Fish & Shellfish Immunology 38 (2014) 25–33

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Adaptive and innate immune molecules in developing rainbow trout, *Oncorhynchus mykiss* eggs and larvae: Expression of genes and occurrence of effector molecules

Rasmus D. Heinecke*, Jiwan K. Chettri, Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form
12 February 2014
Accepted 14 February 2014
Available online 21 February 2014

Keywords:
Ontogeny
Immune system
Rainbow trout
Egg
Gene expression

ABSTRACT

The ontogenetic development of the immune system was studied during the egg phase and the early post-hatch period of rainbow trout. Quantitative real-time PCR (qPCR) was used to assess the timing and degree of expression of 9 important immune relevant genes and EF1-α. Further, immunohistochemical staining using monoclonal antibodies was applied on rainbow trout embryos and larvae in order to localize five different protein molecules (MHCII, CD8, IgM, IgT and SAA) in the developing tissue and immune organs. Maternally transferred transcripts of EF1-α mRNA were detected in the unfertilized egg. Early onset of expression was seen for all immune genes at very low levels. The amount of mRNA slowly increased and peaked around and after hatching. The highest increases were seen for MHCII, C3, C5 and SAA. Immunohistochemistry using five monoclonal antibodies showed positive staining from day 84 post fertilization. Skin, gills, intestine, pseudobranch and thymus showed reactivity for MHCII, thymus for CD8, gill mucus for IgT and pseudobranch and cartilage associated tissue for SAA. The importance of detected factors for early protection of eggs and larvae is discussed.

1. Introduction

Rainbow trout is an important species in the fish farming industry and disease is the most significant limiting factor in the production of this fish species. In order to manage or prevent disease and lower the impact on aquaculture production it is important to increase our understanding of the ontogenetic development of the fish immune system. Rainbow trout is a cold water fish species that produces large eggs and has a long developmental phase from fertilization to hatching.

In salmonids, the development of the thymus and posterior and anterior kidney begins during the early embryonic phase before the larva hatches. The thymus is the first lymphoid organ to appear during the ontogenetic development and it appears as an enlargement of the gill chamber epithelium above the first three gill arches [1,2]. In rainbow trout a thymic primordium can be seen before hatching and at hatching lymphocytes can be observed in the developing thymus [1,3] which is covered by a connective tissue capsule that is impenetrable to waterborne antigens [4,5]. At an early stage when only 15-25 pairs of somites have developed the primitive kidney tubules are formed [6] but large undifferentiated hematopoietic cells are not seen in the kidney until just prior to hatching and only after hatching small and large lymphocytes can be seen in the kidney [1,3].

Maternal transfer of innate immune molecules has been demonstrated to occur in several fish species. In brown trout *Salmo trutta*, mRNA transcripts of lysozyme was detected in the unfertilized egg [7] and in rainbow trout, coho salmon *Oncorhynchus kisutch* and Chinook salmon *Oncorhynchus tshawytscha*, lysozyme activity has been demonstrated [8,9] and it was suggested that lysozyme is an early innate defense molecule protecting the oocyte and embryo against vertically transmitted bacterial diseases. Maternal transfer of IgM and several complement components have been observed in rainbow trout [10] and it was speculated that complement and IgM could act in concert to protect the developing embryo [11]. Interestingly, it was recently shown that phosvitin, a nutritional reserve protein for the developing embryo and a major constituent of the

* Corresponding author. Tel.: +45 35333136; fax: +45 35332742.
E-mail addresses: rdh@sund.ku.dk (R.D. Heinecke), jkc@sund.ku.dk (J.K. Chettri), kub@sund.ku.dk (K. Buchmann).

http://dx.doi.org/10.1016/j.fsi.2014.02.010
1050-4648/

maternally transferred yolk substance, acts as an antimicrobial protein by inhibiting the growth of different species of pathogenic bacteria [12]. In the same study it was also demonstrated that phosvitin binds directly to bacteria and is a multivalent recognition molecule binding to lipopolysaccharide, lipoteichoic acid and peptidoglycan and thus has a dual function as a nutritional protein and as a potent innate defense molecule.

Cells positive for membrane IgM (mIgM) appear in the rainbow trout kidney 4–5 days after hatching [3] but another study on single-cell suspensions demonstrated cells positive for surface IgM 8 days before hatching [10]. Ontogenetic studies on the expression of different immune molecules in salmonids have revealed that transcription is initiated quite early after fertilization. In salmon, the expression of complement factor C3 started at day 14 post fertilization (p.f.) [13] and in rainbow trout several components of the complement system were found as early as 7 days post fertilization when the early embryo develops inside the egg [11].

We have investigated the ontogenetic development of the rainbow trout immune system at the very early embryonic stages starting from fertilization to after hatching. This was done by measuring the relative expression of a set of important innate and adaptive immune genes and demonstrating immune related proteins by immunohistochemistry during the egg phase and the early post-hatch period.

2. Materials and methods

2.1. Rearing and sampling of eggs and larvae

Unfertilized and fertilized rainbow trout eggs were collected at Fousing fish farm (Jutland) and subsequently transported on ice to the fish facilities at the Laboratory of Aquatic Pathobiology. The unfertilized eggs were snap frozen in liquid nitrogen and stored at −80 °C until use. The fertilized eggs were slowly acclimatized to 8 °C and gently transferred to the egg incubator. The incubator was temperature controlled to 8 °C ($\bar{x} = 8.2 \pm 1.0$) and the water was continuously aerated. For quantitative real-time PCR (qPCR), whole eggs and larvae were individually sampled at alternating even days from before fertilization at day 0 to hatching at day 46, corresponding to 382° days (d.d.) and after hatching to day 84. The number of time point samples obtained and processed was three (from day 2–38) and five (at day 0 and from day 40–84). For immunohistochemical staining, samples of whole eggs and larvae were taken at four sampling points (at day 26, 38, 40 and 84), corresponding to 215, 316, 333 and 695 d.d.. One sample was processed at each sampling point.

2.2. Extraction of total RNA and cDNA production

From each sample consisting of one whole egg or larva, total RNA was extracted and purified using the phenol-chloroform method [14]. Tri Reagent (Sigma-Aldrich, Cat. No. T9424, Denmark) was used according to the protocol of the manufacturer. Briefly, each egg or larva was homogenized in a FastPrep®-24 instrument (MP Biomedicals) in a 1.5 ml microcentrifuge tube containing 1 ml of Tri Reagent and approximately 60 mg of 425–600 μm glass beads (Sigma-Aldrich, Cat. No. G8772, Denmark). After homogenization, 200 μl of chloroform was added to the homogenate, mixed and incubated for 3 min. The samples were centrifuged at 4 °C for 15 min at 12,000 *g*. The upper aqueous phase (~600 μl) containing the RNA was pipetted off and mixed with 500 μl 2-propanol to precipitate the RNA. To pellet the precipitated RNA the sample was centrifuged at 4 °C for 10 min at 12,000 *g*. The supernatant was poured off and the pellet was washed three times in 75% EtOH and re-dissolved in 20–50 μl RNase/DNase (Invitrogen Cat. No 10977, Denmark) free water. To remove any contaminating gDNA, the purified RNA was treated with DNase I (DNase I, Fermentas Cat. No EN0521, Denmark). Using a NanoDrop spectrophotometer (Saveen and Werner Aps, Denmark) the purity and concentration of the RNA was measured. The integrity of the RNA was assessed by running a subsample of 2 μl of each sample in a 1.5% agarose gel and the resulting bands were inspected visually. The samples of purified intact RNA from single whole eggs or larvae were kept at −80 °C until use. The TaqMan® Reverse Transcription kit (Applied Biosystems Cat. No. N8080234, Denmark) with random hexamer primers was used for reverse transcription (RT) and first strand cDNA generation. The cDNA was generated in 20 μl reactions using 800 ng RNA/reaction. The resulting solution of 20 μl cDNA was diluted 1:10 by addition of 180 μl RNase/DNase free water. The RT-PCR reactions were run on a T3 Thermocycler (Biometra, Germany) under the following conditions: 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min. Controls containing no reverse transcriptase enzyme were made for all samples under the same conditions.

2.3. Real-time quantitative PCR

The qPCR reactions were set up using 6.25 μl Brilliant® II master mix (Agilent Technologies, cat. No. 600804), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 0.5 μl fluorescent probe (5 μM), 2.25 μl RNase/DNase free water and 2.5 μl cDNA template. The 12.5 μl qPCR reactions were run in 96 well plates on a Stratagene Mx3005P real-time thermal cycler (AH Diagnostics, Denmark). The reactions were initiated by hot start at 95 °C for 15 min followed by 45 cycles of 95 °C for 30 s and 60 °C for 30 s. To check for gDNA contamination all the samples were run as no reverse transcriptase (no RT) controls using IgM primers and probes. Also, 10 samples taken from each of the early, middle and late developmental phases were pooled into three new no RT control samples which were run as no RT controls in each gene assay as well as three no template controls. The fluorescent probes (TAG Copenhagen, Denmark) were dual labeled with BHQ-1 and FAM. The genes studied by qPCR were IgM, IgT, MHCII, TCR β, CD8, CD4, C3, C5 and Serum Amyloid A (SAA). Also, expression analysis was performed on EF1-α to evaluate EF1-α as an internal reference gene. Details of primers and probes are shown in Table 1.

2.4. Immunohistochemistry

The embryos and larvae sampled for immunohistochemistry were fixed in 4% formaldehyde in PBS for 24 h at 4 °C and subsequently transferred to 70% EtOH until embedding in paraffin. Before embedding the samples in paraffin they were first dehydrated in increasing grades of EtOH and cleared in xylene. Tissue sections of 4 μm were cut on a microtome (Leica RM2135, Leica Microsystems, Germany) and placed on adhesive glass slides (SuperFrost® Plus Menzel-Gläser, Germany) and dried for 24 h at 40 °C. Prior to staining, the slides were deparaffinized in xylene and rehydrated in decreasing grades of EtOH (99.9–70%). The tissue sections were incubated for 10 min in 1.5% H_2O_2 in Tris-buffered saline (TBS) to inhibit endogenous peroxidase. Heat induced epitope retrieval was performed by boiling slides for 15 min in a Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0) in a microwave oven. Slides were cooled for 15 min and transferred to TBS at room temperature (RT). The antibody staining was done using a Shandon rack set up with coverplates (Axelab, Denmark). The tissue was blocked for unspecific binding of antibodies by incubating 10 min in 2% bovine serum albumin (BSA) in TBS. The tissue was subsequently covered with primary antibody diluted in 1% BSA in TBS and incubated at 4 °C overnight. Details of the antibodies used can be viewed in Table 2. Next, the slides were incubated 5 min in TBS at RT and HiDef