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# Characterization of three pro-inflammatory cytokines, TNFα1, TNFα2 and IL-1β, in cage-reared Atlantic bluefin tuna *Thunnus thynnus*

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ABSTRACT

Atlantic bluefin tuna (BFT) (*Thunnus thynnus*) is of great economic significance for world aquaculture and therefore it is necessary to ensure optimal and sustainable conditions for the farming of this species. Intensive culture of fish may be limited by infectious diseases that can impact on growth performance and cause heavy losses. However, to date there are no reports of cloning and expression analysis of any major immune genes of Atlantic BFT although some immune genes are known in other BFT species. Therefore the aim of this study was to characterize the first cytokine molecules in Atlantic BFT, through: 1) Isolation of full-length cDNA and gene sequences of TNFα1, TNFα2 and IL-1β, 2) comparison of these molecules to known sequences in other vertebrates, especially teleost fish, by multiple sequence alignment, phylogenetic tree analysis and homology modeling; 3) Quantification of *in vivo* expression of these cytokines in selected tissues in reared BFT over the duration of the farming process. The results indicated that these three cytokines could have value for monitoring Atlantic BFT health status. Curiously, the liver seemed to be an important site of cytokine production during poor health conditions in this species, perhaps reflecting its role as an important organ involved in fish defenses.

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## 1. Introduction

Bluefin tunas (BFT) are the largest of the Thunnus species (Scombridae) and are characterized by a long life span, wide geographic distribution and endothermy [1]. Three species are known, namely Atlantic BFT (*Thunnus thynnus*), Southern BFT (*Thunnus maccoyii*) and Pacific BFT (*Thunnus orientais*). Since their introduction into Mediterranean aquaculture in the early nineties, Atlantic BFT have become the most valuable finfish aquaculture product currently known, with more than half of the world's total production concentrated in the Mediterranean Sea [2]. In Croatia, the first BFT culture began two decades ago, and currently 50% of the total national fisheries export goes to the Japanese market. Tuna aquaculture is a capture-based activity, where wild caught tuna are cultured in marine cages for a specific period of time, in order to increase the commercial value by increasing their protein and fat content. Farms are supplied with fish of 8–15 kg that are then kept in cages for prolonged periods (usually one and a half years but up to two or three years), depending on their initial size and market requests. During that period unpredictable environmental factors, microorganisms and unbalanced diet can contribute to the onset of disease in the tuna [3]. Although disease problems have rarely been reported in adult BFT, juvenile BFT are highly susceptible to various pathogens [4,5], with stress associated with intensive farming conditions possibly affecting individual fish immunocompetence and growth performance [6,7]. To date, most research carried out on tuna immunology has been focused on the Pacific and Southern BFT [8–13], with no reports of the cloning and expression analysis of any important immune genes in Atlantic BFT.

The innate immune response is the first line of host defense against pathogenic organisms [14], helping to control infection until the adaptive immune response develops to provide specific immunity [15]. After detection and recognition of pathogens, the innate immune system initiates and activates other components via the release of cytokines, small cell signaling proteins that act as intercellular mediators. One large family of structurally related cytokines involved in the innate immune response are the 'Tumor

*Abbreviations:* BFT, bluefin tuna; TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; NW, North-West; TACE, TNFα converting enzyme; RACE, rapid amplification of cDNA ends; BLAST, Blast Local Alignment Search Tool; ORF, open reading frame; UTR, untranslated region; ARE, AU-rich elements; bp, base pairs; 3D, three-dimensional; NK, natural killer cells; NKT, natural killer T cells; LPS, lipopolysaccharide; PBL, peripheral blood lymphocytes.

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necrosis factor (TNF) Ligand Superfamily', whose members have a wide range of diverse activities, such as inflammation, apoptosis, cell proliferation and stimulation of different aspects of immunity [16]. A member of this family, Tumor necrosis factor alpha (TNFα), is a major mediator of pro-inflammatory and antimicrobial defense mechanisms, able to eliminate various pathogens by inducing a variety of cellular responses such as phagocytosis and chemotaxis, and is considered an excellent biomarker and health indicator for both mammals and fish [16–19]. TNFα is currently one of the most well-studied fish cytokines, having been described in several teleost fish [9,13,20–34]. 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) [35–37]. 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 "jelly-roll" β-structure [38].

The interleukin-1 (IL-1) family of cytokines, with eleven members in mammals, is another major mediator of inflammation and can induce the expression of a wide variety of non-structural, function-associated genes during infection [39]. IL-1β is a particularly important component of the inflammasome and is produced by a variety of cells, mainly blood monocytes and tissue macrophages. It plays a key role in the host response to microbial invasion, tissue injury and even autoimmune diseases [40] due its ability to enhance phagocyte activity, macrophage proliferation, lysozyme synthesis and leukocyte migration [41] and has also been characterized in various fish species [13,39,42–53]. IL-1β is expressed as a 30 kDa non-functional precursor in mammals, that is fully activated only after it is proteolytically cleaved into a 17.3 kDa mature peptide by a cysteine protease, IL-1β converting enzyme (ICE), associated with transport out of the cell [54–56]. The mature peptide, as with other IL-1 family members, contains 12 β-sheets that form a β-trefoil structure [57].

In this study we have characterized the first cytokine molecules in Atlantic BFT, through: 1) Isolation of full-length cDNA and gene sequences of Atlantic BFT TNFα1, TNFα2 and IL-1β, 2) Comparison of these molecules to known sequences in other vertebrates, especially teleost fish, and 3) Quantification of their *in vivo* expression in selected tissues in reared BFT over the duration of the farming process, in order to evaluate their importance as potential biomarkers for tuna aquaculture.

## 2. Materials and methods

### 2.1. Atlantic BFT sampling

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. Atlantic BFT were sampled three times during the rearing process (total $N = 29$). The first group comprised juvenile fish (8–10 kg) 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 in the farming cage (newly caught). The second group were reared juvenile BFT with wounds and lesions on the skin that led to mortalities in some instances during the acclimation period (damaged BFT). Lastly, the third group were tuna reared for one and a half years and sampled at harvest time (farm-acclimated BFT).

Fish necropsy included assessment of gross pathology and histopathology, bacteriology and parasitology as previously described [3,5]. Damaged fish showed signs of septicemia (data not shown).

**Table 1**
Oligonucleotide primers used to clone and/or amplify the Atlantic bluefin tuna (BFT) TNFα1, TNFα2, IL-1β and β-actin genes.

| Name | Nucleotide sequence (5′ → 3′) | Use |
|---|---|---|
| bftTNF1-F | CCAGGCRGCCATCCATTTAGAAG | |
| bftTNF1-R | CGCTGACCTCACCGCGCTCATCAG | |
| mβactin-F | ATCGTGGGGCGCCCCAGGCACA | Primers used to obtain initial fragments |
| mβactin-R | CTCCTTAATGTCACGCACGAT TTC | |
| bftTNF2-F | TGAATGCAAGGTAGCGCTGGATG | |
| bftTNF2-R | TGGTCTGGTCGGAACTTGTGGCG | |
| 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 genomic DNA |
| bftTNF2-gF | AGGAAACACACAACGCAGAG | |
| bftTNF2-gR | AGGCAAACACACCAAAGAAGG | |
| bftIL1-gF | GGGATAACCAACCAAACTAACAGAAC | |
| bftβactin-rtF | CAGGGAGTGATGGTGGGTATGG | |
| bftβactin-rtR | GAAGGTCTCGAACATGATCTGGGTC | |
| bftTNF1-rtF | GAAAACGTCTCACACCTCTCAGCC | |
| bftTNF1-rtR | CAGCTGAAACACGGCACCAA | Primers for expression studies |
| bftTNF2-rtF | CAGTGGAATGGAAAAATCAGG | |
| bftTNF2-rtR | CTTCACAGTATGGCTCAGATGG | |
| bftIL1-rtF | GAAATGAGATGCAACGTGAGCG | |
| bftIL1-rtR | CACTTTGCTCCTCTAAAATGCTGTCC | |

### 2.2. cDNA production

Liver and head kidney tissues were extracted from 29 fish (9 newly caught BFT, 10 damaged BFT, 10 farm-acclimated BFT) and stored in RNAlater (Qiagen) at −20 °C until RNA extraction. Total RNA was extracted from 50 to 100 mg of tissue using Tri Reagent (Sigma Aldrich, USA) following the manufacturer's instructions 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. Prior to cDNA synthesis, total RNA was treated with 1 unit/μl RNase free DNase I (Fermentas Life Sciences, Germany) following the manufacturer's instructions. cDNA was then synthesized from 5 μg of total RNA using Bioscript™ (Bioline, UK) with oligo dT, following the manufacturer's instructions, and used as a template for PCR and real-time PCR. Production of cDNA for 3′ and 5′ RACE was performed also from 5 μg of total RNA, but using a GeneRacer™ Kit (Life

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