The acute phase repsonse of Atlantic cod (*Gadus morhua* L.): Humoral response and changes in the gene expression of immune related genes

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Bráðasvar í þorski (*Gadus morhua* L.*)*: Vessabundið svar og breytingar á tjáningu ónæmistengdra gena.

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Ritgerð til meistaragráðu í Líf- og læknavísinum

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Thesis for the degree of Master of Science

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Ágrip

Atlandshafsþorskur er efnahagslega mikilvægur fiskur og með minnkun stofnsins síðastliðna áratugi hefur áhugi á þorskeldi aukist hjá þjóðum kringum Norður-Atlandshafið. Í þorskeldi lifa aðeins 10-40% seiðanna af fyrstu 14 vikurnar. Margar ástæður eru fyrir lágri lifun hjá lirfunum, ein ástæðan eru sýkingar. Þess vegna eru rannsóknir á ónæmiskerfi og ónæmisvörnum þorsks mikilvægar.

Bráðasvar er svar líkamans við áverkum, meiðslum og sýkingum. Það felur í sér seyti bráðapróteina eins og pentraxína, ensímtálma, járnbindi- og komplement þátta. Tvær aðal gerðir pentraxína eru Serum amyloid P (SAP) og C-reactive prótín (CRP). Aðal munur milli þessara tveggja prótína er að CRP hefur kalsíum háða sækni í fosfórkólín en SAP ekki.

Í þorski hefur tveimur gerðum af CRP pentraxínum verðið lýst, CRP-PI og CRP-PII. N-enda amínósýru raðgreining hefur sýnt að CRP-PI er líkara SAP en CRP og að CRP-PII samsvarar CRP hjá öðrum fisktegundum en sýnir mikinn breytileika í gerð á milli einstaklinga. Rannsóknir á hlutverki pentraxína í bráðasvari hjá fiskum eru ennþá fáar.

Bráðasvar var framkallað í þorski með því að sprauta terpentínu í vöðva en aðferðin er þekkt aðferð til að framkalla bráðasvar. Blóði var safnað fyrir sermismælingar og sýnum úr lifur, nýrum og milta var safnað fyrir mælingar á genatjáningu. Magn CRP-PI, CRP-PII, kortisóls, IgM, og heildar próteinmagn voru mæld, auk þess sem virkni náttúrulegra mótefna og ensímtálma var mæld í sermi yfir 7 daga tímabil Niðurstöður sýndu að terpentínan jók magn kortisóls í sermi sem náði hámarki 72 klst eftir sprautun. Magn IgM í sermi var marktækt meira 24 klst eftir sprautun hjá terpentínuhóp en hjá viðmiðunarhóp en 168 klst eftir sprautun var magn í terpentínuhóp marktækt lægra en hjá viðmiðunarhóp. Niðurstöður sermismælinga benda því til að kortisól gæti haft bælandi áhrif á aðra ónæmisþætti í sermi og að pentraxín séu ekki dæmigerð bráðaprótein í þorski.

Tjáning CRP-PI, CRP-PII, C3, ApoLP A-I, IL-1β, transferrin, cathelicdin og hepcidin genanna var mæld í nýrum og milta yfir 7 daga tímabil. Öll genin voru stöðugt tjáð í báðum líffærum og tjáning þeirra jókst eftir meðhöndlun með terpentínu. Aukin tjáning CRP-PI, CRP-PII, ApoLP A-1 og C3 var takmörkuð við nýrun en tjáning á hepcidin var aðeins marktækt aukin í milta. Genatjáning transferrin, IL-1β og cathelicidin jókst í báðum líffærum. Niðurstöður benda til að pentraxín og ApoLP A-1 hafi hlutverk sem snemmbúnir miðlarar bráðasvars í nýrum og ásamt boðefninu IL-1β gætu þau hafa örvað kortisól losun og C3 og transferrin tjáningu. Cathelicidin og hepcidin gætu haft hlutverk í bráðasvari en eru líklega mikilvægari í ónæmisvörnum gegn sýkingum.

CRP-PII genið var raðgreint og niðurstöður sýndu breytileika í amínósýrum og kirnum milli einstaklinga. Þessi breytileiki var þó ekki nægur til að skýra munin sem áður hafði verið lýst á stærð og fjölda banda CRP-PII sem greindust í ónæmisþrykki.

Abstract

Atlantic cod (*Gadus morhua* L.) is an economically valuable fish and with decreasing fish stocks in recent decades interest in cod aquaculture has increased in countries around the North Atlantic. In cod farming only 10-40 % of the cod fry survive the first 14 weeks after hatching. There are many reasons for the losses, for example, infections are a common problem. Studies of the immune system and immune defence are therefore highly relevant.

Acute phase response (APR) is a systemic reaction of the body to injury, trauma or infection. The APR results in the secretion of acute phase proteins (APP) like pentraxins, antiproteases, tranferrin and some complement components. Pentraxins consist of two main types, serum amyloid P (SAP) and C-reactive protein (CRP) and the main difference between CRP and SAP is that CRP has a high calcium-dependent affinity for phosphocholine while SAP has not.

In cod two pentraxins have been described, CRP-PI and CRP-PII. The N-terminal amino acid sequence of CRP-PI shows closer homology to SAP than CRP whereas CRP-PII has been shown to be homologous to CRP of other fish species and showed an unusual degree of heterogeneity between individual cod. Studies of the role that pentraxins play in the APR of fish are still scarce.

APR was experimentally induced in Atlantic cod by intramuscular (i.m.) injection of turpentine oil, which is a known method to produce APR. Blood was collected for serum isolation as well as samples from liver, head kidney and spleen for gene expression analysis. The concentrations of the CRP-PI, CRP-PII, cortisol, total protein and IgM, as well as the natural antibody activity and anti-trypstin activity were examined in the serum over a period of 7 days. The turpentine injection significantly increased the level of cortisol which peaked 72 hours after the injection. The IgM concentration was significantly increased after 24 hours but was reduced 168 hours after the injection compared to the control group. The results indicated that the increase in cortisol may have suppressive effects on some humoral immune parameters and that pentraxins are not typical APPs in cod.

The expression of the genes encoding CRP-PI, CRP-PII, C3, ApoLP A-I, IL-1β transferrin, cathelicidin and hepcidin was examined in the head-kidney and the spleen over a period of 7 days. All genes were constitutively expressed and their expression increased following the turpentine injection. The increased gene expression of the pentraxins, ApoLP A-1 and C3 was restricted to the anterior kidney while the expression of hepcidin was restricted to the spleen. The expression of transferrin, IL-1β and cathelicidin increased in both organs. The results suggest that the pentraxins and ApoLP A-1 may act as early APR mediators in the anterior kidney which, with the inflammatory cytokine IL-1β, induce cortisol release and stimulate C3 and transferrin expression. Cathelicidin and hepcidin may play a role in the APR of cod but are probably more important in the immune defence against infection.

Sequencing of the CRP-Pll gene was carried out. The results showed nucleotide polymorphisms and differences in amino acid composition between individual fish. These differences alone cannot explain the differences in the number of bands, size and relative density of cod CRP-PII observed by Western blotting.

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Abbreviations

ApoLP A-I	Apolipoprotein A-I
AMP	Antimicrobial peptide
APR	Acute phase reaction
APP	Acute phase protein
cDNA	Complementary DNA
CRP	C-reactive protein
Ct	Cycle threshold
dNTPs	Deoxynucleoside triphosphates
EF1α	Elongation factor 1α
ELISA	Enzyme-linked immunosorbent assay
HDL	High density lipoproteins
Hpi	hour after (post) injection
IgM	Immunoglobulin M
IL-1β	Interleukin 1β
i.m.	Intramuscularly
LPS	Lipopolysaccharide
NaOH	Sodium hydroxide
n.s.	No significant change
OD	Optical density
qPCR	Quantitative real time PCR
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with tween-20
PCR	Polymerase chain reaction
RIN	RNA Integrity number
RQ	Relative quantity
RT	Reverse transcription
RTqPCR	Reverse transcription followed by quantitive real time PCR
SAP	Serum amyloid protein
SAA	Serum amyloid A
SD	Standard deviation

1 Introduction

1.1. The Atlantic cod

Atlantic cod (*Gadus morhua L.*) is one of the most important commercial fish species in the North Atlantic (Jonsson & Palsson, 2006). The main fishing grounds are as shown in Figure 1, and include the rich fishing areas around Iceland. Cod is primarily a bottom living fish but can live from a depth of a few meters down to 600 m or even more. Optimal water temperature for wild cod is 4-7°C but it can survive in temperatures from 0°C to 16°C or even 20°C for a short time (Perez-Casanova, et al., 2008a). It can also tolerate a wide salt range (Lambert, et al., 1994). Cod feeds mainly on invertebrates and on other fish, like capelin (*Mallotus villosus*). In Icelandic waters the main spawning ground is off the southwest coast of Iceland. Spawning takes place from late March until the beginning of May after which post-spawners and larvae move north along the west coast of Iceland (Jonsson & Palsson, 2006).

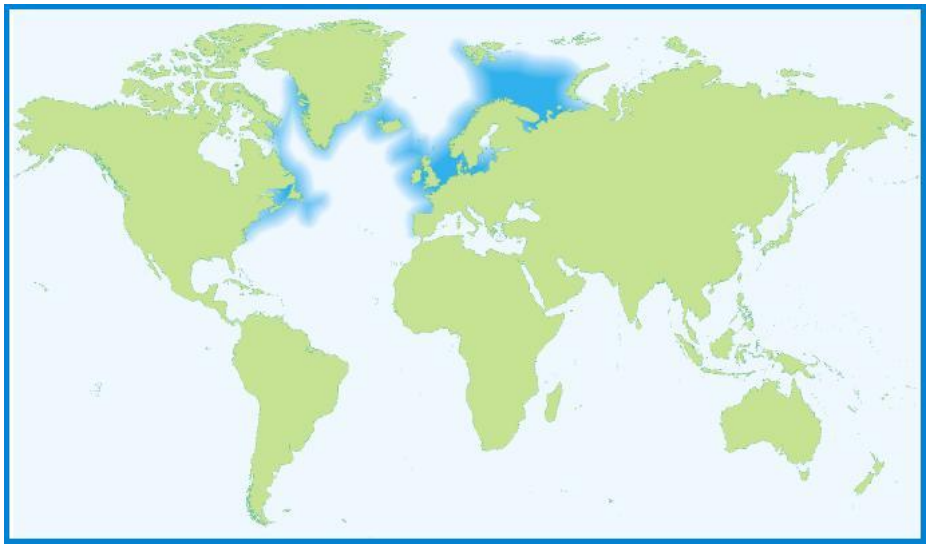

Figure 1: The distribution (blue zones) of Atlantic cod (*Gadus morhua* L.) in the North-Atlantic.

Cod fishing has been, and still is, a very important factor in Iceland´s economy. However, in the last 2-3 decades cod fishing in Icelandic waters has decreased from 460 thousand tons in 1981 to 130 thousand tons in 2007-2008 (Gunnarsson, 2007). It is not expected that cod fishing will increase significantly in the near future because recruitment has been relatively poor. The same situation has been observed in the rest of the North Atlantic and overall cod fishing has decreased from 3.9 million tons in 1968 to 840 thousand tons in 2005 (Gunnarsson, 2007).

Following this decrease in cod fishing the interest in cod farming has increased and it is hoped that cod will become an important new species for the aquaculture industries around the North Atlantic (Gjerde, et al., 2004). In Iceland, experimental cod farming has been carried out since 1992 at the Marine Institute´s Experimental Station at Stadur, Grindavík. In 2003 the company Icecod was established for the sole purpose of developing hatchery production and selective breeding of cod. In 2004 more than 20 cod hatcheries were in production in Norway, Canada, USA, the United Kingdom

and Iceland and the production was about 6 million cod fry (Gunnarsson & Steinarsson, 2007; Steinarsson, 2004).

Cod farming is not trouble free and only 10-40% of the cod fry survive the first 14 weeks after hatching. However, the losses are even greater under natural conditions where about 1 of every 1000 larvae survive the first 10 weeks. There are many reasons for the losses in cod farming, for example nutritional deficiency, contamination, fluctuations in heat and salinity, and malformation. Parasitic, bacterial and viral infections are also common problems in cod aquaculture as in the commercial farming of other species (Steinarsson, 2004). Studies of the immune system and immune defence of cod are therefore highly relevant to cod farming, the aim being to improve survival and the general welfare.

1.2. The immune system of cod

The immune system of cod is not fully competent until about 2-3 months after hatching (Schrøder, et al., 1998). Hence, cod larvae depend on innate cellular and humoral immune parameters like phagocytes, various lectins, antibacterial parameters and the complement system, for defence against infection during the first 2-3 months after hatching (Lange, et al., 2005; Magnadóttir, et al., 2004).

At the Institute for Experimental Pathology, Keldur, research on the immune system and immune responses of cod has been carried out since around 1992. The work at Keldur, and work by other research groups, has shown that the immune system of Atlantic cod is markedly different from other teleost fish species like, for example, the salmonids. Cod generally produces limited specific antibodies in response to immunisation or infection (Lund, et al., 2006; Magnadóttir, et al., 2001; Pilström, et al., 2005; Star, et al., 2011). This makes traditional vaccination difficult and commonly results in poor protection. It is interesting that despite this deficiency, cod is not more sensitive to infection in its natural environment than other fish species (Pilström, et al., 2005). The immune defence of cod has been shown to be characterised by non-specific cellular response like granuloma formation (Magnadóttir, et al., 2002) and cod commonly has a high level of natural or non-specific antibody (immunoglobulin M (IgM)) in serum (Lund, et al., 2006; Magnadottir, et al., 2009; Ronneseth, et al., 2007). Similarly, the level of other humoral parameters like pentraxins and anti-proteases is relatively constant and high (Gisladottir, 2008; Gisladottir, et al., 2009; Magnadottir, et al., 2010).

During the last few years, the emphasis at Keldur has been on studying the acute phase response of cod and the initial response to infection and acute phase induction (Gisladottir, et al., 2009; Magnadottir, et al., 2010). The present work is a continuation and extension of this area of research.

1.3 Acute phase response

The acute phase response (APR) is a systemic reaction of the body to injury, trauma or infection. It has been defined as a rapid, orchestrated, physiologically induced response, which basic aim is to restore homeostasis. APR involves many organs of the body including the immune, neuroendocrine, hepatic, hematopoietic, musculo-skeletal and central nervous systems (Bayne & Gerwick, 2001; Gruys, et al., 2005). The APR can result in local reactions including, for example, increased permeability of blood vessels, accumulation of neutrophils and marcrophages, and stimulation of

fibroblasts as well as systemic reactions including, for example, fever, pain, increased secretion of some hormones, activation of the complement system, increased synthesis and secretion of acute phase proteins (APP) (Bayne & Gerwick, 2001). Secretion of APP is conducted by plasma-borne signals or so-called pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNFα), toll-like receptors (TLRs) and CCAAt/enhancer binding proteins (C/EBPs) (Bayne & Gerwick, 2001; Lin, et al., 2007). The APR varies from one species to another both regarding the type of APP involved and also with respect to the magnitude and rapidity of change in plasma concentrations of a particular APP during APR (Ballou & Kushner, 1992). Studies have suggested that fish have muted APR that is delayed compared to mammals (Russell, et al., 2006) The activation of the APR in fish is frequently associated with the inflammatory response particularly in response to pathogen (Bayne, et al., 2001; Gerwick, et al., 2007; Martin, et al., 2006). Research on cod has shown that the pentraxins CRP-PI and CRP-PII do not act as typical acute phase proteins after infection (Magnadottir, et al., 2010).

1.4 Acute phase proteins

Roughly 40 plasma proteins have been defined as acute phase proteins (APP) (Black, et al., 2004). These can have various functions, for example opsonization, activating complement and modulating the host´s immune response (Gruys, et al., 2005).

An APP is called positive if its concentration increases by 1.25 fold or more in plasma following APR, but negative if its concentration decreases by 1.25 fold or more (Bayne & Gerwick, 2001). Three groups of positive APPs have been defined: 1) proteins with an increase of about 1.5 fold (complement factor C3 is an example of this group), 2) proteins with an increase of two to three fold (an example of this group is haptoglobin), and 3) proteins with a rapid increase of up to 5-fold to 1000-fold. The pentraxins (CRP and SAP) and SAA are examples of proteins belonging to group 3. The APPs can act differently from one species to another. For example, α_2-macroglobulin is not an APP in humans whereas a homologous protein in rat, α_2-macrofetoprotein, is an APP (Kushner, 1982). Similarly, an APP may be a positive APP in one species while being a negative APP in another. For example, transferrin is a negative APP in the liver of Sea bass (*Dicentrarchus labrax*) and most mammalian species, but is a positive APP in rainbow trout (*Oncorhynchus mykiss*) and chicken (Bayne & Gerwick, 2001; Neves, et al., 2009). Most APPs are synthesized in hepatocytes after cytokine stimulation, although some are synthesized by other cell types like monocytes, endothelial cells, fibroblasts and adipocytes (Gruys, et al., 2005; Steel & Whitehead, 1994). In rainbow trout, APPs are synthesized in head kidney macrophages, spleen lymphocytes and peripheral blood lymphocytes including hepatocytes (Liu, et al., 2004). The majority of the classical APPs have been shown to be strongly up-reglulated in channel catfish (*Ictalurus punctatus*) during infection with *Edwardsiella ictaluri* (Peatman, et al., 2007). In rainbow trout, APPs have been shown to be reduced in serum concentration after turpentine injection (Liu, et al., 2004). The APPs examined in the present study will be described below.

1.4.1 Pentraxins

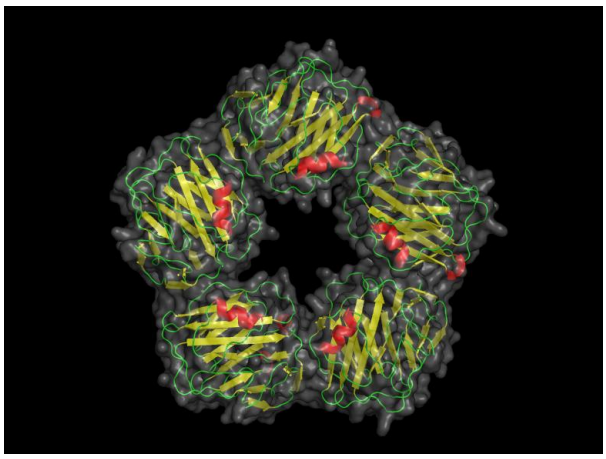

Figure 2: Pentraxins

Penxtraxins, showing the pentrameric structure.

http://en.wikipedia.org/wiki/File:CRP_pretty.png (27.06.11)

The pentraxins have been highly conserved through evolution. The two main types of pentraxins are serum amyloid P (SAP) and C-reactive protein (CRP) The pentraxins consist of five polypeptides that form a pentagonal structure (Bayne & Gerwick, 2001; Jensen, et al., 1995). The main difference between CRP and SAP is that CRP has a high calcium-dependent affinity for phosphocholine while SAP does not. SAP on the other hand recognizes phosphoethanolamine, agarose, some galactosyl galactosides, zymosan, phosphomannans and sulphated glycoaminoglycans (Cartwright, et al., 2004; Tennent & Pepys, 1994). Both CRP and SAP share the requirement for Ca^{2+} for ligand-binding activity (Huong Giang, et al., 2010). Pentraxins in mammals are normally present in a low concentration in serum (< 1 µg mL^{-1}) but can increase in concentration up to 1000-fold during APR (Volanakis, 2001). In fish, pentraxins are commonly present in higher concentrations in serum and show only a slight increase or decrease in concentration following APR (Ellis, 2001). The normal concentration of plasma CRP differs widely between species. For example, the level in mammals can vary from 0.02 µg mL^{-1} in mouse to more than 500 µg mL^{-1} in rat (Ballou & Kushner, 1992). In fish the concentration of CRP can vary from 54 µg mL^{-1} in snapper (*Pagrus auratus*) serum to 400 µg mL^{-1} in dogfish (*Mustelus canis*) serum (Cook, et al., 2003; Robey, et al., 1983). The concentration of the total pentraxins in cod serum has been reported to be about 140 µg mL^{-1} (Magnadottir, et al., 2010).

The role of pentraxins in fish is not fully understood. It is expected that they play an important role in defence mechanisms but more research is needed in this area (Ellis, 2001). In mammals their role in APR has been known for more than seven decades (Ablij & Meinders, 2002). In fish it was first described in plaice (*Pleuronectes platessa*) in 1973 and was at the same time the first APP to be described in fish (Baldo & Fletcher, 1973). CRP is primarily synthesized in hepatocytes. Synthesis in neurons, atherosclerotic plaques, monocytes and lymphocytes has also been reported (Black, et al., 2004).

In rainbow trout which was injected with turpentine oil to generate an APR response, a decrease in CRP concentration was demonstrated after 1 day and the level remained low for 14 days (Liu, et al., 2004). Another study, which focused on the functions of fish pentraxins, showed that when trout was injected with chemicals of environmental importance the concentration of CRP-like molecules in plasma increased (Winkelhake, et al., 1983).

In cod, pentraxin, a CRP-like protein, was first describe by Lund and Olafsen in 1998 (Lund & Olafsen, 1998). In 2008, Gisladottir *et al*. described two CRP-like proteins, CRP-PI and CRP-PII in cod (Gisladottir, et al., 2009). CRP-PI was shown to be equivalent to the CRP-like protein described by Lund and Olafsen (Lund & Olafsen, 1998) but its amino acid sequence was more homologues to SAP in various species like Wolffish (*Anarhichas lupus*) (Lund & Olafsen, 1998), halibut (*Hippoglossus hippoglossus*) (Lund & Olafsen, 1998), Atlantic salmon (*Salmo salar*) (Lund & Olafsen, 1998) and rainbow trout (Murata, et al., 1994) than to CRP (Gisladottir, 2008; Gisladottir, et al., 2009). However, it was phosphorcholin specific, which is characteristic of the CRP type pentraxins (Gisladottir, et al., 2009; Lund & Olafsen, 1998). The CRP-PII protein, which was first described by Gisladottir et al. (Gisladottir, et al., 2009), was a new identity. It showed amino acid sequence homology with CRP from other species and showed an unusual degree of heterogeneity in subunit size and density between individual cod (Gisladottir, et al., 2009).

1.4.2 Apolipoprotein A-I

The major role of lipoproteins in all vertebrates is transport of cholesterol and fatty acid in blood. High density lipoproteins (HDL) are one of the main constituents of fish serum (Delcuve, et al., 1992; Ndiaye, et al., 2000; Powell, et al., 1991). Apolipoprotein (ApoLP) A-I is one of the major proteins of HDL. The protein, which is a single chain 28 kD molecule, is synthesized in the liver and the small intestine (Stoffel, 1984). The ApoLP A-I protein has been isolated from a variety of species like human, rat, chicken, salmon and trout (Boguski, et al., 1985; Byrnes, et al., 1987; Delcuve, et al., 1992; Law & Brewer, 1984; Powell, et al., 1991). ApoLP A-I has many roles including lipid transport and uptake, reversing cholesterol transport, binding of lipopolysaccharide (LPS), antioxidant activity and antimicrobial activity (Concha, et al., 2003; Ndiaye, et al., 2000). Research has shown that ApoLP A-I has antimicrobial activity against gram negative and gram postive bacteria (Concha, et al., 2003; Concha, et al., 2004). Apolipoprotein A-I appears to have a complement C3 regulatory role in cod (Magnadóttir & Lange, 2004). Apolipoprotein expression has been found to be increased in the blood leukocytes of cod after intra peritoneal vaccination with heat-killed *Listonella anguillarum* (Caipang, et al., 2008a).

1.4.3 Complement protein C3

The complement system is important in both innate and adaptive immune defence and has many functions including, opsonisation, direct killing, regulation of the immune response and mediation of inflammation and hence, is one of the first lines of defence against pathogens. The three pathways of the complement system are: the antibody-dependent classical pathway, the antibody-independent alternative pathway, and the lectin pathway. These pathways can all terminate in the lytic pathway. The complement component C3 is the central component of the complement system (Holland &

Lambris, 2002; Lange, et al., 2004; Sunyer & Lambris, 1998). C3 is mostly synthesized in liver but it has been shown that it is also synthesized in other cells and tissues (Morgan & Gasque, 1996). It has been suggested that the alternative pathway may be an important defence mechanism of fish (Ellis, 2001). It has been shown that some pathogenic bacteria in fish are resistant to complement killing (Ellis, 1999). The complement component C3 is also involved in APR. Research on carp (*Cyprinus carpio* L) showed up-regulation of C3 expression in the liver following infection with the parasite *Ichthyophthirius multifiliis* (Gonzalez, et al., 2007). C3 gene expression has been shown to be reduced in liver of rainbow trout 14 days after primary infection with *Yersina ruckeri* 01 and increased 8 hours after re-infection (Raida & Buchmann, 2009).

1.4.4 Interleukin-1β

IL-1β is a member of the interleukin-1 family of cytokines. It is also a member of the β-trefoil family of cytokines (Secombes, et al., 1999; Secombes, et al., 2001). Other members of the interleukin 1 family are IL-1α, IL-1 receptor antagonist (IL-1ra) and IL-18 (Secombes, et al., 1999). Cytokines are important in regulating the host defence network and during immune response. They also mediate the differentiation and balance between T_H1 –T_H2 response which, is important to mount an effective immune response (Savan & Sakai, 2006). IL-1β is one of the pro-inflammatory factors that regulate acute phase protein synthesis and has been shown to induce proliferation, differentiation, maturation and other cellular activities (Bayne & Gerwick, 2001; Mathew, et al., 2002). It is mainly synthesized in monocytes and macrophages but it is also produced in many other cell types (Dinarello, 1988). Research on rainbow trout showed that IL-1β expression was increased in head kidney leukocytes and macrophages following stimulation with LPS (Pleguezuelos, et al., 2000). Other studies have shown that IL-1β expression is induced in rainbow trout by LPS both *in vitro* and *in vivo* (Zou, et al., 2000). IL-1β expression in cod was upregulated during an increase in environmental temperature (thermal stress) (Perez-Casanova, et al., 2008a) and expression of IL-1β in cod was upregulated following infection and other APR induction (Seppola, et al., 2008).

1.4.5 Transferrin

Transferrin is the major iron binding protein in vertebrate serum and is mainly synthesized in the liver (Lambert, et al., 2005; Yang, et al., 1984). It is secreted into the blood but it shows significant expression in organs like brain and kidney (Lambert, et al., 2005). Its main role is in iron metabolism by binding and transporting iron (Lambert, et al., 2005). Transferrin has a high-affintiy iron binding property and can produce a hypoferremic response by a rapid depletion of serum iron. This is an important innate humoral defence mechanism since the serum iron is necessary for the growth of many pathogenic and non-pathogenic bacteria (Dietrich, et al., 2010; Ellis, 2001; Langston, et al., 2001). Some pathogenic bacteria (e.g. *Aeromonas salmonicida*) can, however, overcome the lack of iron with specific mechanisms (Ellis, 1999, 2001).

Transferrin can be either a positive or a negative acute phase protein (APP). It has been shown to be a positive APP in rabbits and rats as well as in rainbow trout (Bayne & Gerwick, 2001). In a study on Sea bass, Neves et al. (Neves, et al., 2009) showed that while transferrin decreased in the liver after infection an increase was seen in the brain. They suggested that locally synthesized transferrin in

the brain might play a role in the host defence mechanisms (Neves, et al., 2009). Caipang et al. (Caipang, et al., 2010) have shown that the expression level of transferrin in the gill epithelial cells of cod was significantly increased 3 hours after infection with *A. salmonicida* and *V. anguillarium* but had returned to pre-infection level 24 hours after the infection.

1.4.6 Antimicrobial peptides

Antimicrobial peptides (AMPs) have been discovered in bacteria, protozoa, fungi, plants and animals and show great diversity throughout the animal and plant kingdoms (Zasloff, 2002, 2007). They are essential in defences against pathogens and important components of the innate immune system in metazoans (Ruangsri, et al., 2010; Zasloff, 2002). There are many different AMPs and cathelicidin is one family of AMPs which is, for example, found in humans where they are well studied (Zasloff, 2002, 2007). Cathlelicidins have been described in several fish species like Atlantic cod, rainbow trout, Atlantic salmon, arctic charr (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta fario*) (Chang, et al., 2005; Chang, et al., 2006; Maier, et al., 2008). Maier et al. (Maier, et al., 2008) have demonstrated that there are at least three cathelicidin genes in cod. They also showed that cod, which was infected with *Aeromonas salmonicida* subsp. *achromongenes* (Asa) showed upregulation in cathelicidin expression 24 h post-infection, which suggested that cathelicidin might play a role in the innate immunity of cod (Broekman, et al., 2011; Maier, et al., 2008). Research on cod eggs and during larval development (up to 68 days post hatching) has shown that cathelicidins are present in the eggs and during the development and show varied expression depending on feeding regimes (Broekman, et al., 2011).

Hepcidin is an antimicrobial peptide and its possible role as a health marker in fish has recieved increased attention in recent years (Douglas, et al., 2003; Shi & Camus, 2006). In general, hepcidin expression in fish appears to be induced by bacterial infection or vaccination, viral challenge and iron overload, seen, for example, in rockbream (*Oplegnathus fasciatus*) (Cho, et al., 2009) and sea bass (Rodrigues, et al., 2006). Research on channel catfish has shown that the expression level of hepcidin was increased in liver after infection (Hu, et al., 2007).

1.5 Other relevant humoral parameters

1.5.1 Total serum protein

If captive cod are maintained at optimum conditions, and on a constant and adequate diet, the total serum protein concentration is expected to be constant (Magnadóttir, et al., 2001). However, the total serum protein concentration of cod and other fish species has been shown to be affected by season, size and temperature (Langston, et al., 2002; Magnadottir, et al., 1999a; Magnadóttir, et al., 1999b).

1.5.2 Cortisol

Cortisol is the primary corticosteroid in teleosts (Harris & Bird, 2000; Mommsen, et al., 1999). Cortisol is an essential component of trauma response and in plasma the concentration can rise dramatically in response to stress (Mommsen, et al., 1999). Research on the effect of cortisol on the immune system has shown that cortisol has depressive effects on a number of immune responses (Espelid, et al.,

1996; Harris & Bird, 2000; Maule, et al., 1989; Nagae, et al., 1994). Cortisol has been shown to have regulatory effects on the immune system of fish. It acts as an inhibitor of some parts of the immune system and enhances other parts (Weyts, et al., 1999). Pérez-Casanova et al (Perez-Casanova, et al., 2008a) have shown that plasma cortisol in cod was increased at 16°C, compared to the control group, when water temperature was slowly increased. At 18°C the plasma cortisol level had returned to the initial level. Research on pheripheral blood cells from salmon has shown a suppressive effect of cortisol on LPS mitogenesis (Espelid, et al., 1996).

1.5.3 IgM and natural antibodies

The level of immunoglobulins (IgM) in cod serum is relatively high (2 – 20 mg mL^{-1}) and the natural antibody activity is high (Magnadottir, et al., 2009). Natural antibodies are produced in the absence of gene-rearrangement but show species characteristic specificity for different antigens like certain self-associated molecular patterns and haptenated (TNP/DNP) proteins. They can also show reactivity for non-self associated molecular patterns like LPS, viral and parasitic products (Avrameas & Ternynck, 1995; Bohn, 1999; Casali & Schettino, 1996; Lacroix-Desmazes, et al., 1998; Magnadóttir, 2006; Pashov, et al., 2002). Natural antibodies are important in maintaining homeostasis, in clearing away apoptotic cells and in tumour defence (Boes, 2000; Bohn, 1999; Magnadóttir, 2006; Mevorach, et al., 1998). Research on cod has shown that infection slightly reduces IgM concentration in serum but has no effect on natural antibody activity (Magnadottir, et al., 2010). Increases in water temperature have been shown to increase expression of IgM-light chain but have no effect on IgM-heavy chain (Perez-Casanova, et al., 2008a).

1.5.4 Anti-proteases

Various anti-proteases are present in fish serum, for example α1-antitrypsin and α_2 macroglobulin (Bowden, et al., 1997; Hjelmeland, 1983). Their activity is an important element in humoral defence and homeostasis. Anti-proteases can inhibit or delay growth of bacteria that secrete toxic proteases as a part of their pathogenicity, such as *Aeromonas salmonicida ssp. achromogenes* (Guðmundsdottir, 1998). Anti-proteases have been described as APPs in several mammals and the role of α_2 macroglobulin in the APR of fish has also been demonstrated (Bayne & Gerwick, 2001). Anti-protease activity has been measured in several groups of cod. These studies have indicated that the anti-protease activity is relatively high and may be of special importance in cod's defence at low environmental temperature (< 7°C) (Magnadottir, et al., 1999a). Infection was shown to have slightly suppressive effects on the anti-trypsin activity of cod (Magnadottir, et al., 2010).

1.6. Real-time polymerase chain reaction

Quantitative real time PCR (qPCR) was the key analytical method used in this study to measure the changes in gene expression during APR. Quantitative real time PCR is an extension of the PCR method (figure 3) developed in the 1980s by Kary Mullis (Mullis, 1990) allowing targeted amplification of specific DNA sequences (Valasek & Repa, 2005). The components of a standard PCR reaction are the template (DNA), polymerase, deoxynucleoside triphosphates (dNTPs), salts and buffer and short

single stranded oligonucleotides, i.e. primers (Markham, 1993; Powledge, 2004). A standard PCR reaction has three basic steps; 1) In the first step the template is “denatured” or disrupted into single strands of DNA by heating to 90- 96°C. 2) In the second step, performed at ca 50-65 °C, primers bind to their target sequence and polymerases are recruited. 3) In the third elongation step the polymerase synthesizes new DNA by reading the template from the primer binding sites and adding to it complementary nucleotides. Each new DNA helix which is formed is composed of one original strand and a new complementary strand (Powledge, 2004).

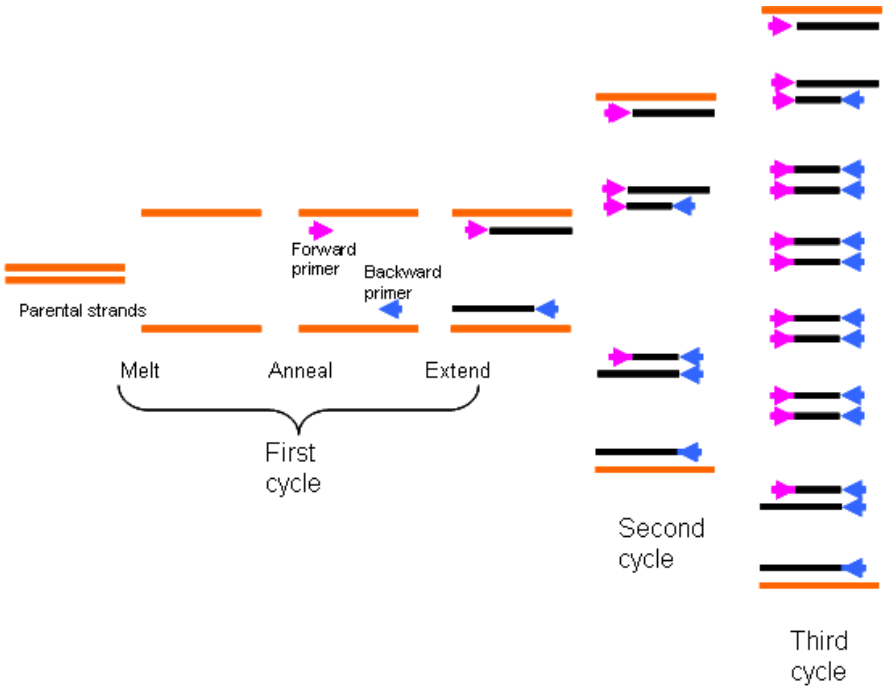

Figure 3: Polymerase chain reaction (PCR)

The figure is schematic presentation of the PCR process. A standard PCR reaction has three basic steps, a denaturation step, an annealing step and an elongation step.

http://www.obgynacademy.com/basicsciences/fetology/genetics/ (19.04.2011)

Quantitation of the formation of DNA during a PCR reaction, or “real time” monitoring of a PCR reaction, was first reported by Higuchi *et al*. in 1993 (Higuchi, et al., 1993). They used ethidium bromide, a common fluorescent dye that binds nucleic acids and fluoresces under ultraviolet light, to monitor the increase in PCR product during the PCR reaction. The advantage of real time PCR, compared to standard analysis of PCR reactions by agarose electrophoresis, is that it allows the detection and measurement of the amplification products generated during each cycle of PCR, thus enabling the detection of smaller difference in target copy number (Ginzinger, 2002; Houghton & Cockerill Iii, 2006).

Today, the most common methods used to detect the progress of PCR reactions in “real-time” employ TaqMan probes, molecular beacons or the SYBR Green dye. The TaqMan method relies on short oligonucleotides, or probes, which have a fluorescent reporter dye attached to their 5´ end and a

fluorescence quencher attached to their 3´ end. These probes bind to the DNA target between the PCR reaction primers during the annealing step of the PCR reaction. During amplification from the primer binding sites, the polymerase cleaves the probe into nucleotides causing the separation of the fluorescent reporter dye and the fluorescence quencher resulting in a fluorescent signal when excited by light of a particular wavelength (Ginzinger, 2002; Houghton & Cockerill Iii, 2006; Valasek & Repa, 2005). Molecular beacons are single-stranded oligonucleotide probes with a hairpin structure. Like TaqMan probes, they contain both a fluorophore and a quencher. Molecular beacons work like switches, when the hairpin is "closed" the fluorophore and quencher are close together and there is no fluorescence. When the hairpin is open the quencher and fluorophore are separated and fluorescence is possible (Tan, et al., 2005). The SYBR Green method uses a cyanine dye that has non-specific binding affinity to double-stranded DNA. During the progress of the PCR reaction the SYBR green dye binds to newly formed double stranded PCR product and emits a fluorescent signal upon excitation which is more than 1000 times greater than that emitted by the unbound dye (Houghton & Cockerill Iii, 2006; Valasek & Repa, 2005).

The real time PCR method is very useful as a sensitive tool to detect pathogens such as bacteria, viruses or fungi, it is also used for sensitive detection of mutations and single nucleotide polymorphisms (Valasek & Repa, 2005). Reverse transcription (RT) followed by quantitative real time PCR (RTqPCR) is a favoured method for quantification of mRNA (via cDNA). However, the method is not flawless, and several things need to be taken into consideration in order to obtain valid data. One of the main problems is normalisation of data between samples. The most common, and simplest, method is to use the expression of a "reference gene" also known as a "housekeeping gene", but total starting RNA, or genomic DNA, quantity are also used to normalise the data (Bustin, et al., 2009; Bustin, et al., 2005; Huggett, et al., 2005). The use of a reference gene to normalise the data aims at removing or reducing differences due to sampling variability and technical variability, such as in the reverse transcription step. There is no single gene that can act as a reference gene for all species or different tissues in a given species (Bustin, et al., 2009; Olsvik, et al., 2005). Reference genes must fulfil certain criteria. Their expression has to be fairly constant over time and development of the tissue in question, and must be minimally changed in response to treatments. Due to these limitations, each experiment may require the use of a new reference gene for each new animal or tissue (Bustin, et al., 2009; Olsvik, et al., 2005). The quality of the RNA is also very important for RTqPCR, if the quality is insufficient, e.g. if the RNA is degraded or contains genomic DNA contamination, results will be affected (Bustin, et al., 2009; Bustin & Nolan, 2004; Olsvik, et al., 2005).

2 Aims of the study

Aims of the study were to examine the acute phase respones (APR) and to sequence the CRP-PII gene in Atlantic cod. The study was composed of the following milestones:

1. The induction of APR in cod using turpentine injection and examination of the effect on serum parameters and gene expression in the spleen and the head kidney.
    - The serological analysis involved analysis of:
        - Serum protein concentration
        - Serum cortisol concentration
        - Serum Pentraxin (CRP-PI and CRP-PII) concentration
        - Serum IgM concentration
        - Natural antibody activity
        - Anti-trypsin activity
    - The Gene expression analysis in the head kidney and the spleen involved analysis of:
        - Pentraxins (CRP-PI and CRP-PII)
        - Apolipoprotein AI
        - Complement C3
        - Interleukin 1-β
        - Transferrin
        - Cathelicidin
        - Hepcidin

2. Sequencing of the CRP-PII gene to try to explain the heterogeneity of CRP-PII between individual cod observed in Western blotting experiments.

3 Materials and methods

3.1. Experimental design: Turpentine injection, a study carried out at Keldur in September 2008

Fifty four cod, mean weight 89.5g, were obtained from the Marine Institute's Experimental Station at Staður, Grindavík, and transported to Keldur where they were kept at 8°C in three 0.17 m^3 tanks and allowed to acclimatize for a few days. Twenty-four fish were injected intramuscularly (i.m.) with 1 mL kg^{-1} body weight of turpentine oil (Slippfélagið, Iceland) and thirty control fish received no treatment. Six fish of the untreated group were killed by anaesthesia (with 50 mg mL^{-1} of tricaine methanesulfonate) and sampled before the turpentine injections and then six fish were sampled from each group at 1 hour, 24 hours, 3 days (72 hours), and 7 days (168 hours) after the turpentine injection. Blood was collected from the caudal vessel for serum isolation. The blood was allowed to coagulate overnight at 4°C. Serum was collected following centrifugation at 400xg for 10 min and stored at -80°C. Samples from liver, head kidney and spleen were collected in RNA later solution (Ambion, TX, USA) and stored at -80°C.

3.2. Serological analysis

3.2.1. Serum protein concentration - Bradford assay

Serum protein concentration was measured according to Bradford´s method (Bradford, 1976) using a protein assay kit from Thermo Scientific (IL, USA). The procedure was according to the manufacturers instructions. Briefly, serum was diluted 1/50 and 5 µL of the dilutions were incubated on a 96 well non-absorbent, flat-bottomed microtray (Nunc, Denmark) for 30 min at room temperature with 300 µL of reagent and optical density (OD) read at 600 nm. All samples were measured in duplicates. A standard graph was plotted using a bovine serum albumin (BSA) standard (Thermo Scientific, IL, USA) and the protein concentration of the serum samples extrapolated from this graph.

3.2.2. Cortisol

Serum cortisol levels were measured using a Cortisol ELISA kit (Neogen Corp, KY, USA) according to the manufacturer's instructions. Briefly, 100 µL serum and 1 mL ethylene ether were mixed in a glass tube, vortexed for 30 sec and then left for a few minutes to allow the liquid phases to separate. The organic phase was collected into a clean tube, frozen in liquid nitrogen and evaporated in a SpeedVac centrifuge. The resulting residue was dissolved in 100 µL of diluted extraction buffer (from the kit). Ten µL of this solution were added to 990 µL of diluted extraction buffer and vortexed. Subsequently, 50 µL of the extracted serum samples, the standard provided by the kit and blank samples (buffer only) were added in duplicates to the assay tray provided, which was coated with anti-cortisol rabbit antibodies. Then 50 µL of the enzyme conjugate provided were added and the tray incubated at room temperature for 1 hour. After incubation the content was discarded and the trays washed with 3 x 300 µL $well^{-1}$ of wash buffer followed by incubation for 30 min with 150 µL $well^{-1}$ of substrate at room temperature. Finally 50 µL $well^{-1}$ of 1 N HCl were added and OD read at 450 nm. A standard graph

was plotted using results of the cortisol standards from the kit and the cortisol concentration of the samples calculated from this graph, as described by the kit's manufacturer.

3.2.3. Pentraxin (CRP-PI and CRP-PII) concentration

An ELISA method devised for the estimation of CRP-PI and CRP-PII has been described elsewhere (Magnadottir, et al., 2010). Briefly, microtrays (96-well, Maxisorp, Nunc) were coated with 100 µL $well^{-1}$ of polyclonal, monospecific anti-CRP-PI or anti-CRP-PII purified mouse immunoglobulins, prepared according to the description by Gisladottir et al (Gisladottir, et al., 2009), 10 µg mL^{-1} in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, Sigma-Aldrich, Germany) and incubated over night at 4°C. The trays were blocked with 0.1 % semi skimmed milk powder in coating buffer (100 µL $well^{-1}$) for 1 hour at room temperature. Serum samples (50 µL $well^{-1}$), diluted 1/500 and 1/2000 in phosphate buffered saline (PBS, Sigma) containing 0.05% tween 20 (PBS-T) were added to the trays as well as 8 five-fold serial dilutions in PBS-T of purified CRP-PI (from 240 µg mL^{-1}) or CRP-PII (from 960 µg mL^{-1}) to generate the respective standard graphs, and incubated for 2 hours at room temperature. Biotin labelled anti-CRP-PI antibody or anti-CRP-PII antibody (prepared at Keldur using an ECL protein biotinylation kit from Amersham Biosciences, UK) diluted 1/100 in PBS-T was added (50µL $well^{-1}$) and incubated for 1 hour at 37°C. Next, streptavidin-labelled alkaline phosphatase (DAKO, Denmark) diluted 1/1000 in PBS-T, was added (50 µL $well^{-1}$) and incubated for 1 hour at 37°C. The microtrays were washed three times with PBS-T between each step. Finally, p-nitrophenylphosphate substrate (Sigma) was added (100 µL $well^{-1}$) and incubated for 30 minutes at room temperature. The reaction was stopped with 3M NaOH (50 µL $well^{-1}$). OD was read at 405 nm. The quantity of the CRP-PI or CRP-PII in the serum samples was calculated from the respective standard graphs.

3.2.4. IgM concentration

The ELISA method described by Israelsson et al. (Israelson, et al., 1991) was used with some modifications. Maxisorp microtrays (see above) were coated with 10 µg $well^{-1}$ in coating buffer (see above) of purified, polyclonal rabbit anti-cod IgM antibody (a gift from the late professor L. Pilström, Department of Medical Immunology, Uppsala University, Sweden) and incubated overnight at 4°C. Trays were blocked with 1% bovine serum albumin in coating buffer for 1 h at room temperature. Serial dilutions of standard cod IgM (prepared at Keldur) and the test serum in PBS-T, 50 µL $well^{-1}$, were incubated for 2 h at 37°C and followed by 50 µL $well^{-1}$ of mouse polyclonal anti-cod IgM antibody diluted in PBS-T (prepared at Keldur) for 1 h at 37°C. The trays were then incubated with 50 µL $well^{-1}$ of alkaline phosphatase conjugated rabbit anti-mouse Ig's (Dako, Denmark), diluted in PBS-T, for 1 h at 37°C. Washing, substrate incubation and development and the reading of the plates was as described in section 3.2.3 above.

A standard graph was plotted using the results from the standard IgM and the quantity of the IgM in serum was extrapolated from this.

3.2.5. Natural antibody activity

Natural antibody activity was measured using an ELISA method previously described (Magnadottir, et al., 2009; Magnadottir, et al., 1999a). Briefly, MaxiSorp microtrays were coated overnight at 4°C with 5 µg well^{-1} of trinitrophenyl conjugated bovine serum albumin (TNP-BSA) (prepared at Keldur) diluted in coating buffer. Blocking was with 0.1% semi-skimmed milk powder in coating buffer for 1 h at room temperature. Serum samples, diluted 10^{-2} in PBS-T, were incubated overnight at 4°C, followed by incubation for 1 h at 37°C, first with mouse anti cod IgM antibody and then with alkaline phosphatase conjugated goat anti mouse Ig antibody (Dako), both diluted in PBS-T as described above. Washing, developing and reading of the trays was as described in section 3.2.3 above. Antibody activity was expressed as the OD_{405nm} value of serum diluted 10^{-2} after subtracting the blank values (PBS-T in place of serum).

3.2.6. Anti-trypsin activity

Anti-protease activity was measured using a modification of the method described by Bowden et al. (Bowden, et al., 1997; Magnadottir, et al., 2010). Twenty µL of serum (in duplicates) were incubated with the same volume of standard trypsin solution (5 mg ml^{-1}, Sigma T-7409) in 1.5 ml microcentrifuge tubes for 10 min at room temperature. To this was added 200 µL PBS and 250 µL of 2% azocasein (Sigma) and incubated for 1 h at room temperature after thorough mixing, followed by the addition of 500 µL of 10% trichloroacetic acid, mixing, and further incubation for 30 min at room temperature. The mixture was centrifuged at 6000 xg for 10 min and 100 µL transferred to a 96 well, flat-bottomed, non-absorbent microtray (Nunc) containing 100 µL well^{-1} of 1N NaOH. OD was read at 450 nm. The blank contained PBS in place of serum and trypsin and the 100% reference wells contained PBS in place of serum. After subtracting the value of the blank the percentage inhibition of trypsin, compared to the reference sample, was calculated for each sample. The anti-trypsin activity was expressed as the % inhibition.

3.3. Gene expression analysis

3.3.1. Isolation of RNA

Total RNA for reverse transcription and quantitative real time PCR assays (RT-qPCR) was isolated with the NuceloSpin® RNA/Protein kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The quantity, and purity, of the RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, NC, USA). The quality of the RNA was determined on Agilent RNA 6000 nano chips on a 2100 Bioanalyser (Agilent, USA, CA). RNA integrity numbers (RIN) of 8 and higher were taken as a measure of acceptable RNA quality. RNA was stored at -80°C.

3.3.2. Removal of genomic DNA

In addition to the on-column DNase digestion step included in the NuceloSpin® RNA/Protein kit an additional DNAse treatment was performed on isolated total RNA with the DNA-free^{TM} Kit (Ambion). In brief, RNA (10µL) was diluted in RNase free-H_2O (40 µL). Five microliters of 10x DNase I buffer and 1 µL rDNase I were added and the solution mixed gently. The solution was incubated at 37°C for 30

min. Next, 5.5 µL DNase Inactivation Reagent was added, mixed well and incubated at room temperature for 2 min. The solution was mixed occasionally during these 2 minutes. Finally, the solution was centrifuged at 10.000xg for 1.5 min and the cleaned RNA transferred to a fresh tube for storage at -80°C.

3.3.3. Synthesis of cDNA

Complementary DNA (cDNA) was prepared from cleaned total RNA with the RevertAid^{TM} First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions. Each reverse transcription (RT) reaction consisted of total RNA (variable between reactions from 500 ng to a maximum of 2000 ng), 5 µM Oligo$(dT)_{18}$ primer, 1 mM dNTP Mix, 1x Reaction buffer, 1 unit Ribolock^{TM} RNAse inhibitor and 10 units RevertAid^{TM} M-MulV Reverse Transcriptase in a total volume of 20 µL. The samples were incubated at 42°C for 60 min followed by 5 min at 70°C. After RT, cDNA samples were stored at -80 °C. For each RNA sample a reaction lacking reverse transciptase (-RT) was performed for use in subsequent qPCR reactions to verify that the RNA samples did not include genomic DNA. To confirm that the total RNA samples used were completely free of genomic DNA all cDNA and –RT samples were tested in PCR reactions using primers for EF1α (Table 1) and AmplitaqGold polymerase (AppliedBiosystems, USA, CA);

Reaction solution:

1 µL cDNA
0.2 µL forward primer (stock solution: 20 µM)
0.2 µL reverse primer (stock solution: 20 µM)
2.0 µL $MgCl_2$ (stock solution: 25 mM)
2.0 µL 10 x PCR buffer
2.0 µL dNTPs (stock solution: 2 µM)
0.1 µL AmplitaqGold
12.5 µL ddH_2O

PCR reaction conditions (hot start PCR):

94 °C for 10 min (polymerase activation)

PCR cycling conditions (30 cycles)

95 °C for 15 sec
55 °C for 15 sec
72 °C for 1 min and 30 sec

Post cycling hold

72°C for 7 min

3.3.4. Primer and probe design for qPCR

The sequences of the primers and probes used in this study are listed in Table 1. The primers for ubiquitin, elongation factor 1α (EF1α), IL-1β, cathelicidin, and hepcidin have been described

previously (Broekman, et al., 2011; Furnes, et al., 2009; Olsvik, et al., 2008; Seppola, et al., 2008; Solstad, et al., 2008). Results from amino acid sequence analyses (Gisladottir, et al., 2009) of cod CRP-PI and CRP-PII were used to construct primers and amplify, clone, and sequence CRP-PI and PII cDNA (Gisladottir et al. unpublished results). The primers and probes for CRP-PI and CRP-PII used in this study were designed based on these sequences with the Primer3 software (Rozen & Skaletsky, 2000). Primers for complement component C3, transferrin, and apolipoprotein A-I were designed in a similar manner based on published sequences (GenBank IDs: AY739672.1, L40370.1, and AY739673.1, respectively). All primers and probes were obtained from TAG Copenhagen (Denmark). Prior to their use they were tested on serial dilutions of cDNA (5 points, 2-fold dilution with each point in triplicate) to determine their amplification efficiency (Eff%, Table 1) according to the formula: $E_x=^{10(-1/slope)}$-1. The cDNA template used for the serial dilutions was selected from the samples used in the study.

Table 1: Primers and probes used for quantitative real time PCR assays in the study.

The table shows, for each assay, the nucleotide sequences and modifications of the primers and probes, the PCR amplicon sizes in basepairs (bp.), the annealing/extension temperature (°C) of the PCR reaction, and the PCR efficiency (Eff%).

Gene	Primer/probe nucleotide sequence (5´-3´)	Amplicon size	°C	Eff%	Reference
CRP-PI	Forward: GGCTACTCGCACCCGTATAA Reverse. CATGTGCCACAGATGGAGAC Probe: FAM-CGTGGCTGATTTCCCCGAGC-BHQ	141	55	100.5	This study
CRP-PII	Forward: AAAGGGAAGCGTGAGGTCATC Reverse: CGCGCCACACGTTGAGT Probe: FAM-CGCACGCGGGACTTCGACG-BHQ	70	60	101.1	This study
ApoA-I	Forward: CTCTTGCTCTTGCCCTTCTG Reverse: GGCACTGTCCTTCACCTGAT Probe: FAM-ATCCGATGCACCCAGCCAGC-BHQ	128	60	86.9	This study
Complement C3	Forward: AGTGGGAAACTACGCACCTT Reverse: TCGACCAACTTTCTGTGCAT Probe: FAM-TGCGAGCTATCTCATCATGGGCA-BHQ	117	60	93.7	This study
IL-1β	Forward: GGAGAACACGGACGACCTGA Reverse: CGCACCATGTCACTGTCCTT	50	60	93.4	(Seppola, et al., 2008)
Transferrin	Forward: GAGCTCCCATCGACAGCTAC Reverse: CAAACCCAGCAGAGGAGAAG Probe: FAM-CACGCTGTGGTTAGCCGCGT-BHQ	150	60	102.2	This study
Hepcidin	Forward: CCAGAGCTGCGGATCGA Reverse: AAGGCGAGCACGAGTGTCA	100	60	95	(Solstad, et al., 2008)
Cathelicidin	Forward: GGTTGAAACTGTCTATCCAGAGG Reverse: AACTCTTGTGCAGGGAATGTC	77	60	98.9	(Broekman, et al., 2011)
EF1α	Forward: ATGTGAGCGGTGTGGCAATC Reverse: TCATCATCCTGAACCACCCTG	72	55	92.8	(Furnes, et al., 2009)
Ubiquitin	Forward: GGCCGCAAAGATGCAGAT Reverse: CTGGGCTCGACCTCAAGAGT	69	60	102.4	(Olsvik, et al., 2008)

3.3.5. Real-time quantitative PCR

Real time PCR assays for specific genes (Table 1) were performed using a Step-One Plus realtime-PCR instrument (AppliedBiosystem). The Maxima^{TM} probe qPCR master mix (2x) (Fermentas) was used in reactions for CRP-PI, CRP-PII, transferrin, C3 and ApoLP A-1 but in the reactions for ubiquitin, EF1α, IL-1β and cathelicidin, the Maxima^{TM} SYBR green qPCR master mix (2x) (Fermentas) was used. Reverse transcribed cDNA was diluted with nuclease free H_2O prior to use in qPCR reactions and assigned a concentration unit corresponding to the concentration of total RNA in the RT reaction, e.g. 4 ng cDNA corresponded to 4 ng of input total RNA in the RT reaction. The PCR reactions for CRP-PI and CRP-PII, in the spleen and transferrin in the head-kidney and the spleen contained the following components:

10 µL PCR master mix,
0.5 µL TaqMan probe (stock: 10µM),
0.9 µL forward primer (stock: 20 µM),
0.9 µL reverse primer (stock: 20µM),
2 µL cDNA template (4 ng)
and 5.7µL ddH_2O.

The PCR reactions for CRP-PI and CRP-PII in the head-kidney, and C3 and ApoLPA-1 in the head-kidney and the spleen contained the following components:

10 µL PCR master mix,
0.5 µL TaqMan probe (stock: 10µM),
0.9 µL forward primer (stock: 20 µM),
0.9 µL reverse primer (stock: 20µM),
2 µL cDNA template (48 ng)
and 5.7µL ddH_2O.

The qPCR reaction conditions for CRP-PI, CRP-PII, transferrin, C3 and ApoLP A-1 were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 60 sec at X°C (the annealing temperature, X°C, varied for different primer pairs, see Table 1).

The qPCR reactions for ubiquitin, EF1α, IL-1β and cathelicidin contained:

10 µL PCR mastermix,
0.3 µL forward primer (stock: 20 µM),
0.3 µL reverse primer (stock: 20 µM),
2 µL DNA template (4 ng),
and 7.4 µL ddH_2O.

The qPCR reaction conditions for ubiquitin, EF1α, IL-1β and cathelicidin were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 60 sec at X°C (the annealing temperature, X°C, varied for different primer pairs, see Table 1). The amplification of a single PCR product, and absence of primer dimers, was verified by melt curve analysis according to default conditions of the

StepOnePlus qPCR machine and the StepOne v2.1 software. All qPCR assays where the cDNA concentration was 48 ng were performed in duplicate (technical replicates), whereas assays where cDNA concentrations were 4 ng were performed in triplicate. Assays for each gene were performed on one sample, per organ, from each individual included in the study for that gene as shown in Table 2.

Table 2: Number of samples for each time point of the study.
Deviations are shown in parenthesis. The lower number of samples for CRP-PII in the kidney is due to omission of samples with no amplification.

Kidney	**0 h**	**1 h**	**24 h**	**72 h**	**168 h**
Turpentine		7	5 (CRP PII : 2)	5	4 (CRP PII : 3)
Control	5	6	4 (CRP PII : 2)	5 (CRP PII : 4)	5 (CRP PII : 4)
Spleen					
Turpentine		7	5	5 (IL1b: 4)	3
Control	5	6	5 (Hepcidin: 4)	5	5

3.4. Data analysis.

Real time PCR data were collected and processed with the StepOne™ v2.1 software (AppliedBiosystems, CA, USA). The qPCR data for each gene, in either kidney or spleen, were calibrated against a control fish from 0 h which was selected as the fish with the median cycle threshold (Ct) value. Relative quantity (RQ) values compared to this fish were calculated using ubiquitin as a reference gene and an equation described by Pfaffl (Pfaffl, 2001) which includes a correction for primer amplification efficiencies in the calculation of gene expression ratios from Ct values. Ubiquitin has been shown to be a suitable reference gene for the cod tissues in this study (Olsvik, et al., 2008). One-way ANOVA on log transformed RQ values was performed to determine if there were significant changes in the expression of the genes of interest within the control group, or within the turpentine-injected group. Comparisons of the expression of genes between turpentine treated and untreated controls within each time point were performed using the REST 2009 software (QIAGEN, Germany) (Pfaffl, et al., 2002), which is based on the Pfaffl equation described above. For statistical calculations in the REST 2009 software, randomization was conducted with 6000 permutations and the cut off for significance was $P = 0.05$. GraphPad InStat 3 (Demo version) was used for statistical analysis in the serological analysis and the qPCR analyses (ANOVA). The Mann Whitney test was used to examine the difference between two distinct groups in the serological analysis with $P < 0.05$ set as the critical value of significance. Statview v5.0.1, Microsoft® Office Excel, and Microsoft® Office Publisher were used for image processing.

3.5. Sequencing of the CRP-PII gene

3.5.1. DNA Isolation and cloning of genomic sequences of CRP-PII

Berglind Gisladottir, at the Keldur Institute for Experimental Pathology, previously amplified and cloned the genomic sequence of the CRP-PII gene from 13 individuals into TOPO vectors (pCR®4-TOPO vector; TOPO TA Cloning® Kit for Sequencing (Invitrogen K4595-01, CA, USA)), 2-6 clones per individual giving a total of 78 clones with an average insert length of 1400 base-pairs (bp) (Gisladottir et al. unpublished data). The cloning procedure, in brief, consisted of DNA extraction from liver samples of individuals, that displayed different CRP-PII band patterns in protein Western blots with a Chelex/proteinase K extraction method. The DNA was used as a template for PCR with CRP-PII specific primers (below) that were designed based on results from amino acid sequence analyses (Gisladottir, et al., 2009) of cod CRP-PII. The PCR was carried out using Long PCR Enzyme Mix (which contains a mixture of Taq and proofreading Pfu polymerases).

Forward primer (CRP-PII_For):5'-GTGTTCCCCGAGGAGACCGCCAA-'3

Reverse primer (CRP-PII_Rev): 5'- GCTTGTATCTAGGGAGCTTT-'3

3.5.2. Sequencing

Plasmids with inserts were sequenced either with the CRP-PII primers (see above) or sequencing primers which were included in the TOPO TA kit (TOPO TA Cloning® Kit for Sequencing (Invitrogen K4595-01, CA, USA). Cycle sequencing reactions were done with the Big Dye Terminator v3.1 kit (AppliedBiosystems) and analyzed on an ABI Prism 310 capillary sequencing machine. A sequencing reaction consisted of the following reagents:

1 µL BigDye (v3.1)
1.5 µL Buffer (5x)
1.6 µL primer (2µM)
X µL DNA template (200-400ng)
X µL dH_2O up to 10µL

The cycle sequencing reaction conditions were:

Step 1 (denaturing) 95°C for 5 minutes
Step 2 (denaturing) 95°C for 15 seconds
Step 3 (annealing) 50°C for 15 seconds
Step 4 (elongation) 60°C for 4 minutes
Step 2-4 was repeated 30 times.

Before analysis on the ABI Prism 310 capillary sequencing machine, the sequencing reactions were processed. Briefly, 40 µL of 75 % isoproponol were added to the reaction and mixed briefly. This mix was incubated for 15 min at room temperature (RT) and then centrifuged 30 min at 18.800 x g (RT). Following centrifugation, the supernatant was discarded and 100 µL of 75 % isopropanol were added and the solution mixed briefly and centrifuged again for 5 min at 18.8000 x g (RT) and the

supernatant discarded. To remove residual isopropanol the samples were then heated with an open lid at 95 °C for 1 min. The DNA pellet was dissolved in 15 µL of Hi-Di^{TM} Formamide (Applied Biosystems), mixed briefly and heated at 95 °C for 2 min. Subsequently the sample was analysed on the ABI Prism 310 machine

Sequence alignments and editing were performed using the Sequencher software (Gene codes Corporation).

4 Results

4.1. The experimental fish

A lesion at the site of injection and intramuscular haemorrhage was seen in all the turpentine injected fish within 1 - 2 hours (Figure 4). Three of the turpentine injected fish died during the experimental period, all within an hour from the injection.

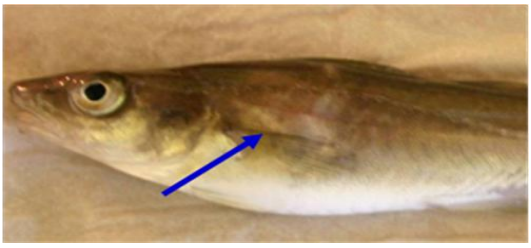

Figure 4: Cod injected with turpentine oil.

The arrow shows the resulting skin lesion and haemorrhage at the site of injection.

4.2. Serological analysis

4.2.1. Total protein concentrations in serum

The results for the total protein concentration analysis of serum are shown in Figure 5. There was no statistically significant difference between the turpentine injected fish and the untreated control fish at any time point and the mean values varied from 22.2 mg mL^{-1} to 37.2 mg mL^{-1}. At the start of the experiment the mean and standard deviation (SD) protein level was 37 $\pm$ 13 mg mL^{-1} and lower levels, albeit statistically insignificant, of about 31, 23, 23 and 33 mg mL^{-1} were seen in the control fish after 1, 24, 72 and 168 hours respectively. Similar levels and an overall reduction in protein concentration were also seen in the turpentine injected fish.

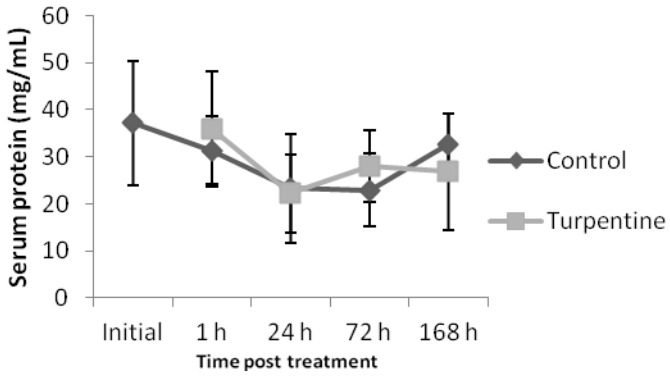

Figure 5: Results of the serum protein analysis

The serum protein concentration (mean $\pm$ standard deviation (SD)) of untreated control fish and fish injected i.m. with turpentine and sampled at different times after the injection (n= 4 - 7). There was no significant difference in the serum protein concentration between the two groups at any of the time points or when compared to the initial value (at 0 hour). The time points following injection shown on the x-axis are not drawn to scale.

4.2.2. Cortisol

The results of the serum cortisol analysis are shown in Figure 6. The mean concentration ranged from about 18 to 460 ng mL^{-1}. At 72 hours after the turpentine injection the concentration of cortisol was significantly higher in the injected fish than in the untreated control fish (26 fold, p=0.02). At other time points there was no significant difference between the two groups and the cortisol levels in the control fish did not change significantly during the experimental period.

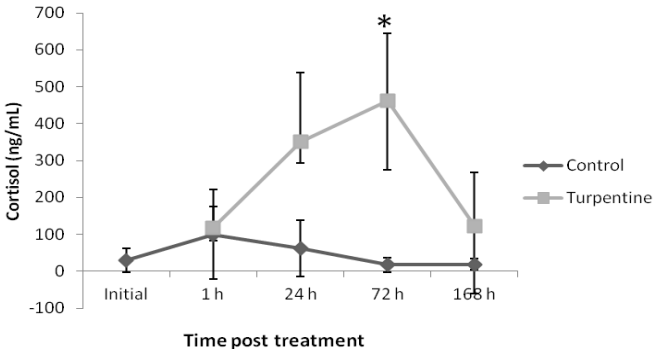

Figure 6: Results of the serum cortisol analysis

The serum cortisol concentration (mean ± SD) of untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection (n= 4 - 6). The asterisk (*) indicates a significant difference (P<0.05) in the cortisol levels of the turpentine treated fish compared to the control fish at the same timepoint. The time points following injection shown on the x-axis are not drawn to scale.

4.2.3 Pentraxin CRP-PI

The results for the CRP-PI analysis are shown in Figure 7. Great individual variation in CRP-PI levels were observed in both groups. The mean value varied from about 18 to 54 µg mL^{-1}. There was no significant difference between the turpentine injected fish and the untreated control fish at any matched time point.

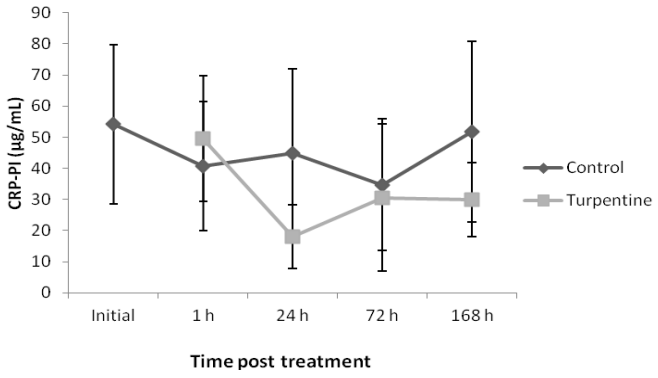

Figure 7: Results of the serum CRP-PI analysis

The serum CRP-PI concentration (mean ± SD) of untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection (n= 4 - 7). The time points following injection shown on the x-axis are not drawn to scale.

4.2.4 Pentraxin CRP-PII

The results for the serum CRP-PII analysis are shown in Figure 8. The mean value varied from 29.2 to 47.7 µg mL^{-1}. Individual variation in CRP-PII levels were not as marked as in the case of CRP-PI. There was no significant difference between the turpentine injected fish and the untreated control fish at any matched time point.

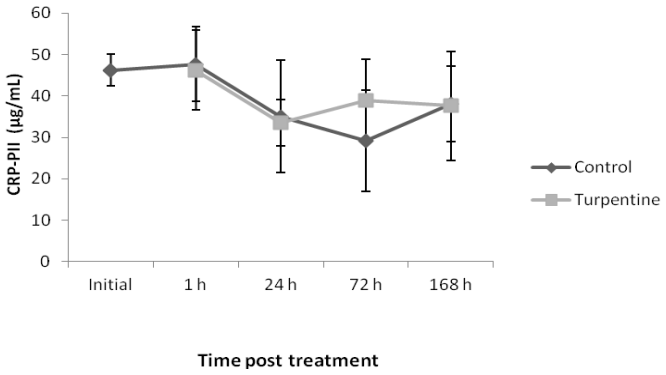

Figure 8: Results of the serum CRP-PII analysis

The serum CRP-PII concentration (mean ± SD) of untreated control fish or fish injected i.m.with turpentine and sampled at different times after the injection (n= 4 - 7). The time points following injection shown on the x-axis are not drawn to scale.

4.2.5. Total IgM

The results of the IgM analysis are shown in Figure 9. The mean IgM concentration varied from 2.6 to 8.7 mg mL^{-1}. At 24 h the IgM level of the turpentine injected fish was significantly higher than of the control fish at same time point or 5.4 + 2.1 and 2.6 ± 1.4 mg ml^{-1}, respectively (p=0.038). At 168 h the IgM level of the turpentine injected fish was significantly lower than that of the control fish at the same time point or 4.0 ± 1.3 and 8.7 ± 3.1 mg ml^{-1}, respectively (p=0.029).

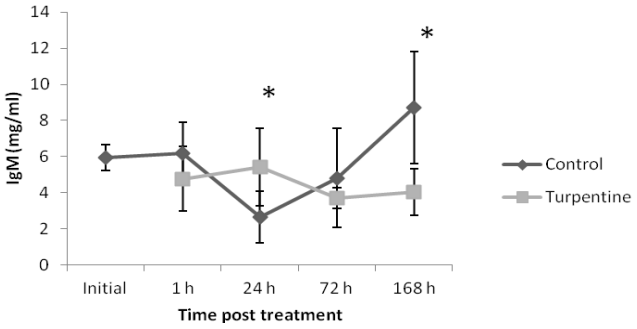

Figure 9: Results of the serum IgM analysis

The serum IgM concentration (mean ± SD) of Atlantic cod, untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection (n= 4 - 7). Asterisks (*) indicate a significant difference (P<0.05) in the IgM level of the turpentine treated fish compared to the control fish at the same timepoint. The time points following injection shown on the x-axis are not drawn to scale.

4.2.6. Natural antibody activity

The results of analysis of the natural antibody activity are shown in Figure 10. There was no statistically significant difference between the natural antibody activity of the treated and the untreated fish at any time point. The mean OD values were from 0.43 to 0.71 The mean natural antibody activity at the start of the experiment (0 h) was 0.534 ± 0.126 OD and at the end of the experiment (at 168 h) the mean natural antibody activity of the control fish and the treated fish was 0.587 ± 0.185 OD and 0.490 ± 0.084 OD respectively.

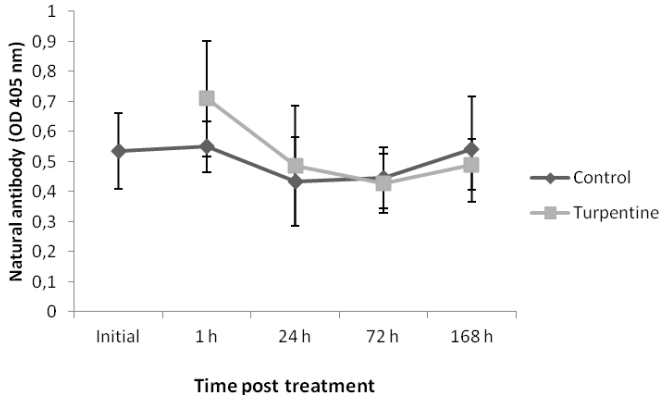

Figure 10 : Results of the serum natural antibody acitvity analysis

The natural antibody (anti-TNP-BSA) activity (mean ± SD) of untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection (n= 4 - 7). No significant difference in natural antibody activity was observed between time matched control and turpentine injected fish. The time points following injection shown on the x-axis are not drawn to scale.

4.2.7. Anti-protease activity

The results of the analysis of the anti-trypsin activity are shown in Figure 11. The mean anti-trypsin activity varied from about 30 to 57% inhibition. At the start of the experiment the mean activity was 41.8 ± 4.4% inhibition. At all sampling times the turpentine injected fish showed lower anti-trypsin activity when compared to the time-matched control fish. There was no statistically significant difference between the anti-protease activity of the treated and the untreated fish at any time point. The highest anti-trypsin activity of 56.6% trypsin inhibition was seen in the control fish after 168 h and the lowest activity of 29.7% was observed in the turpentine injected fish after 24 h.

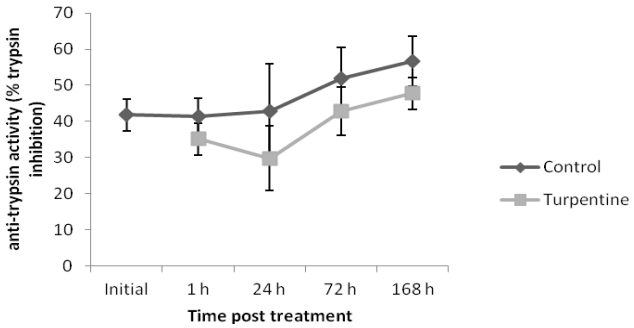

Figure 11: Results of the serum anti-trypsin activity

The anti-trypsin activity (mean ± SD) of untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection (n= 4 - 7). There was no statistically significant difference between the anti-protease activity of the treated and the untreated fish at any time point The time points following injection shown on the x-axis are not drawn to scale.

4.2.8 Summary of the serological analysis

A summary of the serological results at matched sampling times is shown in Table 3. The turpentine injection had limited effect on the serum concentration other than cortisol, which showed 26 fold increased at 72 h after injection and IgM, which showed 2 fold increased after 24h and 2.2 fold decreased after 168 h.

Table 3: Summary of the serological analysis

The table shows statistically significant increase (+) or decrease (-) in the 7 parameters tested in the turpentine injected fish compared to the control fish at the same time point. Non-significant change is indicated by n.s. The table also shows the fold change between the turpentine injected group and the control group and the degree of significance (p).

Parameter/time	1 hour	24 hours	72 hours	168 hours
Total protein	n.s.	n.s.	n.s.	n.s.
Cortisol	n.s.	n.s.	+ (p=0.02) (26fold)	n.s.
CRP-PI	n.s.	n.s.	n.s.	n.s.
CRP-PII	n.s.	n.s.	n.s.	n.s.
IgM	n.s.	+ (p=0.038) (2 fold)	n.s.	- (p=0.029) (2.2 fold)
Nat. antibodies	n.s.	n.s.	n.s.	n.s.
Anti-trypsin	n.s.	n.s.	n.s.	n.s.

4.3. Gene expression analyses

4.3.1 Preparation

4.3.1.1. Assays of RNA quality

RNA quality was assayed on an Agilent 2100 Bioanalyzer. RNA Integrity Numbers (RIN) ≥8 were considered acceptable for RTqPCR analyses. Samples with RIN values <8 were omitted from the study. All spleen samples had RIN values ≥8. The same was true for all head kidney samples, except one (RIN=7.7, Figure 12a, lane 2 (sample 27k)). All the liver samples had RIN values <8.

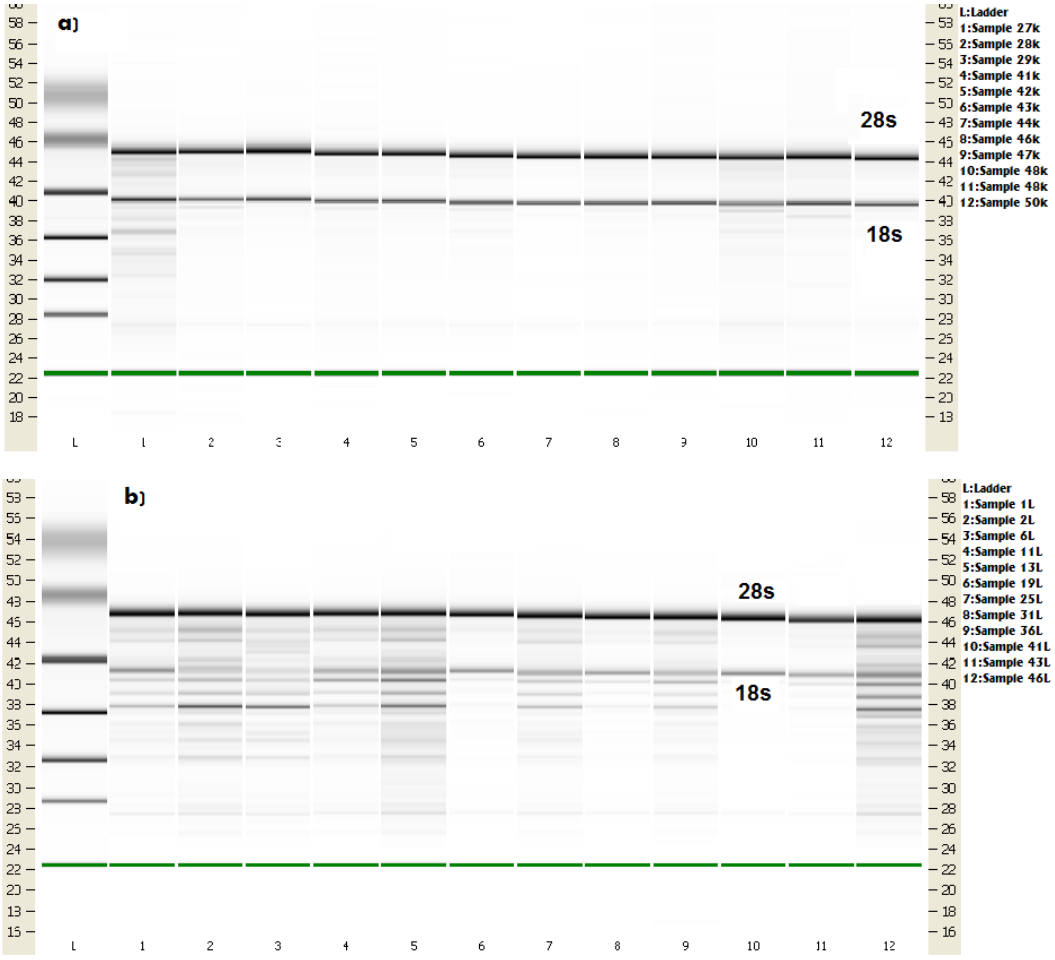

Figure 12: Bioanalyzer analyses of RNA quality

An example of Bioanalyzer analyses of RNA quality in a) 12 head kidney samples and b) 12 liver samples. The 28S and 18S ribosomal RNA (black) bands are observed for all samples. The other bands (grey) represent RNA break-down products.

4.3.1.2. Assays of RNA purity

Total RNA samples were subject to two DNase treatments. First “on column” during the RNA isolation and then in a subsequent step after RNA isolation. Figure 13 shows an example of results from PCR analysis on reverse transcribed RNA which was treated in this manner. Reverse transcription was performed for each sample with, or without reverse transcriptase (-RT). The reverse transcriptions were used as template in PCR reactions with ubiquitin and EF1α primers (Table 1). The lack of

amplification in the –RT samples shows that the total RNA samples used for the reverse transcription were essentially free of contaminating genomic DNA.

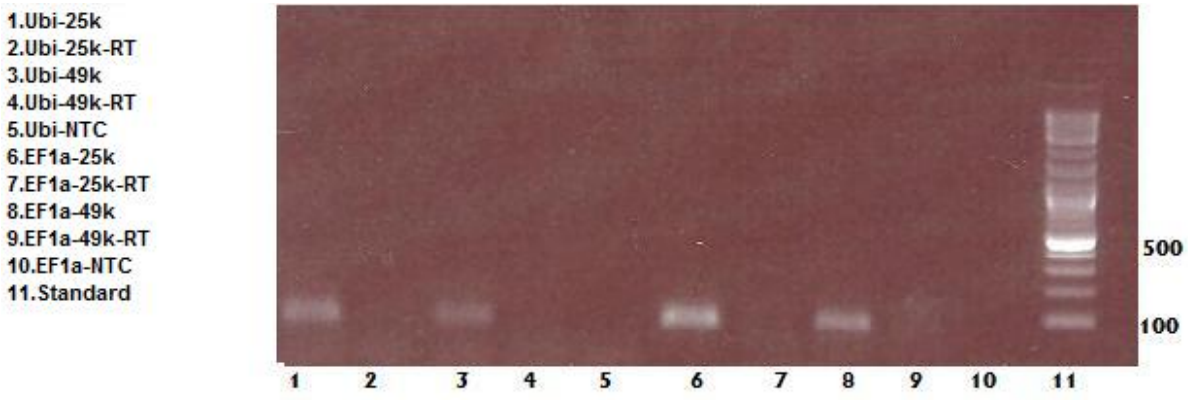

Figure 13: Electrophoresis of PCR products from PCR reactions.

Electrophoresis of PCR products from PCR reactions (ubiquitin (lanes 1-5) or EF1α (lanes 6-10) primers) on reverse transcribed total RNA with or without (-RT) reverse trancriptase. The absence of amplification product in –RT samples indicates that the total RNA samples did not contain much genomic DNA contamination.

4.3.1.3. Validation of reference genes

The choice of the two reference genes, ubiquitin and EF1α, was based on results of Olsvik et.al (Olsvik, et al., 2008) and Furnes et.al (Furnes, et al., 2009). To validate the use of these genes as reference genes in this study their expression was examined in samples from each time point. The results are shown in Figure 14. Ubiquitin was relatively stable in both head kidney (Ct variance=0.24) (Figure 14c) and spleen (Ct variance=0.30) (Figure 14d). EF1α expression was relatively stable in the spleen samples (Ct variance=0.32) but varied considerably (Ct variance= 2.61) in the head kidney samples. Based on this, ubiquitin was used as a reference gene in the study.

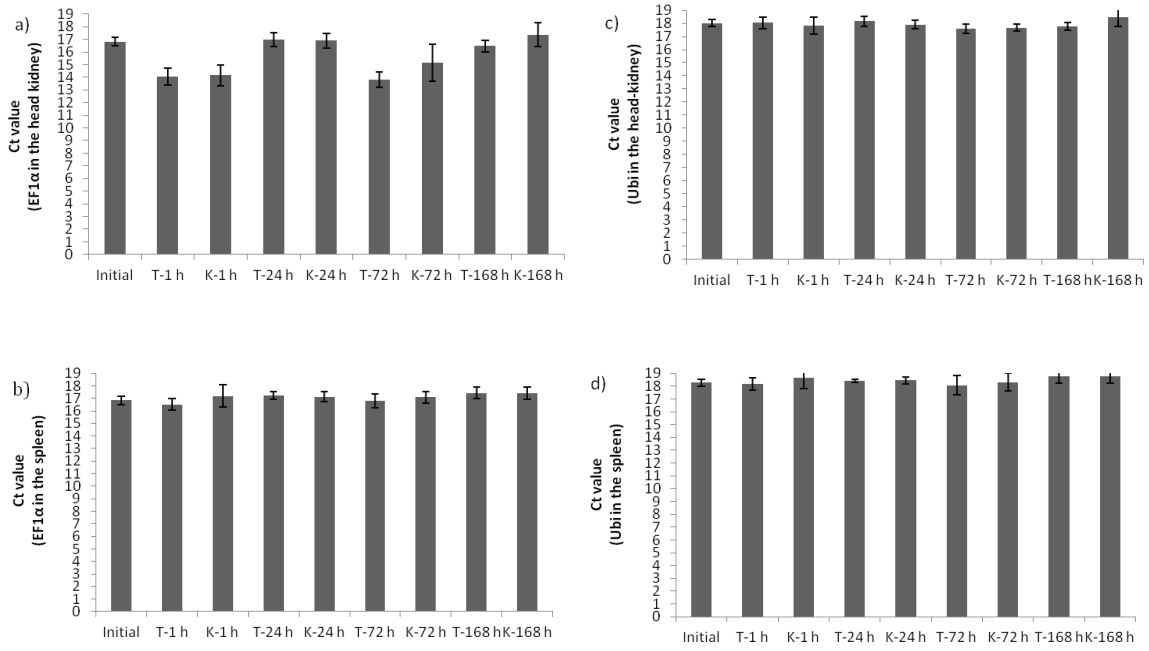

Figure 14: The cycle threshold (Ct) values of untreated control fish and turpentine injected fish.

Histograms showing the cycle threshold (Ct) values of untreated control fish and turpentine injected fish for a) RTqPCR of EF1α in head kidney (Ct variance=2.61), b) RTqPCR of EF1α in spleen (Ct variance=0.32), c) RTqPCR of ubiquitin in head kidney (Ct variance=0.24), and d) RTqPCR of ubiquitin in spleen (Ct variance = 0.30). The fish was sampled over a period of 168 h (7 days). The bars represent the mean values of 3-7 fish and the error bars show the standard deviations of the means. The time points following injection shown on the x-axis are not drawn to scale.

4.3.2. Expression of the target genes

The expression of all target genes included in the study was measured using reverse transcription of total RNA followed by quantitative real-time PCR (RTqPCR). The results from these measurements are shown in Figures 15 and 16 as box and whisker plots of logarithmically transformed relative quantities (log(RQ)) of gene expression. The qPCR data for each gene examined, in either kidney or spleen, were calibrated against a control fish from 0 h which was selected as the fish with the median Ct value for that gene at 0 h. Relative quantity (RQ) values compared to this fish were calculated using ubiquitin as a reference gene and an equation described by Pfaffl (Pfaffl, 2001). Considerable individual variation was seen in the expression of all the test genes throughout the study resulting in relatively large standard error values for mean expression.

To determine the effects of turpentine injection on gene expression in injected fish, the expression in injected fish was compared to that of control fish harvested within the same timepoint, i.e. 1 h, 24 h, 72 h, or 168 h. This was done with the REST 2009 software as described in materials and methods.

4.3.2.1 Differences in the gene expression of CRP-PI, CRP-PII, ApoLP A-I, and C3 between turpentine injected and untreated controls.

One hour after turpentine injection there was a statistically significant difference in the anterior kidney of injected fish, compared to control fish harvested at the same time point, for CRP-PI gene

expression (4.9 fold higher, *P*=0.013), CRP-PII gene expression (24.5 fold higher, *P*=0.004), and ApoLP A-I gene expression (7.1 fold higher, *P*=0.007) as shown in Figs. 15 A, C, and E, respectively. At other time points there was no statistically significant difference in the expression of these genes between the two groups in the kidney. In the spleen there was no statistically significant difference at any time point (Figs. 15 B, D, F, and H). The expression of the complement C3 gene was also only affected in the anterior kidney of injected fish and was significantly elevated (8.9 fold, *P*=0.016), compared to controls, at 72 h.

4.3.2.2 Differences in the gene expression of IL-1β, transferrin, hepcidin, and cathelicidin between turpentine injected and untreated controls

In turpentine injected fish there was a significant difference in IL-1β and cathelicidin gene expression at 72 h, compared to controls, in both the anterior kidney (IL-1β: 7.8 fold higher, *P*=0.023; cathelicidin: 2.2 fold higher, *P*=0.027) and the spleen (IL-1β: 22.4 fold higher, *P*=0.006; cathelicidin: 4.1 fold higher, *P*<0.001) (Figs. 16 A, G, B, and H, respectively). Transferrin gene expression was also significantly elevated in both organs, in the anterior kidney at 72 and 168 h (6.3 fold higher, *P*=0.012 and 8.6 fold higher, *P*=0.018, respectively) and in the spleen at 72 h (9.9 fold higher, *P*=0.008) (Figures 16. C, and D). Hepcdin gene expression only differed from controls in the spleen at 24 h (3.2 fold higher, *P*=0.04) (Figure 16. F). Within other time points the expression of IL-1β, transferrin, hepcidin, and cathelicidin was not significantly affected in the kidney, or the spleen, of turpentine injected fish compared to the controls.

4.3.2.3 Stability of gene expression in untreated controls

Immune- or acute phase-relevant genes should be “stable” in the untreated fish at all time points throughout the study. Any deviation from this could indicate that the fish in the study had, for example, been subject to external stress, infection, or variation in water quality. The expression of the genes of interest included in this study was compared between time-points by one-way ANOVA, both in control fish and turpentine injected fish. The results (Table 4) show that in the control fish there was no significant difference in the expression of the genes analyzed in this study between time points, with the exception of ApoLP A-I in the kidney.

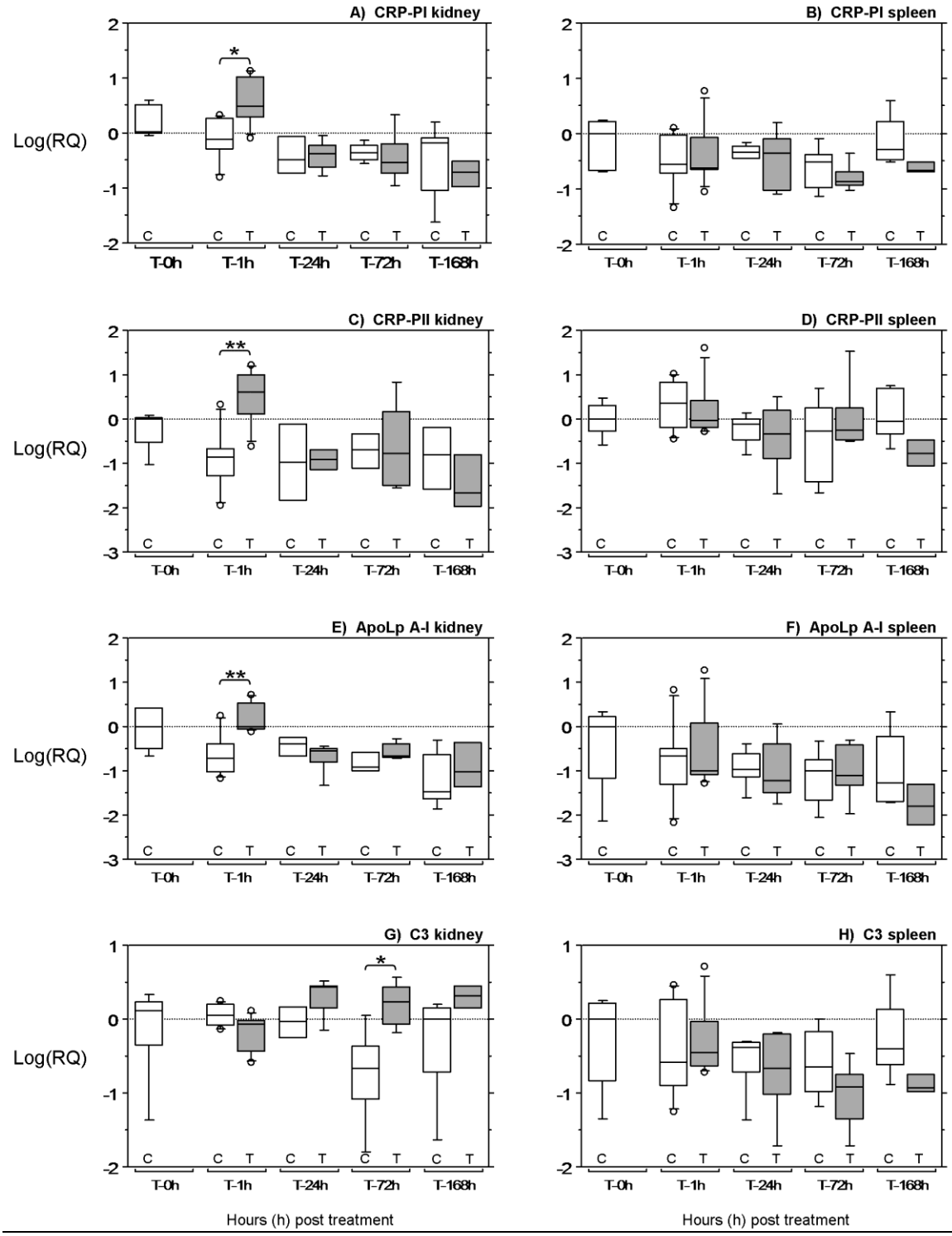

Figure 15: The expression of CRP-PI, CRP-PII, ApoLP A-I and C3 measured by Real Time PCR

Box and whisker plots that show the gene expression (log(RQ)) of CRP-PI, CRP-PII, ApoLP A-I, and C3 in the anterior kidney and spleen of turpentine injected cod (gray boxes) and untreated controls (empty boxes) for each time point (0 h, 1 h, 24 h, 72 h, and 168 h) of the sampling period. The asterisks indicate a statistically significant difference as calculated with REST2009 (* $p<0.05$, ** $p<0.01$; for precise *P* values see Table 4) in gene expression of turpentine injected fish compared to controls within time points. The bottoms of the boxes in the plot represent the 25th percentile and the top the 75th percentile; the band within the boxes shows the median. The whiskers show the 10th percentile and the 90th percentile. Values that fall above the 90th percentile and below the 10th percentile are presented as open circles.

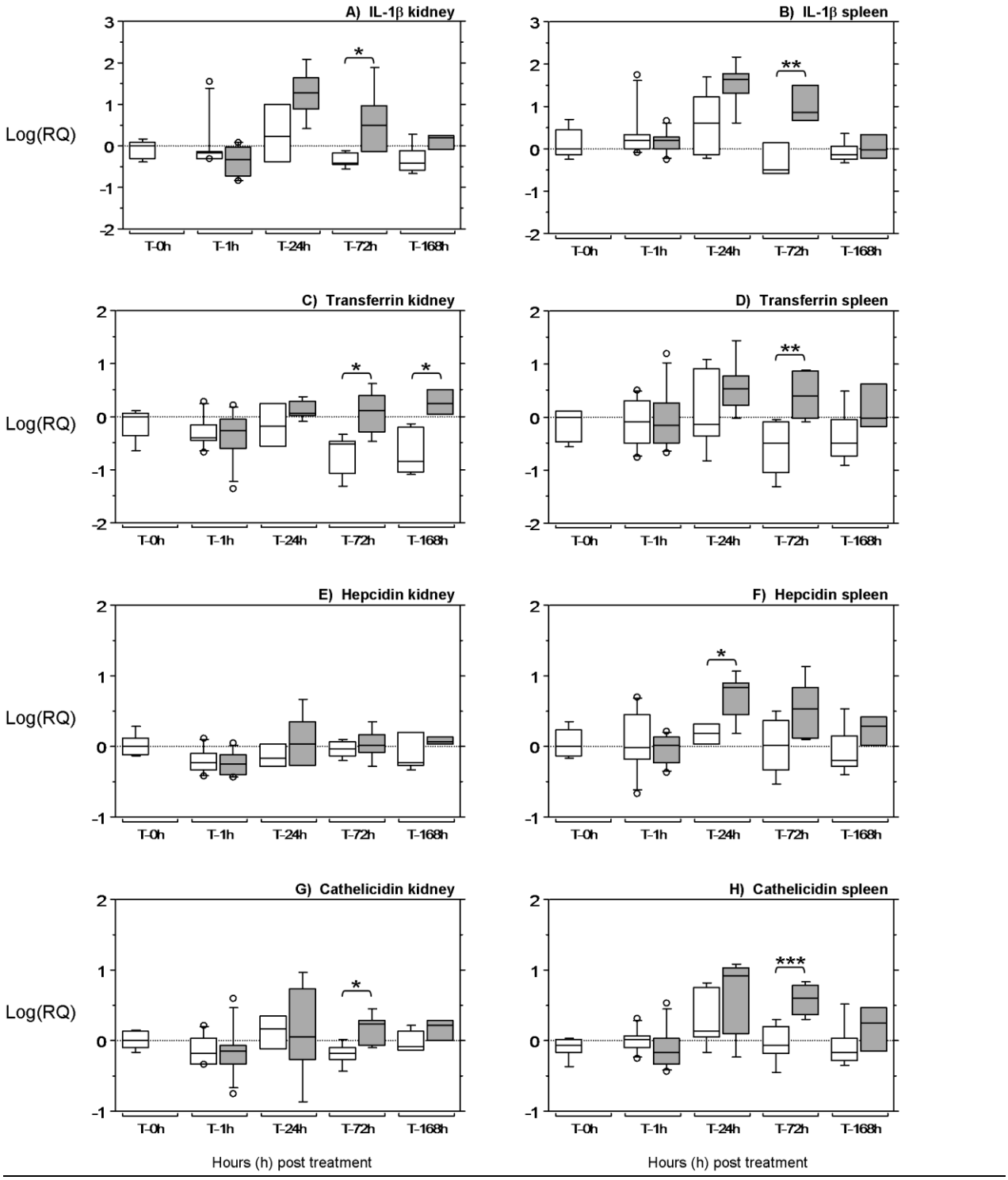

Figure 16: The expression of IL-1β, transferrin, hepcidin and cathelicidin by Real Time PCR

Box and whisker plots that show the gene expression (log(RQ)) of IL-1β, transferring, hepcidin and cathelicidin in the anterior kidney and spleen of turpentine injected cod (gray boxes) and untreated controls (empty boxes) for each time point (0 h, 1 h, 24 h, 72 h, and 168 h) of the sampling period. The asterisks indicate a statistically significant difference as calculated with REST 2009 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; for precise *P* values see Table 4)) in gene expression of turpentine injected fish compared to controls within time points. The bottoms of the boxes in the plot represent the 25th percentile and the top the 75th percentile; the band within the boxes shows the median. The whiskers show the 10th percentile and the 90th percentile. Values that fall above the 90th percentile and below the 10th percentile are presented as open circles.

4.3.3 Summary of the gene expression analysis

A summary of the gene expression results at matched sampling times is shown in Table 4. The CRP-PI (4.9 fold), CRP-PII (24.5 fold) and ApoLP A-I (7.1 fold) showed increased ecpression in head kidney at 1 h after injection. The C3 showed 8.9 fold increased 24 h after injection. IL-1β showed 7.8 fold increased in the head kidney and 22.4 fold increased in the spleen 72 h after injection. Transferrin showed 6.3 fold increased at 72 h and 8.6 fold increased at 168 h after injection in the head kidney and 9.9 fold increased at 72 h after injection in the spleen. Cathelicidin showed 2.2 fold increased in at 72 h after injection in the spleen and 4.1 increased at 72 h in the spleen. Hepcidin showed 3.2 fold increased at 24 h after injection in the spleen.

Table 4: Summary of the gene expression analysis

The table shows the results from one-way ANOVA analyses of gene expression between time points in the controls and turpentine injected fish. It also shows the results (fold change and *P*-value) from comparisons of gene expression in injected and control fish within timepoints as calculated with the REST2009 software. No significant difference is indicated by n.s. a) Shows the results for CRP PI, CRP PII, ApoLP AI and C3. B) Shows the results for IL-1β, transferrin, hepcidin and cathelicidin.

a) Gene expression of CRP-PI, CRP-PII, ApoLP-AI and C3.

Gene	**Organ**	**One way ANOVA**		**Fold change and *P* value treated vs. control within timpoints**			
		Control	**Treated**	**1h**	**24h**	**72h**	**168h**
CRP PI	Kidney	*F*(4,20)=2.15, *P*=0.112	*F*(4,21)=10.87, *P*<0.001	4.9 *P*=0.013	n.s.	n.s.	n.s.
	Spleen	*F*(4,21)=1.37, *P*=0.278	*F*(4,20)=1.17, *P*=0.352	n.s.	n.s.	n.s.	n.s.
CRP PII	Kidney	*F*(4,16)=0.68, *P*=0.615	*F*(4,17)=4.62, *P*=0.015	24.5 *P*=0.004	n.s.	n.s.	n.s.
	Spleen	*F*(4,21)=1.31, *P*=0.298	*F*(4,20)=1.45, *P*=0.254	n.s.	n.s.	n.s.	n.s.
ApoA-I	Kidney	*F*(4,20)= 4.05, *P*=0.015	*F*(4,21)=6.14, *P*=0.002	7.1 *P*=0.007	n.s.	n.s.	n.s.
	Spleen	*F*(4,21)=0.45, *P*=0.771	*F*(4,20)=1.53, *P*=0.232	n.s.	n.s.	n.s.	n.s.
C3	Kidney	*F*(4,20)=1.64, *P*=0.203	*F*(4,21)=2.03, *P*=0.128	n.s.	n.s.	8.9 *P*=0.016	n.s.
G	Spleen	*F*(4,21)=0.35, *P*=0.843	*F*(4,20)=2.00, *P*=0.134	n.s.	n.s.	n.s.	n.s.

b) Gene expression of IL-1β, transferring, hepcidin and cathelicidin.

Gene	Organ	One way ANOVA		Fold change and P value treated vs. control within timepoints			
		Control	Treated	1h	24h	72h	168h
IL-1b	Kidney	$F(4,20)=1.20$, $P=0.340$	$F(4,21)=8,48$, $P<0.001$	n.s.	n.s.	7.8 $P=0.023$	n.s.
	Spleen	$F(4,21)=2.11$, $P=0.116$	$F(4,19)=10.86$, $P<0.001$	n.s.	n.s.	22.4 $P=0.006$	n.s.
Transferrin	Kidney	$F(4,20)=2.53$, $P=0.073$	$F(4,21)=2.58$, $P=0.067$	n.s.	n.s.	6.3 $P=0.012$	8.6 $P=0.018$
	Spleen	$F(4,21)=1.29$, $P=0.308$	$F(4,20)=1.65$, $P=0.200$	n.s.	n.s.	9.9 $P=0.008$	n.s.
Hepcidin	Kidney	$F(4,20)=0.99$, $P=0.434$	$F(4,21)=1.95$, $P=0.140$	n.s.	n.s.	n.s.	n.s.
	Spleen	$F(4,20)=0.23$, $P=0.920$	$F(4,20)=5.77$, $P=0.003$	n.s.	3.2 $P=0.040$	n.s.	n.s.
Cathelicidin	Kidney	$F(4,20)=1.62$, $P=0.210$	$F(4,21)=0.67$, $P=0.617$	n.s.	n.s.	2.2 $P=0.027$	n.s.
	Spleen	$F(4,21)=1.78$, $P=0.171$	$F(4,20)=4.86$, $P=0.007$	n.s.	n.s.	4.1 $P<0.001$	n.s.

4.4 Sequence analysis of CRP-PII

Previously, Gisladottir et al (Gisladottir, 2008) described individual variation in the number and size of CRP-PII reactive proteins in Western blots of cod serum. In an attempt to find an explanation for this variability the genomic sequence of the CRP-PII gene from 13 individual was PCR amplified and cloned into TOPO vectors. Sequencing of these clones revealed that they were of equal length and highly homologous, although there was some variation in their nucleotide sequence between individual fish (figure 17 and table 5).

```
                                                  ac
ttcgcccttgtgttccccgaggagaccgccaacagcttcgtagagctgttttcccgccaag       60
gagctcagcctgaacgccttcacgctctgcctgaggttcgccacggagctcaaagggaag       120
                                  a  t  t
cgtgaggtcatcctcttcgcctaccgcacgcgggacttcgacgaactcaacgtgtggcgc       180
    ca  c
gagaaggatggcaggtgagctgctggattccaaatgttccctgaaaagatatgttgtcca       240
tgaaatcattccggaacgaatgccctttattcatgtggaattaatttaaaaaatcattca       300
aagaaaacaagtcgaaacgtagaatcattttaataaaacgtatgtagggaaaacatagat       360
ttgagatgcatgaaatggttaattactacaaaacaaagaagttcttcatgacatcctcag       420
aacatggattctgactgcctctcttctatcgtttaattcagtgaggacgtaattggccgt       480
gttcaaatctaatttaaaaaaataattttaagcaaatcgggcaaaaattaaatgctaact       540
ggcacctttccatcaactgatcggcagcgaataagcgaggctaagaaaagcctataagtg       600
gtcgtcatggttttcccatggatgagatgagaggaatactttggcctcctcatggttcca       660
cacgtaggccctacctgtttgatgctaccagcttcattttcaaacggtaggagagaatct       720
gggaaggcaccagcattggaaaagcctaataacaactcagaagggttgcatacgcgtgac       780
tgaacatgaaaaggtttacgactgaaacccgagcagcggtctggggttcatagaagcaaa       840
aaatcacctttgagttccaactaacccacatgattttctgagccgatactcgtccacatc       900
tgtgtattgtgttcacggcgtgtgtttgttgttttttttcaggctgtccctctacctgagc       960
ggaccgggggctctctttgaggttccggagcttggaccgctggcgaaccacgtctgtttg       1020
acctgggagtcgctgacggggcgaaccacgatgtacgtcaacggccggagttctgcgagc       1080
cagatcttccagaggggccacagggtgcggccgggggggaaagattatcctgggccaggac       1140
                                  g
cccgacaacttccttggagacttccaggccaagcagagttttgtgggagagatcttcggg       1200
gtgaacatgtgggactacgttctgccttcagtcgcaattcagctgctgtccactggggaa       1260
                                                        g
gatttctctgatgccaacgtccttgactgggccacggtgactcttatgcccactgggagc       1320
             ta
gctgttgagtacaagtaaagctccctagatacaagcaagggcgaat             1366
```

Figure 17: The Consensus nucleotide sequence of the sequenced CRP-PII clones with polymorphic nucleotides shown in red.

Table 5: A summary of the nucleotide variability in the genomic sequence between individuals.
Note: there was variability in the nucleotide sequence of different clones from fish nr. 9, as indicated by 2 possible nucleotides per location.

Fish/bases	49	50	152	154	156	185	186	189	1171	1313	1335	1336
6	A	C	G	G	C	A	G	T	A	G	T	A
7	T	T	G	G	C	A	G	T	G	A	G	T
8	T	T	G	G	C	A	G	T	A	A	G	T
9	T	T	A - G	T- G	C- T	A- C	G- A	T- C	G	A	G	T
11	A	C	G	G	C	A	G	T	A	G	T	A
12	T	T	G	G	C	A	G	T	A	A	G	T
13	T	T	G	G	C	A	G	T	G	A	G	T
14	A	C	G	G	C	A	G	T	A	A	G	A
15	T	T	G	G	C	A	G	T	G	A	G	T
18	A	C	A	T	T	C	A	C	A	G	T	A
24	T	T	G	G	C	A	G	T	G	A	G	T
25	A	C	G	G	C	A	G	T	A	G	T	A
29	T	T	G	G	C	A	G	T	G	A	G	T

Nine of the twelve nucleotide polymorphisms (table 5) detected in the CRPPII clones resulted in amino acid change (figure 18 and table 6). Interestingly, one of them (nucleotide 1336) resulted in a STOP codon.

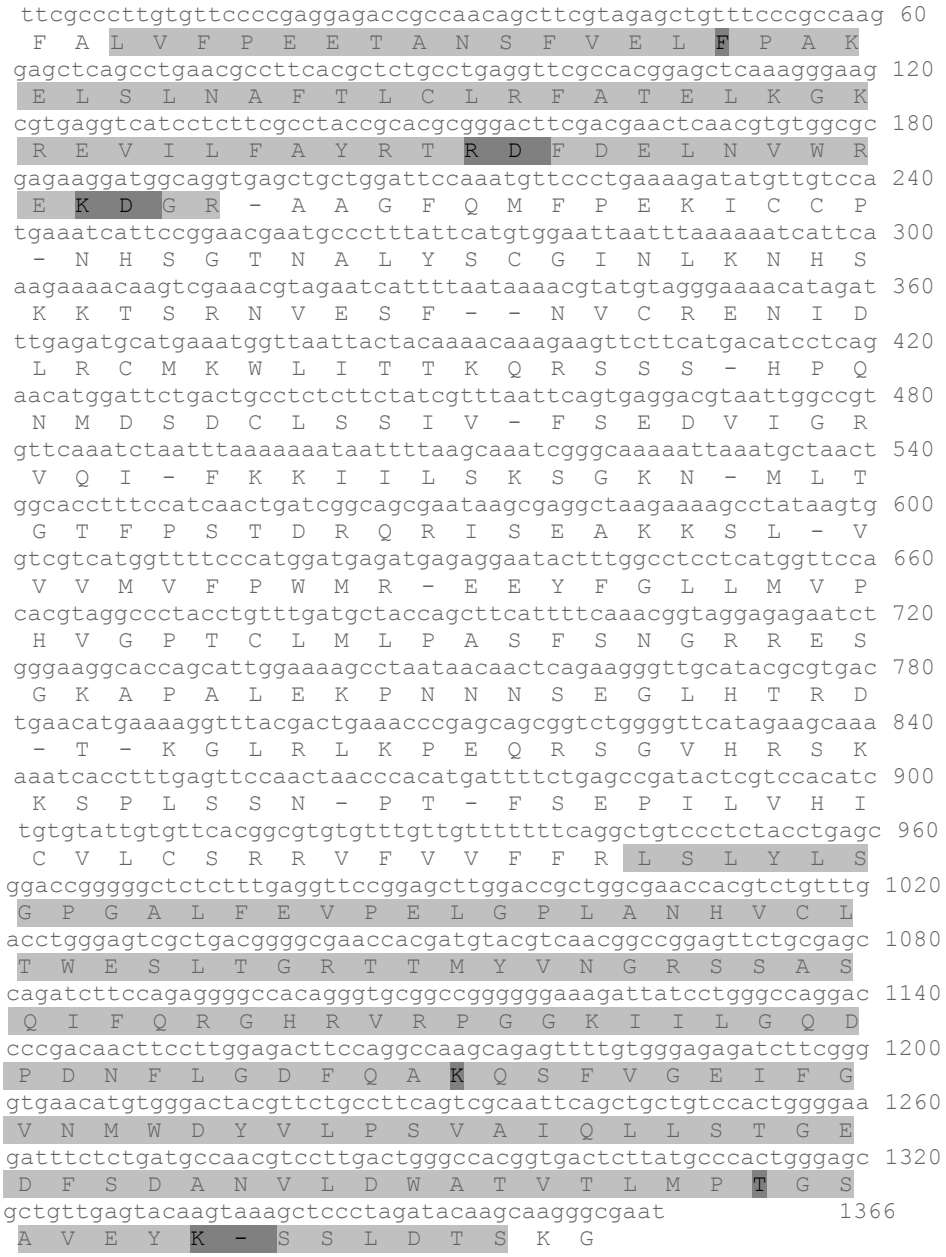

Figure 18: The nucleotide sequence and deduced amino acid sequence of CRP-PII protein in cod according to the sequenced clones.

The highlighted (yellow) amino acids indicate the exons of the genomic DNA sequence. The amino acids highlighted with red were variable between individuals as shown in table 6.

Table 6: Amino acid variability between individuals based on nucleotide sequence.

Note: The variability in amino acids between fish. Fish nr. 9 also showed variability between clones, therefore 2 amino acids are shown. The charges of the amino acids are shown as (+) for positively charged amino acids and (-) for negatively charged amino acids.

Fish / bases	49-51	151-153	154-156	184-186	187-189	1171-1173	1312-1314	1333-1335	1336-1338
6	T	R (+)	D (-)	K (+)	D (-)	K (+)	A	N	K (+)
7	F	R (+)	D (-)	K (+)	D (-)	E (-)	T	K (+)	STOP
8	F	R (+)	D (-)	K (+)	D (-)	K (+)	T	K (+)	STOP
9	F	Q	D -Y	K-T	D (-)	E (-)	T	K (+)	STOP
11	T	R (+)	D (-)	K (+)	D (-)	K (+)	A	N	K (+)
12	F	R (+)	D (-)	K (+)	D (-)	K (+)	T	K (+)	STOP
13	F	R (+)	D (-)	K (+)	D (-)	E (-)	T	K (+)	STOP
14	T	R (+)	D (-)	K (+)	D (-)	K (+)	T	K (+)	K (+)
15	F	R (+)	D (-)	K (+)	D (-)	E (-)	T	K (+)	STOP
18	T	Q	Y	T	D (-)	K (+)	A	N	K (+)
24	F	R (+)	D (-)	K (+)	D (-)	E (-)	T	K (+)	STOP
25	T	R (+)	D (-)	K (+)	D (-)	K (+)	A	N	K (+)
29	F	R (+)	D (-)	K (+)	D (-)	E (-)	T	K (+)	STOP
Total line	F	R (+)	D (-)	K (+)	D (-)	K (+)	T	K (+)	STOP

The results show a difference between individual, when the charged of the amino acid is considered. The positively charged amino acids are show as (+) and negatively charged amino acid as (-). Other amino acids are neutral.

5 Discussion

The acute phase response (APR) is a systemic reaction of the body to injury, trauma or infection. The response can result in the secretion of acute phase proteins (APPs) like the pentraxins (CRP and SAP) (Bayne & Gerwick, 2001). The main aim of the present study was to examine the effects of acute phase induction in cod on serological parameters including the stress hormone cortisol and several immune parameters, as well as the effects on expression of several possible APP genes, like the pentraxins and the antimicrobial peptides cathelicidin and hepcidin.

Many methods, like LPS or turpentine oil injection, have been used to induce APR (Carroll, et al., 2005; Fujiki, et al., 2001; Kushner & Feldmann, 1978; Liu, et al., 2004; Russell, et al., 2006). In this study turpentine injection was used to produce acute phase response in cod.

The increase of cortisol observed in the serum of the turpentine injected cod, and the trauma and tissue injury in the form of swelling and bleeding under the skin at the injection site, were visible signs that the cod was affected by the turpentine injection and would be expected to produce a systemic acute phase response.

The result of this turpentine injection experiment demonstrated that cod shows a different response to turpentine injection than to infection or vaccination (Caipang, et al., 2008a; Caipang, et al., 2008b; Caipang, et al., 2010; Magnadottir, et al., 2010).

The results also showed that individual fish differed from each other in their response to turpentine injection. The same varied response has been seen in other fish species, for example in rainbow trout in response to viral, bacterial and fungal inflammatory agents (Gerwick, et al., 2002).

5.1 Serology

The concentration of cortisol in serum had increased and reached a maximum level at 72 hours after the turpentine injection. After 168 hours the level had returned to approximately the base level. The control fish showed relatively low cortisol levels at all sampling times indicating that the experimental conditions and the handling of the fish involved, did not by themselves induce a stress response. The pre-injection cortisol levels seen were very similar to the resting levels reported in other studies of cod (Magnadottir, et al., 2010; Perez-Casanova, et al., 2008a; Pérez-Casanova, et al., 2008b). The increase of cortisol in this study was considerably higher and peaked earlier than was previously reported in cod infected with *Aeromonas salmonicida* ssp. *achromogenes* when a maximum 4.8 fold increase was observed after 6 days (Magnadottir, et al., 2010). The increase following turpentine injection was comparable to that of juvenile cod subjected to acute thermal challenge, which showed a 19- or 35- fold increase depending on the size of the fish (Perez-Casanova, et al., 2008a). The time-lag, from the induction of APR until the peak in serum/plasma cortisol level is observed can vary greatly depending on the type of induction, the fish species and physiological and environmental conditions. For example, haddock (*Melanogrammus aeglefinus*), a fish species closely related to cod, showed a peak in plasma cortisol levels 2 weeks after a daily handling stress for 4 weeks. After 3 weeks the level had declined to a level that was not significantly different from the pre-stress values (Hosoya, et al., 2007). Rainbow trout, on the other hand, showed an increase in cortisol level 1 hour

after being subjected to a confinement stress and a maximum peak was seen after 2 days (Cairns, et al., 2008). This may indicate that in general gadoid species are relatively slow responders to APR induction, an observation that is supported by other work on cod (Pérez-Casanova, et al., 2010). However, it has also been show that after short-term overcrowding the cortisol response peaked after 2 hours (Caipang, et al., 2008b).

The result of the total serum protein analysis in this study revealed no difference between the control group and the turpentine injected group but slightly reduced protein levels were observed in both groups from 24 hours onwards compared to the initial status. This may reflect the restricted feeding employed compared to the previous feeding regimes at the Marine Institute´s Experimental Station at Staður, Grindavík, since nutritional status and food consumption as well as various physiological and environmental factors have been shown to influence the protein concentration of fish serum (Love, 1981; Magnadottir, et al., 1999a; Melingen, et al., 1995). Caiping et al. (Caipang, et al., 2008a) found no difference in the serum protein concentration between non-vaccinated cod and cod that had been vaccinated with heat-killed *Listonella anguillarum*. A different situation was recorded in plaice since intra peritoneal injection (i.p.) with turpentine resulted in as much as 74% reduction in serum protein levels within 18 days compared to control fish, which were injected with saline (White, et al., 1981).

The total mean concentration of the pentraxins CRP-PI and CRP-PII in cod serum in the present experiment was approximately 100 µg ml^{-1}. Overall the pentraxin concentration was lower than had been determined previously in cod of similar size, which was kept under similar conditions (Magnadottir, et al., 2010). This difference may be the result of seasonal variation since the present group was sampled in the autumn while the previous group was sampled in the early spring. Great individual variation was seen in the concentration of the two pentraxins, especially of CRP-PI. This is comparable to what has previously been found in cod (Magnadottir, et al., 2010). The level of CRP-PI was also generally higher than that of CRP-PII although this difference was not statistically significant. The concentration of CRP-PI and CRP-PII in serum was not significantly affected by the turpentine injection, with only a slight suppression of their levels compared to the initial status. The suppressive effects were more marked in the case of the CRP-PI but neither protein seems to act as a typical APP.

Pentraxins are the classical positive APP in mammals and used, for example, as bioindicators of cardiovascular disease in human medicine (Bayne & Gerwick, 2001; Cray, et al., 2009). The role of pentraxins in the APR and the immune response of fish is not clear and seems to vary, from one species to another as well as being dependent on the type of acute phase induction used. In a comparable study where rainbow trout was injected with turpentine oil the serum CRP concentration was significantly reduced 1 day after injection compared to the initial value and the reduction persisted for up to 14 days (Liu, et al., 2004). Other research on rainbow trout has revealed different responses to different chemicals. For example, the serum level of CRP in rainbow trout increased significantly reaching a maximum at 3 days after exposure to metriphonate and then decreased to below normal levels, while the level of CRP had decreased significantly at 14 days after exposure to potassium permanganate (Kodama, et al., 2004). Hoover *et al*. in a study of rainbow trout demonstrated the pattern recognition function of fish pentraxins and a potential role in the pathogenesis of furunuculosis

(Hoover, et al., 1998). Research has shown that the serum level of CRP increased in common carp and two different carp lines, Polish K and Israeli D, after infection with *Aeromonas hydrophil,* however no increase was seen in the common carp after injection with *E. coli* lipopolysaccharide (LPS) serotype 0111:B4 (MacCarthy, et al., 2008). The CRP levels in major carp (*Catla catla*) increased 2.8-3.5 fold following exposure to pollutants (Paul, et al., 1998). An increase in the serum concentration of CRP-like protein was also reported in channel catfish following turpentine injection while the level was reduced following infection and a low temperature shock (Szalai, et al., 1994).

The results of the IgM analysis in serum showed that the concentration of IgM was significantly increased at 24 hours after the turpentine injection and was significantly reduced after 168 hours compared to the time-matched control fish. The reduced IgM concentration following the peak in cortisol activity suggests that cortisol can have suppressive effects on the IgM concentration. This agrees with both *in vivo* and *in vitro* studies that have demonstrated the suppressive effects that cortisol administration can have on the number of B-lymphocytes and IgM secretion in fish (Espelid, et al., 1996; Saha, et al., 2004). In infected cod the IgM concentration was shown to be suppressed at 6 days post infection compared to the time-matched control fish, which coincided with the maximum cortisol response (Magnadottir, et al., 2010).

The result of the analysis of the natural antibody activity in serum showed no difference between the control group and the turpentine injected group at any time point. In a previous study infection was shown to suppress the natural antibody activity at 6 days post infection compared to the time-matched control fish (Magnadottir, et al., 2010). There appears therefore to be some difference in the effects that APR induced by turpentine injection can have on natural antibody activity and IgM concentration of cod compared to APR induced by infection.

The result of the analysis of the anti-trypsin activity in serum showed no statistically significant difference, albeit the turpentine injected cod showed slightly lower anti-trypsin activity at all sampling times compared to the control group. Although not statistically significant using the Mann Whitney test this may indicate that the parameters that contribute to the antitrypsin acitivity (like α1-anti-trypsin and α2-macroglobulin) could act as negative APPs in cod and that their activity is possibly not affected by increased cortisol levels. In mammals and fish, both α1-antitrypsin and α2-macroglobulin have been described as weak positive or negative APPs (Bayne & Gerwick, 2001; Sitjà-Bobadilla, et al., 2006). In a previous study, infection was shown to have a similar suppressive effect on the anti-trypsin activity of cod (Magnadottir, et al., 2010).

It might be that the cortisol had suppressive effects on other factors in serum. It is known that cortisol has supressive effects on a number of immune response in fish (Fast, et al., 2008; Harris & Bird, 2000).

5.2 Gene expression

The material for reverse transcription quantitative PCR (RTqPCR) needs to be of good quality, so the results will not be adversely affected (Bustin & Nolan, 2004; Olsvik, et al., 2005). When the quality of the RNA extracted from the liver, head kidney and spleen was determined the results showed that the RNA from the liver tissue was substantially degraded and could not be use for RTqPCR analysis.

However, the quality of the head kidney and spleen RNA was acceptable. The difference in the RNA quality between the liver and the other tissues is probably due to the poor stability of the relatively fat liver tissue during storage in the RNA later solution. Liver samples harvested in another study that were snap-frozen in liquid nitrogen and stored at -80°C until used produced good quality RNA (data not presented).

The cod pentraxins, CRP-PI and CRP-PII, and the six other immune parameters examined were all constitutively expressed in both the anterior kidney and the spleen and their expression was augmented by the injection of turpentine. An exception to this was the CRP-PII expression in the anterior kidney of three individual fish from the treated group and four from the control group where no ampification was observed. A possible explication for this is that the CRP-PII levels in these fish was not high enough for the real time PCR assay to detect the expression of CRP-PII.

The results showed a certain pattern of response with respect to the timing of the maximum response and whether the response was restricted to the anterior kidney or the spleen or seen in both organs. In the anterior kidney the gene expression of the pentraxins, CRP-PI and CRP-PII, ApoLP A-1 and complement component C3 was significantly increased but no significant difference was seen in the spleen. The peak in expression of the pentraxins and ApoLP-A1 was seen early, at 1 hour after (post) injection (hpi), while the expression of C3 occurred later, at 72 hpi. A significant increase in the expression of the antibacterial peptide cathelicidin, the pro-inflammatory cytokine IL-1β, and transferrin was seen in both organs at a later stage, at 72 hpi. Hepicidin expression was only induced in the spleen.

In the present study the expression of the pentraxins CRP-PI and CRP-PII was significantly increased 1 hour after turpentine injection in the head kidney compared to the control fish. This was in contrast to the serum protein measurements of the pentraxins in the same fish. The relative increase in gene expression of CRP-PII was 5 fold higher than the relative increase in expression of CRP-PI. This suggest that both types of cod pentraxins may act as early mediators of APR in the head kidney.

A few studies have examined the expression of the pentraxins genes in fish tissues and with variable results. Research on rainbow trout has revealed that SAP-like pentraxin expression was reduced in the liver after confinement stress (Talbot, et al., 2009). Other research has shown no difference in the expression of pentraxins in rainbow trout liver after primary and secondary infections with *Yersina ruckeri* 01 (Raida & Buchmann, 2009). In Zebrafish (*Danio rerio*) the expression of CRP-like pentraxins in internal organs has been shown to increase at various time-points following *A. salmonicida* or *S.aureus* infection (Lin, et al., 2007).

The gene expression of ApoLP A-I was significantly elevated in the head kidney at 1 hour after injection suggesting that it may also to act as an early mediator of APR in cod. Research on carp shows that apolipoprotein has antimicrobial activity against gram negative and gram positive bacteria in teleost fish (Concha, et al., 2003). Another study on rainbow trout supports the view that ApoLP A-I plays an important defensive role against bacterial disease (Villarroel, et al., 2007). It has been shown that ApoLP A-I might have a regulatory role in the complement system by affecting the formation of the membrane attack complex (MAC, C5b-9) (Hamilton, et al., 1993). Inhibition of the MAC cascade

through the binding of C3 has also been suggested to take place in cod under certain conditions (Magnadóttir & Lange, 2004).

The role of the pentraxins and ApoLP-AI as early mediators in the APR of cod probably involves the activation of the complement system by stimulating the C3 expression, which peaked later in the kidney at 72 hpi. One of the known functions of CRP is the activation of the complement system (Ballou & Kushner, 1992; Mold, et al., 1999). In mice, CRP does not behave as an APP but it protects mice against bacterial infection and inflammatory conditions (Mold, et al., 1999). This is achieved by the activation of the classical complement pathway through ligand-binding to the complement factor C1q but compared to the activation by C3 by antibody-antigen complexes this results in more limited inflammatory effects and hence, reducing the damaging effects of inflammation (Black, et al., 2004; Mold, et al., 1999). This function has been demonstrated experimentally in rainbow trout (Nakanishi, et al., 1991). The complement C3 protein is one of the first lines of defence against pathogens (Sunyer & Lambris, 1998). C3 has been shown to be a positive APP in both mammals and common carp (Bayne & Gerwick, 2001; Gabay & Kushner, 1999; Gonzalez, et al., 2007) but the role of C3 in other fish species appears to be more varied in Eurasian perch (*Perca fluciatilis*) it was shown that handling stress did not affect C3 expression in the spleen (Milla, et al., 2010) , which agrees with the present result and down regulation was, for example, reported in the liver of vaccinated rainbow trout (Gerwick, et al., 2007). In common carp the expression of C3 in liver has been shown to increase, showing peak expression at 24 hours after infection with *Ichthyophthirius multifiliis* (Gonzalez, et al., 2007).

These early mediators, the pentraxins and ApoLP A-I, could also be involved in the cortisol release from the neuroendocrine cells of the kidney, which peaked at 72 hpi in the same fish, as discussed above. This might have been *via* induction of the gene expression of the pro-inflammatory cytokine, IL-1β, as seen in the present study. The expression of IL-1β was significantly increased at 72 hpi in the spleen and in the kidney. Interleukin-1β is a pro-inflammatory cytokine, which has been shown to regulate acute phase protein synthesis (Bayne & Gerwick, 2001). The increase in the IL-1β expression following an acute phase induction in the present study is in agreement with other studies of fish. IL-1β expression has been found to be increased in the blood leukocytes of cod after vaccination with heat-killed *Listonella anguillarum*. In the same study transferrin increased after 3 and 10 days and ApoLP A-I was increased after 3 and 7 days (Caipang, et al., 2008a). Caiping et al. (Caipang, et al., 2010) have shown that the expression of IL-1β in gill epithelial cells was significantly increased at 3 hours after infection with *A. salmonicida* and *V. anguillarium*. In the same study the expression of transferrin increased after infection. The relatively slow maximum induction of IL-1β expression (72 hpi) observed in the present study is an agreement with previous findings for cod (Caipang, et al., 2008a; Perez-Casanova, et al., 2008a). Expression of IL-1β has been seen to increase in the spleen after vaccination with a *Vibrio anguillarium* antigen while in the same study the transferrin expression increased after the vaccination (Caipang, et al., 2009). Faster (within hours) induction of IL-1β expression has been reported during *in vitro* stimulation of cod leukocytes (Caipang, et al., 2010; Lazado, et al., 2010; Seppola, et al., 2008). Research has shown that cortisol can suppress IL-1β expression of cultured rainbow trout and carp leukocytes concurrently with the

suppressed proliferation of the fish leukocytes (Engelsma, et al., 2002; Verburg-Van Kemenade, et al., 1999; Weyts, et al., 1998a; Weyts, et al., 1998b). Research on head kidney leucocytes of rainbow trout showed that 100 and 320 ng mL^{-1} doses of cortisol inhibited LPS-induced IL-1β expression (Zou, et al., 2000). The increase in serum cortisol reaching a maximum concentration at 72 hpi seen in the present study and, at same time, the suppressed respiratory burst activity of the anterior kidney phagocytes (Magnadottir, et al., 2011) would, hence, be expected to have suppressed the IL-1β expression in the anterior kidney and later in the spleen.

The expression of transferrin was significantly increased at 72 and 168 hours after injection in the head kidney and after 72 hours in the spleen. This result suggests that transferrin may be an important parameter in the APR of cod and its gene expression, at least in the kidney, is apparently not affected by the cortisol release. Research has shown that transferrin can be either a positive or a negative acute phase protein (Bayne & Gerwick, 2001). The results in the present study as well as in other studies show that expression of transferrin in different organs varies depending on the stimulant used. Transferrin expression has been shown increased in 2 studies of cod (Caipang, et al., 2008a; Caipang, et al., 2009) and in the liver of channel catfish (Peatman, et al., 2007). Talbot et al. (Talbot, et al., 2009) showed that there was no change in the expression of transferrin in the liver of rainbow trout after exposure to a confinement stressor.

Changes in the gene expression of the two antimicrobial peptides, hepcidin and cathelicidin were relatively limited. The expression of cathelicidin was significantly elevated at 72 hpi in the spleen and kidney. Both the constitutive expression and the turpentine induced expression of cathelicidin remained relatively low in the present study, which suggests that cathelicidin may not play a major role in the APR of cod involving inflammatory reaction and marked cortisol release. Cathelicidin is an antimicrobial peptide (AMP) which has a role in defence against pathogens (Maier, et al., 2008). Caiping et al. (Caipang, et al., 2010) have shown that the expression of cathelicidin in gill epithelial cells was significantly increased 3 and 24 hours after infection by *A. salmonicida* but not by *V. anguillarium*.

The expression of hepcidin was only significantly elevated in the spleen at 24 hours after injection. Hepicidin is an antimicrobial peptide and research suggests that it is important in the first line of defence against pathogens (Solstad, et al., 2008). A relatively slow (24 h) upregulation of hepcidin expression was observed in the anterior kidney of cod following bacterial and viral mimic injections (Poly I:C) (Solstad, et al., 2008) which is comparable to the present results. Other research revealed a faster (within 3 h) induction of hepcidin expression following *in vitro* bacterial infection of cod epithelial cells (Caipang, et al., 2010). In Zebrafish the expression of hepcidin in internal organs has been shown to be increased following *A. salmonicida* or *S.aureus* infection (Lin, et al., 2007). The limited change seen in anterior kidney expression in the present study might reflect the suppressive effects of the cortisol release. Other studies have shown that, for example, handling stress, which induced cortisol release had suppressive effects on the hepcidin expression in the spleen of Eurasian perch (Milla, et al., 2010).

The differences in the gene expression observed in the two organs, the anterior kidney and the spleen are to be expected in view of subtle functional and structural difference between the two

organs. The anterior kidney, the spleen and the thymus are the major immune organs of teleosts. The respective roles of the anterior kidney and the spleen vary from one species to another. Both the anterior kidney and the spleen are important scavenging and haematopoietic organs but the spleen is generally considered to play a secondary role to the anterior kidney (Dalmo, et al., 1997). The posterior kidney also has endocrine functions and forms a part of the hypothalamus-pituitary-interrenal-axis, an important element in maintaining homeostasis (Engelsma, et al., 2002).

As might be expected, the results show that effects of the turpentine injection on the pentraxin expression was faster and more marked at the genetic level than at the protein secretion level. The pentraxins, in particular CRP-PII, although not a typical APP in serum appear, with ApoLP A-I, to be involved in the early APR, possibly inducing cortisol release and an increased gene expression of IL-1β, C3 and transferrin at a later stage. The increase in cortisol probably had suppressive effects on other immune factors.

5.3 Sequencing

In an attempt to explain the heterogeneity of CRP-PII demonstrated by the variability in size, density and number of anti-CRP-PII antibody reactive bands seen in Western blots of cod serum (Gisladottir, 2008), the CRP-PII genomic DNA was cloned and sequenced . The sequence revealed that the gene has at least 2 exons and 1 intron. Unpublished data indicated that the gene might be even larger or 3 exons (Einar Arnason, personal communication).

The results of the sequencing showed that the clones were of equal size although variablitiy was seen in nucleotides and amino acid sequences between individuals. This suggests that there is a single CRP-PII gene. If there had been more than one gene one would expect them to be of uneven lengths. The difference that was observed in the base sequence was approximately 50/50 between clones, which means that the difference can hardly be classified as PCR error.

Some information, or indications, can be gained by comparing the variations in the amino acid content of CRP-PII from different individuals. In some instances an amino acid with a different charge was substituted and in other instances the variability was in the presence or absence of a STOP signal and, if absent, in the presence of the sequence Asn(N) –Lys(K)-Ser(S). The sequence Asn-X-Ser/Thr, where X can be any amino acid except proline, signifies a possible N-linked glycosylastion site (Shakin-Eshleman, et al., 1996).

Hence, the results of the sequence analysis are not sufficient to explain fully the variability seen in the number of CRP-PII bands seen in Western blotting but they suggest the following three possible explanations for the variation:

1) It is due to variable splicing of the gene resulting in variable composition of exons. This could be examined by systematic PCR analysis of cDNA.
2) It is due to fragmentation of the (secreted) protein i.e. proteases might digest the protein into smaller units and variations in the amino acid sequences could influence the digestion sites.

3) It is due to the presence or absence of the STOP codon, when absent an N-linked glycosylation is possible which could explain the difference seen in Western blotting. However, limited glycosylation of CRP-PII was demonstrated by a treatment with PNGase (Gisladottir, et al., 2009)

6 Conclusion

The results of the present study show that the response of cod to turpentine injection was limited and the injection did not have a great effect on the serum concentration of factors other than cortisol. It might be that the cortisol increase had suppressed the effect on the other serum factors. The protein measurements did not indicate that the pentraxins acted as typical acute phase proteins in serum. However, the results of the gene expression analyses do suggest that the pentraxins, as well as ApoLP-A1, may act as early APR mediators in the anterior kidney which, with the inflammatory cytokine IL-1β, might have induced the cortisol release and stimulated C3 and transferrin expression. Cathelicidin and hepcidin may play a minor role in the APR of cod and are probably more important in the immune defence against infection. The results indicate that although cod is known to show limited adaptive immune response the innate response of cod appears similar to that of other fish species like rainbow trout and channel cat fish. The difference that was seen in the nucleotide and amino acid sequences between individual fish cannot by itself explain the observed heterogeneity of CRP-PII.

In future studies it would be interesting to repeat the experiment with a stimuli of bacterial or viral origin and using a larger number of fish in each group. It would also be interesting to examine to expression of pentraxins, and of other immune parameters in the liver and in other organs that are known to be involved in the immune response of cod.

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The acute phase response of Atlantic cod (*Gadus morhua*): Humoral and cellular response

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ABSTRACT

Intra-muscular injection of turpentine oil was used to induce acute phase response (APR) in Atlantic cod (*Gadus morhua* L.). The effects on the serum cortisol, total protein, IgM and pentraxin concentration were examined as well as the effects on natural antibody, anti-trypsin and leukocyte respiratory burst activity. The turpentine injection resulted in a 26 fold increase in the cortisol level after 72 h. Slightly reduced serum protein level in both groups was attributed to the restricted feeding during the experimental period. The IgM serum concentration was significantly reduced after 168 h in the turpentine treated fish while the natural antibody activity was not affected. The anti-trypsin activity was initially suppressed but recovered to normal levels at the end of the experiment. The turpentine injection had little effect on the serum level of the pentraxins, CRP-PI and CRP-PII. The respiratory burst activity was significantly suppressed after 72 h.

It is concluded that 1) cod shows a relatively slow humoral and cellular response to APR induction, 2) the increase in serum cortisol level may be the key modulator of the mainly suppressive effects on the immune parameters and 3) pentraxins are not typical acute phase proteins in cod.

1. Introduction

Atlantic cod is an economically valuable fish and with decreasing fishing stocks in recent decades interest in cod aquaculture has increased in countries around the North Atlantic [1]. Various problems commonly associated with fish farming have been experienced and had to be overcome in cod aquaculture, including fish diseases often linked to the high density and stressful conditions of fish farming. Considerable losses during the early stages of cod rearing have also been experienced, losses due to, for example, technical problems, problems with feeding and infections. Studies of the immune defence of cod at different developmental stages are therefore highly relevant, the basic aim being to improve health and disease resistance and to develop successful prophylactic measures for cod in aquaculture.

Several studies have indicated that cod's immune response to infection or vaccination is different and possibly deficient when compared to, for example, the salmonids [2]. This applies especially to the specific antibody response of cod, which is generally low or absent [3–5]. Innate humoral and cellular parameters are probably important in cod's immune defence and there are indications that cod's phagocytes play a principal role in defence against infectious diseases. The respiratory burst activity of cod's leukocytes is relatively high [6] and granuloma formation is a characteristic feature of cod's cellular response to bacterial infection. Within 2–3 weeks from infection the pathogen has been encapsulated by macrophages and epithelioid cells with a thin enclosing rim of fibroblast cells [7,8].

Some humoral parameters of cod have been characterized at our institute and their role in immune response or ontogeny studied [3,9–11]. Most recently the classical acute phase proteins, the pentraxins, have been isolated from cod serum. Two types of CRP-like pentraxins, referred to as CRP-PI and CRP-PII, have been characterized and their possible role in early infection investigated [12,13].

Acute phase response (APR) refers to homeostatic processes triggered by injury, infection or other trauma and involves all the major systems of the body including the innate immune system and the neuroendocrine system. Associated with an APR is a significant change, negative or positive, in the plasma levels of

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so-called acute phase proteins (APPs) like the pentraxins, anti-proteases, transferrin and some complement components [14].

The objective of this study was to induce acute phase response in cod using turpentine intra-muscular injection [15,16] and examine the effects on the level of pentraxins and several other humoral parameters in serum. The effect on the serum cortisol level and on the respiratory burst activity of cod's kidney leucocytes was also examined.

2. Materials and methods

2.1. Fish and sampling

2.1.1. Experiment 1: Serological and gene expression studies

Fifty four cod, mean weight 89.5 g, were obtained from the Marine Institute's Mariculture Laboratory, Stadur, Grindavík, and transported to the Institute at Keldur where they were kept at 8 °C in three 170 L tanks in well aerated sea water, salinity 32‰. Feeding was kept at minimum during the experimental period. After acclimatizing for few days, 24 fish were injected intramuscularly (i.m.) with 1 ml kg^{-1} body weight of turpentine oil and 30 fish received no treatment. Six fish were killed by anaesthesia and sampled before the turpentine injections and then six fish on average from each group after 1 h (h), 24 h, 72 h (3 days) and 168 h (7 days) following the injection.

Blood was collected from the caudal vessel of anaesthetized fish and allowed to coagulate overnight at 4 °C. Serum was then collected by centrifuging at 400 × g for 10 min and stored at −80 °C. Organs were also collected for gene expression studies.

2.1.2. Experiment 2: Leukocyte isolation and respiratory burst activity

The source and maintenance of the cod was as described above. Forty cod, mean weight 125 g, were used, twenty fish were injected with turpentine as described above and 20 received no treatment. Fourteen fish from each group were used in this study. One to three fish from each group were killed by anaesthesia after 1 h, 5 h, 24 h, 72 h, 144 h (6 days) and 240 h (10 days) and the kidney collected for leukocyte isolation.

2.2. Total protein concentration

A protein assay kit from Thermo Scientific (IL, USA) was used. Samples were measured in duplicates, a standard graph plotted using bovine serum albumin standard from the kit and the protein concentration of the serum samples extrapolated from this graph.

2.3. Cortisol concentration

A cortisol ELISA assay kit from Neogen Corp. (KY, USA) was used. Samples were measured in duplicates, a standard curve was plotted using a cortisol standard from the kit and the cortisol concentration of the samples calculated from this graph, as described by the kit's manufacturer.

2.4. IgM concentration

The ELISA method, described by Israelsson et al. [17], was used with some modifications. Maxisorp microtrays (96 well, Nunc, Denmark) were coated with 10 μg $well^{-1}$ in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6, Sigma) of purified, polyclonal rabbit anti-cod IgM antibody (a kind gift from the late professor L. Pilström, Department of Medical Immunology, Uppsala University, Sweden) and incubated overnight at 4 °C. Blocking was with 1% bovine serum albumin in coating buffer for 1 h at room temperature (22 °C). Serial dilutions of standard cod IgM (prepared at our Institute) and the test serum in phosphate bufferd saline (PBS, Sigma, USA) containing 0.05% tween 20 (PBS-T), 50 μl $well^{-1}$, were incubated for 2 h at 37 °C and then with 50 μl $well^{-1}$ of mouse polyclonal anti-cod IgM antibody (prepared at our Institute) for 1 h at 37 °C, followed by 50 μl $well^{-1}$ of alkaline phosphatase conjugated rabbit anti-mouse Ig's (Dako, Denmark) for 1 h at 37 °C, both diluted in PBS-T. Finally, 100 μl $well^{-1}$ of p-nitrophenyl substrate solution (Sigma) was added and the reaction stopped after 30 min at room temperature with 50 μl $well^{-1}$ of 3 N NaOH and optical density (OD) read at 405 nm. A standard graph was plotted using the results from the standard IgM and the quantity of the IgM in serum was extrapolated from this. Extensive washing in PBS-T was carried out between each step.

2.5. Natural antibody activity

Natural antibody activity was measured using an ELISA method previously described [18,19]. Briefly, MaxiSorp microtrays were coated overnight at 4 °C with 5 μg $well^{-1}$ of trinitrophenyl conjugated bovine serum albumin (TNP-BSA) (prepared at our Institute) diluted in coating buffer. Blocking was with 0.1% semi-skimmed milk powder in coating buffer for 1 h at room temperature. Serum samples in duplicates, diluted 10^{-2} in PBS-T, were incubated overnight at 4 °C, followed by incubation for 1 h at 37 °C, first with mouse anti-cod IgM antibody and then with alkaline phosphatase conjugated goat anti-mouse Ig antibody (Dako), both diluted in PBS-T as described above. Washing, developing and reading of the trays were as described above. Antibody activity was expressed as the OD_{405nm} value of serum diluted 10^{-2} after subtracting the blank values (PBS-T in place of serum).

2.6. Anti-protease activity

Anti-protease activity was measured using a modification of the method described by Bowden et al. [18,20]. Twenty microlitre of serum (in duplicates) were incubated with the same volume of standard trypsin solution (5 mg ml^{-1}, Sigma T-7409) in 1.5 ml microcentrifuge tubes for 10 min at room temperature. To this was added 200 μl PBS and 250 μl of 2% azocasein (Sigma) and incubated for 1 h at room temperature after thorough mixing, followed by the addition of 500 μl of 10% trichloroacetic acid, mixing, and further incubation for 30 min at room temperature. The mixture was centrifuged at 6000 g for 10 min and 100 μl transferred to a 96 well, flat-bottomed, non-absorbent microtray (Nunc) containing 100 μl $well^{-1}$ of 1 N NaOH. Optical density was read at 450 nm. The blank contained PBS in place of serum and trypsin and the 100% reference wells contained PBS in place of serum. After subtracting the value of the blank the percentage inhibition of the trypsin compared to the reference sample was calculated for each sample. The anti-trypsin activity was expressed as the % inhibition.

2.7. Pentraxin concentration

ELISA methods devised for the estimation of CRP-PI and CRP-PII have been described elsewhere [13]. Briefly, MaxiSorp microtrays were coated with polyclonal mouse anti-CRP-PI or anti-CRP-PII purified immunoglobulins (prepared at our Institute [12]), 10 μg $well^{-1}$ in coating buffer overnight at 4 °C. Blocking was with 0.1% skimmed milk powder in coating buffer for 1 h at room temperature. Purified standard CRP-PI or CRP-PII protein in serial five-fold dilutions and serum samples diluted 1/500 and 1/2000, both diluted in PBS-T and tested in duplicates, were incubated for 2 h at room temperature. Biotin labelled anti-CRP-PI or anti-CRP-PII mouse Ig's (prepared at our Institute using an ECL protein

biotinylation kit from Amersham Biosciences, UK) diluted in PBS-T was added and incubated for 1 h at 37 °C. This was followed by alkaline phosphatase-linked streptavidin (Dako) diluted in PBS-T and incubated for 1 h at 37 °C. Washing, developing and reading of the trays were then as described above. A standard graph was plotted using the results for the purified standard CRP-PI or CRP-PII and the quantity of the CRP-PI or CRP-PII in serum was extrapolated from this graph.

2.8. Respiratory burst activity of isolated kidney leukocytes

2.8.1. Leukocyte isolation

A sample from the head kidney was aseptically removed and put into a sterile petri dish containing 8 ml of Hank's balanced salt solution (HBSS, Invitrogen, Denmark) adjusted to 370 mOsm by adding 1.2 g NaCl, 1.4 g $NaHCO_3$, 0.33 g glucose per litre and containing 100 IU (international units) penicillin ml^{-1} and 50 IU streptomycin ml^{-1}. The tissue was thrust through a steel mesh (mesh screen 40, Sigma) to produce single cell suspension, transferred to conical test tubes and kept on ice for 5 min, allowing tissue remains to settle. The cell suspension was centrifuged (400 × g for 10 min at 8 °C), the supernatant discarded and the cells gently suspended in 8 ml of HBSS and centrifuged again. This was repeated twice and any remains of red blood cells removed. Finally the pellet was re-suspended in 1 ml of HBSS and 10 μl of a 1/50 dilution were placed on a Neubauer hemocytometer for standard cell counting and the number of cells ml^{-1} estimated.

2.8.2. Analysis of respiratory burst activity

The assay used was Amplex red Hydrogen Peroxide Assay (Kit A-12212, Molecular Probes, Leiden, The Netherlands), a one step fluorometric method. A stock solution of Amplex red substrate was prepared according to the manufacturer's instructions. Three solutions were mixed in conical test tubes wrapped in aluminium foil: 1) 25 μM Amplex Red reagent in 1× Reaction Buffer, 2) 25 μM Amplex Red reagent and 0.05 U ml^{-1} horseradish peroxidase (HRP) in 1× Reaction buffer, 3) 25 μM Amplex Red reagent, 0.05 U ml^{-1} HRP and 0.8 mM PMA (phorbol 12-myristate 13-acetate) in 1× Reaction buffer. One hundred microlitre $well^{-1}$ of the reaction mixture from each tube was put in triplicates in a microtray and 20 μl (ca. 2 million cells) of the cell solution added to each well, except in wells representing negative controls.

Fluorescence was measured using excitation at 530 nm and fluorescence detection at 595 nm at intervals for up to 3 h. The trays were kept protected from light at 9 °C. Background fluorescence of a non-H_2O_2 control reaction was subtracted from each value. The reading taken at 30 min was used in the figure presented.

2.9. Statistical analysis

For statistical analysis the StatView™ analysis system for Windows was used. Unpaired *t*-test was used to examine the statistical difference between the groups. $p \leq 0.05$ was set as the critical value of significance.

3. Results

A lesion at the site of injection and intra-muscular haemorrhage was seen in all the turpentine injected fish within 1–2 h. Three of the turpentine injected fish died during the experimental period in Experiment 1, all within an hour from the injection. In Experiment 2 three of the turpentine injected fish were lost, two died within 1 h from injection and one within 5 h from injection.

3.1. Cortisol concentration

The results of the cortisol analysis are shown in Fig. 1. Individual variation in cortisol levels of both groups was considerable. The mean (and standard deviation (SD)) cortisol level of the untreated control fish at the start of the experiment was 29.5 ± 32.9 ng ml^{-1}. A slight, but statistically insignificant increase was seen in the control fish after 1 and 24 h, which subsided to normal levels after 72 and 168 h. In the turpentine injected fish the cortisol concentration had increased significantly ($p = 0.0474$) after 24 h and reached a peak value of 461 ng ml^{-1} after 72 d ($p = 0.0272$), which was about 26 fold the level of the untreated fish at this time point. At the end of the experiment the cortisol levels of the turpentine injected fish were not significantly different from that of the time-matched control fish or compared to the initial value.

3.2. Total protein concentration

The results of the protein analysis are shown in Table 1a. The difference in the serum protein concentration between the untreated control fish and the turpentine injected fish was not statistically significant at any matched time point. Compared to the initial serum protein concentration lower levels, albeit statistically insignificant, were seen in both the control fish and the turpentine injected fish at all other sampling times.

3.3. IgM concentration

The results of the IgM analysis are shown in Fig. 2. The mean IgM concentration at the start of the experiment (0 h) was 5.9 ± 0.7 mg ml^{-1}. Only at 168 h was there a statistically significant difference between the two groups. At this time point the control fish showed higher IgM concentration than the treated fish or 8.7 ± 3.1 ml^{-1} compared to 4.0 ± 1.2 mg ml^{-1} of the turpentine treated fish ($p = 0.032$). Compared to the initial IgM level reduced IgM concentration was observed in the control group after 24 h (2.6 ± 1.4 mg ml^{-1}; $p = 0.0012$) and in the turpentine injected fish after 72 h (3.7 ± 0.6 mg ml^{-1}; $p = 0.0004$) and 168 h (4.0 mg ml^{-1}; $p = 0.0157$).

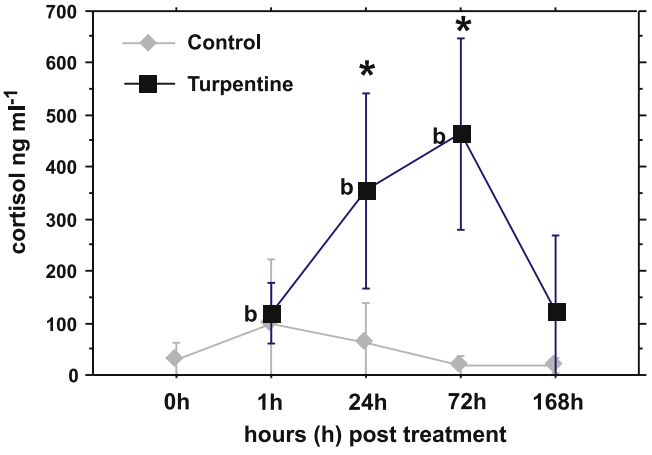

Fig. 1. The serum cortisol concentration (mean ± SD) of Atlantic cod, untreated control fish or fish injected intramuscularly (i.m.) with turpentine and sampled at different times after the injection ($n = 4$–6). Asterisk indicates a significant difference ($p < 0.05$) in the cortisol level of the turpentine treated fish compared to the control fish at the same time point. Letter (b) indicates a significant change in the cortisol level of the turpentine treated fish compared to the initial status (0 h).

Table 1
The total protein concentration (a) and natural antibody activity (b) (mean ± SD) of serum from untreated control fish and fish injected i.m. with turpentine, sampled before the injection and at four time points after the injection.

Parameters/ Hours (h)	a. Total serum protein		b. Natural antibody activity	
	Control fish	Turpentine fish	Control fish	Turpentine fish
Initial	37.2 ± 13.2		0.534 ± 0.126	
1 h	31.3 ± 7.2	35.9 ± 12.3	0.549 ± 0.086	0.709 ± 0.191
24 h	23.3 ± 11.5	22.2 ± 8.3	0.386 ± 0.121	0.508 ± 0.186
72 h	22.9 ± 7.7	28.1 ± 7.7	0.446 ± 0.102	0.428 ± 0.099
168 h	32.7 ± 6.6	26.9 ± 12.3	0.587 ± 0.185	0.490 ± 0.084

3.4. Natural antibody activity

The results of the natural antibody activity analysis are shown in Table 1b. There was no statistically significant difference between the natural antibody activity of the treated and the untreated fish at any matched time point or when compared to the pre-treatment level. Some reduction in the natural antibody activity was seen in the turpentine injected fish towards the end of the experiment which was statistically significant ($p = 0.0137$) between the samples collected at 1 h (0.709 ± 0.191) and at 72 h (0.428 ± 0.099).

3.5. Anti-trypsin activity

The anti-trypsin activity is shown in Fig. 3. At the start of the experiment the mean activity was 41.8 ± 4.4% trypsin inhibition. At all sampling times the turpentine injected fish showed lower anti-trypsin activity when compared to the time-matched control fish. This difference was statistically significant at 1 h and 72 h ($p = 0.036$ and $p = 0.035$ respectively). At 1 h and 24 h the anti-trypsin activity of the turpentine injected fish was also significantly reduced when compared to the initial level ($p = 0.018$ and $p = 0.168$ respectively). Compared to the initial level the anti-trypsin activity of the control fish showed significantly higher anti-trypsin activity at 72 h and 168 h ($p = 0.0348$ and $p = 0.002$ respectively). The highest anti-trypsin activity of 56.6% trypsin inhibition was seen in the control fish after 168 h and the lowest activity of 29.7% was observed in the turpentine injected fish after 24 h.

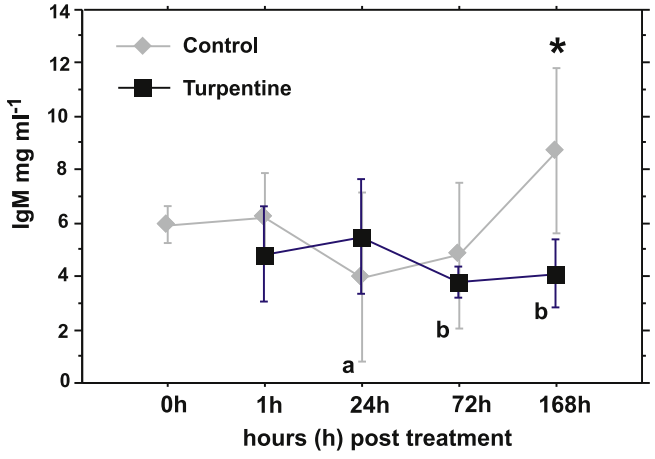

Fig. 2. The serum IgM concentration (mean ± SD) of Atlantic cod, untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection ($n = 4–7$). Asterisk indicates a significant difference ($p < 0.05$) in the cortisol level of the turpentine treated fish compared to the control fish at the same time point (168 h). Letters (a, b) indicate a significant difference in the cortisol level of the control fish (a) or the turpentine treated fish (b) compared to the initial status (at 0 h).

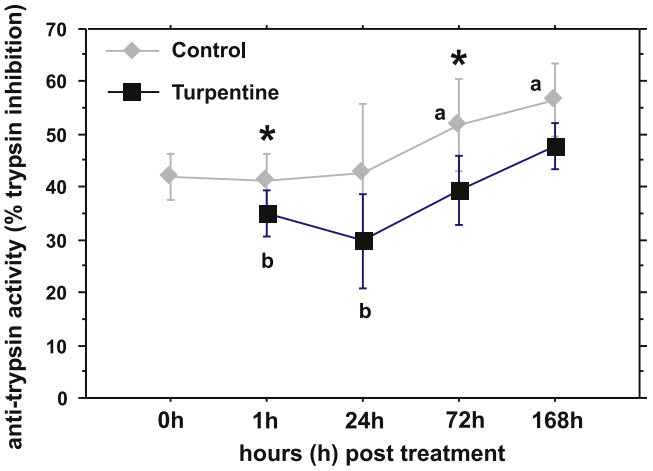

Fig. 3. The anti-trypsin activity (mean ± SD) of Atlantic cod, untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection ($n = 4–7$). Asterisk indicates a significant difference ($p < 0.05$) in the cortisol level of the turpentine treated fish compared to the control fish at the same time point (1 h and 72 h). Letters (a, b) indicate a significant difference in the cortisol level of the control fish (a) or the turpentine treated fish (b) compared to the initial status (at 0 h).

3.6. Pentraxin concentration

3.6.1. CRP-PI

The results of the CRP-PI analysis are shown in Fig. 4. Considerable individual variations were observed in the CRP-PI serum level of cod. The mean and SD of the CRP-PI concentration of untreated fish at the start of the experiment was 54.1 ± 25.5 μg ml^{-1}. The CRP-PI level the untreated control fish and the turpentine injected fish was not significantly different at any matched time point and CRP-PI level of the control fish did not change significantly during the experiment compared to the initial level. At 24 h the turpentine treated fish showed significantly reduced CRP-PI level compared to the initial status (18.1 μg ml^{-1}, $p = 0.0164$).

3.6.2. CRP-PII

The results of the CRP-PII analysis are shown in Fig. 5. Individual variations in CRP-PII level were not as marked as in the case of

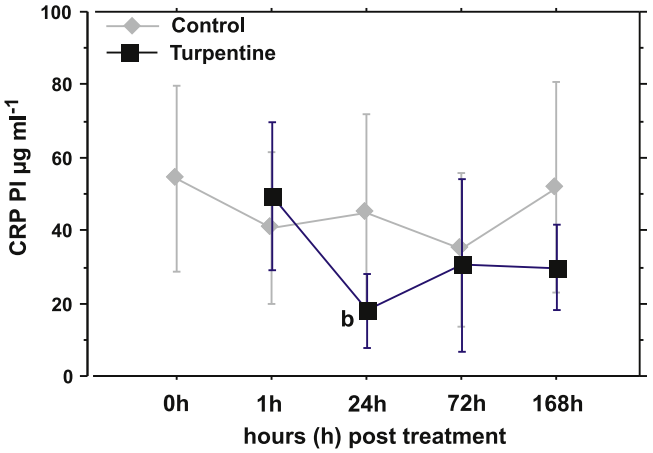

Fig. 4. The serum CRP-PI concentration (mean ± SD) of Atlantic cod, untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection ($n = 4–7$). Letter (b) indicates a significant change in the cortisol level of the turpentine treated fish compared to the initial status (0 h).

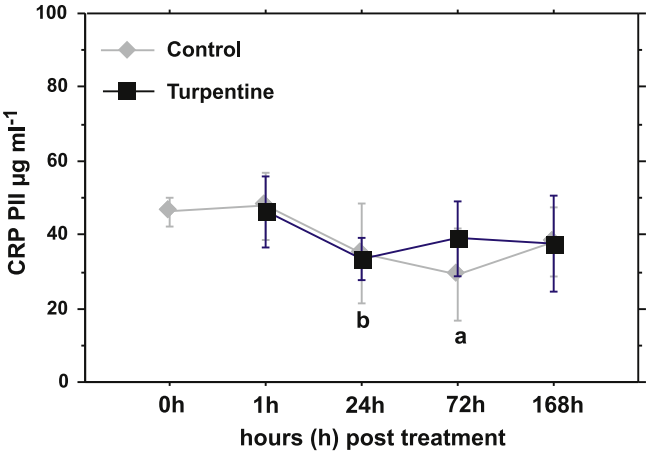

Fig. 5. The serum CRP-PII concentration (mean ± SD) of Atlantic cod, untreated control fish or fish injected i.m. with turpentine and sampled at different times after the injection ($n = 4–7$). Letters (a, b) indicate a significant difference in the cortisol level of the control fish (a) or the turpentine treated fish (b) compared to the initial status (at 0 h).

CRP-PI. The CRP-PII level at the start of the experiment was 46.2 ± 3.8 µg ml^{-1}. The CRP-PII level of the untreated control fish and the turpentine injected fish was not significantly different at any time point. Compared to the initial CRP-PII level of untreated fish, the control fish showed a significantly reduced level after 72 h (29.1 ± 12.3 µg ml^{-1}; $p = 0.01$) and the turpentine treated fish after 24 h (38.9 ± 10 µg ml^{-1}; $p = 0.0016$).

3.7. Respiratory burst activity

The results of analysis of the respiratory burst activity are shown in Fig. 6. The limited number of samples at each sampling time limits the statistical analysis of the data obtained. The respiratory burst activity of the control fish and the turpentine injected fish was similar and relatively high at 1 h and similar but relatively low at 5 h after injection. Both groups showed some recovery of activity after 24 h but after 72, 144 and 240 h the turpentine injected fish showed a reduced activity compared to the untreated control fish. This reduction was statistically significant at 144 and 240 h after the injection ($p = 0.0014$ and $p = 0.0119$ respectively).

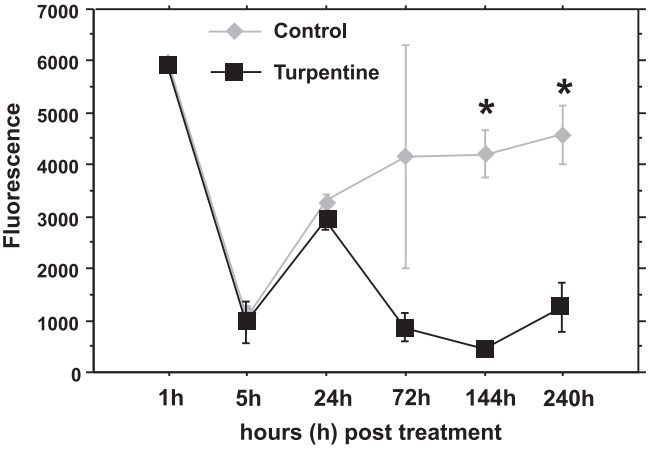

Fig. 6. The respiratory burst activity (mean + SD) of Atlantic cod phagocytes isolated from the kidney of untreated control fish or fish injected i.m. with turpentine and sampled at different times after injection ($n = 1–3$). Asterisk indicates a significant difference ($p < 0.05$) in the cortisol level of the turpentine treated fish compared to the control fish at the same time point (144 h and 240 h).

4. Discussion

In this study intra-muscular injection of turpentine oil was used to induce acute phase response (APR) in cod and the effects on humoral and cellular parameters examined. Turpentine is one of several agents that have been used experimentally to induce APR in mammals and fish and the dosage used in the present study was comparable to previously reported dosages for fish [15,16,21,22]. APR can also be generated by other inflammatory agents like bacterial LPS [16] as well as by stress, injury or infection [23–25]. The relatively fast appearance of an inflammatory response seen at the site of injection indicated that APR had been successfully generated in the present study. The accompanying raised cortisol level was a further sign of APR.

Maintenance of the fish under the experimental conditions and the handling involved did not by itself induce a marked stress response in the fish as shown by the relatively low cortisol level of the control fish, which was similar to the resting level reported in other studies of cod [13,26,27]. The turpentine injection caused a significant increase in the cortisol level reaching a maximum level of about 26 fold concentration after 72 h, before returning to the base level after 168 h. This increase in cortisol was considerably higher and peaked earlier than was previously reported in cod infected with *Aeromonas salmonicida* ssp. *achromogenes* [13] and was comparable to that of juvenile cod after being subjected to acute thermal challenge [27].

Various studies have shown that the time-lag from the induction of APR until a peak in cortisol concentration is observed and the magnitude of the response varies greatly depending on the type of induction, fish species and physiological and environmental conditions [13,26–33]. In general, gadoid species, like cod and haddock, appear to be relatively slow stress responders, i.e. a peak in cortisol observed within days, compared to, for example, rainbow trout which shows a peak in cortisol within hours [28,31–33]. The results of the present study are in agreement with this observation. Several studies have also shown that stress and the serum cortisol level can have significant effects on different immune parameters and immune defence of fish as will be referred to below [34–37].

The turpentine injection and the raised cortisol level did not affect the total serum protein concentration. However, slightly reduced levels were observed in both groups from 24 h onwards. This reduction is probably due to the restricted feeding throughout the experimental period compared to the previous feeding regimes at the Mariculture Laboratory. Nutritional status is known to be one of many important modulators of the serum protein concentration of fish serum [38]. This overall lower protein concentration of the experimental fish may have contributed to some of the changes in serum parameters observed, as mentioned below.

A different situation was recorded in plaice (*Pleuronectes platessa*) since an intra peritoneal injection with turpentine resulted in a reduced serum protein level compared to control fish injected with saline, the turpentine injection possibly affecting the appetite of the fish [22].

The mean immunoglobulin concentration of the cod serum was 5–6 mg ml^{-1} or on average about 19% of the total serum protein concentration. While comparable values have been seen in other studies of cod, this is relatively high compared to other fish species like the salmonids [8,39]. Some reduction in the IgM levels was observed in both the control and the turpentine injected fish after 24–72 h, probably concurrent with the overall lower protein concentration seen at these time points. After 168 h, however, the IgM concentration was significantly reduced in the turpentine treated fish compared to the time-matched control fish and also compared to the initial level. This suggests that the APR induction

resulted in a relatively late reduction in the IgM levels probably induced by the raised cortisol level. Both *in vivo* and *in vitro* studies have demonstrated the suppressive effects that cortisol administration can have on the number of B-lymphocytes and IgM secretion in fish, which agrees with the present results [36,37].

The natural antibody activity, which in the present work was determined by the anti-hapten activity of cod antibodies [19], was not significantly affected by the APR induction and neither the increased cortisol concentration nor the overall reduced serum protein concentration seemed to affect the natural antibody activity. Similarly, the significant reduction in the IgM concentration after 168 h did not (or only to a limited degree) appear to influence the natural antibody activity. In a previous study infection was shown to suppress both the serum IgM concentration and the natural antibody activity after 144 h compared to the time-matched control fish [13]. There may therefore be a subtle difference in the effects that APR induced by turpentine injection or by infection can have on the IgM concentration and natural antibody activity i.e. on the TNP hapten affinity of cod IgM.

The suppressed anti-trypsin activity of the turpentine injected cod and the, at least partial, recovery to control levels at the end of the experiment the suggests that the parameters that contribute to the anti-trypsin activity (like α1-anti-trypsin and α2-macroglobulin) may act as negative APPs in cod and that their activity is possibly not affected by the increased cortisol levels. This was also the pattern observed during early stages of infection [13]. The anti-trypsin activity of fish serum is primarily attributed to the activity of α1-anti-trypsin and α2-macroglobulin [20,40], which can inhibit or suppress bacteria that rely on toxic proteases for their pathogenicity [41]. Both α1-anti-trypsin and α2-macroglobulin have been described as a weak positive or negative APP in mammals and fish [14,42].

A great individual variation was seen in the serum concentration of the two pentraxins in cod, especially of CRP-PI, which made statistical analysis unreliable. The level of CRP-PI was also generally slightly higher than that of CRP-PII. These findings were in agreement with a previous study of cod [13]. The turpentine injection had little but slightly suppressive effect on the serum level of the cod pentraxins and in the case of CRP-PII some reduction was also seen in the control fish after 72 h.

Pentraxins are the classical positive APP in mammals and used, for example, as bioindicators of cardiovascular disease in human medicine [14,43]. The normal level of pentraxins in fish in often relatively high or >100 μg ml^{-1} in fish like channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*) and here cod compared to <1 μg ml^{-1} in humans [14,16]. The role of pentraxins in the APR and the immune response of fish is not clear and seems to vary from one species to another and also depending on the type of acute phase induction used. For example, an increase in the serum concentration of CRP-like protein was reported in channel catfish following turpentine injection while the level was reduced following infection and a low temperature shock [16]. On the other hand, reduced serum levels of CRP were seen in rainbow trout following turpentine injection [15] and elevated CRP levels in major carp (*Catla catla*) following exposure to pollutants [44]. In a study of rainbow trout Hoover et al. demonstrated the pattern recognition function of fish pentraxins and potential role in the pathogenesis of furunculosis [45]. In the present study, neither of the two pentraxin types of cod was found to act as typical an APP although, as suggested by the low level of CRP-PI observed after 24 h, CRP-PI may function as a weak or minor negative APP.

In view of the relatively consistently high level of pentraxins in cod serum and ligand-binding property [12] a role in pathogen defence seems likely but remains to be verified. Individual heterogeneity with regard to the sub-unit forms of CRP-PII has been described, partly due to variable glycosylation [12]. In the present study no change in the molecular pattern of CRP-PII was observed following APR (results not shown) as has been described in the molecular pattern of CRP from rohu (*Labeo rohita*) exposed to pollution [46].

The limited number of fish contributing to the respiratory burst (RB) activity data in Experiment 2 as well as great individual variations limited the statistical analysis of the results. However, the suppressed RB activity after 144 and 240 h compared to the time-matched control fish seems conclusive. Cortisol was not measured in Experiment 2 but assuming that the cortisol level peaked after 72 h as in Experiment 1, it is likely that cortisol was the main inducer of reduced phagocytic activity following turpentine injection. Modulation of cellular immune response by cortisol has been demonstrated in various fish species. Suppressed phagocytic activity due to acute stress or administration of cortisol has, for example, been demonstrated in salmon and rainbow trout [34–36].

In conclusion, the induced APR resulted in a marked increase in serum cortisol and had limited but largely suppressive effects on the immune parameters examined. Overall the response was relatively slow. In the case of reduced IgM concentration and RB activity it seems likely that cortisol was the prime modulating factor. The cod pentraxins did not show typical APP activity.

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The acute phase response of cod (*Gadus morhua* L.): Expression of immune response genes

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ABSTRACT

An acute phase response (APR) was experimentally induced in Atlantic cod (*Gadus morhua* L.) by intramuscular injection of turpentine oil. The change in the expression of immune related genes was monitored in the anterior kidney and the spleen over a period of 7 days. The genes examined were two types of pentraxins, apolipoprotein A1 (ApoA-I), the complement component C3, interleukin-1β (IL-1β), transferrin, cathelicidin, and hepcidin. All genes were constitutively expressed in both organs and their expression amplified by the turpentine injection. A pattern of response was observed both with respect to the organ preference and to the timing of a maximum response. The increased gene expression of the pentraxins, ApoA-I and C3 was restricted to the anterior kidney, the gene expression of IL-1β, cathelicidin, and transferrin increased in both organs, while hepcidin gene expression was only significantly increased in the spleen. The pentraxins and ApoA-I appear to be early mediators of APR in cod, possibly stimulating C3 and IL-1β response, while the antimicrobial peptides may play a minor role. The increase in transferrin gene expression in both organs, and apparent indifference to cortisol release associated with the turpentine injection, suggests that this could be a typical acute phase protein in cod.

1. Introduction

Acute phase response (APR) is an important homeostatic process, an innate response to tissue injury, infection or stress involving physiological and immune processes as well as the endocrine system [1–3]. APR is commonly characterized by a marked change in the plasma levels of the so-called acute phase proteins (APPs). The change in these proteins can be positive or negative, minor or major [1,3,4]. The archetypal mammalian APPs are the pentraxins, C-reactive protein (CRP) and serum amyloid P (SAP). Various other proteins have been classified as APPs, for example serum amyloid A, transferrin, complement component C3 and α-2-macroglobulin [1]. Pro- and anti-inflammatory cytokines like interleukin (IL) -1β, IL-6 and tumour necrosis factor-α are also key elements in APR [5,6].

APR has been studied in several fish species and a number of APPs have been identified in fish [1]. Pentraxins, either a single type or both types, have been isolated and characterized in several fish species like plaice (*Pleuronectes platessa* L.) [7], rainbow trout (*Oncorhynchus mykiss*) [8], channel catfish (*Ictalurus punctatus*) [9], carp (*Cyprinus carpio*) [10] and salmon (*Salmo salar*) [11]. Recently two types of CRP-like pentraxins, referred to as CRP-PI and CRP-PII, were isolated and characterized from cod serum [12].

Studies of the role that pentraxins play in the APR of fish are still rare. By injecting fish with turpentine, bacterial lipopolysaccharide or other inflammatory agents or, for example, subjecting fish to confinement stressor, APR has been induced in species like plaice, channel catfish and rainbow trout with varying effects on the level of CRP, SAP or other plasma proteins [9,13,14]. For example, turpentine injection resulted in an eighteen fold increase in serum levels of CRP in channel catfish [9] while reduced serum levels of CRP were seen in rainbow trout and both CRP and SAP levels were reduced in plaice [7,15]. Infection also seems to have varied effects on the serum pentraxin level in different fish species [14,16,17]. The change in the gene expression of pentraxins has been studied in zebrafish (*Danio rerio*), rainbow trout and other salmonids following infection, inflammation or stress induction with varying results [16,18,19]. Various humoral parameters of cod have been

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studied at our institute and their participation in immune response, ontogeny or reaction to inherent or environmental changes examined [20–23]. Recent emphasis has been on examining the early APR of cod to infection and turpentine injection with particular attention to the possible role played by the classical acute phase proteins, the pentraxins. The results so far indicated that neither CRP-PI nor CRP-PII act as typical APPs in cod serum [17,24].

The present paper describes the effects of an acute phase induction by turpentine injection on the gene expression of the pentraxins and several other immune parameters in the anterior kidney and spleen of cod.

2. Materials and methods

2.1. Fish and sampling

Fifty four cod, mean weight 89.5 g, were divided between three 170 L tanks of sea water and kept at 8 °C. The fish was acclimatized for few days before treatment. On day 0 five fish, 1–2, from each tank were killed by anaesthesia using 50 mg mL^{-1} of tricaine methane sulfonate (TMS, Aqua Life, Syndel Laboratories Ltd. Vancouver, Canada) and samples from the anterior kidney, spleen, and liver collected in RNAlater® solution (Ambion, TX, USA) and stored at −80 °C. Eight fish from each tank were then sedated using 30–40 mg mL^{-1} of TMS, marked by cutting a section from the caudal fin and injected intramuscularly with 1 mL kg^{-1} body weight of filtered (0.45 μm, Millipore, MA, USA) turpentine oil (Slippfelagid, Iceland). The rest of the fish received no treatment. After 1 h, 24 h, 72 h and 168 h 1–3 injected and untreated fish from each tank (total 4–7 fish) were sampled as described above. Blood samples collected from this fish at the same time points were used in an analysis of serological parameters [24].

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from tissue samples with the NucleoSpin® RNA/Protein kit (Macherey–Nagel, Germany) according to the manufacturer's protocol which includes an on-column DNase digestion step. Additional DNase treatment was performed on all total RNA samples with the DNA-free™ kit (Ambion, TX, USA) following the manufacturer's instructions. The quantity, and purity, of the total RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, DE, USA). The integrity of the RNA was determined using Agilent RNA 6000 nano chips (Agilent, CA, USA) on a 2100 Bioanalyzer (Agilent, CA, USA). RNA integrity numbers of 8 and higher were taken as a measure of acceptable RNA quality. Extracted total RNA was stored at −80 °C (Note: The liver samples were found to be unusable due to degradation, despite sampling into RNAlater® and subsequent storage at −80 °C. This is possibly due to the high lipid content of the cod liver, which could impair the diffusion of RNAlater® into the tissue. In a separate experiment cod liver samples snap-frozen in liquid nitrogen yielded intact RNA). Complementary DNA (cDNA) was prepared from anterior kidney and spleen total RNA with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions. Each reverse transcription (RT) reaction consisted of total RNA (variable between reactions from 500 ng to a maximum of 2000 ng), 5 μM Oligo$(dT)_{18}$ primer, 1 mM dNTP Mix, 1x kit reaction buffer, 1 unit Ribolock™ RNase inhibitor and 10 units RevertAid™ M-MulV reverse transcriptase in a total volume of 20 μL. The samples were incubated at 42 °C for 60 min followed by 5 min at 70 °C. For each reverse transcribed RNA sample a corresponding, identical, reaction lacking reverse transcriptase was performed and used in subsequent PCR reactions to verify the absence of genomic DNA in the total RNA. After RT, cDNA samples were stored at −80 °C.

2.3. Primer and probe design

The sequences of the primers and probes used in this study are listed in Table 1. The primers for ubiquitin, IL-1β, cathelicidin, and hepcidin have been described previously [25–28]. Results from amino acid sequence analyses [12] of cod CRP-PI and CRP-PII were used to construct primers and amplify, clone, and sequence CRP-PI and PII cDNA (Gisladottir et al. unpublished results). The primers

Table 1
Primers and probes used for quantitative real time PCR assays in the study. The table shows, for each assay, the nucleotide sequences and modifications of the primers and probes, the PCR amplicon sizes in basepairs (bp.), the annealing/extension temperature (°C) of the PCR reaction, and the PCR efficiency (Eff%).

Gene	Primer/probe nucleotide sequence (5′-3′)	Amplicon size	°C	Eff%	Reference
CRP-PI	Forward: GGCTACTCGCACCCGTATAA Reverse. CATGTGCCACAGATGGAGAC Probe: FAM-CGTGGCTGATTTCCCCGAGC-BHQ	141	55	100.5	This study
CRP-PII	Forward: AAAGGGAAGCGTGAGGTCATC Reverse: CGCGCCACACGTTGAGT Probe: FAM-CGCACGCGGGACTTCGACG-BHQ	70	60	101.1	This study
ApoA-I	Forward: CTCTTGCTCTTGCCCTTCTG Reverse: GGCACTGTCCTTCACCTGAT Probe: FAM-ATCCGATGCACCCAGCCAGC-BHQ	128	60	86.9	This study
Complement C3	Forward: AGTGGGAAACTACGCACCTT Reverse: TCGACCAACTTTCTGTGCAT Probe: FAM-TGCGAGCTATCTCATCATGGGCA-BHQ	117	60	93.7	This study
IL-1β	Forward: GGAGAACACGGACGACCTGA Reverse: CGCACCATGTCACTGTCCTT	50	60	93.4	[27]
Transferrin	Forward: GAGCTCCCATCGACAGCTAC Reverse: CAAACCCAGCAGAGGAGAAG Probe: FAM-CACGCTGTGGTTAGCCGCGT-BHQ	150	60	102.2	This study
Hepcidin	Forward: CCAGAGCTGCGGATCGA Reverse: AAGGCGAGCACGAGTGTCA	100	60	95	[28]
Cathelicidin	Forward: GGTTGAAACTGTCTATCCAGAGG Reverse: AACTCTTGTGCAGGGAATGTC	77	60	98.9	[25]
Ubiquitin	Forward: GGCCGCAAAGATGCAGAT Reverse: CTGGGCTCGACCTCAAGAGT	69	60	102.4	[26]

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and probes for CRP-PI and CRP-PII used in this study were designed based on these sequences with the Primer3 software [29]. Primers for complement component C3, transferrin, and apolipoprotein A-I were designed in a similar manner based on published sequences (GenBank IDs: AY739672.1, L40370.1, and AY739673.1, respectively). All primers and probes were obtained from TAG Copenhagen (Denmark). Prior to their use they were tested on serial dilutions of cDNA (5 points, 2 fold dilution with each point in triplicate) to determine their amplification efficiency (Eff%, Table 1) according to the formula: $E_x = 10^{(-1/\text{slope})} - 1$. The cDNA template used for the serial dilutions was selected from the samples used in the study.

2.4. Quantitative real time PCR

Quantitative real time PCR (qPCR) analyses were performed on a StepOne Plus™ real time PCR instrument (AppliedBiosystems, CA, USA). The qPCR reactions for CRP-PI, CRP-PII, transferrin, C3, and ApoA-I contained the following components: 1x Maxima® probe qPCR master mix (Fermentas, Canada), 250 nM probe, 900 nM forward and reverse primers, and cDNA template in a total reaction volume of 20 μL. Reverse transcribed cDNA was diluted with nuclease free H_2O prior to use in qPCR reactions and assigned a concentration unit corresponding to the concentration of total RNA in the RT reaction, e.g. 4 ng cDNA corresponded to 4 ng of input total RNA in the RT reaction. The amount of cDNA used in the probe reactions was 4 ng, except for the qPCR analyses of CRP-PI, CRP-PII, C3, and ApoA-I in the anterior kidney samples and C3 and ApoA-I in the spleen samples; in these cases 48 ng of cDNA were used. The reaction conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at X°C (X: annealing/extension temperature, Table 1). The qPCR reactions for other genes in Table 1 contained 1x SYBR green qPCR master mix (Maxima® SYBR green qPCR master mix (Fermentas, Canada) for ubiquitin, IL-1β and cathelicidin and Power SYBR green PCR Master Mix (AppliedBiosystems, CA, USA) for hepcidin), 300 nM forward and reverse primers, cDNA template (4 ng (ubiquitin, IL-1β and cathelicidin) or 10 ng (hepcidin)) in a total reaction volume of 20 μL. The reaction conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at X°C (for temperature see Table 1) followed by melting curve analysis according to default settings with the StepOnePlus™ instrument, i.e. 15 sec at 95 °C followed by 1 min incubations starting at 60 °C and increasing with 0.3 °C increments to 95 °C with a fluorescence read after each 1 min incubation. All qPCR assays where the cDNA concentration was 48 ng were performed in duplicate (technical replicates), whereas assays where cDNA concentrations were 4 ng were performed in triplicate. Assays for each gene were performed on one RNA sample, per organ, from each individual included in the study for that gene (Table 2).

2.5. Data analysis

Real time PCR data was collected and processed with the StepOne™ v2.1 software (AppliedBiosystems, CA, USA). The qPCR data for each gene, in either kidney or spleen, were calibrated against a control fish from 0 h which was selected as the fish with the median cycle threshold (Ct) value at that time point. Relative quantity (RQ) values compared to this fish were calculated using ubiquitin as a reference gene and an equation described by Pfaffl [30] which includes a correction for primer amplification efficiencies in the calculation of gene expression ratios from Ct values. Ubiquitin was chosen because previous studies [26,31] indicate that it is a suitable reference gene for the cod tissues used in this study. One-way ANOVA analyses on LOG (base 10) transformed RQ values (Log_{10}(RQ)) were performed to determine if there were significant changes in gene of interest expression between time points for the control fish, or the turpentine-exposed fish. Comparisons of the expression of genes between turpentine treated and untreated controls within each time point were performed using the REST 2009 software (QIAGEN, Germany) [32] which is based on the Pfaffl equation described above. For statistical calculations in the REST 2009 software, randomization was conducted with 6000 permutations and the cut off for significance was $P = 0.05$. Microsoft® Office Excel and Publisher were used for image processing. Statview v 5.0.1 was used to construct box and whisker plots and for statistical analyses.

Table 2
Number of samples for each time point of the study. Deviations are shown in parenthesis. The lower number of samples for CRP-PII in the kidney is due to omission of samples with no amplification.

	0 h	1 h	24 h	72 h	168 h
Kidney					
Turpentine		7	$5^{(\text{CRP-PII}: 2)}$	5	$4^{(\text{CRP-PII}: 3)}$
Control	5	6	$4^{(\text{CRP-PII}: 2)}$	$5^{(\text{CRP-PII}: 4)}$	$5^{(\text{CRP-PII}: 4)}$
Spleen					
Turpentine		7	5	$5^{(\text{IL-1b}: 4)}$	3
Control	5	6	$5^{(\text{Hepcidin}: 4)}$	5	5

3. Results

As previously described [24], a visible lesion and intramuscular haemorrhage was seen within 1–2 h at the site of injection in all the turpentine injected fish. The test gene expression data were normalized to the expression of ubiquitin. Ubiquitin expression in the tissue samples used in the study was relatively stable (Fig. 1) compared to the test genes and there was no difference in ubiquitin gene expression between time points and treatments, neither in the head kidney nor the spleen (ANOVA, head kidney: $F(8,37) = 1.846$ $P = 0.100$, spleen: $F(8,37) = 1.007$ $P = 0.447$).The results for relative gene expression in the controls and turpentine injected fish within each time point throughout the study are shown in Figs. 2 and 3 as Log_{10}(RQ) values. The expression of the genes of interest included in the study was compared between time points by one-way ANOVA, both in control fish and turpentine injected fish. The results (Table 3) show that in the control fish there was no significant difference in the expression of the genes analyzed in this study between time points, with the exception of ApoA-I in the kidney (T-0h vs. T-168h $P < 0.01$ (Tukey–Kramer post-test)).

3.1. Differences in the gene expression of CRP-PI, CRP-PII, ApoA-I, and C3 between turpentine injected and untreated controls

As shown in Fig. 2 and Table 3, 1 h after turpentine injection there was a statistically significant increase in the anterior kidney of injected fish, compared to control fish sampled at the same time point, for CRP-PI gene expression (4.9 fold higher, $P = 0.013$), CRP-PII gene expression (24.5 fold higher, $P = 0.004$), and ApoA-I gene expression (7.1 fold higher, $P = 0.007$) as shown in Fig. 2 (A), (C), and (E), respectively. At other time points there was no statistically significant difference in the expression of these genes between the two groups in the kidney. In the spleen there was no statistically significant difference at any time point (Fig. 2 (B), (D), (F), and (H)). The expression of the complement C3 gene was also only affected in

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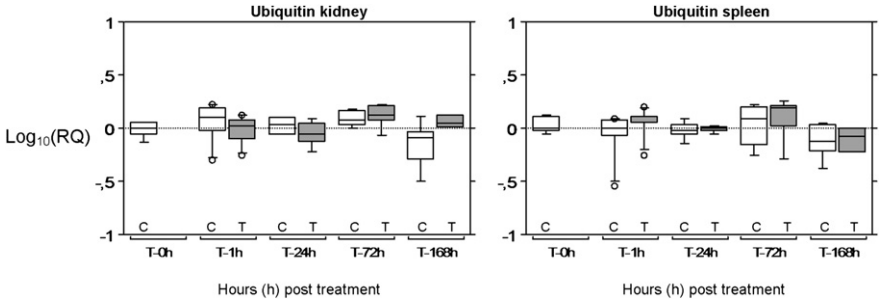

Fig. 1. Box and whisker plots that show the relative gene expression (Log_{10}(RQ)) of ubiquitin in the anterior kidney and spleen of turpentine injected cod (grey boxes, *T* on *x*-axis) and untreated controls (empty boxes, C on *x*-axis) for each time point (0 h, 1 h, 24 h, 72 h, and 168 h) of the sampling period. In the head kidney samples the average Ct value for ubiquitin was 17.95 (Standard deviation (SD) 0.49, Ct range = 17.26–19.64, variance = 0.23), in the spleen the average Ct value was 18.40 (SD 0.55, Ct range = 17.52–20.21, variance = 0.30). The lower boundary of the boxes in the plot represents the 25th percentile and the top the 75th percentile; the band within the boxes shows the median. The whiskers show the 10th percentile and the 90th percentile. Values that fall above the 90th percentile and below the 10th percentile are presented as open circles.

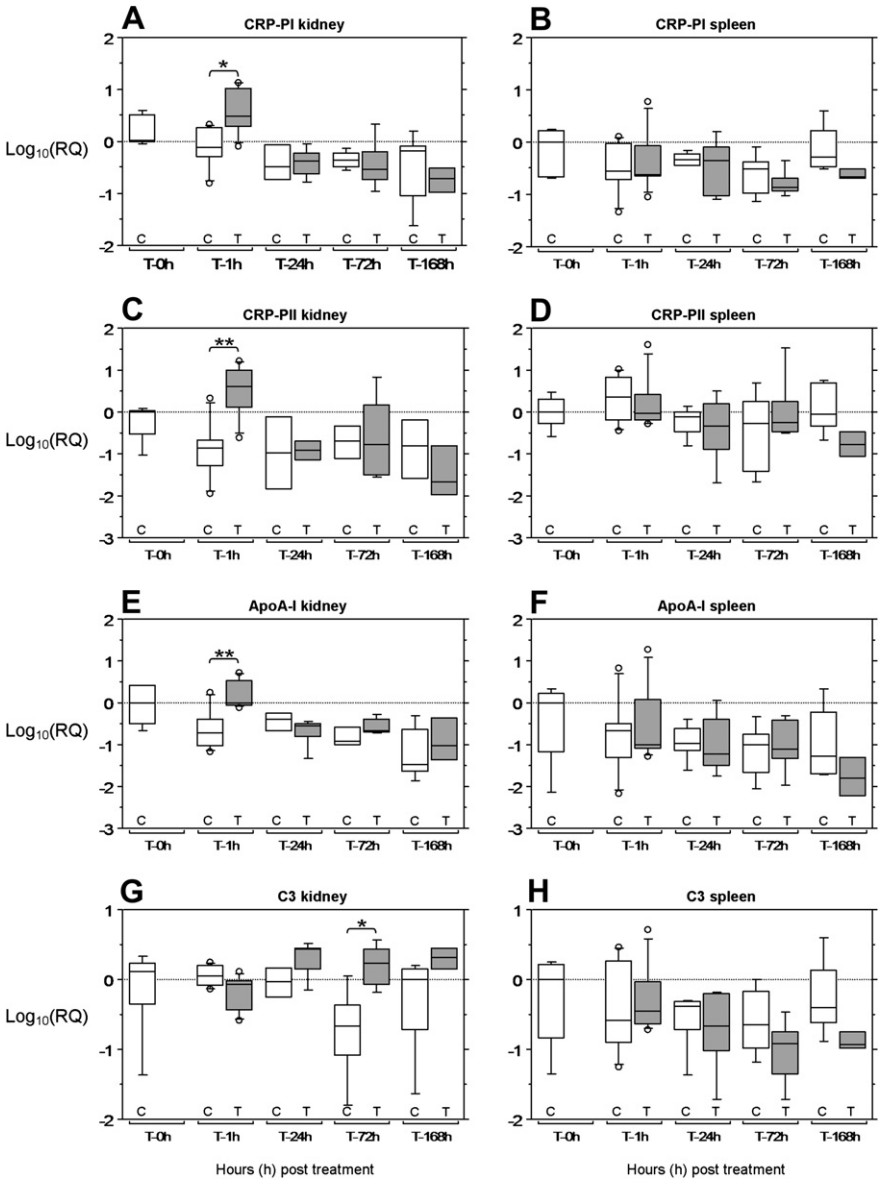

Fig. 2. Box and whisker plots that show the relative gene expression (Log_{10}(RQ)) of CRP-PI, CRP-PII, ApoA-I, and C3 in the anterior kidney and spleen of turpentine injected cod (grey boxes, *T* on *x*-axis) and untreated controls (empty boxes, C on *X*-axis) for each time point (0 h, 1 h, 24 h, 72 h, and 168 h) of the sampling period. The asterisks indicate a statistically significant difference between treated and untreated fish within time points as calculated with the REST 2009 software (*$P < 0.05$, **$P < 0.01$; for precise *P* values see Table 3). For details on the box and whiskers presentation see Fig. 1.

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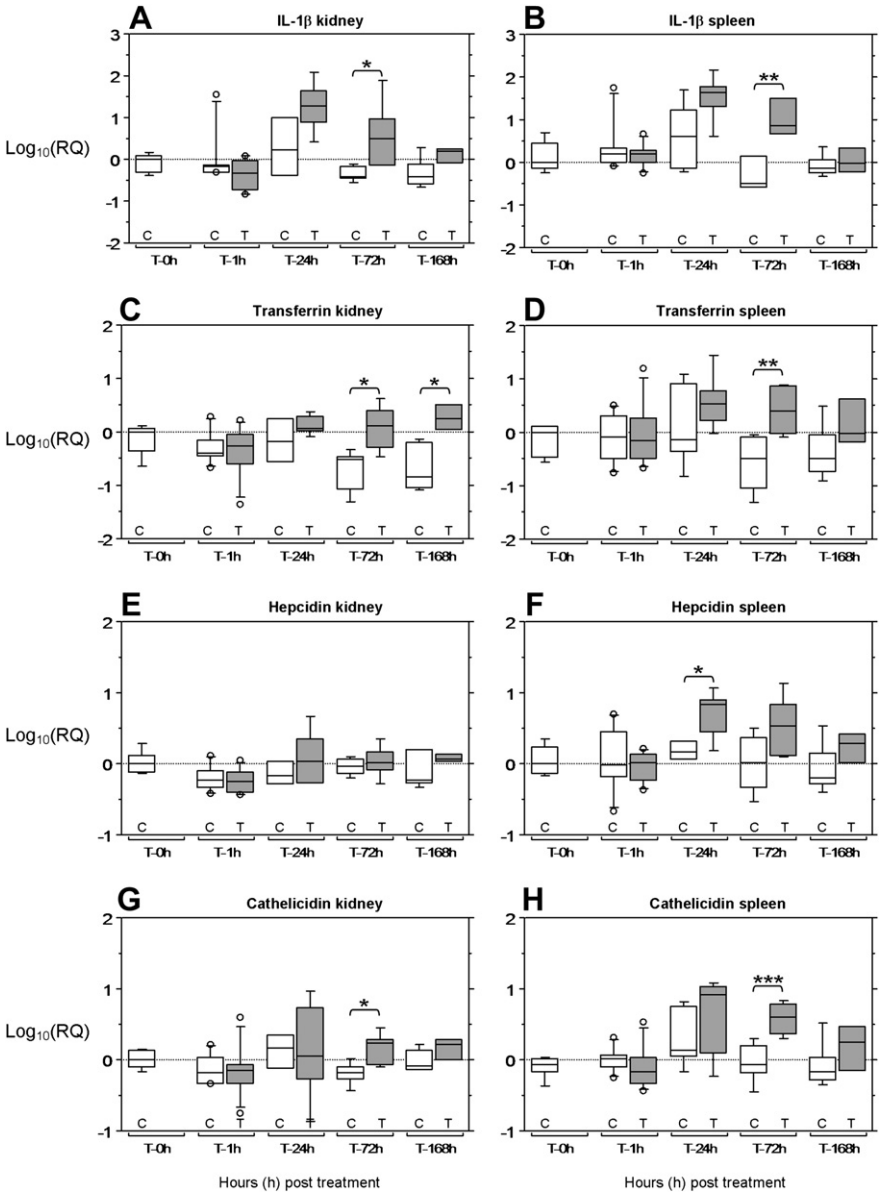

Fig. 3. Box and whisker plots that show the relative gene expression (Log_{10}(RQ)) of IL-1β, transferrin, hepcidin, and cathelicidin in the anterior kidney and spleen of turpentine injected cod (grey boxes, *T* on *x*-axis) and untreated controls (empty boxes, C on *x*-axis) for each time point (0 h, 1 h, 24 h, 72 h, and 168 h) of the sampling period. The asterisks indicate a statistically significant difference as calculated with REST 2009 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; for precise *P* values see Table 3) in gene expression of turpentine injected fish compared to controls within time points. For details on the box and whiskers presentation see Fig. 1.

the anterior kidney of injected fish and was significantly elevated (8.9 fold, $P = 0.016$), compared to controls, at 72 h.

3.2. Differences in the gene expression of IL-1β, transferrin, hepcidin, and cathelicidin between turpentine injected and untreated controls

In turpentine injected fish there was a significant increase (Fig. 3 and Table 3)in IL-1β and cathelicidin gene expression at 72 h, compared to controls, in both the anterior kidney (IL-1β: 7.8 fold higher, $P = 0.023$; cathelicidin: 2.2 fold higher, $P = 0.027$) and the spleen (IL-1β: 22.4 fold higher, $P = 0.006$; cathelicidin: 4.1 fold higher, $P < 0.001$) (Fig. 3 (A), (G), (B), and (H), respectively). Transferrin gene expression was also significantly elevated in both organs, in the anterior kidney at 72 and 168 h (6.3 fold higher, $P = 0.012$ and 8.6 fold higher, $P = 0.018$, respectively) and in the spleen at 72 h (9.9 fold higher, $P = 0.008$) (Fig. 3 (C), and (D)). Hepcidin gene expression only differed from controls in the spleen at 24 h (3.2 fold higher, $P = 0.04$) (Fig. 3 (F)). Within other time points the expression of IL-1β, transferrin, hepcidin, and cathelicidin was not significantly affected in the kidney, or the spleen, of turpentine injected fish compared to the controls.

4. Discussion

In the present study the change in gene expression of the cod pentraxins, CRP-PI and CRP-PII, and six other immune parameters was monitored during an acute phase response (APR) of cod induced by an intramuscular injection of turpentine oil.

The eight genes examined in the study were all constitutively expressed in both the anterior kidney and the spleen and their

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Table 3
The table shows the results from one-way ANOVA analyses of gene expression between time points in the controls and turpentine injected fish. It also shows the results (fold change and *P* value) from comparisons of gene expression in injected and control fish within time points as calculated with the REST 2009 software. No significant difference is indicated by n.s.

Gene	Organ	One-way ANOVA		Fold change and *P* value treated vs. control within time points			
		Controls	Treated	1 h	24 h	72 h	168 h
CRP-PI	Kidney	$F(4,20) = 2.15$, $P = 0.112$	$F(4,21) = 10.87$, $P < 0.001$	4.9 $P = 0.013$	n.s.	n.s.	n.s.
	Spleen	$F(4,21) = 1.37$, $P = 0.278$	$F(4,20) = 1.17$, $P = 0.352$	n.s.	n.s.	n.s.	n.s.
CRP-PII	Kidney	$F(4,16) = 0.68$, $P = 0.615$	$F(4,17) = 4.62$, $P = 0.015$	24.5 $P = 0.004$	n.s.	n.s.	n.s.
	Spleen	$F(4,21) = 1.31$, $P = 0.298$	$F(4,20) = 1.45$, $P = 0.254$	n.s.	n.s.	n.s.	n.s.
IL-1b	Kidney	$F(4,20) = 1.20$, $P = 0.340$	$F(4,21) = 8{,}48$, $P < 0.001$	n.s.	n.s.	7.8 $P = 0.023$	n.s.
	Spleen	$F(4,21) = 2.11$, $P = 0.116$	$F(4,19) = 10.86$, $P < 0.001$	n.s.	n.s.	22.4 $P = 0.006$	n.s.
Transferrin	Kidney	$F(4,20) = 2.53$, $P = 0.073$	$F(4,21) = 2.58$, $P = 0.067$	n.s.	n.s.	6.3 $P = 0.012$	8.6 $P = 0.018$
	Spleen	$F(4,21) = 1.29$, $P = 0.308$	$F(4,20) = 1.65$, $P = 0.200$	n.s.	n.s.	9.9 $P = 0.008$	n.s.
C3	Kidney	$F(4,20) = 1.64$, $P = 0.203$	$F(4,21) = 2.03$, $P = 0.128$	n.s.	n.s.	8.9 $P = 0.016$	n.s.
	Spleen	$F(4,21) = 0.35$, $P = 0.843$	$F(4,20) = 2.00$, $P = 0.134$	n.s.	n.s.	n.s.	n.s.
ApoA-I	Kidney	$F(4,20) = 4.05$, $P = 0.015$	$F(4,21) = 6.14$, $P = 0.002$	7.1 $P = 0.007$	n.s.	n.s.	n.s.
	Spleen	$F(4,21) = 0.45$, $P = 0.771$	$F(4,20) = 1.53$, $P = 0.232$	n.s.	n.s.	n.s.	n.s.
Cathelicidin	Kidney	$F(4,20) = 1.62$, $P = 0.210$	$F(4,21) = 0.67$, $P = 0.617$	n.s.	n.s.	2.2 $P = 0.027$	n.s.
	Spleen	$F(4,21) = 1.78$, $P = 0.171$	$F(4,20) = 4.86$, $P = 0.007$	n.s.	n.s.	4.1 $P < 0.001$	n.s.
Hepcidin	Kidney	$F(4,20) = 0.99$, $P = 0.434$	$F(4,21) = 1.95$, $P = 0.140$	n.s.	n.s.	n.s.	n.s.
	Spleen	$F(4,20) = 0.27$, $P = 0.894$	$F(4,20) = 5.77$, $P = 0.003$	n.s.	3.2 $P = 0.040$	n.s.	n.s.

expression augmented by the injection of turpentine. A definite pattern of gene expression was observed both with respect to the timing of the maximum response and to whether the response was limited to the anterior kidney or the spleen or seen in both organs. Thus, a statistically significant increase in the gene expression of the pentraxins (CRP-PI and CRP-PII), ApoA-I and complement component C3 was restricted to the anterior kidney. The increase in the expression of the pentraxins and ApoA-I appeared early, at 1 h after the induction (hpi) while the maximum expression of C3 occurred later, at 72 hpi. A significant increase in IL-1β, transferrin, and cathelicidin was seen in both spleen and kidney, in all cases at 72 hpi, and for transferrin also at 168 h. A significant difference in the gene expression of the antibacterial peptide, hepcidin, was restricted to the spleen showing maximum response at 24 h. One-way ANOVA of gene expression in control fish between time points throughout the study period revealed no significant differences in the expression of the genes analyzed in this study (Table 3), with the exception of ApoA-I in the kidney. The expression of immune- or acute phase-relevant genes should be stable in untreated fish under ideal experimental conditions. Therefore, the ANOVA results suggest that the gene expression in the fish in the study was not significantly influenced by external factors such as handling stress.

The pentraxins, ApoA-I and C3 have all been described as APPs and the pentraxins are the classical positive APPs of mammals and some fish [1,9,33]. Previous studies of cod have shown that neither CRP-PI nor CRP-PII act as typical plasma APPs during early infection or following APR induced by an inflammatory agent (turpentine) [17,24]. The results of the present study, however, suggest that pentraxins may act as early mediators of APR in cod. ApoA-I also seems to act as an early mediator of APR in cod. ApoA-I is the protein constituent of the high-density lipoproteins (HDL), which are abundant in fish plasma [21,34]. As well as taking part in lipid metabolism, ApoA-I has been classified as an APP in fish [1,35,36]. Caipang et al. [37] have, for example, demonstrated a significant upregulation of ApoA-I in blood leukocytes from cod 24 h after intra-peritoneal vaccination with heat-killed bacteria. The role of the pentraxins and ApoA-I as early mediators in the APR of cod probably involves the activation of the complement system by stimulating the C3 gene expression, which peaked later in the kidney at 72 hpi. Activation of the complement system is one of the basic functions attributed to the pentraxins [38,39] and has, for example, been demonstrated experimentally in rainbow trout [40]. ApoA-I is believed to have a regulatory role in activation of the complement system by affecting the formation of the membrane attack complex (MAC, C5b-9) [41]. An inhibition of the MAC cascade through the binding of C3 has also been suggested to take place in cod under certain conditions [21]. C3 is the central component of the three complement pathways and, hence, a key element in innate and adaptive immune defence and homeostasis [42]. C3 is commonly classified as a positive APP in mammals [1,43] while its role in the APR of fish appears to be more varied and a down regulation of C3 was, for example, reported in the liver of vaccinated rainbow trout [44].

These early mediators, the pentraxins and ApoA-I, could also be involved in the cortisol release from the neuroendocrine cells of the kidney, which, in the same fish, was shown to peak at 72 hpi [24]. This may have been via induction of gene expression the pro-inflammatory cytokine, IL-1β. As mentioned above, a statistically significant increase in IL-1β gene expression was seen in the kidney and spleen at 72 hpi. The increase in IL-1β gene expression following an acute phase induction is in agreement with other studies of fish, including cod, which have demonstrated the importance of IL-1β as the major pro-inflammatory cytokine secreted from activated phagocytes [27,37,45] as well as a communicator with the neuroendocrine system [2]. The relatively slow maximum induction of IL-1β gene expression (72 hpi) observed in the present study is in agreement with previous findings for cod [37,46,47]. The stress hormone, cortisol, has been shown to suppress IL-1β gene expression of cultured rainbow trout and carp leukocytes concurrently with the suppressed proliferation of the fish leukocytes [6,48]. The serum cortisol level observed in our previous study of the same fish [24] reached a maximum concentration at 72 hpi and, at the same time, suppressed respiratory burst activity of anterior kidney phagocytes. This would, hence, be expected to have suppressed the IL-1β gene expression in the anterior kidney and the spleen as seen in the present study.

Transferrin, hepcidin and cathelicidin are antimicrobial proteins or peptides as well as APPs and both transferrin and hepcidin assert their antimicrobial activity in fish through their regulation of iron homeostasis [1,49]. An increase in transferrin gene expression was statistically significant in both organs at 72 hpi and stayed significantly elevated in the kidney throughout the experiment. Transferrin, therefore, appears to be an important parameter in the APR of cod and its expression, at least in the kidney, is apparently not affected by the cortisol release. These results are in agreement with other studies of fish. Thus, an increase in transferrin gene

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expression was seen in blood- and spleen leukocytes of cod following an intra-peritoneal injection of heat-killed bacteria and showed a similar time span as in the present study [37,46]. An upregulation of transferrin gene expression has also been demonstrated in channel catfish and sea bass following bacterial infection [50,51] while induced stress did not have adverse effects on transferrin gene expression in rainbow trout [19].

Changes in the gene expression of the two antimicrobial peptides, hepcidin and cathelicidin were relatively limited. Hepcidin is an antimicrobial peptide and its possible role as a health marker in fish has received increased attention in recent years [52,53]. In general hepcidin gene expression in fish appears to be induced by bacterial infection or vaccination, viral challenge and iron overload, seen, for example, in rockbream (*Oplegnathus fasciatus*) [54] and sea bass [55]. A relatively slow (24 h) upregulation of hepcidin gene expression, comparable to the present results, was observed in the anterior kidney of cod following bacterial and viral mimic injections (Poly I:C) [28] while faster (within 3 h) induction of hepcidin gene expression has been seen following *in vitro* bacterial infection of cod epithelial cells [45]. The limited change seen in anterior kidney hepcidin gene expression in the present study might reflect the suppressive effects of the cortisol release. Another study has shown that, for example, handling stress, which induced cortisol release had suppressive effects on hepcidin gene expression in the spleen of Eurasian perch (*Perca fluviatilis*) [56].

The changes in the gene expression of cathelicidin showed a peak response later than hepcidin (at 72 hpi). Cathelicidin is a well characterised antimicrobial peptide in vertebrates and has also been identified in various fish species [57–59]. Studies of cathelicidin gene expression in cod under different conditions have demonstrated its antibacterial activity against fish pathogens and, hence, a role in innate defence [58]. Recent studies by Broekman et al. [25] have also indicated an active role in cod larvae development. The relatively small fold changes in the gene expression of both hepcidin and cathelicidin suggests that the antimicrobial peptides may not play a major role in the APR of cod when it involves inflammatory reaction and marked cortisol release.

The differences in the gene expression observed in the two organs, the anterior kidney and the spleen, are to be expected in view of subtle functional and structural differences between the two organs. The anterior kidney and the spleen, as well as the thymus, are the major immune organs of teleosts but their respective roles in an immune response vary from one species to another. Both the anterior kidney and the spleen are important scavenging and haematopoietic organs but the spleen is generally considered to play a secondary role to the anterior kidney [60]. The posterior kidney also has endocrine functions and forms a part of the hypothalamus-pituitary-interrenal-axis, an important element in maintaining homeostasis [2].

In our previous study of the same fish it was shown that APR induction by turpentine injection had rather limited and generally suppressive effects on humoral immune parameters, including the pentraxins. At the same time an increase in serum cortisol was significant and coincided with the reduced respiratory burst activity of the anterior kidney leukocytes [24]. However, the results of the gene expression analyses in the present study suggest that the pentraxins and ApoA-I may act as early APR mediators in the anterior kidney which, with the inflammatory cytokine IL-1β, induce cortisol release and stimulate C3 and transferrin expression. The antibacterial peptides seem to play a minor role in the APR of cod and are probably more important in the immune defence against infection.

The results indicate that of the two pentraxin types in cod, CRP-PII plays a more active role in the acute phase response of cod than CRP-PI. The results of the present and the previous study [24] also reveal the complex link between the induction of gene expression and the matching protein translation.

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