

## CYTOKINE GENES IN FISH

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### EXTENDED ABSTRACT ONLY – DO NOT CITE

Considerable progress has been made in recent years on the cloning and sequencing of fish cytokine genes (Secombes et al., 1998; Secombes et al., in press). In some cases the fish genes have been the first non-mammalian genes isolated and have given great insight into cytokine gene evolution. The increased knowledge currently accruing on cytokine regulation of immune responses within fish coupled with their potential future use as antimicrobials for disease control within the aquaculture industry (Secombes and Scheerlinck, 1999), makes fish cytokine biology a rapidly expanding arena.

#### **Interleukin-1**

IL-1 $\beta$  has been fully sequenced in several teleost species, including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), seabass (*Dicentrarchus labrax*) and carp (*Cyprinus carpio*), and has been partially sequenced in plaice (*Pleuronectes platessa*), goldfish (*Carassius auratus*), and turbot (*Scophthalmus maximus*). The sequences lack a signal peptide in common with mammalian molecules but differ in lacking an identifiable "cut-site" for ICE, required for generation of the biologically active mature peptide in mammals (Zou et al., 1999; Fujiki et al., 2000a). The salmonid sequences translate into a smaller precursor than in carp (260 vs 276 amino acids) and have one fewer exon (6 vs 7). Expression studies have shown that the transcription of the gene is induced by injection of LPS or challenge with Gram negative bacteria, or by stimulation of cultured leucocytes with LPS, with temperature and cortisol markedly affecting expression *in vitro* (Zou et al., in press). Both a second allele and a second IL-1 $\beta$  gene have been found in trout, and PCR studies have confirmed expression of both

genes upon stimulation with LPS. That a second IL-1 $\beta$  gene occurs in other fish species has also been confirmed (in goldfish). Sequencing of the 5' flanking region of the trout gene has revealed a number of possible transcription factor binding sites, including those for NF $\kappa$ B, CRE, PU-1, SP-1, NF-IL6 and  $\gamma$ -IRE. The bioactivity of recombinant trout IL-1 $\beta$  has also been examined, and shown to induce expression of immune genes in trout macrophages, and to induce proliferation of D10.g4.1 cells (Scapigliati, pers. comm). The dogfish (*Scyliorhinus canicula*) IL-1 $\beta$  cDNA has also been sequenced recently in our laboratory and will be discussed in detail.

### **Transforming growth factor- $\beta$**

Studies on TGF- $\beta$  have shown that all three isoforms are present in bony fish (Laing et al., 2000a). The fish equivalent of TGF- $\beta$ 1 and TGF- $\beta$ 3 are widely expressed in adults, whilst TGF- $\beta$ 2 has to date only been shown to be expressed in the heart of adult carp (Sumathy et al., 1997). The genomic organisation of trout TGF- $\beta$ 1 is different to that of other vertebrate TGF- $\beta$ 's (Daniels and Secombes, 1999), and preliminary data from the other fish isoforms suggest this may be peculiar to TGF- $\beta$ 1. Recently an inverse relationship between TGF- $\beta$  transcript production and bactericidal activity of kidney macrophages has been shown in triamcinolone-treated hybrid striped bass (Harms et al., 2000).

### **Chemokines**

Several chemokine and chemokine receptors have also been isolated in bony fish (Dixon et al., 1999; Fujiki et al., 1999; Daniels et al., 1999), with the latter representing receptors for both CC and CXC chemokines. A gene equivalent to IL-8 has also been sequenced in the lamprey (*Lampetra fluviatilis*) with some 32-33% amino acid identity to mammalian IL-8 genes and 40% identity to the chicken gene EMF-1 (Najakshin et al., 1999). It was found by sequencing clones representing the most abundant component of a blood leucocyte library after subtraction with liver cDNA. To date expression and bioactivity analysis of these molecules has still to be performed.

### **Other**

Most recently TNF $\alpha$  has been sequenced in the Japanese flounder (Hirono et al., 1999) and rainbow trout (Laing et al., 2000b), In addition, a pre-B cell enhancing factor has been sequenced in carp (Fujiki et al., 2000b). These molecules have

45% and 72% nucleotide identity and 45% and 86% amino acid identity to the equivalent human genes, respectively. The pre-B cell enhancing factor is known to be expressed in head kidney and peritoneal leucocytes but sites of TNF $\alpha$  expression have yet to be determined. The genes for a number of other cytokine receptors have also been sequenced in various bony fish, including the PDGF receptor, M-CSF receptor (CSF1R) and IL-2 receptor gamma chain ( $\gamma$ C) (How et al., 1996 ; Wang and Secombes, 2000).

### **Concluding Remarks**

Clearly we are only at the tip of the iceberg with respect to the isolation and study of cytokine genes in lower vertebrates. The next few years will see increasing numbers of genes being sequenced and the production of more recombinant molecules for bioactivity testing. Hopefully such advances will allow a further leap in our understanding of cytokine evolution, by producing probes to search for equivalent molecules in invertebrates, although perhaps only functional analogues will be present in some groups (Beschinn et al., 1999).

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**ENVIRONMENTAL EFFECTS ON THE ONTOGENY  
OF NON-SPECIFIC AND SPECIFIC DEFENCES  
IN TURBOT LARVAE**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Flatfish are economically important in European fisheries and are becoming increasingly significant in aquaculture. Turbot have been identified as the most suitable species of flatfish for rearing in aquaculture in Northern Europe because of its ease of growth and high market value (Munro, 1995). The growth of aquaculture has clearly shown that infectious disease is an important limiting factor in the production of flatfish. Prior to metamorphosis larvae are extremely susceptible to a number of viral and bacterial diseases, particularly *Vibrio* and *Aeromonas* sp., which are frequently associated with turbot larvae (Bergh, 1997; Novoa and Figueras, 1996). One of the problems of intensive culture of turbot is the variation in the survival rate of the larvae during early rearing stages. This is characterised by high, though variable mortality rates which make final survival rates unpredictable (Padros, 1996; Munro 1995).

Increased knowledge of the turbot immune system, and the effects of environmental conditions such as temperature and nutritional effects, on the appearance of defence mechanisms during ontogeny will aid the introduction of new strategies to cope with the problem of high mortality. It may also have a major impact on the recruitment of turbot in the natural environment and in

fisheries. Despite recent advances in salmonid immunology there is relatively little information available on the immune system of flatfish at a molecular level. Since turbot larvae are very small, a molecular approach is ideal to monitor early gene expression of non-specific and specific defences.

In order, to determine the effects of environmental conditions on the ontogeny of non-specific and specific defences in turbot larvae, probes are being developed to genes of the turbot immune system, concentrating on genes already characterised in other fish species, where the immunological relevance is very apparent.

The main approach which has been taken, in order to develop probes and obtain partial sequences of the turbot immune genes is PCR based homology cloning. Known sequences are aligned to reveal sites of conservation for primer design. Following PCR with degenerate primers based on these regions, products of the correct predicted size are cloned and sequenced and analysed for homology. However, the cDNA used as a template is also an important consideration, and must be from tissues or cells likely to express the gene of interest, as gene expression can be tissue specific and many immune genes are not expressed constitutively. For example, transferrin and Rag-1 are expressed predominantly in the liver and expression of IL-1 $\beta$  requires induction. Stimulation can be easily achieved *in vivo* by bacterial challenge, as with an attenuated (aroA-) strain of the Gram negative bacterium *Aeromonas salmonicida*, or *in vitro* by incubation of head kidney leucocytes with lipopolysaccharide (LPS).

To date partial sequences have been obtained for turbot interleukin 1 beta (IL-1 $\beta$ ), transforming growth factor beta 1 (TGF $\beta$ 1), recombinase activating gene 1 (Rag-1) and Transferrin. The nucleotide identity of these sequences to other fish species is shown in Table 1.

Preliminary expression studies looking at immune gene development in turbot eggs and newly hatched larvae have been carried out using probes to IL-1 $\beta$ , TGF $\beta$ 1 and Rag-1. The results showed that TGF $\beta$ 1 was expressed in eggs (day 1 – day 4 post-fertilisation) but not in the early larval stages (day 5 at hatching – day 7). There was no IL-1 $\beta$  or Rag-1 expression detectable at any stage over this early period of development.

TABLE 1: Percent nucleotide identity of partial sequences of immune genes in turbot to those of other fish species.

Gene	Length Sequenced	% Nucleotide Identity
IL-1 $\beta$	197bp	80% Plaice
		72% Rainbow Trout
TGF $\beta$ 1	185bp	94% Plaice
		81% Rainbow Trout
		79% Goldfish
Rag-1	552bp	82% Rainbow Trout
		74% Goldfish
		74% Zebrafish
Transferrin	871bp	72% Japanese Flounder
		67% Rainbow Trout
		66% Atlantic salmon

In a second larval rearing study, larval turbot were reared at 10°C, 14°C and 18°C under (largely) bacterial free conditions. Larvae reared at 10°C were incubated in LPS 24 hours prior to sampling. Expression of IL-1 $\beta$  was detected from 1-4 days after hatching in larvae stimulated with LPS but not in unstimulated samples. This is consistent with our previous work which showed that turbot IL-1 $\beta$  required induction by *in vitro* stimulation of head kidney leucocytes with LPS or by challenge (intraperitoneal) with *Aeromonas salmonicida*. Expression of the Rag-1 gene was not detected at this stage of development and is presumably a later event.

Probe development is currently on-going for other turbot immune genes.

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**ANTIBACTERIAL PROTEINS IN SKIN MUCUS  
FROM RAINBOW TROUT**

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**EXTENDED ABSTRACT ONLY -DO NOT CITE**

Teleost fish, in common with higher vertebrates, have the ability to mount adaptive 'memory' type immunity against invasive micro-organisms. Unlike mammals, however, adaptive immunity in fish is dependent upon temperature, fish age or level of development. Thus, most teleosts spend a large part of their life without the protection conferred by antibodies and so rely on the non-specific responses of the blood or mucosal surfaces to deal with opportunistic infection. A key part of innate immune defences in animals is the expression of a repertoire of antimicrobial peptides. These are low molecular weight (<10 kDa) proteins that display broad spectrum microbicidal activity (Boman, 1995). Whilst they have been extensively studied in many vertebrate and invertebrate animals, their presence in teleosts has received only scant attention.

To investigate the presence of such proteins in skin secretions of rainbow trout, *Oncorhynchus mykiss*, surface mucus was collected from instigated adult trout and subjected to ethanol precipitation, 2.5 % trifluoroacetic acid extraction, batch cation exchange on CM-Sepharose and reverse phase HPLC on 20 % acetonitrile gradients and a C<sub>18</sub> column (Smith et al., 2000). Fractions were assayed for antibacterial activity by the double layer radial diffusion procedure of Lehrer et al. (1991) using mainly the Gram positive bacterium, *Planococcus citreus*, and lyophilised cell

walls of *Micrococcus luteus*, as test agents. Fractions of interest were run on Tris-tricine 16 % SDS polyacrylamide gels and silver stained by standard procedures.

These experiments revealed that *O. mykiss* mucus contains at least five low molecular weight antimicrobial proteins. One is a conventional lysozyme with a molecular mass of ca 14 kDa and a pI 9.0. Another is an unusual muramidase with a molecular mass of ca 14 kDa but a pI of ca 6.0. Three other non-muramidase proteins are also present. One is a peptide of ca 3 kDa, active against Gram positive bacteria. Its partial amino acid sequence, obtained by Edman degradation, indicates that it has an amphipathic  $\alpha$ -helical secondary structure; a feature shown by many other animal antimicrobial peptides. However, it does not appear to be expressed consistently in trout mucus. By contrast, two other antimicrobial proteins in *O. mykiss* mucus, which have molecular masses of ca 6.6 kDa and 7.9 kDa, respectively, occur more constantly throughout the year. One of these (the 6.6 kDa peptide) has been isolated by reverse phase HPLC and subjected to mass spectrometry and partial N-terminus amino acid sequence analyses. It has a molecular mass of 6,621 Da and the first 10 residues are identical to a member of the High Mobility Group (HMG) family of proteins from mammals (Landsman et al, 1986). Broth micro-dilution antibacterial assays (Giacometti et al., 2000) have revealed that this protein is active against both Gram positive and Gram negative bacteria, is inhibited by high salt concentrations and is non-lytic. The fifth protein fractionated by reverse phase-HPLC is a peptide of 7,959 Da, as determined by mass spectrometry. Internal sequence data, obtained after trypsin digestion, show that it contains repeats of a GGHD motif, known to be present in holotricin, an antibacterial peptide previously purified from coelopteran beetles by Lee et al. (1995).

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**INTERFERON INDUCED GENES  
IN THE RAINBOW TROUT**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

The vertebrate interferon system provides an early line of cellular defence against viral infection by inducing the production of proteins that inhibit virus replication. Mx proteins have been shown to be part of these intracellular mediators of viral resistance (see for review: Samuel, 1991).

In the rainbow trout, three Mx cDNA have been cloned and sequenced (Leong *et al.*, 1998). At the amino acid level, RBTMx1 and RBTMx3 are greater than 96 % identical and RBTMx2 differs slightly from the other two with an 88.2 %

identity to RBTMx3 and a 90.6 % identity to RBTMx1. It is not known whether these isoforms are encoded by one of more loci.

Fish had been shown to have interferon activity (De Kinkelin *et al.*, 1982) but to date interferon has neither been purified nor its coding nucleic acid sequence determined. In this study we used Mx gene as a marker of interferon activity in the rainbow trout *Oncorhynchus mykiss*.

### Expression of mx *in vitro*

An RT-PCR system for Mx genes has been developed. Primer pairs were designed from the published cDNA sequences in order to specifically amplify the three Mx forms. Genomic DNA was used as template for PCR and the products were cloned and sequenced. The three Mx sequences obtained appeared to include one or two introns, making the primers suitable for expression studies by RT-PCR. The specificity and inducibility of Mx genes were confirmed using these primer pairs for *in vitro* studies. The rainbow trout cell line RTG2 was induced by poly I:C or Interferon Containing Supernatants (ICS) for 12, 24 or 48 hours. ICS induced transiently Mx1 and Mx3 after 12 hours of incubation. PolyI:C induced Mx1 and Mx3 later, after 24 hours of incubation (Fig. 1).

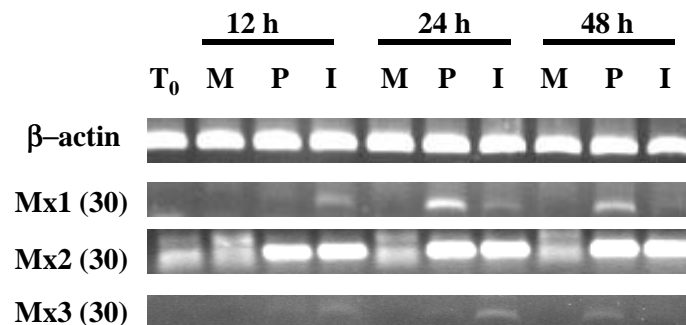


Figure1: Time course of induction of Mx expression in RTG2 cells by ICS or polyI:C. The number of PCR cycles is indicated in brackets. T<sub>0</sub>, control before incubation, M, Medium, P, PolyI:C 20 µg.ml<sup>-1</sup>, I, ICS diluted 2 fold

Induction of Mx1/3 and Mx2 have different time course patterns, and adds to argument they may have different biological functions as suggested by the cellular location of the proteins (Leong, 1998).

### **Mx expression in DNA-vaccinated fish**

Expression of Mx1 transcripts was investigated in fish which were previously injected with a DNA vaccine directed against the surface glycoprotein of the VHS virus (Lecocq-Xhonneux *et al.*, 1994). Eight weeks after vaccination, Mx was expressed in Liver and head kidney of vaccinated fish, but not in the controls (injected with vector alone).

### **Regulation sequence of mx gene**

As a way to simplify later analysis of interferon production, the promoter of the interferon-induced gene Mx1 has been investigated. Five genomic libraries were constructed using the Genewalker Kit (Clontech). Briefly, genomic DNA was purified and digested with five different blunt-end restriction enzymes (PvuII, StuI, DraI, EcoRV and ScaI) giving, after ligation of an adaptor on 3' and 5' ends, five genomic libraries. Reverse primers were designed in the 5' end of the Mx1 cDNA and used in nested PCR with the adaptor primers. Hot start and touchdown technologies were used to increase the specificity of amplification. Long distance PCR was also carried out with a mix of *Taq* and *Pfu* polymerases.

Two fragments of 620 bp and 356 bp were obtained, cloned and sequenced. The two fragments appeared to belong to the same contig. As a verification of the sequence, a forward primer were designed within this new sequence and used with a reverse primer located on the transcribed region of the gene. PCR was carried out on genomic DNA extracted from different individuals and gave a product with the expected size.

The sequence obtained has the typical structure of a promoter of an interferon induced gene: a TATA box located 30 based upstream of the putative site of initiation of transcription, a highly conserved 13 bp-long Interferon Stimulating Response Element (ISRE, see Fig 2) and a Sp1 site, responsible for induction by viruses.

Consensus	RGAAANNGAAASY
Rainbow trout	TGAAAGTGAAACA *****
Mouse	AGAAAC-GAAACT
Mouse 202	GGAAATTGAAAGC
Human 2',5'AS	GGAAAC-GAAACC
Human 6-16	GGAAAATGAAACT
Human 56 kDa	GGAAAGTGAAACT
Human ISG15	GGAAACCGAAACT
Human ISG54	GGAAAGTGAAACC
Human factor B	GGAAACAGAAACT
Human IFN- $\alpha$ 1	AGAAATGGAAAGT

Figure 2: Alignment of the Interferon Stimulating Response Element (ISRE) in rainbow trout with mammalian ISRE present in the regulating sequence of various interferon induced genes. Codes for the consensus sequence are as followed: R = A or G, Y = C or T, S = G or C, N = A, C, G or T.

This structure is similar to the mouse promoter for Mx1 (Hug *et al.*, 1988) with the difference that in rainbow trout there is a single ISRE longer than the five ISRE motifs present in the murine promoter. The ISRE shows a complete identity with the human promoter for the 56 kDa-interferon induced-2',5' oligoadenylate synthetase, another interferon-induced gene. Only the first and last base of the trout ISRE does not match the consensus (Friedman and Stark, 1985) and seem to be inverted.

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## **IPN RECOMBINANT VACCINES**

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### **EXTENEDED ABSTRACT ONLY – DO NOT CITE**

IPN has allegedly been responsible for high (30%) mortalities in post-smolt Atlantic salmon in Norway and Shetland but experimental infection of fish with the IPN virus isolated from these outbreaks has failed to induce any signs of disease. However, one Norwegian group has claimed to induce IPNV-related mortality but the conditions have not been published.

Vaccination efficacy trials have been based on the ability of vaccinated fish to clear the virus after challenge. Two recombinant IPNV proteins have been expressed in different systems: The IPN VP3 protein and a truncated form of the VP2 protein were expressed in *E. coli*, the yeast *Pichia pastoris*, the fish cell line Chinook salmon Embryo Cells(CHSE), and the mammalian cell line Chinese Ovary cells(CHO). Fish were immunised with 100ug antigen in Montanide adjuvant by ip injection. Fish were blood sampled 8-14 weeks later and then challenged by ip injection of IPNV. Three and 10 weeks after challenge fish were sampled for serum antibody (ELISA and virus neutralisation) and kidney (for presence of culturable virus).

All vaccines induced anti-IPNV antibodies detectable by ELISA. The *E.coli* – expressed antigens did not induce virus neutralising antibodies (the other antisera have not yet been tested in this assay). Three weeks following challenge, the *E.coli* – immunised fish showed a marked increase in antibody titres (by ELISA) and this antisera was now able to neutralise the virus (other

antisera have not yet been tested). Sera from control fish was negative for antibody by both assays, both before and after challenge.

Following challenge, IPNV was isolated from some individuals in all groups of fish (n=10) except the group immunised with the yeast recombinant VP2 vaccine. This antigen also appeared to be the most immunogenic in that it induced antibody responses in a higher proportion of fish than the other test vaccines.

**DNA VACCINATION DURING  
LOW TEMPERATURE  
AND PARTIAL STARVATION**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Intramuscular DNA vaccination against fish rhabdoviruses has been outstandingly successful when fish have been vaccinated under optimal conditions. Such conditions do not always apply to fish farms e.g. in winter or when fish are fed for maintenance. How DNA vaccines might work under these situations was examined in goldfish (*Carassius auratus L*), (Russell et al., 2000). To prevent the use of dangerous pathogens a plasmid which encodes the reporter betagalactosidase (b-gal) was used because 1) the limiting dose of this plasmid for antibody production by goldfish was known (Kanellos et al.,1999); and 2) the rate of destruction of the transfected b-gal positive myofibres is a measure of cytotoxic T cell activity in mice (Davis et al.,1997).

Goldfish, weighing 5g to 15 g, were kept at 15 °C for 1 month and then water temperatures were adjusted up to 24 °C or down to 9 °C and the fish were left to acclimatise for 1 month (Bennett et al., 1998) when they received 500ng plasmid by injection into the epaxial muscle behind the dorsal fin. Fish at 9°C were then shifted down to 7°C and then to 5 °C at intervals of 28 days. At 24°C b-gal-positive fibres appeared after 1 week and were destroyed by 2 weeks (Figure 1).

Antibody became detectable at 2 weeks and remained at a moderate level. At 15°C fibre destruction and antibody production occurred more slowly, at 4-8 weeks, and antibody became very high at 18 weeks. At ≤9°C, the production of antigen in muscle rose to a maximum between 4 to 18 weeks and antibody rose to a moderate level over this time (Figure 1).

These differing kinetics suggested that at 24 °C fish behaved like mice in that cytotoxic T cells caused the destruction of fibres as B cells started to produce antibody at 1 to 2 weeks after DNA injection. At ≤9°C antibody was made without the accompanying destruction of b-gal-positive fibres as if T but not B cells were less active at this temperatures, as suggested by hapten-carrier experiments with carp (Avtalion et al., 1976). The only example of cyprinid fish making antibody at 5°C is to bacterial flagellin (Azzolina et al., 1978) which is a T-independent antigen in mice. Fish lack IgG and so their sustained antibody production might require less T cell activity than mice.

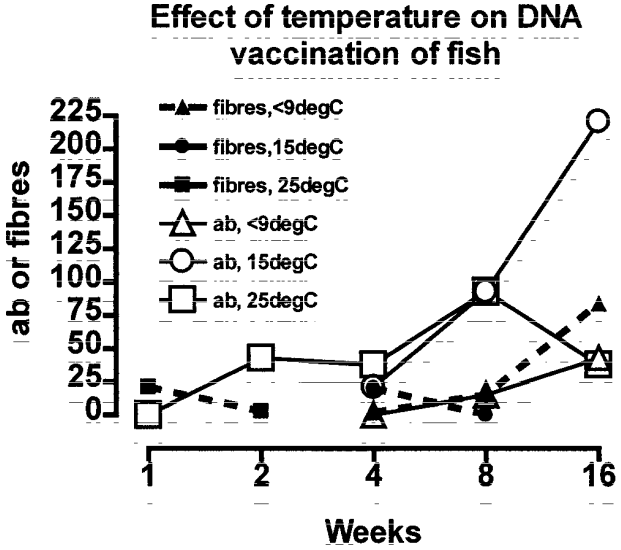


Figure 1.

When temperature is lowered fish eat less and so fish at 22 °C were put on a reduced diet of 0.33% bodyweight of goldfish food flakes (Tetra) each day compared to their usual intake of 3% for growth. The fish on 0.33% lost 20% of their weight in 6 weeks and then had to be killed under the terms of our Home Office license. The fish on 3% food gained 50% body weight. The fish on 1% food increased 14% bodyweight. Plasmid was injected at 2 weeks into the experiment. At 4 weeks fish on all 3 diets had antigen positive fibres and at 6 weeks, the end of the experiment, all fish had serum antibody (Figure 2).

The conclusions of this work were that intramuscular DNA vaccination works when fish are at low temperatures or on low food intake and so DNA might be a better means of immunising fish than with exogenous antigen. Future work could extend the range of temperatures and food intake and examine whether cytokines and dietary factors can improve the immune response under limiting conditions of food or temperature.

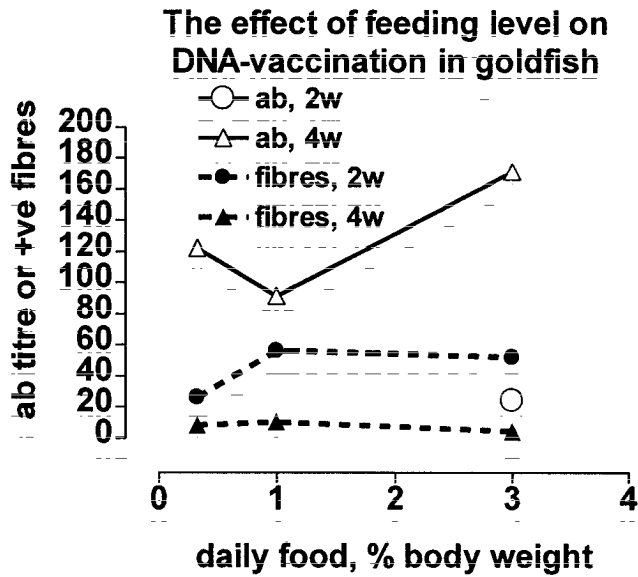


Figure 2

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**PROTECTIVE EFFICACY OF  
VHSV DNA VACCINATION  
IN RAINBOW TROUT**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Viral haemorrhagic septicaemia (VHS) has severe effects on farmed rainbow trout, resulting in significant losses. Despite efforts over the past two decades using traditional approaches, no successful vaccine has been developed (Lorenzen & Olesen, 1997). DNA vaccination is a potentially successful vaccine strategy whereby protection is induced through administration of pathogen-derived genes into the animal. Good protection is achieved as the DNA vaccine appears to be processed and presented in a similar manner to that of a natural infection (Donnelly *et al.* 1997). To date, DNA vaccination has proved successful in rainbow trout vaccinated against the G (glycoprotein) gene of infectious haematopoietic necrosis virus (IHNV) (Anderson *et al.* 1996) or VHSV (Lorenzen *et al.* 1998). In this study, rainbow trout were vaccinated with

VHS-G DNA vaccine in order to determine how early protection against VHSV could be achieved.

Rainbow trout (mean weight 4.5g; n=150/group) were injected intramuscularly (i.m.) with either 0.5 µg pCDNA3-vhsG or 0.5 µg pCDNA3 in 30 µl Tris/EDTA (TE). Control groups were injected with either TE alone, 2µg inactivated VHSV or left untreated. Fish were challenged by bath at either 1 week, 4 weeks or 8 weeks post vaccination (p.v.) using VHSV virus (Voldbjerg strain;  $1 \times 10^5$  50%  $\text{ml}^{-1}$  TCID<sub>50</sub>). Dead fish were collected daily and cumulative mortalities were calculated over a 4 week period. Blood samples were taken from fish surviving challenge and sera was analysed for neutralizing activity using the 50% PNT test (Olesen & Jorgensen 1986). Mx gene expression was examined in vaccinated, but unchallenged fish (Dr B Collet, University of Aberdeen).

When fish were challenged 1 wk p.v., there was no significant protection in any of the treatment groups (Table 1). Fish receiving the pCDNA3-vhsG vaccine showed a high level of protection when challenged 4 weeks p.v and only 22 % mortalities were recorded. In contrast, mortalities of 86 % or greater were found in all other groups (Table 1). When fish were challenged 8 weeks p.v., a similar result was achieved where mortality amongst DNA-vaccinated fish was 14% but high in all other groups. Vaccination with 2 µg inactivated VHSV failed to provide any protection in fish challenged at either 4 or 8 weeks p.v. (Table 1). No significant mortalities were recorded in vaccinated, unchallenged fish (data not shown).

Table 1. Cumulative mortalities in VHSV DNA vaccinated rainbow trout following challenge at various times post vaccination

Group Treatment	Cumulative mortalities (%) following challenge		
	1 week p.v.*	4 week p.v.	8 week p.v.
0.5 µg pCDNA3- vhsG	60	22	14
0.5 µg pCDNA3 Tris/EDTA	74	98	78
2 µg inactivated VHSV	86	88	96
Untreated	54	86	88
	74	90	92

\*p.v.=post-vaccination

This study demonstrates that VHSV DNA vaccination induces significant protection as early as 4 weeks p.v. with 0.5 µg of DNA. At 8 weeks p.v., doses as low as 0.1 µg DNA is protective (Lorenzen *et al.* 1999). Work is currently in progress to determine if protection correlates with the presence of neutralising antibodies and if a "threshold" level of DNA is required to induce protection at various stages after vaccination.

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**USE OF POLY(DL-LACTIDE-CO-GLYCOLIDE)  
MICROPARTICLES AS ADJUVANTS IN ATLANTIC SALMON**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Vaccination of atlantic salmon in the Scottish salmon farming industry generally involves intraperitoneal injection of individual fish using vaccines containing oil adjuvants. This method is thought to confer good protection for considerable lengths of time. However, this method is labour intensive and causes a considerable amount of stress to the fish, since the fish have to be anaesthetized, handled, injected and returned to clean water to recover. The size of the fish also has to be considered since injecting fish weighing less than 15g is difficult. Other potential problems with injection immunisation are adhesion formation, the use of potentially toxic adjuvants, temporary reduced feeding, potential puncture of intestines and wound creation (Ellis, 1988). It is therefore desirable to use safer adjuvants and also to develop immunisation strategies which are less stressful and labour intensive. Over the past thirty years in human medicine there has been an interest in the use of biodegradable polymers as adjuvants and vaccine delivery systems both for oral and non-oral routes (O'Hagan *et al*, 1993, Jones *et al*, 1996). One of the most thoroughly researched polymers is that of poly(DL-lactide-co-glycolide) (PLG), which has a safe record of use within medical and veterinary uses (Visscher *et al*, 1987, Vert *et*

al, 1991). Used as an adjuvant it is thought to act by effective presentation of the vaccine to antigen processing cells and as a depot for prolonged release. Our work concentrates on the use of PLG as an adjuvant and the potential of PLG as a carrier for oral vaccine delivery in atlantic salmon.

PLG microparticles were prepared using a commercially available lactide/glycolide polymer (Medisorb, USA) incorporating a commercially available furunculosis vaccine (AVL, UK). Three different grades of PLG microparticles were prepared incorporating vaccine and were subsequently injected or orally intubated into atlantic salmon (weighing 10-15g, 80 per group) at three doses of PLG (100µg, 10µg or 1µg PLG per fish). Control groups were ip injected or orally intubated with blank PLG microspheres (containing PBS), PBS or 100µl of oil adjuvanted furunculosis vaccine (AVL, UK). Fifty fish per group were challenged at week 13 post immunisation with a virulent strain of *Aeromonas salmonicida* MT1326D, which had previously been passaged through atlantic salmon to increase its virulence. 30 fish per group were bled at week 22 post immunisation and serum antibody titres determined by a bacterial agglutination test.

In both the ip injected and orally intubated groups no significant protection was conferred by any of the PLG constructs upon experimental challenge. Significant protection was achieved using the oil adjuvanted vaccine. RPS values are as shown in Table 1. Upon testing sera from the ip injected groups by bacterial agglutination, titres recorded from the oil adjuvanted ip injected group were consistently high, whereas the sera from all bar three fish in the PLG construct groups failed to produce recordable agglutinating titres.

Our initial studies have shown that we were unable to record agglutinating antibody titres in fish immunised either by ip injection and oral intubation of the PLG/vaccine and that these groups failed to produce significant protection against experimental challenge with a virulent strain of *Aeromonas salmonicida*. Future work will concentrate on improving encapsulation and optimising release of the vaccine.

Table 1: RPS values for experimental groups upon experimental challenge with *Areomonas salmonicida*

<b>Group</b>	<b>RPS ip injection</b>	<b>RPS Oral intubation</b>
PLG Blanks	-9.4	-13.6
PLG Low IV 100µg/fish	12.5	-59.1
PLG Low IV 10µg/fish	12.5	-36.4
PLG Low IV 1µg/fish	-37.5	-50
PLG 2A 100µg/fish	28.1	-54.5
PLG 2A 10µg/fish	9.4	-40.9
PLG 2A 1µg/fish	-25	-40.9
PLG 4A 100µg/fish	-12.5	-13.6
PLG 4A 10µg/fish	-28.1	4.5
PLG 4A 1µg/fish	-9.4	4.5
Furovac oil adjuvant	37.5	81.8

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## **THE EFFECT OF ENVIRONMENTAL SALINITY AND HOST SPECIES**

*(Oncorhynchus tshawytscha, O. kisutch, O. mykiss)*

### **ON THE PATHOGENESIS OF *Loma salmonae***

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### **EXTENDED ABSTRACT – DO NOT CITE**

*Loma salmonae* (Microspora) is a gill disease causing morbidity and mortality of farm-reared salmonids (*Oncorhynchus*), particularly chinook salmon, in the Pacific Northwest region of North America (Hauk 1984; Kent et al., 1989). A high mortality rate and susceptibility to secondary infections often make chinook salmon a difficult host species for researching *L. salmonae*. A freshwater rainbow trout model has been developed to study pathogenesis and pathobiology of *L. salmonae* infections (Speare et al., 1998). Rainbow trout experience less *L. salmonae*-associated mortality than chinook salmon and are less susceptible to secondary infections (Speare et al., 1998). However, host species and environmental factors such as salinity may be important the transmission and proliferation of *L. salmonae*. The effects of host species and salinity on the duration and intensity of *L. salmonae* infections have not been formally examined. The purpose of this research was to determine if host species and environmental salinity affected the duration and intensity of *L. salmonae* infections.

This research was conducted in the Fish Health Wet Laboratory of the Pacific Biological Station (Fisheries and Oceans Canada) in Nanaimo, British Columbia. Eighty fish from each of three species, chinook salmon, coho salmon and rainbow trout (RBT) were examined. Forty fish of each species were reared

in freshwater (0 - 5 parts per thousand (ppt)) and forty fish were reared in seawater (22 - 24 ppt). Each fish was infected, *per os* with approximately 50 000 *L. salmonae* spores from infected chinook salmon gills. All three species were cohabited in each seawater and freshwater 750-litre deep oval fiberglass tanks.

Examination of gills was completed every two weeks post-exposure (PE) to infection to assess the percentage of fish infected which was characterised by the presence of spore-filled cysts (xenomas) within the gill filaments. Lethal gill samples were taken at week 7 and 8 PE for histology to assess the intensity of infection in terms of the number of xenomas per gill arch. The three species were compared within each salinity. Each species was also compared at the different salinities. Interactions between species and salinity were also examined.

Chinook salmon and rainbow trout began developing visible gill xenomas by week 5 PE while coho salmon did not begin to develop xenomas until week 6 PE. Rainbow trout had cleared infection by week 9 PE while infections in chinook and coho salmon persisted. The percentage of infected fish increased over time and then decreased in the case of rainbow trout (Table 1). The percent infection over time in seawater and freshwater did not differ substantially. The mean intensity of infection, measured in xenomas per gill arch, was highest in week 8 PE chinook salmon in freshwater (130) and lowest in week 8 PE rainbow trout in seawater (2). In general, chinook salmon had the highest intensity of infection followed by coho salmon and rainbow trout (Table 2). The intensity of infection may be dependent upon interactions between species and salinity.

The longer duration and higher intensity of *L. salmonae* infections in chinook salmon may explain why *L. salmonae*-associated mortality is more of a problem in chinook salmon aquaculture. Rainbow trout appear to be more resistant to *L. salmonae* infections. This makes rainbow trout a favourable model for studying pathogenesis, since mortality is rare and the complete life cycle may be examined (Speare et al., 1998). The resistance of rainbow trout to mortality from *L. salmonae* infections may be of interest as researchers attempt to find methods for control of epizootics in aquaculture.

Table 1: Effects of host species and salinity on the percentage of *L. salmonae* infections over time, expressed as the percent of xenoma-positive fish examined.

	seawater						freshwater					
week PE	2	5	6	7	8	9	2	5	6	7	8	9
<b>chinook</b>	0	33	90	100	100	100	0	40	90	100	100	100
<b>coho</b>	0	0	90	100	100	90	0	0	90	100	100	67
<b>RBT</b>	0	40	100	38	57	0	0	33	80	60	50	0

Table 2: Effects of host species and salinity on the intensity of *L. salmonae* infections over time, expressed as the mean number of xenomas per gill arch

	seawater		freshwater	
week PE	7	8	7	8
chinook	96	48	116	130
coho	40	20	32	29
RBT	5	2	15	6

The duration and intensity of *L. salmonae* infections is dependent upon species and salinity. Chinook and coho salmon had longer durations of infection while rainbow trout had a comparatively shorter duration of infection. Chinook salmon

had a higher intensity of infection compared to coho salmon and rainbow trout which may explain higher mortality in *L. salmonae*-infected chinook salmon. Salinity does not appear to affect the duration of *L. salmonae* infections but it may affect the intensity of infection and may also be dependent upon species. It is important to realise that host species and salinity are important factors in the transmission, proliferation and pathogenesis of *L. salmonae*.

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**EFFECT OF FOOD DEPRIVATION  
ON BKD AND FURUNCULOSIS**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Anorexia is a common sign of bacterial and viral diseases and is thought to be a consequence of the disease process. However, this inappetence may be an active portion of the host defense system (Murray et al. 1978, 1979). There is evidence with some bacterial fish pathogens that feed withdrawal can significantly reduce mortality (Damsgård et al., 1998; Wise and Johnson, 1998). We tested this idea by withdrawing or reducing food from juvenile chinook salmon (*Oncorhynchus tshawytscha*) during *Renibacterium salmoninarum*

(causative agent for bacteria kidney disease, BKD) and *Aeromonas salmonicida* (causative agent for furunculosis) epizootic. Fish in the BKD experiment were first purposely stressed and then fed either a full ration, half of that or fasted for six weeks (Pirhonen et al. 2000). Fish in that experiment were naturally infected with BKD. In the furunculosis experiment fish were infected by introducing five infected cohabitants into the tanks of 50 naïve fish which were either fed or fasted for one month.

At the termination of the BKD experiment, feed intake of the fish was evaluated by X-radiography after feeding all groups to complete satiation and the amount of BKD p57 antigen in the kidneys was measured by ELISA to assess effects of infection on feeding rates. Only a few individuals in each treatment died during the experiment, but the proportion of fish with detectable antigen concentration increased as ration level decreased. Within each treatment, fish with undetectable concentrations of p57 antigen ate significantly more than fish with elevated antigen levels. Exponential regressions were fitted for each ration level describing the decrease of appetite as levels of antigen concentrations increased. The data indicate that even fish that were quite sick as judged from their relatively high antigen concentrations can still feed, and that previous food deprivation can increase the feed intake to some extent in the sick fish (Pirhonen et al. 2000).

In the furunculosis experiment one month after exposure to *A. salmonicida*, disease specific mortality was low (5.0 % and 13.3 % in fed and fasted groups, respectively); there was no mortality in uninfected control fish. While few fish had detectable *A. salmonicida* in the kidney, at the termination of the experiment an average of 18.5 % and 65.0 % of the fish in fed and fasted groups, respectively, had this bacterium in or on mucus. Feed intake was measured by X-radiography at days 16 (fed groups) and 32 (all groups). Feed intake as well as growth were unaffected by exposure to bacteria. However, food consumption was greater when fasted fish exposed to *A. salmonicida* were offered a meal than in those infected individuals that had been eating. Our results suggest that during a chronic infection of furunculosis fasting would not increase immunocompetence.

These results may be relevant for application of medicated diet, as it seems possible that fasting of sick fish before administration of medicated ration could increase the probability that also sick individuals would eat.

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**COMPARISON OF PHYSIOLOGICAL AND BIOCHEMICAL  
PARAMETERS IN RAINBOW TROUT, COHO  
AND ATLANTIC SALMON FOLLOWING  
INFESTATION WITH SEA LICE (*LEPEOPHTHEIRUS SALMONIS*)**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

A number of strategies to control sea lice have been used over the last decade with limited success. A better understanding of host-parasite relationships is essential in order to develop more effective control measures (for the future). Natural resistance to sea lice infestation has been observed in coho salmon; however the mechanism remains unknown (Johnson and Albright, 1992). We are examining the response of sea lice (*Lepeophtheirus salmonis*) to different host species and the response of different host species to sea lice. Our focus is on biochemical parameters of salmonid mucus and physiological parameters of salmonid blood during infestation with sea lice.

**Methodology**

Three salmonid species (rainbow trout, coho and Atlantic salmon) were cohabited in four tanks for two weeks to acclimate. The tanks were separated

into two test and two control tanks and approximately 100-150 infective copepodids/fish were added to each test tank. On days 0 (prior to infestation), 1, 3, 7, 14, and 21, post infestation, blood and mucus samples were collected. Cortisol, glucose, hematocrit, electrolytes and protein were measured in the blood as previously described (Bowers et al, 2000). Alkaline phosphatase, lysozyme, and protease activities were analyzed in the mucus as previously described (Ross et al, 2000).

#### *Live lice incubations*

Incubations were carried out by adding live lice for up to one hour in test tubes containing either seawater or mucus from rainbow trout, coho salmon, Atlantic salmon or winter flounder. Lice were then removed and protease activity in seawater and mucus was measured using zymography.

#### *Statistical methods*

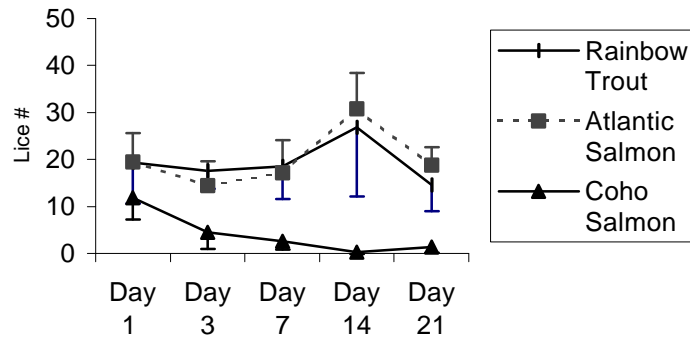
Analyses of variance (ANOVAs) were performed to observe tank effects during the trial (1-way), and to compare blood and mucus parameters among species, day and condition (test/control) (3-way).

## **Results**

#### *Sea lice counts*

There were no significant differences in lice load on any day between rainbow trout and Atlantic salmon. Lice counts were significantly lower on coho salmon than on Atlantic salmon and rainbow trout (Figure 1). The number of lice on coho salmon steadily decreased over time with only 3 total lice found on 10 fish on day 14. On day 21 the number of lice increased on coho salmon while they decreased on rainbow trout and Atlantic salmon.

Figure 1: Lice counts on the three salmonid species over the course of the experiment



#### *Blood Physiology*

Several blood parameters were examined including cortisol, glucose, osmolality, electrolytes, protein and hematocrit. Only hematocrit, protein and glucose levels were analyzed for coho salmon. Electrolytes such as sodium, chloride and potassium showed no significant differences between infected and control fish or between species. Sodium levels ranged between 150-165 mmol/L in both Atlantic salmon and rainbow trout. Chloride levels ranged between 120-135 mmol/L and potassium levels fluctuated between 0.8-1.5 mmol/L in both Atlantic salmon and rainbow trout. Blood osmolality, cortisol and glucose levels also showed no significant difference between infected and control fish or between the two species. Osmolality ranged from 320-340 mosm/L while glucose levels stayed between 4-8 mmol/L. Cortisol levels ranged anywhere from 0-200 nmol/L but mostly hovered between 20-40 nmol/L for all three species.

Protein and hematocrit levels did not vary significantly between infected and control fish but did vary between species. Coho salmon had significantly higher hematocrit levels ( $p < 0.05$ ) than the other two species while rainbow trout had

significantly higher protein levels ( $p < 0.05$ ) than the other two species over the course of the study.

#### *Mucus Biochemistry*

Alkaline phosphatase levels were significantly higher ( $p < 0.05$ ) in infected Atlantic salmon compared to controls on days 3 and 21, while no significant differences were noticed between infected and control fish in coho salmon and rainbow trout. There were no significant differences in alkaline phosphatase levels between Atlantic salmon and coho salmon, but they both differed significantly from rainbow trout on day 21 ( $p < 0.05$ ). There was a significant interaction between species, day and condition in lysozyme activity. Lysozyme levels were significantly higher in infected rainbow trout than control fish throughout, and infected coho also had significantly higher lysozyme activities compared to control fish on day 21. Quantitative measurements of protease activity (azocasein hydrolysis) did not differ significantly between species or control and infected fish except on day 21 when infected coho salmon had significantly less protease activity compared to controls. Qualitative measurements of protease activity (zymography) showed a significant increase in low molecular weight proteases in infected rainbow trout and Atlantic salmon. These proteases were secreted by the louse as shown by Ross et al (2000).

#### *Live lice incubations*

Atlantic salmon and rainbow trout mucus appeared to stimulate protease release from lice more consistently (75-80% positive responses) ( $n=20$ ) than seawater or coho salmon and flounder mucus (20-30% positive responses) ( $n=20$ ) as determined by the appearance of lice-derived low molecular weight protease bands on zymograms.

### **Conclusions**

Almost all lice were sloughed off from coho salmon between days 7 and 14, consistent with the findings of Johnson and Albright (1992). Lice numbers on day 21 increased on coho salmon. The lice on coho salmon at day 21 had probably migrated from the other species. Sea lice have been observed to move

from one host to another (Jacobsen, 1993). The lack of change in blood physiology suggests that cortisol, and other stress responders measured, are not involved in the coho salmon inhibitory response to *L. salmonis* challenge. However, the above results suggest that differences in skin mucus between species may play a role in resistance to sea lice.

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**PHYSIOLOGICAL, BIOCHEMICAL AND  
HISTOLOGICAL APPROACHES TO EXAMINING  
SEA LICE-SALMON INTERACTIONS**

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Sea lice infections are a major concern for salmonid aquaculture operations. Laboratory models have been established to examine interactions between sea lice (*Lepeophtheirus salmonis*) and Atlantic salmon (*Salmo salar*) as part of an effort to develop alternate strategies for sea lice control. We have developed methods to grow sea lice from eggs and culture them to the infective stages in tanks under controlled conditions. Sea lice have been maintained on salmon in our re-circulation system for as long as 7 months. Experiments conducted to date include 4 major areas: 1) production of sea lice eggs and assessment of their viability, including production of maximum numbers of infective copepodids, 2) effects of sea lice on the development of chronic stress and suppression of host salmon defence mechanisms, 3) identification of factors (eg. enzymes) in mucus that change during the course of infection and characterization of their source and roles, and 4) examination of resistance of recovered and naive Atlantic salmon to sea lice. A review of our research to date is presented.

*Development of standardized methodology enabling consistent and repeatable infections of salmon with sea lice under laboratory conditions*

Salmon for physiological and pathological studies are obtained as smolt from certified hatcheries and smoltified in the Atlantic Veterinary College Aquatic Animal Facility by increasing salinity over a 1 week period to sea water strength (30 ppt) and maintained at 10EC under controlled conditions. A system of obtaining *L. salmonis* eggs from aquaculture sites and culturing them to the infective copepodid stage in the laboratory has been developed. Consequently, sea lice have been maintained on salmon in a re-circulation system for as long as 7 months. The numbers of surviving adult females sea lice and their egg-strings decreased over the experimental period (Mustafa et al., 1999). Eggs from these parasites also lost their hatching ability and ability to develop into infective copepodids. Currently, attempts are being made to increase culture viabilities by examining potential areas of stress and trying various modifications to the culture system.

Effects of sea lice on the development of chronic stress and suppression of host salmon defence mechanisms; identification of factors (eg. enzymes) in mucus that change during the course of infection and characterization of their source and roles

Infection of Atlantic salmon with sea lice in a laboratory setting induces a chronic stress response with a resultant increase in plasma cortisol and a suppression of non-specific defence mechanisms (Bowers et al., in press; Mustafa et al., 2000). The fish were not stressed during the larval stages of lice development, but plasma cortisol levels were elevated and non-specific immune responses depressed when the sea lice became mobile pre-adults and adults. The stress response could be measured well before any physical damage, such as skin lesions, was observed.

Biochemical changes in the mucus were examined since the mucus is the primary site of interaction between salmon and *L. salmonis*. Mucus protease activity was observed to increase over the course of a sea lice infection in Atlantic salmon (Ross et al., in press). Low molecular weight, 17 to 22 kDa, trypsin-like proteases were of particular interest and were determined to be derived from the sea lice (Firth et al., submitted). A cDNA library has been constructed from whole pre-adult *L. salmonis* and several clones have been partially sequenced to yield an expressed sequence tag (EST) library. Comparisons of ESTs with sequence databases resulted in 56% of the ESTs having similarity to known genes from other organisms. Chymotrypsin and

trypsin genes have been identified and a number of full-length trypsin clones have been obtained (Johnson et al., submitted). Comparison of the sequence of the trypsin found in the salmon mucus with the above genes will be carried out in the future. It is proposed that the trypsin is being secreted by the sea lice into the mucus to either aid in feeding and/or to help the sea lice in avoiding the host immune system by digesting humoral immune factors.

Changes in protein glycosylation state were observed in plasma samples from infected Atlantic salmon. Two plasma proteins were identified in 2-D gel electrophoresis as having an increased number of sialic acid side chains in infected fish. Sialic acid moieties on glycoproteins are important in the regulation of protein and cell turnover and changes in the sialylation state of these plasma proteins may be an important trigger or indicator of the health status of the fish. Identification of the sialylated proteins in plasma is underway.

At present we are comparing the responses of rainbow trout, Atlantic and coho salmon to infections with *L. salmonis*. It is known that coho salmon, in comparison to Atlantic salmon, are particularly resistant to sea lice (Johnson and Albright, 1992), but the mechanism for this resistance is not known. Rainbow trout are also being included due to their increasing commercial potential (partly because they are relatively resistant to the ISA virus). The development of chronic stress, suppression of host defence mechanisms, and changes in host mucus and serum biochemistry are being studied. Results from this study are being presented at this Congress (Fast et al., this volume).

Rainbow trout were just as susceptible to sea lice infections as Atlantic salmon and exhibited a similar stress and immune response. Fish with *L. salmonis* infections had a significant suppression in macrophage respiratory burst activity and phagocytic capacity once all sea lice had reached the pre-adult and adult stages. Macrophage function remained suppressed for an additional 60 days. Trout were exposed to spores of the gill microsporidian pathogen *Loma salmonae* after sea lice reached the adult stage. Gill xenoma counts revealed that sea lice infected fish had 2.5 times more xenomas than did non-infected fish. This study supported the hypothesis that sea lice infections decrease non-specific immune responses such that fish have increased susceptibility to subsequent infections (Mustafa et al., submitted).

Morphological studies were carried out based upon a novel technique for preserving carbohydrate complexes for light and electron microscopy which we

developed at the Atlantic Veterinary College (Horne and Sims, 1998). There is a thinning and >washing out= of mucus on adult Atlantic salmon when they have a significant burden of sea lice. This may lead to increased osmoregulatory stress, which is observed with sea lice challenge. A detailed study of the light and electron microscopy of mucus distribution on Atlantic salmon is currently being analyzed: Five Atlantic salmon were carefully sampled at 13 sites each for epidermis and its mucous coat. The resulting 65 sites are being compared for thickness of skin and mucus, abundance of mucus-producing cells, and the ultrastructural features of the mucus.

#### *Resistance of recovered and naive Atlantic salmon to sea lice*

During the establishment of a reproducible sea lice infestation model, it was observed that Atlantic salmon experience decreased numbers of sea lice following an initial infection. Based on this observation, an experiment has been designed to investigate the response of Atlantic salmon to sea lice by determining the degree of parasite rejection and susceptibility of recovered fish to subsequent infections. Results to date show that salmon have significantly lower intensities of infection compared to controls during the subsequent infections, indicating that salmon may develop some protective immunity in reinfested fish.

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