 2007), monogenean (Sigh *et al*. 2004), copepod (Cavello *et al*. 2009) or digenean (Mladineo and Block 2010) infections. Interestingly, sanguinicolid digenean *Cardicola orientalis* failed to induce any of the target cytokines in gills, indicating the

probability of modification of host local immune response induced by the parasite (Polinski *et al*. 2014). On the other hand, infection with monogenean ectoparasite *N. melleni* led to up-regulation of pro-inflammatory cytokines in *S. lalandi* spleen, suggesting host systemic response against the monogenean (Reyes-Becerril *et al*. 2017). *P. appendiculatus* significantly induced ABFT IL-1β at the site of infection, as reported in copepod infections in striped trumpeter (Cavello *et al*. 2009) and Atlantic salmon (Fast *et al*. 2006) (Figure 3.21A). *D. katsuwonicola* caused significant up-regulation of ABFT TNFα2 (Figure 3.21A) in contrast to the same species infecting PBFT gills, where IL-1β and TNFα1 were up-regulated, but TNFα2 expression has not been evaluated (Mladineo and Block 2010). Although this digenean in two closely related hosts did cause immune reaction in gills, the expression profiles of targets differed. Expression of cytokines in response to parasite infection clearly depends on host and parasite species, and consequently on the severity of the damage caused by the parasite. Therefore, versatility of the immune responses in the closely related host species and against the same group of parasites residing in the same location, is also influenced by many intrinsic (host) and extrinsic (parasite) factors: genetic background of host resistance, parasite pathogenicity and the degree of tissue damage caused, the phase and duration of infection, the size and age of the host and sequence of the exposure. Finally, ABFT moderate inflammatory response failed to seriously endanger neither of parasites examined in this study. This might be attributed to the tendency of pro-inflammatory cytokines to decrease and stabilise at late times post infection to avoid further tissue damage caused by excessive inflammatory response (Alvarez-Pellitero 2008; Pérez-Cordón *et al.* 2014).

4.4. Pathohistological analysis of ABFT infected gill filaments

Pathohistological analysis of two parasitic species presented in this study aimed to further investigate the host-parasite interactions through observation of cellular immune response. Difference in activated cell types between the two parasitic species reflected differential target expression in the infected tissue. Numerous mast cells found at *D. katsuwonicola* parasitation site can be related to significant up-regulation of TNFα, whereas tissue-destructive *P. appendiculatus* infection with goblet cells proliferation is accompanied by IL-1β induction.

4.4.1. Pathohistology of *D. katsuwonicola*-infected gills

Adult didymozoids are a particular group of digeneans because they are usually found encysted in the host tissues instead of being attached in the organ lumen. Their plasticity of morphological traits and parasitation sites includes an enormous range of body sizes, shapes and tissue localizations (Mladineo *et al*. 2010) also reflected in a wide array of reactions inflicted by the hosts. Formation of a connective-tissue capsule with aggregation of macrophages around juvenile and adult trematodes is the most common type of parasite sequestration from the healthy host tissue (Dezfuli *et al*. 1997).

ABFT tissue reaction consisted of a relatively thin and loose fibrous capsule, overlaid by proliferating squamous epithelium, abundantly interspersed by mast cells, eosinophils and goblet mucous cells in distinctive areas (Figure 3.22A). A peripheral nerve in close vicinity of the encysted afferent filament artery and didymozoids (Figure 3.22A) indicates that the parasite might stimulate the blood flow through the infected site, as suggested by Dezfuli *et al.* (2005). Consequently, one can speculate whether *Didymosulcus* employs its neighbouring artery only for juvenile migration towards its predicted site, and/or relays on it for further nutrition. On the other hand, the drawback of blood vessel vicinity for the parasite is that it enables inflow of immune cells from the circulation (Figure 3.22A). In this study, numerous mast cells, eosinophils and few rodlet and lymphocyte-like cells were located at the site of *D. katsuwonicola* cyst, visible also in the connective-tissue capsule and its anastomosing capillaries (Figure 3.22C). High number of mast cells is usually found at the parasitation site, especially inside, and/or in close proximity of capillaries as shown in this case (Sfacteria *et al*. 2015). The migration of mast cells normally occurs within microcirculation of gills, enabling them to move rapidly across the gills to the site of infection (Powell *et al*. 1990). Interestingly, in human skin mast cells appear to be predominant source of pre-formed TNF released upon inflammatory stimulus (Walsh *et al*. 1991) and corresponding to the significant up-regulation of this target observed in gill tissue surrounding the didymozoid. As for rodlet cells, unexpectedly low number was found surrounding didymozoid cyst compared to the copepod (Figure 3.22C). Although it has been suggested that rodlet cells have a functional role in teleost host defense against parasites (Reite and Evensen 2006, Secombes and Wang 2012), in didymozoid-infected ABFT gills there was no clear interaction between innate immune cells and scarce number of rodlet cells. The last conspicuous

cellular types observed surrounding the cyst were numerous and active mucous goblet cells in the peripheral cyst part (Figure 3.22D). Cytokines, like IL-1 and TNFα, have been suggested to induce secretions of the mucus (Buchmann 1999), which enables mechanical cleansing of pathogen as well as a stable media for secretion of different immune mediators. It can also contain substances with biostatic and biocidal activity as immunoglobulins, complement factors, lysozyme, proteases and lectins (Angeles Esteban 2012). Lastly, although goblet cells are probably not directly involved against *Didymosulcus* infection and are present in fewer numbers in comparison to copepod infection, their proliferation might indicate correlation with measured expression of target genes.

4.4.2. Pathohistology of *P. appendiculatus*-infected gills

While didymozoid infection is slowly developing and evokes mobilisation of mainly eosinophilic-granulated cell types, *P. appendiculatus* infection is tissue-damaging, acute and characterised by hemorrhage and extensive proliferation of rodlet and mucous goblet cells (Figure 3.23C). Similarly, *P. appendiculatus*-infected SBFT gills also showed signs of hemorrhage with minor hyperplasia or fibroplasia (Adams *et al*. 2017). However, unlike ABFT, SBFT response to copepod infection was predominately characterized by eosinophilic inflammatory response, and no rodlet or goblet cells were reported at the site of infection. Specific alteration in copepod-infected ABFT gills was observed in form of cells undergoing death process, although it was not possible to differentiate between necrotic and apoptotic changes (Figure 3.23E and F). Whilst didymozoids in general do not impose major mechanical compression on the tissues that might induce necrosis (Perera 1992; Perera 1994), as confirmed in this study, copepods that attach by clamping tissues consequently do (Figure 3.23A). In contrast to necrosis as an externally induced cell injury, apoptosis is a protective mechanism of cells that ensures a death program triggered by detection of an extra or intracellular unfavourable condition, i.e., deprivation of growth factors, DNA damage, or infection. It leads to increased caspase and endonuclease activities, cleavage of target substrates, nuclear condensation, DNA fragmentation, and cell shrinkage (Guillermo *et al*. 2009), aiming at “altruistic suicide” of the host cell, before the invading organism has a chance to multiply and disperse throughout other host cells (James and Green 2004). Interestingly, significant expression of TNFα would be expected in the tissue undergoing

necrotic/apoptotic changes as in copepod infection, especially because of its ability to induce NF-kB-mediated apoptosis (Reyes-Cerpa *et al*. 2012), but in contrast it was measured in didymozoid infection that lacks such alterations. This suggests that copepod-induced cell death is not dominated by this cytokine as it was previously suggested in cell line models (Gaur and Aggarwal 2003). It also supports the fact that TNF-induced cell death plays only a minor role compared to its overwhelming functions in the inflammatory process and that its death-inducing capability is weak compared to other family members (such as FAS receptor or apoptosis antigen 1) (Gaur and Aggarwal 2003). Acute tissue-destructive inflammatory response in copepod infection supported by IL-1β up-regulation was dominant over cell-death signalling and TNFα expression. Induction of IL-1β in copepod-infected gills may also be associated with numerous mucus secreting goblet cells due its ability to stimulate the release of mucin in these cells (Deplancke and Gaskins 2001). In addition, mammalian goblet cells also express inflammasome, a multiprotein complex involved in the production of active mature IL-1β (Birchenough *et al*. 2015).

5. Conclusions

Two TNFα isoforms are present in Atlantic bluefin tuna (ABFT): TNFα1 and TNFα2. Both isoforms showed good homology to other known TNFα's with the highest amino acid identity with PBFT TNFα molecules. ABFT TNFα2, like in Pacific bluefin tuna (PBFT), lacks ARE or endotoxin-responsive elements typical for teleost cytokines. Low amino acid identity and phylogenetic separation of ABFT TNFα1 and TNFα2 suggest their distinct physiological roles. ABFT TNFα1 and TNFα2 share the same tertiary structure as mammalian homologues, but with different receptor-ligand binding mechanisms.

ABFT IL-1β showed the highest identity with Southern bluefin tuna (SBFT) IL-1β. Presence of instability motifs (ATTTA) within IL-1β introns suggests their possible influence on regulation of mRNA splicing and/or expression. ABFT IL-1β also undergoes incomplete splicing which is often seen in other teleosts IL-1β. Homology modeling revealed that ABFT IL-1β has similar tertiary structure as its human homologue, but binds differently to its receptor.

ABFT IL-1β and TNFα2 were significantly induced *in vitro* by PAMPs and *D. katsuwonicola* and *P. appendiculatus* protein extracts, as well as during natural gill infection with two parasites. This suggests that they play an important role in host defense against wide array of pathogens. Contrary, ABFT TNFα1 showed relatively poor up-regulation following both *in vitro* stimulation and parasite infection, but had stronger expression then TNFα2 in caged ABFT suffering infections and/or injuries for a prolonged period of time. Expression of these immne mediators in parasites PE-stimulated PBL (*in vitro* model) was congruent to that in infected tissues (*in vivo* model), diverging only in respect to parasite species. Patohistological changes differed between encysted didymozoid and actively-attached copepod, being congruent with the expression profiles of targets in the gills. Interestingly, fold change of targets' expression was far more elevated when PBL culture was stimulated by purified PAMPs (LPS and Poly I:C) then by the total extract of parasite protein extracts, even when two doses (1 μg/ml and 10 μg/ml*)* were used. This might be related to a lower antigenicity of parasites resulting in a mild host immune response that is able to restrict the parasite to its attachment site or enclosed within the cyst, but fails to endanger its survival in a larger extent. It also suggests that the host-parasite

interaction balances between moderate innate immunity response and evading mechanisms adopted by a digenean and copepod.

This study has confirmed that TNFα1, TNFα2 and IL-1β could be used as biomarkers for monitoring of ABFT health status. Furthermore, their transcriptional regulation in both natural and controlled conditions should continue to be investigated in order to better understand the physiological context important for tuna welfare and adapt optimal rearing practices for future ABFT cultivation.

6. References

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7. Summary

Atlantic bluefin tuna ABFT, *Thunnus thynnus*, is one of the largest *Thunnus* species with high economic significance for Croatian industry. However, little is known about its innate immune system and defense mechanisms and to date there are no reports of cloning and expression analysis of any major immune genes of ABFT. Therefore, within this thesis the first cytokine molecules in ABFT (TNFα1, TNFα2 and IL-1β) are cloned and compared to known sequences in other vertebrates, especially teleost fish. In order to evaluate putative significance of ABFT TNFα1, TNFα2 and IL-1β as biological markers, their expression levels were monitored and described in selected tissues, at various stages of two years-long farming process. To evaluate ABFT TNFα1, TNFα2 and IL-1β induction by PAMPs and potential role in response to bacterial and viral infections, their expressions were evaluated in simulated acute infection using peripheral blood leukocytes stimulated *in vitro* with LPS and Poly I:C. Lastly, the role of three cytokines in acute and chronic parasitic infection was examined during natural infection with *Pseudocycnus appendiculatus* (Copepoda) and *Didymosulcus katsuwonicola* (Digenea), as well as during leukocyte exposure to total protein extracts isolated from two parasite species. As an additional record to support latter molecular results, a pathohistological analysis of *D. katsuwonicola* and *P. appendiculatus* was performed on semithin sections of infected gill filaments.

ABFT TNFα mRNA molecules were comprised of a 744 bp (TNFα1) and 738 bp (TNFα2) open reading frame (ORF) that encoded a protein of 247 (TNFα1) and 245 (TNFα2) amino acids, showing good homology to other known TNFα's with highest amino acid identity with PBFT TNFα sequences. ABFT TNFα2 3' UTR contained no ARE or endotoxin-responsive elements, implying rather unique situation when compared to other teleost TNFα2 mRNAs. ABFT TNFα1 and TNFα2 shared only 39% of amino acid identity, suggesting that they have distinct phylogenetic separation and physiological roles. The coding region of the ABFT IL-1β mRNA sequence comprised of 724 bp encoding 246 amino acids with highest identity with SBFT IL-1β. Instability motifs (ATTTA) were also found in the ABFT introns, except intron 3, implying a possibility that these intron instability motifs influence or have a role in regulating splicing of pre-RNA as well as mRNA expression. Within mRNA derived

from damaged ABFT liver and head kidney tissues, a second, incompletely spliced transcript was detected containing exons 1-5 plus intron 1.

Induction of ABT IL-1β and TNFα2 by PAMPs and protein extracts from *D. katsuwonicola* and *P. appendiculatus*, as well as during natural infection with the two parasites, suggests their important role in inflammation, being engaged in controlling parasite infections, in contrast to ABFT TNFα1. Targets' expressions in general followed congruent pattern in parasites PE-stimulated PBL (*in vitro* model) and in host tissue (*in vivo* model), diverging only in respect to parasite species. Although ABFT TNFα1 showed relatively poor up-regulation following *in vitro* stimulation and parasite infection, it had stronger expression then TNFα2 in caged ABFT suffering infections and/or injuries for prolonged period of time. Cellular innate response to the digenean *D. katsuwonicola* showed rather chronic character, resulting with a moderate inflammatory reaction that fails to seriously endanger digenean existence. Copepod *P. appendiculatus*, attached to the gill epithelium by clamping, caused direct tissue disruption with undergoing necrotic or apoptotic processes, and extensive proliferation of rodlet and goblet cells. Differential expression patterns of target cytokines in tissue surrounding two parasites and *in vitro* PBL model suggest that quality and quantity of tuna immune response is conditioned by parasite adaptive mechanisms and pathogenicity.

8. Sažetak

Atlantska plavoperajna tuna *Thunnus thynnus* jedan je od najvažnijih hrvatskih izvoznih proizvoda riboprerađivačke indutrije, pridonoseći s 50% nacionalnog izvoza prema japanskom tržištu. Uzgoj tune u Hrvatskoj se bazira na ulovu (*capture-based aquaculture*), što podrazumijeva hvatanje juvenilne tune (8-15 kg) iz prirode i njen uzgoj u kavezima tijekom sljedeće dvije godine ili koliko je potrebno da postigne izlovnu masu od najmanje 30 kg i optimalni udio masti i proteina u mišićju. Tijekom takvog produženog uzgojnog razdoblja nepredvidivi okolišni čimbenici, izloženost nametnicima i drugim patogenim mikroorganizama te neuravnotežena prehrana pogoduju pojavi i širenju bolesti. Za razliku od odrasle, juvenilna tuna je vrlo osjetljiva na infekcije, posebno u fazi prilagodbe životu u kavezu kada je imunološko stanje jedinki već opterećeno stresom uzrokovanim ulovom, prijevozom i prijelazom u kaveze. U usporedbi s drugim komercijalno značajnim vrstama, još se uvijek malo zna o imunološkom sustavu i njegovoj regulaciji u ove, za akvakulturu iznimno važne vrste.

Prvi cilj ove studije bio je kloniranje čitave sekvence mRNA i gDNA pro-inflamatornih citokina TNFα1, TNFα2 i IL-1β atlantske plavoperajne tune te usporedba s istima u ostalih kralješnjaka služeći se tehnikama poravnavanja višestrukih sekvenci, filogenetskim analizama i homolognim 3D modeliranjem. Nadalje, kako bi se ocijenila upotrebljivost triju citokina kao bioloških biljega njihova ekspresija je kvantificirana u odabranim tkivima tijekom tri kritična razdoblja dvogodišnjeg uzgoja. U svrhu evaluacije aktivacije TNFα1, TNFα2 i IL-1β u atlantske plavoperajne tune različitim imunostimulansima te njihove moguće uloge tijekom bakterijskih i virusnih infekcija, njihova ekspresija se kvantificirala na modelu akutne infekcije koristeći leukocite krvi stimulirane *in vitro* s LPS-om i Poly I:C-om. Na poslijetku, uloga triju citokina tijekom kronične i akutne infekcije nametnicima, istražena je modelom infekcije koristeći leukocite krvi stimulirane *in vitro* ekstraktom nametnika kopepodnog račića *Pseudocycnus appendiculatus* i dvorodnog metilja *Didymosulcus katsuwonicola,* kao i tijekom prirodne infekcije škrga dvama nametnicima.

Rezultati su otkrili da kodirajuće regije TNFα mRNA molekula u atlantske plavoperajne tune sadrže 744 bazna para (bp) (TNFα1) i 738 bp (TNFα2) koje kodiraju proteine sačinjene od 247 (TNFα1)

odnosno 245 (TNFα2) aminokiselina, što je blizu prosječne duljine proteina TNFα u riba (242 aminokiseline). Dobivene sekvence su pokazale relativno visoku sličnost s istim sekvencama u drugih koštunjača, a posebno s TNFα pacifičke plavopeajne tune *Thunnus orientalis*. Zanimljivo, u neprepisujućoj regiji (*untranslated region UTR*) 3' kraja molekule TNFα2 atlantske plavoperajne tune nisu pronađeni elementi karakteristični za 3'UTR TNFα2 drugih koštunjača (*AU-rich elements ARE* i *endotoxin-responsive elements*). Nadalje, međusobna sličnost molekula TNFα1 and TNFα2 iznosi samo 39%, što upućuje na filogenetsku odvojenost dobro vidljivu i na filogenstskom stablu. TNFα1 se tako nalazi u istoj grupi s ostalim pripadnicima najstarijeg reda *Perciformes*, udaljen od TNFα2 i ostalih pripadnika mlađih redova *Cypriniformes* i *Siluriformes*. Ovakva filogenetska separacija, kao i razlike u aminokiselinskim sljedovima upućuju i na moguće različite fiziološke uloge dviju molekula TNFα.

Rezultati homolognog modeliranja pokazali su da su tercijarne strukture TNFα1 and TNFα2 molekula atlantske plavoperajne tune odgovarajuće onima u čovjeka, što je posljedica konzerviranosti ključnih segmenata odgovornih za formaciju sekundarne i tercijarne strukture. Međutim, aminokiseline koje sudjeluju u stvaranju veze receptor-ligand kod čovjeka pokazale su slabu konzerviranost, što upućuje na različite mehanizme receptor-ligand vezivanja između TNFα atlantske plavoperajne tune i njegovog humanog homologa.

Kodirajuća regija IL-1β mRNA molekule atlantske plavoperajne tune sastoji se od 724 bp i kodira protein od 246 aminokiseline. Najveću sličnost dijeli sa proteinom IL-1β južne plavoperajne tune *Thunnus maccoyii* od kojega se razlikuje u devet aminokiselina. Zanimljivo, tzv. '*instability motifs*' u vidu sljedova ATTTA, inače specifični za 5' UTR molecule IL-1β molekule, u atlantske plavoperajne tune su pronađeni i unutar introna. Ovakav položaj sljedova ATTTA upućuje na njihovo moguće sudjelovanje u regulaciji prekrajanja nezrele mRNA IL-1β i/ili u ekspresiji IL-1β. Također su uočeni i mRNA transkripti koji uz eksone 1-4 posjeduju i intron 1. Inače, takvo i slična nepotpuna prekrajanja IL-1β mRNA česta su i kod drugih koštunjača.

Homolognim modeliranjem proteina IL-1β atlantske plavoperajne tune utvrđeno je da molekula ima sličnu tercijarnu strukturu kao i njegov humani homolog, međutim ne stupa u vezu sa svojim

receptorom na isti način, jer posjeduje samo jednu od ukupno sedam aminokiselina za koje je dokazano da sudjeljuju u vezivanju humanog proteina IL-1β s njegovim receptorom.

Aktivacija IL-1β i TNFα2 nakon stimulacije *in vitro* imunostimulansima (LPS i Poly I:C) i proteinskim ekstraktom nametnika *D. katsuwonicola* i *P. appendiculatus*, ali i tijekom prirodne infekcije škrga dvama nametnicima dokazuje kako ova dva citokina igraju važnu ulogu u upalnim procesima i općenito imunološkom odgovoru atlantske plavoperajne tune na različite vrste patogena. TNFα1 je pokazao slab odgovor u modelu *in vitro*, međutim u jetri tuna koje su pokazivale znakove infekcije bio je značajno eksprimiran. Ekspresija ciljnih gena tijekom stimulacije leukocita proteinskim ekstraktima nametnika (model *in vitro*) bila je sukladna onoj tijekom prirodne infekcije istim nametnicima (model *in vivo*), razlikujući se samo u odnosu na vrstu nametnika. U oba slučaja citokini su bili slabo do umjereno eksprimirani, za razliku od stimulacije LPS-om i Poly I:C-om koja je rezultirala njihovom mnogo snažnijom ekspresijom.

Patohistološka analiza zaraženih škržnih filamenata otkrila je kako je stanični odgovor atlantske plavoperajne tune na infekciju dvorodnim metiljem *D. katsuwonicola* kroničnog karaktera te rezultira odvajanjem nametnika u vezivno-tkivnu kapsulu. U neposrednoj blizini nametnika zabilježeni su različiti tipovi stanica imunološkog sustava (mastociti, eozinofilni granulociti, mukozne vrčaste stanice i tzv. 'rodlet cells' štapičaste stanice). S druge strane, kopepodni račić svojim prihvaćanjem za škržne filamente uzrokuje oštećenje epitelnog tkiva popraćeno nekrotičnim/apoptotičnim staničnim procesima te snažnu proliferaciju mukoznih vrčastih stanica i štapičastih 'rodlet'stanica.

Rezultati patohistološke analize zaraženih škržnih filamenata, jačina lokalne ekspresije citokina te slaba antigenost obaju nametnika tijekom stimulacije *in vitro* ukazuju na umjereni imunološki odgovor tune, koji iako ne uspjeva trajno ugroziti opstanak nametnika, pruža učinkovitu zaštitu od širenja infekcije. Jačina i vrsta imunološkog odgovora atlantske plavoperajne tune stoga uvelike ovisi o vrsti i patogenosti nametnika, njegovom razvojnom stadiju i stupnju antigenosti te trajanju same infekcije.

9. Curriculum vitae

PERSONAL INFORMATIONS

Name and surname	**Ivana Lepen Pleić**
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WORK EXPERIENCE

Date (from – until)	2009- present
Institution	Institute for Oceanography and Fisheries, Split
Position	Novice in science, Phd student
Work field	Molecular biology, fish immunology
Date (from – until)	May 2009 – September 2009
Institution	Faculty of Medicine, Department for molecular biology and biotechnology, Rijeka
Position	Project associate
Work field	Molecular biology, cancer research

EDUCATION

Date	2010 - present
Place	Osijek, Croatia
Institution	University J.J. Strossmayer, Postgraduate studies of molecular biosciences
Qualification awarded	to be PhD in molecular biosciences

Date	2001 - 2008
Place	Split, Croatia
Institution	University of Split, Faculty of Sciences and Mathematics
Qualification awarded	Professor of biology and chemistry

PERSONAL SKILLS AND COMPETENCES

Languages	Croatian (mother tongue); English (fluent)
Computer skills	Excellent general knowledge of computer based software and technology, and usage of Microsoft Windows Office Suite programs.
Other	Holder of type B driver's license

TRAINING

Year	2011 (one week training)
Place	Germany
Institution	Bioscience Events & PCR Services BioEPS
Subject and skills covered	Real-time PCR (qPCR Basic Modul)

Year	2011 (one week training)
Place	Split
Institution	Institute for oceanography and fisheries
Subject and skills covered	Phylogeny course

VISITS TO FOREIGN RESEARCH AND EDUCATION INSTITUTIONS

Year	September 2011- January 2012
Place	Scotland, UK
Institution	University of Aberdeen, Department of Zoology
Subject and skills covered	Fish immunology, cloning and expression of immunity related genes

RESEARCH AND OTHER PROJECTS

2009. Regulation of p53 tumor suppressor by ribosomal proteins in physiological and pathological conditions; position: project associate; Unity Through Knowledge Fund

2009-2010. Development of a health genomic profile for the captive Atlantic bluefin tuna (*Thunnus thynnus*); position: project associate; Unity Through Knowledge Fund

2009-2010. Dynamics and pathology of parasitofauna in reared marine fish; position: PhD student; Ministry of Science and Education of the Republic of Croatia

AWORDS AND RECOGNITIONS

- Government scholarship during four years of University education
- Scholarship for PhD students, visit to foreign institutions – Croatian Science Fondation HRZZ

PUBLICATIONS

1. Blažeković K, **Lepen Pleić I**, Đuras M, Gomerčić T, Mladineo I. Three *Anisakis* spp. Isolated from toothed whales stranded in the Eastern Adriatic Sea coast. *Int J Parasitol* 2015; **45**(1): 17-31.

2. **Lepen Pleić I**, Bušelić I, Trumbić Ž, Bočina I, Šprung M, Mladineo I. Expression analysis of the Atlantic bluefin tuna (*Thunnus thynnus*) pro-inflammatory cytokines, IL-1β, TNFα1 and TNFα2 in response to parasites *Pseudocycnus appendiculatus* (Copepoda) and *Didymosulcus katsuwonicola* (Digenea). *Fish and Shellfish Immunol* 2015; **45**: 946-954.

3. **Lepen Pleić I**, Secombes CJ, Bird S, Mladineo I. Characterization of three pro-inflammatory cytokines, TNFα1, TNFα2 and IL-1β, in cage-reared Atlantic bluefin tuna *Thunnus thynnus*. *Fish and Shellfish Immunol* 2014; **36** (1); 98-112.

4. Bošnjak I, **Lepen Pleić I**, Borra M, Mladineo I. Quantification and in situ localisation of abcb1 and abcc9 genes in toxicant-exposed sea urchin embryos. *Environ Sci Pollut Res Int* 2013; **20** (12): 8600-8611.

5. Grubišić L, Šegvić-Bubić T, **Lepen Pleić I**, Mišlov K, Tičina V, Katavić I, Mladineo I. Morphological and genetic identification of spontaneously spawned larvae of captive Bluefin Tuna *Thunnus thynnus* in the Adriatic Sea. *Fisheries* 2013; **38** (9): 410-417.

6. **Lepen I**, Puizina J. FISH mapping of 18S-5.8S-26S rRNA genes and fluorochrome banding in the triploid viviparous onion, *Allium x cornutum* Clementi ex Visiani, 1842. *Acta Biol Cracoviensia Ser Bot* 2011; **53** (1); 111-116.

7. Šegvić-Bubić T, **Lepen I**, Trumbić Ž, Ljubković J, Sutlović D, Matić-Skoko S, Grubišić L, Glamuzina B, Mladineo I. Population genetic structure of the reared and wild gilthead sea bream (*Sparus aurata*) in the Adriatic inferred with microsatellite loci. *Aquaculture* 2011; **318** (3/4); 309-315.

NATIONAL AND INTERNATIONAL CONFERENCES

1. Beg Paklar, Gordana; Džoić, Tomislav; Grbec, Branka; **Lepen Pleić, Ivana**; Mladineo, Ivona; Zorica, Barbara; Čikeš Keč, Vanja; Ivatek-Šahdan, Stjepan. South Adriatic - potential spawning ground for scobrids inferred by genetics and modelling. 26th International union of geodesy and geophysics. Prague, Czech Republic, 2015. (poster presentation)

2. Čikeš Keč, Vanja; Zorica, Barbara; Beg Paklar, Gordana; Džoić, Tomislav; Grbec, Branka; **Lepen Pleić, Ivana**; Mladineo, Ivona; Grubišić, Leon; Ivatek-Šahdan, Stjepan. Incidental spawning of some scombrids in Adriatic or not? XV European Congress of Ichthyology. Porto, Portugal, 2015. (poster presentation)

3. **Lepen Pleić, Ivana**; Bušelić, Ivana; Hrabar, Jerko; Šprung, Matilda; Bočina, Ivana; Mladineo, Ivona. Histopathological alterations induced by gill and skin parasitic infections in the Atlantic bluefin tuna (*Thunnus thynnus*) and its innate response modelled *in vitro*. 17th International Conference on Diseases of Fish and Shellfish. Las Palmas de Gran Canaria, Canary Islands, Spain, 2015. (oral presentation)

4. **Lepen Pleić, Ivana**; Bušelić, Ivana; Trumbić, Željka; Bočina, Ivana; Šprung, Matilda; Mladineo, Ivona. Expression of proinflammatory cytokines IL-1β, TNFα1 and TNFα2 induced by PAMPs and gill parasitic infections in the Atlantic bluefin tuna (*Thunnus thynnus*) peripheral blood leukocytes and gills. 17th International Conference on Diseases of Fish and Shellfish / Mladineo. Las Palmas de Gran Canaria, Canary Islands, Spain, 2015. (oral presentation)

5. Blažeković, Kristina; **Lepen Pleić, Ivana**; Gomerčić, Tomislav; Đuras, Martina; Mladineo, Ivona. Preliminarno istraživanje raznolikosti i distribucije pripadnika zoonotičnog roda *Anisakis* spp. u morskih sisavaca Jadrana. Simpozij parazitarne zoonoze i teški metali u kopnenih i morskih predatora. Zagreb, Hrvatska, 2013. (oral presentation)

6. Bošnjak, Ivana; **Lepen Pleić, Ivana**; Borra, Marco; Benevenuto, Giovanna; Mladineo, Ivona. Učinak bisfenola A (BPA) na gensku ekspresiju i morfologiju embrija morskog ježinca. Proceedings of the 2nd International Symposium "Vera Johanides". Zagreb, Hrvatska, 2013. (oral presentation)

7. **Lepen Pleić, Ivana**; Ferri, Josipa; Petrić, Mirela; Pezelj, Roko; Radonić, Ivana; Vladislavić Stanić, Nikolina; Oraić, Dražen; Zrnčić, Snježana; Mladineo, Ivona. Effect of Vetregard on the immunological parameters in the reared European sea bass. 1st International conference of fish and shellfish immunology. Vigo, Spain, 2013. (poster presentation)

8. **Lepen Pleić, Ivana**; Secombes, Christopher; Bird, Steve: Mladineo, Ivona. Characterization of three major cytokines: IL1β, TNFα1 and TNFα2 in reared Atlantic bluefin tuna (*Thunnus thynnus*). 1st International conference of fish and shellfish immunology. Vigo, Spain, 2013. (poster presentation)

9. **Lepen Pleić, Ivana**; Ferri, Josipa; Trumbić, Željka; Petrić, Mirela; Pezelj, Roko; Radonić, Ivana; Vladislavić Stanić, Nikolina; Oraić, Dražen; Zrnčić, Snježana; Mladineo, Ivona.

Effect of mixture composed of β glucans and MOS on the immunological parameters in the reared european sea bass. 16th International Conference on Diseases of Fish and Shellfish Diseases. Tampere, Finland, 2013. (poster presentation)

10. **Lepen Pleić, Ivana**; Secombes, Chris; Bird, Steven; Mladineo, Ivona. Characterization of three major cytokines: IL1, TNF1 and TNF2 in reared Atlantic bluefin tuna *Thunnus thynnus*. 16th International Conference of Fish and Shellfish Diseases. Tampere, Finland, 2013. (poster presentation)

11. Blažeković, Kristina; **Lepen Pleić, Ivana**; Đuras Gomerčić, Martina; Gomerčić, Tomislav; Mladineo, Ivona. Molecular identification of *Anisakis* spp. complex from gastrointestinal tract of stranded cetaceans in Adriatic Sea. European cetacean society. Galway, Ireland, 2012. (oral presentation)