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Short communication

Ontogenetic profile of innate immune related genes and their tissue-specific expression in brown trout, *Salmo trutta* (Linnaeus, 1758)

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ABSTRACT

The innate immune system is a fundamental defense weapon of fish, especially during early stages of development when acquired immunity is still far from being completely developed. The present study aims at looking into ontogeny of innate immune system in the brown trout, *Salmo trutta*, using RT-PCR based approach. Total RNA extracted from unfertilized and fertilized eggs and hatchlings at 0, 1 h and 1, 2, 3, 4, 5, 6, 7 weeks post-fertilization was subjected to RT-PCR using self-designed primers to amplify some innate immune relevant genes (TNF-α, IL-1β, TGF-β and lysozyme c-type). The constitutive expression of β-actin was detected in all developmental stages. IL-1β and TNF-α transcripts were detected from 4 week post-fertilization onwards, whereas TGF-β transcript was detected only from 7 week post-fertilization onwards. Lysozyme c-type transcript was detected early from unfertilized egg stage onwards. Similarly, tissues such as muscle, ovary, heart, brain, gill, testis, liver, intestine, spleen, skin, posterior kidney, anterior kidney and blood collected from adult brown trout were subjected to detection of all selected genes by RT-PCR. TNF-α and lysozyme c-type transcripts were expressed in all tissues. IL-1β and TGF-β transcripts were expressed in all tissues except for the brain and liver, respectively. Taken together, our results show a spatial-temporal expression of some key innate immune-related genes, improving the basic knowledge of the function of innate immune system at early stage of brown trout.

1. Introduction

The innate immune system represents the first line of defense against any tissue damage or pathogen interaction and plays a pivotal role in counteracting the establishment of infection, especially during early stages of development when the acquired immunity is still far from being completely developed. Natural response generally precedes the adaptive response, activates and determines the nature of the acquired response, contributing to maintenance of homeostasis [1].

The classification of the humoral parameters of innate immunity is commonly based on their pattern recognition specificities or effector functions. Thus, some lytic enzymes, such as lysozyme, cause hydrolysis of N-acetylmuramic acid and N-acetylglucosamine of bacterial cell wall peptidoglycans, leading to breakdown of bacteria and playing an important role in defense mechanisms [2]. Lysozyme was shown not only in several tissues of adult fish species [2], but also in oocytes, embryos and larvae of some fishes, as tilapia, *Oreochromis mossambicus* [3], salmonids [4] and sea bass, *Dicentrarchus labrax* [5]. According to Balfry and Iwama [6], maternal lysozyme activity in kidney and serum is highly correlated to lysozyme activity of unfertilized eggs of coho salmon, *Oncorhynchus kisitch*.

Li et al. [7] showed that lysozyme activity in rainbow trout, *Oncorhynchus mykiss*, is higher in embryos derived from cortisol-treated oocytes until 13 days post fertilization than in control group, suggesting an important role of innate immune system in the early cleavage stages of embryonic cells. Moreover, in recent years, lysozyme-encoding genes were detected and the protein from some fishes was cloned [8–12]. Xing et al. [13] have identified two genes encoding two different lysozymes, g-type and c-type, from grass carp, *Ctenopharyngodon idellus*, having only 8.6% similarity of amino acid sequence between them and different lytic activities against fish bacterial pathogens. Both mRNA levels of g- and c-type genes increase as consequence of bacterial infection, g-type lysozyme having a stronger up-regulation than that of c-type.

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According to the authors [13], the g-type lysozyme might be induced for the defense against bacterial infections, while the c-type might be the main molecule for the defense under normal conditions. At the same time, in zebrafish, *Danio rerio*, c-type lysozyme has high homologies with other animal lysozymes, being expressed in macrophage lineage as early as 20 h post-fertilization [14].

Cytokines are soluble messenger molecules of innate and acquired immune system and include interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), colony stimulating factors (CSF) and chemokines [15]. Among them, some of the major cytokines isolated in fish are IL-1β, IL-8, TNF-α, and CC and CXC chemokines, interacting with cells and receptors to generate mucosal antibody and cell-mediated immune responses or involved in phlogistic and homeostatic processes [15].

Most of fish cytokines are identified in biological assays on the basis of their functional similarities to mammalian cytokines [16]. Meijer et al. [17], analyzing zebrafish genomic sequence database for the presence of genes encoding interleukin receptors (IL-R), showed the presence of counterparts for the human IL-1R and IL-18R genes.

In the last years it has become increasingly accepted the assessment of cytokine gene expression as an effective parameters of the immune response evaluation. Indeed, gene expression analysis is used to evaluate cytokine dynamics as consequence of viral [18], parasite [19,20] and bacterial [21,22] infections and of the use of probiotic [23] and dietary supplements [24,25]. According to Raida et al. [22], cytokine gene expression in blood cells of rainbow trout infected with *Yersinia ruckeri* is significantly higher in fish with high bacteremia causing death than in both non-infected control fish and infected fish that survive the infection. Moreover, cytokine gene expression in spleen and head kidney of Japanese flounder, *Paralichthys olivaceus*, is affected by experimental infection with *Nocardia seriolae*. Particularly, both TNF-α and IL-1β expressions are significantly increased during the first hours following infection, reducing their expressions one or few days after infection, depending on the different concentrations of bacterial suspensions [26].

The aim of this paper is to analyze some un-explored innate immune relevant genes during the ontogenic development of brown trout, *Salmo trutta*, further evaluating the tissue-specific expression in adult specimens. This will improve knowledge of the maturation of innate immune system during development of one of the most important species employed in restocking programs of European inland waters, although it is well known that European wild populations of *S. trutta* differ among them in phenotypic and genetic aspects depending on their geographical distribution [27,28]. Thus, in spite of the broad range of different geno- and phenotypic forms its ontogeny was thoroughly described by Killeen et al. [29] who indicated 40 successive steps from fertilization to the end of yolk resorption, thinking out a quantitative score system on the basis of a wide range of developmental features. According to the authors, different environmental temperatures affect the developmental score in a linear way, indicating that the relative durations of subsequent intervals of development are retained.

2. Materials and methods

2.1. Fish and experimental conditions

For the ontogenetic study, adult brown trout, weighing between 400 and 500 g, were collected from a fish farm of a private company in the province of Potenza, Italy. Three pairs of sexually mature female and male were anesthetized with phenoxyethanol (300 μL L^{-1}, Sigma) and stripped individually for egg and sperm collection. After fertilization, embryos of each brood were kept separately at 12 °C (±1 °C) until the end of the experiment that took about 35 days for larval hatching. Unfertilized eggs (UFEs), fertilized eggs (FEs) and hatchlings (Hs) were collected separately at 0 and 1 h, and 1, 2, 3, 4, 5, 6 and 7 weeks post-fertilization. Samples collected at 5, 6 and 7 weeks post-fertilization correspond to Hs at 1, 7 and 14 days from hatching, respectively. Sampling was stopped before starting of exogenous feeding.

For tissue-specific expression study of innate immune genes, adult healthy brown trout, weighting 150–200 g, were taken from a held population with no previous history of detectable infection and deeply euthanized for tissue collection. Tissues from different organs such as muscle, ovary, heart, brain, gill, testis, liver, intestine, spleen, skin, posterior kidney, anterior kidney and blood were aseptically collected.

All samples (UFEs, Fes, Hs and tissues) were collected in TRI reagent (100 mg tissue mL^{-1}, Sigma) and kept at −80 °C until RNA extraction.

2.2. Total RNA isolation and reverse transcription

Total RNA was treated with DNase (Ambion) to minimize the genomic DNA contamination and the RNA concentration was determined by measuring the OD at 260 and 280 nm using NanoDrop (ND-1000, Celbio). The extracted RNA integrity was verified by agarose gel electrophoresis and visualization of the 28 S and 18 S ribosomal RNA. Total RNA (1.0 μg) was used for first-strand cDNA synthesis using thermocycler (MJ mini cycler, BioRad). RNA was incubated with 1 μL of 50 ng μL^{-1} random hexamers, 1.0 μL of 10 mM dNTPs and DEPC water at 65 °C for5 min followed on ice for at least 1 min and subsequently added to the mixture containing 4 μL of 5X buffer, 1 μL of 100 mM DTT, 1 μL of SuperScript III RT (200 U μL^{-1}, Invitrogen) and DEPC water. The reverse-transcription reaction was conducted at 25 °C for 5 min, 50 °C for 50 min followed by termination at 75 °C for 15 min with a temporary holding at 4 °C. RT-reactions lacking reverse transcriptase (RT minus) were also performed to verify that the samples did not contain genomic DNA. The PCR reactions used to amplify immune relevant genes (TNF-α, IL-1β, TGF-β and lysozyme c-type) and β-actin (as housekeeping gene to check integrity of RNA) were performed using primer pairs designed by AlleleID 5.0 software, as reported in Table 1. All amplification reactions consisted of an initial denaturation at 95 °C for 10 min prior to 42 cycles of 95 °C denaturation for 30 s, 54 °C annealing for 45 s and 72 °C extension for 45 s, followed by a final 72 °C extension for 10 min using 0.5 units of AmpliTaqGold DNA polymerase (Applied Biosystems). The generated PCR products (10 μL) were then electrophoresed on 1.8% agarose gel and the band of interest were sequenced. The sequence obtained was subjected to homology search using BLASTn tool (http://www.ncbi.nlm.nih.gov/).

Table 1
Primer sequences used in PCR analysis and length of the amplicons.

NCBI RefSeq	Gene	Sequences (5′–3′)	Length (bp)
NM_001124235	β-actin	GGTATGGAGTCTTGCGGTATC CTGTTGGAAGGTGGAGAGAG	261
AJ278242	IL-1β	CAGGAGGGCAGCAGGGTTCAG GGAGCAGGACAGGTAGAGGTTGG	244
NM_001124357	TNF-α	CCACCATACATTGAAGCAGATTG CCTGGCTGTAAACGAAGAAGAG	197
X99303	TGF-β	TCGCTGGGTGTCCTTTGATG AGTAGTGGTTTGTCGTTTCTTCC	268
X59491	Lysozyme c-type	TTGTTCTCCTGCTTGTGGCTGTG CGTCGGTGTTGCGGTTGGTG	177