The ontogenic development of innate immune parameters of cod (Gadus morhua L.)

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Abstract

The aim of this study was to monitor the ontogenic development of innate immune parameters of cod (Gadus morhua L.) and to determine the presence of maternal IgM. The general protein composition and enzyme activity was also studied. At intervals, samples were collected of fertilized cod eggs and larvae from 3 days after fertilization until 57 days after hatching. Cell lysates were prepared and analysed by Western blotting using antibodies prepared against cod IgM, the complement component C3 and C-reactive protein (CRP) as well as against cod serum proteins and haemoglobin. Antibodies against salmon cathepsins and against several mammalian proteins of immunological significance were also used. Maternal IgM was not detected but C3 and the closely associated apolipoprotein A-I were present from the time of embryo organogenesis. C-reactive protein was not detected and none of the antibodies against mammalian immune parameters cross-reacted with the cod material. Protein and proteomic analysis showed that the major proteins of the egg samples were vitellogenin derived maternal proteins. Other non-vitellogenin maternal proteins, not yet identified, were also detected in the fertilized eggs. Cathepsin was present in all samples, but other enzyme activity was restricted to larval samples from 4 days after hatching when feeding had commenced. Haemoglobin was not detected until 10 days after hatching.

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1. Introduction

Experimental farming of Atlantic cod (Gadus morhua L.) is being carried out in several countries including Iceland and cod farming may soon become a viable industry (Knappskog et al., 1993; Steinarsson and Björnsson, 1999; Björnsson et al., 2001).

The morphological development of embryonic and larval cod is well documented (Frideirsson, 1978; Kj—rsvik et al., 1991; Pedersen and Falk-Pedersen, 1992; Hall et al., 2004). During the 12–14-day period from fertilization till hatching, the eggs go through well defined developmental stages of cell cleavage (0–2 days post fertilization (dpf)), gastrulation (2–3 dpf) and organogenesis (4–10 dpf) (Frideirsson, 1978; Hall et al., 2004). At hatching (10–12 dpf), the yolk sac is prominent and lasts until 3–6 days post hatching (dph). At the same time, the mouth opens and the larvae starts active feeding. During the next 30–40 days, the gradual metamorphosis of cod larvae is completed, basically involving the compartmentation of the alimentary tract and the development of the fins and the jaw structure (Frideirsson, 1978; Pedersen and Falk-Pedersen, 1992; Hall et al., 2004).

During the first 4–8 weeks of cod larval development, major losses (85–95%) are observed in aquaculture. Bearing in mind the massive losses in nature this may be partly an innate, natural phenomenon. However, in aquaculture, these losses are commonly attributed to feeding problems and to infections due to opportunistic or pathogenic microorganisms in association with stress inducing factors like density and handling (Knappskog et al., 1993; Bergh et al., 2001).

Studies of the ontogenic development and activity of non-specific and specific immune parameters are highly
relevant in this context both to evaluate the ability of cod larvae to combat diseases and to examine the effects of possible prophylactic measures. Schröder et al. (1998), studying the ontogeny of the lymphoid organs in cod, showed that immunoglobulin-producing cells were not present until the cod had reached about 33 mm in length, 58 days after hatching at the rearing temperatures of 11.5–17°C. Whether this coincides with the ability to instigate humoral antibody response to an antigenic stimuli is still not known, but it is clear that during the first 2–3 months, the cod is dependent on innate immune parameters for the defence against pathogens or opportunistic agents.

Apart from the innate cellular components like phagocytes and cytotoxic cells, the innate immune system also includes several important humoral components like the complement proteins, lysozyme, lectins, pentraxins, cathepsins, growth inhibitors like transferrin, protease inhibitors, anti-bacterial peptides and anti-viral agents like interferon and Mx proteins (Alexander and Ingram, 1992; Ellis, 2001). The appearance of these components during the ontogeny of fish has not been greatly studied. Recently, Lange et al. (2004a), monitoring the ontogenic development of the complement component C3 in cod using immunohistology, showed that this component was present in different larval tissues at hatching.

In the present study, the fate of maternal proteins and the ontogenic development of inherent proteins was monitored from 3 dpf until 57 dph. Some of these components belong to the humoral immune system, some have enzymatic activity while the function of others is still unknown.

2. Material and methods

2.1. Sampling of eggs and larvae

Samples of fertilized cod eggs and larvae were obtained during the spawning season (April–June) at the Marine Institute’s Experimental Station, Stadur, Grindavík. Details of the rearing temperature, growth rate and sampling are shown in Fig. 1. The eggs were kept at 7°C and the larvae reared at 7–9°C during the first week. The temperature was then increased by 1°C every 2 weeks until reaching 12°C. This temperature was maintained throughout the rest of the sampling period (57 dph). Other details of cod larvae rearing conditions at the Experimental Station have been published elsewhere (Steinarsson and Björnsson, 1999 (with some modifications)) but briefly rotifers (Brachionus plicatilis) were fed from day 3, brine shrimp (Artemia salina) from day 15, and commercial food pellets were gradually introduced about 30–40 dph.

Four egg samples were collected, 3, 7, 9 and 11 dpf, and 12 larval samples, 1 (i.e., during hatching), 2, 4, 10, 11, 15, 22, 29, 36, 43, 50 and 57 dph. Samples were collected in plastic containers, in a minimum of sea water, quickly frozen in liquid nitrogen and then stored at −80°C. The amount collected varied depending on size/age but was in the region of 50–150 eggs/larvae per sample.

2.2. Cell lysate preparation

The method used was based on Weissman’s protocol (Weissman, 1991) with some modifications. Everything was kept on ice using ice-cold solutions. Eggs or larvae were pressed through a stainless steel tea strainer with 1 ml lyses buffer, 50 mM Tris–HCl, pH 7.6, 0.3 M NaCl, 0.5% Triton X-100 (Sigma, USA). The suspension was frozen at −80°C, thawed and centrifuged at 15,000×g for 10 min at 4°C. The supernatant was collected, the protein content estimated using a kit from Pierce (USA) based on the Bradford method (Bradford, 1976) and adjusted to 2 mg ml⁻¹. A sample was removed for an immediate enzyme and protein analysis by SDS–PAGE. A protease inhibitor cocktail (Sigma, P8340) was added to the rest of the preparations, which were then stored at −80°C.

Fig. 1. The sampling of egg and larvae (arrows) from 3 dpf until 57 dph. The growth rate of the larvae is shown and the environmental temperature.
2.3. SDS–PAGE analysis and proteomics

SDS–PAGE analysis was carried out using the Mini-PROTEAN II system from Bio-Rad (USA) according to the manufacturer’s instructions. The method was originally described by Laemmli (1970) and has been described in detail elsewhere (Magnadóttir et al., 1995). The separation gel was 14% and the stacking gel 4.5% acrylamide. For the SDS–PAGE protein analysis and Western blotting, samples were reduced in an equal volume of 0.125 M Tris, pH 6.8, sample buffer containing 0.5% 2-mercaptoethanol and 2% SDS for 2–5 min at 100 °C and 15 μl (15 μg) loaded per well. For enzyme analysis, unreduced samples (sample buffer omitting the mercaptoethanol) were loaded onto a separation gel containing 0.05% gelatine, 0.05% casein or 0.4% collagen according to the method of Heussen and Dowdle (1980).

Following the SDS–PAGE separation, gels analysed for protein were stained with Coomassie Blue or Silver Stain Plus (Bio-Rad). For enzyme analysis, SDS was eluted from the gel with 2% Triton X-100, 0.05 M Tris, pH 8.0, followed by an incubation in 0.05 M Tris, 0.005 M CaCl2, pH 8.0, and the gel then negatively stained with Coomassie Blue. Molecular weight estimation was based on standard graphs of log molecular weight of different standard protein markers (Bio-Rad) and the Rf values, the molecular weight of the unknown protein being interpolated from this graph.

For mass spectrometry based protein identification (proteomics), samples were excised from acrylamide gel following Coomassie Blue staining. This analysis was kindly carried out by K. Bailey, School of Biomedical Sciences, Queen’s Medical Centre, Nottingham, UK.

Table 1a
Mouse antibodies against cod proteins used in Western blotting analysis of egg and larvae samples

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cod serum</td>
<td>Polyclonal, detects several serum proteins</td>
</tr>
<tr>
<td></td>
<td>(Magnadóttir, 1998).</td>
</tr>
<tr>
<td>Anti-cod IgM</td>
<td>Monospecific, polyclonal antibodies</td>
</tr>
<tr>
<td></td>
<td>(Magnadóttir, 1998) and monoclonal anti-heavy-chain antibodies, a present</td>
</tr>
<tr>
<td></td>
<td>from L. Pilström, Uppsala, Sweden.</td>
</tr>
<tr>
<td>Anti-C3</td>
<td>Polyclonal, detects the α- and β-chain of C3 but is not monospecific</td>
</tr>
<tr>
<td></td>
<td>(Lange et al., 2004a,b).</td>
</tr>
<tr>
<td>Anti-CRP</td>
<td>Monospecific, polyclonal antibody that detects C-reactive protein (CRP)</td>
</tr>
<tr>
<td></td>
<td>purified from cod serum according to the method by Lund and Olafsen, 1998</td>
</tr>
<tr>
<td></td>
<td>(unpublished data).</td>
</tr>
<tr>
<td>Anti-haemoglobin</td>
<td>Monospecific, polyclonal antibody prepared against an 18-kDa protein from red blood cell lysate identified by N-terminal amino acid sequence analysis as haemoglobin (unpublished data).</td>
</tr>
</tbody>
</table>

Most of the antibodies were prepared at the Institute for Experimental Pathology, University of Iceland.

Table 1b
Antibodies against non-cod proteins used in Western blotting analysis of egg and larvae samples

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-salmon cathepsin D, H and L</td>
<td>Polyclonal rabbit antibodies, a present from R. Dalmo, Tromsø University, Norway.</td>
</tr>
<tr>
<td>Anti-human lysozyme</td>
<td>Polyclonal rabbit antibody against human lysozyme from Sigma.</td>
</tr>
<tr>
<td>Anti-human α2-macroglobulin</td>
<td>Polyclonal rabbit antibody from Sigma.</td>
</tr>
<tr>
<td>Anti-human α-trypsin protein</td>
<td>Polyclonal rabbit antibody from Sigma.</td>
</tr>
<tr>
<td>Anti-human iNOS</td>
<td>Polyclonal rabbit antibody against inducible nitric oxide synthetase from Sigma.</td>
</tr>
<tr>
<td>Anti-bovine uNOS</td>
<td>Monoclonal antibody against universal NOS, from Affinity BioReagents, USA.</td>
</tr>
</tbody>
</table>

2.4. Western blotting

Following SDS–PAGE separation, proteins were transferred to a nitrocellulose membrane (NC, Hybond-ECL, Amersham Life Science, UK), using the semi-dry MilliBlot Graphite Electroblotter from Millipore (UK) according to the manufacturer’s instructions. Transfer was generally for 1 h at room temperature. Following the transfer, residual sites of the NC membrane were blocked with 0.1% semi skimmed milk powder in 0.1 M Tris-buffered saline, pH 7.8, containing 0.1% Tween20, at 4 °C overnight. Incubations in the primary and the conjugated secondary antibodies, diluted in the blocking solution, were for 1 h at room temperature with extensive washing between each step. The blots were developed using the ECL system from Amersham. Prestained molecular weight markers from Bio-Rad and Invitrogen (Germany) were used for estimating molecular weight as described above.

2.5. Antibodies

The specific primary antibodies used are listed in Tables 1a and b. The anti-cod serum antibody was prepared against serum pool from sexually immature cod and did not detect vitellogenin. Tested against adult cod serum this antibody detected 8–10 proteins, including IgM and apolipoprotein A-I. The secondary peroxidase conjugated anti-mouse or anti-rabbit antibodies were from Dako (Denmark).

3. Results

3.1. Growth rate

Fig. 1 shows the larval growth rate as well as the cultivation temperature and sampling frequency. The larvae were about 2–4 mm in length at the time of hatching and
had reached 20 mm at the end of the sampling period. An increase in growth rate was observed following the introduction of commercial feed (at about 40 dph) and the increase in temperature (to 12°C).

3.2. The protein composition of egg and larvae lysates

The SDS–PAGE analysis of the egg samples (3–11 dpf) and the first 6 larvae samples (1–15 dph) is shown in Fig. 2. From 11 to 15 dph onwards, no major changes were observed in the protein pattern. The first egg sample, 3 dpf, showed three distinct protein bands, approximately 84, 24 and 15 kDa in size. The 84-kDa band (I in Fig. 2) and the 15-kDa band (III in Fig. 2) were present until 4 dph, the density decreasing with time and the 84-kDa protein apparently breaking down to a 66-kDa protein and intermediaries during the egg development. The 24-kDa band (II in Fig. 2) also decreased during egg development and had disappeared at hatching. Silver staining (not shown) revealed additional two to three low molecular weight protein bands (10–14 kDa) present in all egg stages but disappearing at 4 dph. From 4 dph, several protein bands were seen in the larval lysates, the most prominent being a 45-kDa protein (IV in Fig. 2).

3.3. Western blotting analysis of egg and larvae lysates

The Western blotting analysis of the first 10–12 samples is shown in Fig. 3, using antibodies against (a) cod serum protein antibody, (b) anti-cod C3 antibody and (c) anti-salmon cathepsin H antibody. S1: Prestained standards from Bio-Rad, kDa as shown; (1) egg samples collected 3, 7, 9 and 11 dpf; (2) larvae samples collected 1, 2, 4, 10, 11 and 15 dph. I, II and III show the proteins used for proteomic analysis.
proteins and (b) cod complement component C3 and against salmon cathepsin H.

The anti-cod serum protein antibody (Fig. 3a) detected several high molecular weight proteins of diffused appearance, between 50 and 90 kDa, in all the egg samples and in the first larvae samples (1–2 dph). These proteins, as well as a 16-kDa protein, seen only at 3 dph, and an 18-kDa protein detected from 3 dph until 4 dph, were probably of maternal origin. Larval proteins detected by this antibody were a 28-kDa protein seen from 4 dph, a 15-kDa protein, which appeared just before hatching and increased in density as the larvae got older and a major 30-kDa protein, which was first detected 7 dph. Only this 30-kDa protein has been identified so far as apolipoprotein A-I by N-terminal amino acid sequence analysis and screening of a cDNA library (Magnadóttir and Lange, 2004).

The anti-C3 antibody (Fig. 4b) detected a 74-kDa C3 β-chain from 7 dph onward, increasing in density in the larval stages. A 115-kDa α-chain was occasionally seen in the later larval samples.

The anti-salmon cathepsin H antibody (Fig. 4c) detected a 42.5-kDa protein in all the samples. The reaction was faint in the early egg samples but increased after hatching at 2–4 dph. The anti-cathepsin D antibody detected a 40-kDa protein in all the samples showing similar change in relative density as the anti-cathepsin H antibody (not shown). The anti-cathepsin L antibody gave no reaction.

The anti-haemoglobin antibody detected an 18-kDa band from 10 dph (not shown). The anti-cod IgM and the anti-cod CRP antibodies gave no reaction with any of the samples and neither did the non-fish antibodies listed in Table 1b.

3.4. Substrate gel enzyme analysis of egg and larvae lysates

The results of the substrate gel enzyme analysis for gelatinase and caseinase activity are shown for the first 10 samples in Fig. 4a and b. No enzyme activity was detected in the egg lysates or in the larvae lysates at 1 and 2 dph. From 4 dph, similar enzyme activity was seen in all samples (4–57 dph). Gelatinase activity was mainly associated with approximately 40-, 24- and 20-kDa bands; caseinase activity was associated with approximately 27-, 24- and 22-kDa bands.

Table 2
Results of the proteomic analysis of three protein bands excised from SDS–PAGE gel of egg lysate from 3 dpf (see Fig. 2) and a comparison to haddock vitellogenin (Reith et al., 2001)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid sequences</th>
<th>Matching sequences (aa: amino acids)</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. 84 kDa</td>
<td>PMLVAEVR</td>
<td>No match</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LQLAPVQGLNK</td>
<td>Haddock Vitellogenin B aa 806–906: IQALPVQGLNK</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td>TPVLPLAA</td>
<td>No match</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QYEFATELLQTPQLLR</td>
<td>Haddock Vitellogenin B aa 288–304: QYEFATELLQTPQLLR</td>
<td>94.1</td>
</tr>
<tr>
<td></td>
<td>LTNEAQIPEVLNLHLVT</td>
<td>Haddock Vitellogenin B aa 305–321: LTNEAQIPEVLNLHLVT</td>
<td>76.5</td>
</tr>
<tr>
<td>II. 24 kDa</td>
<td>YLPASLLAGLVEGK</td>
<td>Haddock Vitellogenin A aa 1312–1325: YLPASLLAGLVEGK</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>AAAPPAALGEVR</td>
<td>No match</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QVSLLVATSNK</td>
<td>Haddock Vitellogenin A aa 1332–1343: QVSLLVATSNK</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>EYSMATAEGLVVK</td>
<td>Haddock Vitellogenin A aa 1271–1285: EYSMATAEGLVVK</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>LCADGILLSK</td>
<td>Haddock Vitellogenin A aa 1248–1256: LCADGILLSK</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td>PASLLAGLVEGK</td>
<td>Haddock Vitellogenin A aa 1314–1325: PASLLAGLVEGK</td>
<td>91.6</td>
</tr>
<tr>
<td>III. 15 kDa</td>
<td>VQLLINALVEENHW</td>
<td>Haddock Vitellogenin B aa 1210–1222: VQLLINALVEENHW</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>AQGGYQATAYWDPASR</td>
<td>Haddock Vitellogenin B aa 1192–1208: AQGGYQATAYWDPASR</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>NALNPVTILVR</td>
<td>Haddock Vitellogenin B aa 1173–1185: NALNPVTILVR</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>LLATGLVGLGK</td>
<td>No match</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LVETGLVGLGK</td>
<td>No match</td>
<td></td>
</tr>
</tbody>
</table>
The different developmental stages of cod eggs and larvae described by Fridgeirsson (1978) and Hall et al. (2004) were to some extent reflected in the present results. The first egg sample collected 3 dpf, during early gastrulation, showed a different protein pattern from the three egg samples collected during late organogenesis (7–11 dpf), namely the absence of complement proteins and the presence of a 16-kDa unidentified maternal protein detected by the anti-cod serum antibody. Organogenesis signified the appearance of the complement proteins, C3 and apolipoprotein A-I. Other changes, like the disappearance of the vitellogenin derived proteins and the appearance of gelatinase, caseinase and collagenase activity, coincided with the vanishing yolk sac and the start of feeding at 4 dpf.

Maternal IgM was not observed in the eggs or early larval samples using several polyclonal and monoclonal antibodies against cod IgM. Immunohistochemical studies similarly did not detect maternal IgM in comparable samples (unpublished data). The conclusion is that cod does not transfer maternal IgM to the offspring as do several other fish species (Avtalion and Mor, 1992; Takemura, 1993; Takemura and Takano, 1997). This fact, in conjunction with the late appearance of IgM producing lymphocytes (Schroder et al., 1998), emphasises the importance of innate immune defence parameters in the early stages of cod larvae.

The C3 β-chain and the closely associated apolipoprotein A-I were first detected at organogenesis and were present in all egg and larvae samples from then on. The relative instability of the C3 α-chain probably accounts for its absence in most of the samples (Lange et al., 2004b). Previous immunohistological studies by Lange et al. (2004a) demonstrated the presence of C3 in several organs like yolk sac periblast, muscle, liver, kidney and brain, at hatching. The present data therefore extends the detection of the complement system to the pre-hatching, embryonic stages of cod. C3 is the central component of the vertebrate complement system (Sunyer and Lambris, 1998). Involvement in other functions such as organ formation and regeneration has also been indicated (Mastellos and Lambris, 2002). The present findings as well as Lange et al.’s data (2004a) support the idea that C3 may play a supplementary role in the early formation and generation of cod organs. In this context, the early appearance of apolipoprotein A-I and its association with C3 is interesting. Apolipoprotein A-I may function as an inhibitor of the potentially harmful lytic pathway in early development (Magnadottir and Lange, 2004).

No CRP was detected in any of the egg or larvae samples. CRP is a member of the pentraxin family, an acute phase protein in mammals, and has been isolated from several fish species including cod (Lund and Olafsen, 1998). The present results suggest that CRP may not be an important defence agent in the early stages of cod development. This was somewhat surprising since the serum concentration of CRP is relatively high in cod fry (3–4 months past hatching) irrespective of antigenic stimuli (unpublished data).

The major maternal proteins detected in the egg samples were the two types of vitellogenin derived proteins (lipovitellins), A and B, the storage proteins of egg yolk, showing close similarity to vitellogenins of other teleosts (Greeley et al., 1986; Matsubara et al., 1999; Reith et al., 2001). The 84- and 15-kDa proteins probably represent derivatives of the heavy and light chains of vitellogenin B and the 24-kDa protein the light chain of vitellogenin A. The presence of low molecular weight phosvitin derivatives was also indicated following silver staining. The detection of both heavy- and light-chain lipovitellin and phosvitin derivatives throughout the egg development until after hatching is similar to that described in barfin flounder (Matsubara et al., 1999) but different from the situation described in haddock where lipovitellin A derivatives are completely degraded during oocyte maturation (Reith et al., 2001). These differences may reflect the absence of proteolytic enzymes other than cathepsin in the embryonic stages of cod.

The anti-cod serum antibody used in the Western blotting analysis detected some non-vitellogenin proteins in the egg samples that also appeared to be maternal like the 16-kDa protein, only seen at 3 dpf and the 18-kDa protein present until just after hatching. These proteins have not been identified yet. The anti-serum antibody also found three non-maternal proteins: The 30-kDa apolipoprotein A-I and its association with C3 is interesting. Apolipoprotein A-I may function as an inhibitor of the potentially harmful lytic pathway in early development (Magnadottir and Lange, 2004).

Since the appearance of the enzymes (gelatinases, caseinases and collagenases) coincided with the start of feeding, it seems likely that these are primarily digestive enzymes of the alimentary tract. A study of the ontogenetic development of cod larvae by enzyme histology, which has been carried out in concurrence with the present study, has
shown the presence enzymes in the gastrointestinal tract just after hatching (unpublished data). An early appearance of digestive enzymes in the larval stages has also been described in other species (Baglole et al., 1998).

Cathepsins (D and H) were detected in all samples and showed a slight increase after hatching, with the onset of feeding. Cathepsins are important lysosomal enzymes involved in the digestion of the yolk proteins in oocytes, fertilized eggs and the yolk sac (Carnevali et al., 1999, 2001). This process is the energy source of the growing embryo and an increase at hatching has been observed in other fish species (Carnevali et al., 2001). Cathepsins are also normally seen in various non-ovarian tissues of fish (Brooks et al., 1997) and can be involved in cellular degradation of proteins during, for example, post mortem, spawning or starvation (McLay, 1980; Ando et al., 1986; Yamashita and Konagaya, 1990; Martin et al., 2001; Nielsen and Hauch Nielsen, 2001) as well as having bacteriolytic and defensive roles (Aranishi, 1999; Hu and Leung, 2003). The presence of cathepsins in all the samples tested, from 3 dpf until 57 dph, is therefore to be expected and may have both a defensive and protein degrading role in the cod embryo and larva. The cathepsins detected by Western blotting did not show corresponding enzyme activity in the substrate gel. The reason could be a lack of specificity for the substrates used, low activity or inactivation of the enzymes by the treatment involved.

Haemoglobin was not seen at 4 dpf but was detected at 10 dpf. This shows that, although the heart and blood system develop during organogenesis, the red blood cells do not appear until well after the development of the vascular system and well beyond the first feeding (also observed by Fridgeirsson, 1978; Hall et al., 2004).

The present study is a part of an ongoing examination of the early development of cod with emphasis on non-specific immune parameters like the complement system, the leukocytic enzyme activity and phagocytotic activity. These parameters are also being used to evaluate the effects of immunostimulants and infection on the immune system of cod larvae. It is hoped that this knowledge will help in developing prophylactic measures before the specific immune parameters like antibody producing cells have developed.

Acknowledgements

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