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# Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod

Marit Seppola *, Hanne Johnsen, Saskia Mennen, Bjørnar Myrnes, Helge Tveiten

*Nofima Marin, Box 6122, NO-9291 Tromsø, Norway*

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ABSTRACT

The immune system in teleosts is not completely developed during embryonic and larval stages and immune competence is assumed to be restricted. This study is the first to address whether immune transcripts are maternally transferred to offspring and when immune genes are transcriptionally active in Atlantic cod (*Gadus morhua*). In unfertilised eggs, transcripts encoding lysozyme and cathelicidin were found indicating maternal transfer of antibacterial transcripts. Lysozyme activity was also present at this stage suggesting the presence of a functional protein. Transcripts of two other putative antibacterial genes (hepcidin and pentraxin) and antiviral genes (ISG15 and LGP2) were absent in unfertilised eggs. The transcriptional onset of these genes occurred during the gastrula period. Transcripts of the heavy chain constant regions of the immunoglobulin (Ig) D, membrane-associated and secreted form of IgM were absent in unfertilised eggs. Transcription of the heavy chain locus commenced at low levels during the segmentation period indicating the onset of B-cell development. Most innate immune genes showed an increase in transcription around hatch and first feeding, indicating a preparation for increased pathogen exposure at this time. Prior to and during metamorphosis all genes showed a pronounced elevation in transcript levels indicating a further maturation of the immune system during this period.


## 1. Introduction

Atlantic cod (*Gadus morhua*) has received increased focus as a candidate species for aquaculture in recent years. Mass production of eggs and larvae is now routinely carried out, but high and unpredictable mortality during early stages of development often occurs. The high mortality rate in early life may be caused by either poor egg quality or infections with viral or bacterial pathogens. In the fish farming industry, eggs of good quality have been defined as those that exhibit low mortality at fertilisation, eyeing, hatching and first feeding [1]. Embryonic survival and hatching rates, however, provide no information about the regulatory factors and mechanisms that determine egg quality. Poor egg quality can derive from parental genetics, inadequate broodstock nutrition, stress, poor water quality or overripening [2,3], but the physiological and molecular basis for quality still remain obscure [4]. One aspect of egg and larval quality that has been largely overlooked is the possible influence provided by differences in immunological capacity. It is conceivable that protection from pathogens by maternally transferred immune components, or immune competence acquired during early development may provide an important contribution to survival under culture conditions. It is therefore important to establish if and when factors of the immune system are expressed during early ontogeny in Atlantic cod.

There is a substantial period of time in embryonic development prior to the activation of the zygotes own genome. During this period maternal factors are essential and are supplied to the egg during oogenesis. These factors are stored in the form of mRNA, protein or any other biomolecule [5] and are important for the embryo until its own transcriptional machinery is functional. Studies of several fish species have shown that maternal immunoglobulins (Ig) are transferred from mother to progeny [6–11]. These antibodies retain the ability to bind antigen and confer higher survival to the larval offspring [12,13]. Although maternal Ig transfer is not yet established in Atlantic cod [14], maternal deposition of other immune factors may be important. For example, few studies have examined the extent of maternal transfer of innate immune components.

The initiation of the zygotic gene program, or embryonic genome activation (EGA), normally occurs during the so-called mid blastula transition (MBT). In zebrafish (*Danio rerio*) embryo's MBT occurs gradually, starting at cell cycle 9–10 (≈512–1000 cells) and ending at late cycle 13 [15]. Atlantic cod is assumed to follow a

*Abbreviations:* hpf, hours post-fertilisation; wpf, weeks post-fertilisation; wph, weeks post-hatching.

* Corresponding author. Tel.: +47 77 62 90 00; fax: +47 77 62 91 00.
*E-mail addresses:* marit.seppola@nofima.no (M. Seppola), hanne.johnsen@nofima.no (H. Johnsen), saskia.mennen@nofima.no (S. Mennen), bjornar.myrnes@nofima.no (B. Myrnes), helge.tveiten@nofima.no (H. Tveiten).

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similar developmental pattern and the 512–1000 cell stage is reached after 36 hours post-fertilisation (hpf) at 7 °C [16]. However, to our knowledge no detailed studies of either EGA or the onset of embryonic immune gene expression have been carried out in Atlantic cod.

Many fish species, including Atlantic cod, hatch at a poorly developed stage and are exposed to pathogens before their lymphoid organs have matured and adaptive immunity has developed [17]. One study has shown that autologous IgM positive lymphocytes are not present until 8–10 weeks post-hatching (wph) [17] indicating that Atlantic cod may possess other immune components that are important during early development. Candidates may be innate defense components such as the antiviral interferon (IFN) response, the acute phase response, antibacterial proteins and peptides [18–23]. The type I IFN system is considered as the initial innate immune response against viral infections. Type I IFNs are still not identified in cod, but interferon stimulated genes such as ISG15 and LGP2 have been identified [24,25]. In the acute phase response a rapid accumulation of serum proteins such as the pentraxins including C-reactive protein (CRP) and serum amyloid P component (SAP) occurs [26,27]. However, some fish including Atlantic cod possess only one pentraxin gene with similarity to both CRP and SAP [28]. Antibacterial proteins and peptides such as lysozyme, hepcidin and cathelicidin are important in the innate immune defense against microbial invasion and kill bacteria by disrupting the cell membrane [29,30]. These latter genes have recently been identified in cod [31–33].

Identification of several immune genes in cod has provided the basis for study of the transcriptional onset during ontogenesis and to address whether some immune components are of maternal origin. In this study we have monitored the level of maternal transfer and the initiation of transcription of genes involved in the interferon response, acute phase response and antibacterial defense during ontogenesis in Atlantic cod. The present study demonstrates that genes assumed to have an important role in Atlantic cod immune defense are present during early developmental stages.

## 2. Materials and methods

### 2.1. Fish

Atlantic cod eggs were incubated in 32–33‰ seawater at the National Cod Breeding Centre (Kraknes, Tromsø, Norway). Cod eggs were hand stripped *in vitro* and fertilised. Then incubated in 25 L conical incubators at an average temperature of 4.3 °C until 100% hatching was achieved between 455 and 503 hpf. After hatching, larvae were kept at 5 °C for 5 days (503–623 hpf). The temperature was then increased to 10 °C during 5 days (623–911 hpf) and maintained at this temperature for the remaining experiment (911–1631 hpf). Microalgea (Nanochloropsis®, Reed Mariculture) were added to the experimental tanks for the first 10 days post-hatching (dph). Larvae were fed 10 times a day with rotifers enriched with Phosonorse® and Micronorce® from 2 dph. From 25 to 29 dph larvae were fed with a mixture of rotifers and enriched *Artemia* and from 30 to 45 dph larvae were fed with only *Artemia.* Weaning onto dry diet (Alganorse®) commenced at 46 dph. Sampling of unfertilised eggs, fertilised eggs and larvae was performed during 10 weeks. Samples were rapidly submerged in RNAlater (Ambion, Austin, TX, USA) and incubated at 4 °C overnight, then stored at −20 °C.

### 2.2. Isolation of RNA and synthesis of cDNA

5–10 eggs/embryos or 1–3 larvae were pooled and homogenised using MagNA Lyser Green Beads and the MagNa Lyser Instrument (Roche Diagnostics GmbH, Mannheim, Germany). Homogenised samples were treated with 2 mg/ml ProteinaseK (Applied Biosystems, Foster City, CA, USA) and incubated at 37 °C for 90 min. The E.Z.N.A Total RNA kit (OMEGA BIO-TEK, Doraville, GA, USA) was used for RNA isolation using the recommended on-column DNase treatment. All samples were adjusted to an RNA concentration of 1 μg in a total volume of 20 μl, followed by a second in-solution DNase treatment with 1 U RQ1 DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Reverse transcription was performed in a total reaction volume of 40 μl with 10 μl DNase treated RNA using High Capacity RNA-to-cDNA master mix (Applied Biosystems) according to the recommended protocol. The cycling parameters were as following: 25 °C for 5 min, 42 °C for 60 min and 85 °C for 5 min. The cDNA was diluted three times in water for further use in quantitative real time PCR. The sample homogenisations, isolation of RNA and cDNA synthesis were performed in triplicates.

### 2.3. Isolation of RNA and synthesis of cDNA from ovarian fluid and unfertilised eggs

Ovarian fluid and unfertilised eggs were sampled from Atlantic cod immediately following hand stripping. Unfertilised eggs and ovarian fluid were separated by density differences. Unfertilised eggs were rapidly washed three times in 1× phosphate buffered saline (PBS). PBS washed eggs were treated with 100 μg/ml RNase A (Qiagen, Hilden, Germany) in 10 ml 1× PBS for 10 min at room temperature. RNA from ovarian fluid, PBS washed eggs and RNase treated eggs were isolated using the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) with the recommended on-column DNase treatment. cDNA was prepared as described previously. The absence of genomic DNA in the RNA (non-template control, NTC) was verified by all of samples being subjected to reverse transcription with the High Capacity RNA-to-cDNA kit (Applied Biosystems) without Multiscribe reverse transcriptase present.

### 2.4. Real time PCR

Real time PCR was performed in duplicates using the 7900HT Fast Real-Time PCR System and Power SYBR Green PCR Master Mix as recommended by the manufacturer (Applied Biosystems). Real time primers for target genes (lysozyme, cathelicidin, pentraxin, hepcidin, ISG15, LGP2, sIgM, mIgM and IgD) and endogenous control (18S rRNA) are listed in Table 1. NTC was performed for a selection of samples to verify the absence of genomic DNA as described above. All data were analysed with the SDS 2.3 software (Applied Biosystems) and exported to Microsoft Excel for further analysis.

The efficiency of the PCR reactions was close to 100%, determined by analysis of two-fold dilutions of cDNA, allowing the use of $2^{-\Delta\Delta CT}$ method for calculation of relative transcriptional levels [34]. The sample with the lowest transcriptional level served as calibrator and the transcriptional level was set to value 1. From relative quantification values obtained from three pooled samples the mean quantity ± standard error (SEM) was calculated.

### 2.5. Primer design

Primers for the genes ISG15, LGP2 and hepcidin have previously been reported [24,25,32]. The cDNA sequences for g-type lysozyme and cathelicidin have also been reported [31,33], but the real time primers were designed in this study. The pentraxin gene was identified in a subtractive cDNA library [24] and was submitted to the GenBank (accession no. FJ940746).

Transcripts encoding the membrane form of cod Igμ heavy chain (Cμ1-Cμ2-Cμ3-μTM1-μTM2) are generated by splicing the