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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)Stefano Cecchini^{a,*}, Mariateresa Paciolla^{a,b}, Elio Biffali^c, Marco Borra^c, Matilde V. Ursini^b, Maria B. Lioi^a^a Department of Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, Potenza 85100, Italy^b Institute of Genetics and Biophysics "Adriano Buzzati-Traverso", CNR, Naples 80131, Italy^c Molecular Biology Service, Stazione Zoologica Anton Dohrn, Naples 80121, Italy

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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-1 β , TGF- β and lysozyme c-type). The constitutive expression of β -actin was detected in all developmental stages. IL-1 β 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-1 β 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.

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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-1 β , 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-1 β 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 $\mu\text{L L}^{-1}$, 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 $\text{ng } \mu\text{L}^{-1}$ random hexamers, 1.0 μL of 10 mM dNTPs and DEPC water at 65 °C for 5 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-1 β , 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	GGTATGGAGTCTTGGCGTATC CTGTTGGAAGGTGGAGAGAG	261
AJ278242	IL-1 β	CAGGAGGGCAGCAGGGTTCAG GGAGCAGGACAGGTAGAGGTTGG	244
NM_001124357	TNF- α	CCACCATACATTGAAGCAGATTG CTGGCTGTAACGAAGAAGAG	197
X99303	TGF- β	TCGCTGGGTGTCCTTTGATG AGTAGTGGTTTGTCTTTCTCC	268
X59491	Lysozyme c-type	TTGTTCTCTGCTGTGGCTGTG CGTCGGTGTTCGGTGTGTTG	177

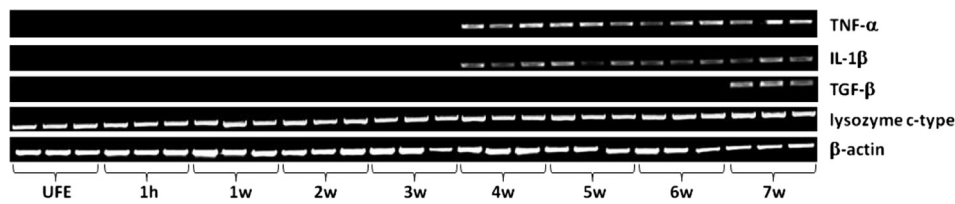


Fig. 1. Ontogenetic appearance of mRNA transcripts of innate immune relevant genes. The corresponding β -actin bands show the quality of mRNA used. (UFE, unfertilized eggs; 1 h and 1 w–7 w, hours and weeks post-fertilization). The samples were run in triplicate.

3. Results and discussion

We assessed the RNA quality and DNA and protein contamination by calculating the $OD_{260\text{ nm}}/OD_{280\text{ nm}}$. Ratios for all types of samples examined ranged from 1.83 to 2.26. The quality of RNA was inspected by agarose gel electrophoresis and all samples showed bands typical of non-degraded RNA (not shown). Furthermore, we checked the integrity of the RNA samples by RT-PCR using β -actin primer pairs and strong amplification products for β -actin gene (261 bp) were found for all samples (Figs. 1 and 2).

To provide clues on the involvement of selected genes during development, we examined their temporal expression in eggs, hatched embryos and larvae of brown trout. TNF- α and IL-1 β transcripts were detected by RT-PCR from 4 week post-fertilization onwards. However, these transcripts were not detected in unfertilized eggs and up to 3 weeks post-fertilization. TGF- β transcript was observed during late stage of trout development: from 7 week post-fertilization onwards. The lysozyme c-type transcript was detected early from unfertilized egg stage onwards (Fig. 1).

In the tissue-specific expression study, constitutive expressions of β -actin, lysozyme c-type and TNF- α transcripts were detected in

all the examined tissues. IL-1 β and TGF- β transcripts were expressed in all tissues except the brain and liver (Fig. 2). Moreover, BLAST analysis revealed a percent similarity ranging from 63 to 90%, at nucleotide sequences level, with *O. mykiss* specie used for gene sequences benchmark for primers design (Fig. 3).

The innate immune system is the fundamental defense weapon of fish at early stage of life and it is the only defense mechanism until the complete development of the acquired immune system [30]. Information on the ontogeny of the fish immune system is largely restricted to a few fish species, making difficult a comparison between different species. Moreover, until some years ago, studies focusing on the ontogenesis of the innate immune system were mainly limited to the first appearance of cells involved in phagocytosis processes or in the appearance and activity of enzymes involved in the innate immunity response [30].

In recent years, with the application of genetic and proteomic methods more knowledge was obtained and major progress can be expected in this field in the future. Thus, in this study, we investigated the spatial-temporal appearance of some innate immune relevant genes (TNF- α , IL-1 β , TGF- β , lysozyme c-type) during the ontogenetic development of brown trout and their tissue-specific

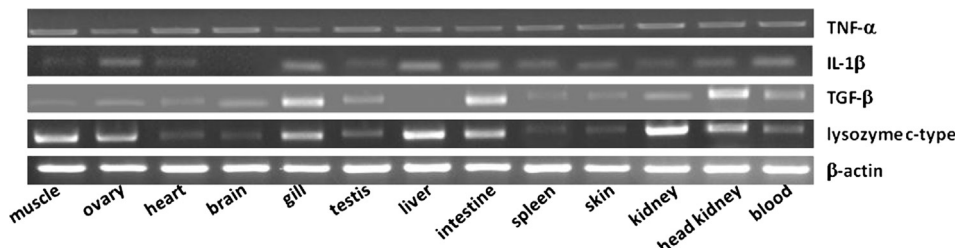


Fig. 2. Expression of different immune relevant genes and corresponding β -actin amplification mRNA in brown trout (*Salmo trutta*) tissues.

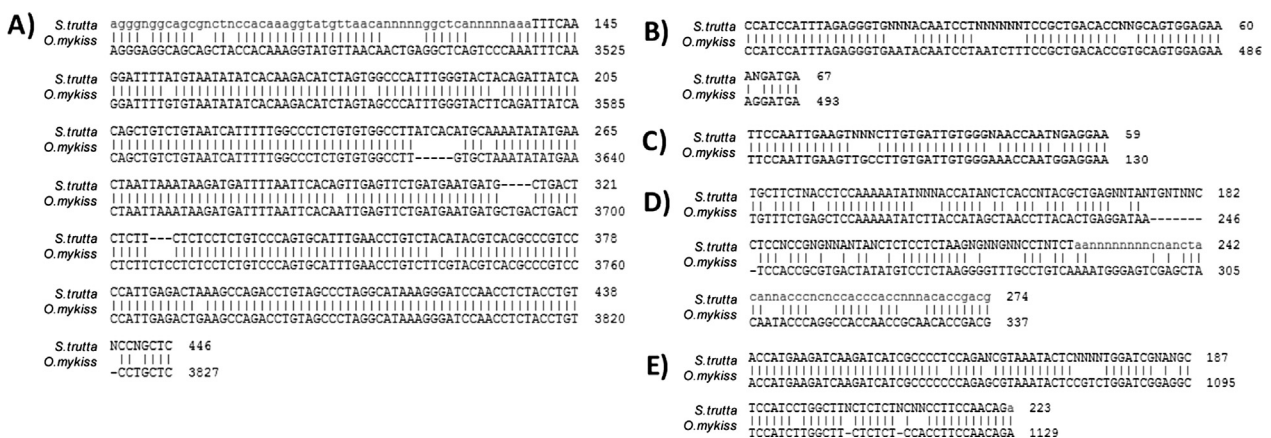


Fig. 3. Nucleotide sequence alignments of the A) IL-1 β , B) TNF- α , C) TGF- β , D) lysozyme c-type, E) β -actin between *S. trutta* and *O. mykiss* species. The comparison of DNA sequences between two species shows the 90% of similarity for IL-1 β , 82% for TNF- α , 89% for TGF- β , 63% for lysozyme c-type, and 86% for β -actin.

expression in adult specimens. The strong amplification products for β -actin found in all samples demonstrated the sample RNA integrity, confirming the use of this gene as an effective house-keeping gene control as shown for other fish species [14,31,32]. As regards the ontogeny of brown trout, our results showed that lysozyme c-type is already expressed from UFEs onwards, which might indicate the maternal deposit. On the contrary, TNF- α and IL-1 β expressions appear in Hs from 4 weeks post-fertilization onwards, about 7 days before hatching, while TGF- β expression appears more belatedly, about 14 days after hatching, indicating the achievement of functional competency by brown trout when fish has to defend from attack by a broad spectrum of invading organisms. According to the developmental stages of brown trout described by Killeen et al. [29], TNF- α /IL-1 β and TGF- β expressions appear when the yolk sac vascularization phase is completed and during the fin fold resorption phase, respectively.

Regarding lysozyme gene expression during fish ontogenesis, Nayak et al. [32] showed that the g-type is already expressed in UFEs of rohu, *Labeo rohita*, whereas the c-type gene expression is delayed to 24 h post-fertilization, similarly to that observed in zebrafish [14]. At the same time, IL-1 β and TNF- α , but not TGF- β , genes are already expressed in embryos of common carp, *Cyprinus carpio*, at 24 h post-fertilization, showing an increase of expression after lipopolysaccharide injection implying a functional embryonic innate defense system [33]. In rainbow trout at the yolk-sac larval stage the expression of only few genes, and among them IL-22, are up-regulated by *Y. ruckeri* infection without mortality, whereas several cytokines (IL-1 β , TNF- α , IL-22, IL-8 and IL-10) are up-regulated by the infection in 3 month-old fry [34]. According to the authors [34], the limited capacity of larval immune system to enhance a response by gene regulation at the transcriptional level seems suggest that at the yolk-sac stage larvae might be covered by protective shield of different immune factors which protect against a broad range of pathogens.

In our tissue-specific expression study, constitutive expressions of lysozyme c-type and TNF- α transcripts were detected in all the examined tissues, whereas IL-1 β and TGF- β transcripts were expressed in all tissues except for brain and liver, respectively. On the contrary, according to literature [35,36] lysozyme c-type is only expressed in the liver and kidney in rainbow trout and in liver and stomach in brill, *Scophthalmus rhombus*, whereas in Japanese flounder and in rohu, it is expressed in head kidney, posterior kidney, spleen, brain and ovary [37] and in posterior kidney and blood [32], respectively. Moreover, for what concerns the g-type, it seems expressed in all examined tissues in rohu and brill [32,36]. Thus, the meaning of the different tissue expression of lysozyme g- and c-type in the studied species remains to be clarified, even if the c-type might be the main molecule for the defense under normal conditions, whereas the g-type lysozyme might be induced for the defense against bacterial infections [13]. This assumption seems to be also supported by Jiménez-Cantizano et al. [36], who observed an increase of g-type transcripts in brill head kidney after lipopolysaccharide and *Photobacterium damsela* subsp. *piscicida* treatments.

Cytokines have a fundamental role in the regulation of fish immune response by binding to specific receptors at the cell membrane. This elicits a cascade of events leading to induction, enhancement or inhibition of several cytokine-regulated genes. Our results, showing cytokine gene-expression in early developmental stages of brown trout, let us suspect a potential involvement of TNF- α , IL-1 β and TGF- β in defense mechanisms against pathogens. Indeed, several papers pointed out the cytokine tissue gene expression as consequence of pathogen infection [20,22,26,38]. Moreover, not only pathogens are able to induce cytokine gene expression improvement. In fact, bath-treated rainbow trout fry

with three different immunostimulants (plasmid DNA, lactoferrin and β -glucan) possessed higher gene expression with regard to the pro-inflammatory cytokines IL-1 β , TNF- α , IL-6 and the anti-inflammatory cytokines IL-10 and TGF- β than control fish [39]. At the same time, rainbow trout fed diet supplemented by *Lactobacillus plantarum*, a probiotic lactic acid bacterium, show an increase of cytokine gene expression in head kidney, improving the survival against *Lactococcus garvieae* infection [40].

In conclusion, our result show that, whereas lysozyme c-type is already expressed in UFEs, allowing us to suspect its major role during the early developmental stage, and TGF- β gene expression is delayed after hatching, TNF- α and IL-1 β gene expressions appear about 7 days before hatching, probably indicating a preparation of the brown trout immune system for the post-hatching period. Even though this paper is to consider a preliminary study concerning the innate immune system development of brown trout, the generated information regarding the spatial-temporal expression of some innate immune-related genes may contribute to the basic knowledge to better understand the functional embryonic and larval innate immune system of brown trout.

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