Aspects of the Atlantic salmon immune response during infection with the salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837)

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by

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Declaration

I hereby declare that this thesis has been composed by myself, and has not been accepted, in full or part, in any previous application for a higher degree. The work of this thesis is a record of my own work; any collaborative work has been specifically acknowledged, as have all sources of information.

Keith Walton

Abstract

Atlantic salmon (*Salmo salar*) were experimentally infected with *Lepeophtheirus salmonis* copepodids and aspects of the host's immune response investigated. Copepodid secretory/excretory product (SEP) produced during early settlement was analysed using fast-protein liquid chromatography (FPLC), sodium dodecyl sulphate (SDS)-electrophoresis and zymography. Following establishment and the appearance of the chalimus stages, the expression of the chemokine interleukin-8 (IL-8) in the heart, spleen, head kidney, fins, liver and pyloric cæca was investigated using real-time (quantitative) PCR (qPCR). Furthermore, the secretions of *L. salmonis* chalimus were analysed for the presence of the prostanoid PGE_2 using commercially available enzyme-linked immunoassay (EIA) kits.

Analysis of copepodid secretory/excretory product suggested that any immunosuppressive component is not proteinaceous in nature. Whilst there was a definite increase in protein concentration of SEP relative to control SEP, further analysis using subtractive chromatographic analysis did not reveal any unique fraction present in either SEP or CSEP that was absent in the other. Interleukin-8 expression levels in tissues changed following *L. salmonis* infection, with heart and spleen showing significant increases in IL-8 gene expression, whilst the head kidney, fins, liver and pyloric cæca showed no significant increase. The increase in splenic IL-8 expression may be linked to its role as one of the major secondary lymphoid organs. However, this is the first record of increase in IL-8 expression in cardiac tissue. The secretions of *L. salmonis* chalimus were found to contain quantifiable levels of PGE_2, albeit in highly variable quantities. This concurs with already published findings for adult *L. salmonis* (see Fast, *et al.* 2004). It is proposed that the chalimus states us the PGE_2 to modulate the hosts' immune response at the site of attachment and feeding.

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Chapter 1
General Introduction

1.1 *Lepeophtheirus salmonis* (Krøyer 1837)

Lepeophtheirus salmonis (Copepoda: Caligidae) is an economically important parasite of sea farmed and wild Atlantic salmon (*Salmo salar*). However, much of the host-parasite interactions remains unknown (Finstad *et al.*, 2000; Ross *et al.*, 2000). Indeed, in an industry that quadrupled its production output between 1988 and 1998 (data from Pike & Wadsworth, 1999) sea lice are the most important pathogen of farmed salmon in the northern hemisphere, costing the Scottish economy between £25 and £30 million per annum, which is approximately 10% of the first sale value (Johnson *et al.*, 2004; Johnson & Fast, 2004*)*.

L. salmonis are dioecious, crustacean, obligate, oviparous ectoparasites of the salmonids, with a direct, holometabolous life cycle. This means that once a population of *L. salmonis* has become established in a fishery, the infestation is maintained by self-infestation and/or cross-infestation (Branson *et al.*, 2000). The life cycle of *L. salmonis* has been described by Johnson and Albright (1991) and Schram (1993). Briefly, the parasite has ten separate life cycle stages: two planktonic naupliar stages, an infective copepodid stage, four chalimus larval stages, two pre-adult stages (where the sexes become macroscopically distinct) and one adult stage. In total there are 8 parasitic stages of *L. salmonis* on the host fish (Johnson and Albright, 1992). Mature females carry fertilised eggs in a pair of egg sacs, each of which can contain between 350 and 500 eggs (Wootten *et al*., 1982; Johannessen, 1978; Heuch *et al.*, 2000). A female can produce up to 11 pairs of eggstrings in a lifetime (Heuch *et al.*, 2000). The transmission of *L. salmonis* is dependent on the copepodid stage and environmental factors such as temperature and salinity, and is time limited since the copepodid does not feed and relies on endogenous energy supplies (cited Pike and Wadsworth, 1999). This has resulted in a suite of behaviours that maximise the likelihood of finding a suitable host. The behaviours can be split into two separate aspects:

(i) those that aid in the orientation of the copepodid towards their hosts' natural environment and (ii) those that assist in host recognition (cited Pike and Wadsworth, 1999).

1.2 Parasitic settlement, attachment & moulting

The successful settlement of *L. salmonis* copepods is of crucial importance in its transmission (Tucker *et al.*, 2000a). Tucker *et al*. (2000a) investigated the effects of temperature and salinity on the settlement and survival of *L. salmonis* on Atlantic salmon and found a clear, positive relationship between water temperature and salinity and the settlement and survival of *L. salmonis*. They reported an increase in survival of greater than 31% with an increase of 5 °C in water temperature from approximately 7 °C to 12 °C and a decrease in salinity of 10 parts per thousand resulted in a 30% decrease in settlement and survival. Tucker *et al*. (2000a) proposed that the change in temperature had a direct effect on the metabolic rate of the copepodid, suggesting that the colder water caused the copepods to be less active than those in the warmer water. This concurs with the findings of Conley and Curtis (1993), who observed increased temperature had a significant effect on the swimming activity and survival of *Salmincola edwardsii*.

Once a suitable host is encountered, initial attachment of the copepodid is by its maxillipeds (Bron *et al.*, 1991). The maxillipeds allow the copepodid to hold on to the fish surface whilst it moves over a small area, probing the surface with the anterior end of the cephalothorax. This allows the first and second antennae to be brought into close contact with the host surface (Bron *et al.*, 1991). Butler (2001) proposed that, if the copepodid settles on a sub-optimal host, it may detach and resettle and attach on an alternative host within a theoretical window period. This is in agreement with the studies of Bron *et al.*

(1991) and Kabata (1979). If settlement occurs outwith this theoretical period then the parasite may not be able to detach but is able to continue the life cycle successfully (Butler, 2001). Once attached, a more durable attachment is formed when the copepodid pierces the fish epithelium with its prehensile second antennae, then producing a frontal filament via a secretion that acts to anchor the parasite to the fish (Bron *et al.*, 1991; Kabata, 1981).

The presence of a frontal filament in the copepodid phase is a feature of most, if not all, siphostomatoid Copepoda (Pike *et al.*, 1993). The frontal filament is present in all chalimus stages and Johnson and Albright (1991) state that some pre-adults of *Caligus clemensi*, *C. spinosus* and most species of *Lepeophtheirus*, are reported to be attached prior to moulting into the adult stage. Anstensrud (1990) reported a similar situation in the pre-adult stages of *Lepeophtheirus pectoralis*. Heegaard (1947 cited by Pike *et al.*, 1993) stated that the frontal filament is formed *de novo* at each moult of the chalimus of *L. salmonis*. This has been supported more recently by evidence from Anstensrud (1990) and Johnson and Albright (1991). Pike *et al.* (1993) therefore surmised that the frontal filament may fulfil a role in temporary attachment of the pre-adult stages as well as its primary role in chalimus attachment.

The structural morphology of the filament has been shown to vary between copepod genera. The appearance of filaments of *C. elongatus* and *L. salmonis* is distinctly different and reflects a seemingly different mode of production and attachment to the host (Pike *et al.*, 1993). The filament of *C. elongatus* is long and slender and fixes directly to the fish scale by a large basal plate. The surface of the filament is smooth and straight for most of its length, forming an almost S-shaped bend as it arises from the anteroventral surface of the cephalothorax and proximally decreasing in diameter (Pike *et al.*, 1993). In comparison, the filament of *L. salmonis* is short and stumpy and inserted into the epidermis

covering the scales where it actively secretes an adhesive along the basement membrane and the epidermal layer (Bron *et al*., 1991). Like *C. elongatus* the surface of the *L. salmonis* filament is usually smooth but it is occasionally contoured. However, unlike *C. elongatus* the frontal filament of *L. salmonis* is considered an integral part of the chalimus' body (Pike *et al.*, 1993; Gonzalez-Alanis *et al.*, 2001).

Once anchored, the copepodid undergoes a series of moults, passing through four chalimus stages before moulting into a motile pre-adult stage (Kabata, 1972; Pike, 1989; Johnson & Albright, 1991). The chalimus stages are fixed, such that their grazing causes a limited area of erosion that is confined to the site of attachment. The erosion causes a local cellular reaction that can be seen as a small black spot to the naked eye due to the presence of melanocytes (Jones *et al.*, 1990).

1.3 Parasite distribution on host

Following experimental infections, Tucker *et al.* (2000a; 2000b) and Tucker *et al*. (2002) reported that the preferential settlement sites in *L. salmonis* infections are the pectoral and dorsal fins. Tucker *et al*. (2000a; 2000b) showed a settlement preference for the pectoral and dorsal fins whilst Tucker *et al.* (2002) found the dorsal fin had a higher parasite density than the pectoral and most other fins, despite its relatively small comparative surface area.

Boxshall (1976) suggested that preferential settlement of *Lepeophtheirus pectoralis* on fins is due to these sites being associated with water currents and that the copepodids move towards the water currents. As such, settlement distribution reflects the attraction of water currents produced by the host and particularly the fins of a fish, which are dynamic zones

of flow, pressure, distribution and thrust (Bone, *et al.*, 1995; Bond, 1996). Tucker *et al*. (2002) propose that fins, with their fin rays, may also provide increased protection as microhabitats for copepodid settlement.

Once settled the copepodid undergoes a series of moults until the transformation from the anchored chalimus IV stage into the pre-adult stage induces a significant change in louse activities and distribution on the host (Mustafa *et al.*, 2000; Grimnes and Jakobsen, 1996). The pre-adults and adults tend to aggregate on the head, dorsal surface and post anal areas where they graze on the epithelial cells and mucus of the host (Branson *et al.*, 2000; Finstad *et al.*, 2000; Grimnes and Jakobsen, 1996). Blood is taken opportunistically if epithelial capillaries are damaged via extensive grazing (cited Pike and Wadsworth, 1999). Mustafa *et al.* (2000) hypothesise that the cephalic and external opercular regions are preferred by the mobile stages of *L. salmonis* as they have thin epidermis with little, if any, scaling. Bron *et al.* (1991) put forward two alternative theories for the distribution of the sea lice. They suggested that the parasites settle on specific areas by homing in on the water currents or, alternatively, attempt to shelter from the water currents in these areas.

1.4 Effects of parasitism with *L. salmonis*

Two forces broadly control infection intensity with *L. salmonis* and the subsequent pathology. The first is the length exposure to the infectious copepodids and the second is the susceptibility of the fish to infection (MacKinnon, 1998). The parameters that affect the host susceptibility include stress levels, nutrition and immunocompetency, all of which have a genetically determined component and are highly interactive (MacKinnon, 1998).

Nolan *et al.* (1999) propose that the damage caused to *S. salar* by *L. salmonis* can be split

into two distinct categories: (i) direct physical damage caused by the parasite feeding on the epithelial cells, blood and mucus and (ii) indirect damage caused by the integrated stress response of the skin and gill epithelia, osmoregulatory consequences and the host's immune system.

1.4.1 Mechanical damage

All of the stages that are attached to the host cause damage by grazing (Branson *et al.*, 2000), with Finstad *et al*. (2000) reporting as few as thirty pre-adult lice being able to kill a post-smolt Atlantic salmon (however, this number may be closer to 60 once mortality and differing effects at different ontogenetic stages (Wagner *et al*., 2008)). Wells *et al*. (2006) have shown that the stress of initial entry into seawater exacerbates the physiological impact of *L. salmonis* infection on sea trout (*Salmo trutta*) smolts, so that even infection levels of 13 lice per fish (approximately 0.35 lice.g^{-1} fish) can increase the chance of morbidity. Grimnes and Jakobsen (1996), however, report limited pathological consequences to the fish, even in high infection intensities, until the lice moult into the pre-adult stage. As the lice moult into pre-adults there is an increase in mechanical damage and a concurrent osmoregulatory breakdown (Grimnes and Jakobsen, 1996). Finstad *et al*. (2000) estimated that between thirty and fifty percent of migrating sea trout post-smolts in areas of intensive salmon farming are killed by grazing *L. salmonis*. Extensive epidermal and submucosal erosion and haemorrhaging are common effects of *L. salmonis* infestation and occasionally there may be sub-epidermal oedema associated with infection (Johnson *et al*., 1996). In extreme cases severe grazing around the cranium can lead to exposure and erosion of the underlying myotomes, sometimes reaching the cranium (Mustafa *et al.*, 2000; Wootten *et al.*, 1982).

As the lice graze on the fish epithelium, body fluids such as blood, lymph and proteins are

lost to the environment, disrupting the osmoregulatory homeostasis between the *milieu* interior and the external marine environment (cited Pike and Wadsworth, 1999) causing an increase in chloride levels as well as a decrease in haematocrit, total protein and albumin levels. The decreased haematocrit may be due to an increase in the plasma ions and a shrinking of erythrocytes and resulting in anaemia. The decrease in total protein and albumin (known as hypoproteinaemia) suggests that anaemia is probably caused by membrane damage and the leakage of blood components into the environment (Grimnes and Jakobsen, 1996). All of the above cause an increase in physiological and osmotic stress (Davies and Rodger, 2000; Grayson *et al.*, 1995; Finstad *et al.*, 2000).

1.5 Stress

When an animal is placed in a stressful situation, such as being parasitised, it actively produces two types of endocrine response: the hypothalmo-pituitary-interrenal (HPI) response that culminates with an increase in plasma cortisol concentration (a primary indicator of stress) and the adrenergic response that leads to an increase in the concentration of the catecholamines, adrenaline and noradrenaline (Mustafa *et al.*, 2000; Sumpter, 1997).

Chronically stressed fish are more susceptible to infection with pathogens than non-stressed fish (MacKinnon, 1998; Roberts and Rodger, 2001). A possible explanation proposed by Mustafa *et al.* (2000) is that stress can cause neuroendocrine and autonomic changes that modulate both the specific and non-specific immune defence mechanisms. Other investigations have also indicated that there is a positive correlation between elevated plasma cortisol concentrations due to stress and immunosuppression (MacKinnon, 1998). The best definition of stress related to fish disease was given by Brett (1958 cited

by Roberts and Rodger, 2001) as "…a stage produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced". The changes that occur in response to environmental stress, such as parasitism, are termed the general adaptation syndrome (GAS). The events comprising GAS are mediated by a hormonal and nervous reaction.

1.5.1 Hypothalmo-pituitary-interrenal (HPI) axis response

The HPI axis is a hormonal cascade reaction that results in the production and secretion of cortisol. Corticotrophin-releasing hormone is the first hormone in the cascade and it controls the release of adrenocorticotrophin (ACTH) from the anterior pituitary. In turn, ACTH stimulates inter-renal tissue to release corticosteroids, particularly cortisol.

Cortisol is a pluripotent hormone whose effects can be advantageous at moderately elevated levels, such as increasing leukocyte migration (Balm, 1997) and plasma glucose concentration (Pankhurst and Van Der Kraak, 1997), but deleterious at highly elevated levels, e.g. promoting apoptosis (cell death) (Nolan *et al.*, 1999). Barton (1997) has reviewed the stress process in fish and describe the following effects: the corticosteroids increase the susceptibility of fish to disease by decreasing the numbers and activity of circulating lymphocytes; elevated concentrations of cortisol cause a reduction in the release of interleukins needed for lymphocyte precursors to differentiate; and cortisol can also alter the affinity of corticosteroid receptors on lymphocytes.

1.5.2 Adrenergic response

The adrenergic response to stress sees chromaffin cells in the kidneys and walls of the

posterior cardinal vein secrete catecholamines. Various physiological stimuli can trigger the mobilisation of catecholamines but acute stress does not appear to deplete catecholamine stores. It is believed that stress stimulates the biosynthesis of replacement catecholamines at a rate equal to its mobilisation (Sumpter, 1997). On the other hand, chronic stress does decrease catecholamine-storing capacity. Fast *et al*. (2008) propose that changes in catecholamine levels observed in *L. salmonis* experimental infections may be caused by an attenuated cortisol response under chronic stress. The purpose of the catecholamines is to lessen the potentially disruptive effects of stress on the animal's physiology via optimisation of cardiovascular and respiratory functions (Sumpter, 1997). Furthermore, stress leads to an increase in the energy demand of the animal as it attempts to resolve the stressor (Barton, 1997). This energy is mobilised by the catecholamines in the form of plasma glucose, a secondary indicator of stress. Plasma glucose concentration increases significantly in lice infestations with a peak in concentration coinciding with the peak in plasma cortisol concentration (Dawson *et al.*, 1999; Mustafa *et al.*, 2000). In fish farms there is no scope to resolve the stressor and this results in a prolonged period of increased metabolism (Pankhurst and Van Der Kraak, 1997). The ultimate consequence of this prolonged increased metabolism is that there is little or no energy for growth (Barton, 1997; Pankhurst and Van Der Kraak, 1997).

1.6 Stress & immunosuppression

The skin epithelium of fish offers good possibilities for evaluating indirect stress effects of ectoparasites on their host (Nolan *et al.*, 1999). Responses such as increased apoptosis of branchial chloride cells and pavements cells of the skin have been shown to be under the control of cortisol and therefore occur in areas not directly affected by the parasite (Nolan *et al.*, 1999).

Nolan *et al.* (1999) concluded from their experiments that many of the epithelial changes witnessed during infection of Atlantic salmon with *L. salmonis* are similar to those described for general stress. They hypothesised that these effects were likely to be mediated by hormones, and in particular, cortisol and catecholamines. Increase in catecholamines in the gill increases branchial blood flow resulting in increased perfusion and blood pressure. It is possible that the lamellar swelling associated with infestation may reflect the action of high levels of catecholamines released as a result of parasite-induced stress (Nolan *et al.*, 1999). Nolan *et al.* (1999) do not believe that the effects associated with the gills are caused by any direct action of the parasite as mobile stages have not been found in these areas.

In the short-term, fish are able to regulate the resultant plasma osmolality and electrolyte concentration imbalance of direct damage by physiological adaptation (cited Pike and Wadsworth, 1999). However, this is energetically demanding and induces further stress. Finstad *et al.* (2000) found that the circulating levels of stress hormones in infected fish are significantly higher than in uninfected fish. Over a prolonged period the multifactorial perturbations to the fish's physiology eventually exceed its limited homeostatic capabilities and result in morbidity and subsequent mortality (cited Pike and Wadsworth, 1999). However, Pike and Wadsworth (1999) do not believe that stress is the sole reason for mortalities in infected fish as the skin only represents approximately twenty percent of the total surface area over which osmoregulation occurs; the other eighty percent is performed by the gills.

Although the majority of pathology associated with *L. salmonis* infestation is caused by the motile stages, Grimnes *et al.* (1996, cited by Finstad *et al.*, 2000) reported changes in the behaviour of Atlantic salmon post-smolts during exposure to *L. salmonis* copepodids.

Finstad *et al.* (2000) interpreted this as anecdotal evidence that sea lice infestations also stress fish at earlier ontogenetic stages. Furthermore, Grimnes and Jakobsen (1996) reported a six-fold increase in 'leaping and rolling activity' in Atlantic salmon during exposure to *L. salmonis* copepodids. Following exposure the authors also reported an epidermal reaction showing as black spots. No such spots were noted on the fish in the control group. From their studies, Grimnes and Jakobsen (1996) concluded that *L. salmonis* copepodids and chalimus stages have almost no effect on the long-term survival of Atlantic salmon post-smolts. They were unable to detect any physiological effects in terms of increased chloride level, and decreased protein and haematocrit levels were found in fish infected with early chalimus stages of the lice. However, they do note that in spite of the limited physiological consequences for post-smolts infected with chalimus larvae, the stress caused seems to be the causal agent for the mortality of at least some of the infected fish.

An up-to-date summary on fish immunity and parasite infections can be found in Alvarez-Pellitero (2008) and a review of the physiological and immunological interactions of *L. salmonis* and their hosts can be found in Wagner *et al.* (2008).

1.6.1 Effect of parasitism with *L. salmonis* conclusions

The damage caused by the mechanical actions of the lice and the damage caused by stress are coupled and the overall pathology of *L. salmonis* infection is a combination of the two. The stress is caused by the presence of the lice feeding and by the loss of exudate and proteins into the environment from the lesions caused by the lice.

1.7 Vaccines

Although the current chemotherapeutic techniques employed against *L. salmonis* on Atlantic salmon are curative, preventative measures are preferable. Thus, immunoprophylaxis (vaccination), is potentially the most efficacious strategy against *L. salmonis* (Jenkins *et al.*, 1993). There are a number of problems associated with chemotherapeutants that can be overcome with vaccines (Ellis, 2001; Raynard *et al.*, 2002):

- As vaccines are prophylactic there are fewer losses from disease
- There is sustained protection
- There are no toxic side-effects to the fish
- There is no accumulation of toxic residues in the tissues of the fish
- There is no withdrawal period
- Adaptation to the vaccine is unlikely but, if it does happen, it can be overcome by incorporating new antigenic components into the vaccine or by modifying existing ones
- There are no environmental implications once the fish have been immunised as no chemicals are discharged into the water.

Furthermore, any vaccine would specifically target salmon parasites whereas chemotherapeutants do not discriminate between its targets. From a financial perspective, the cost of vaccination would be lower than the cost of buying and storing chemotherapeutants, assuming antigens could be expressed as recombinant proteins or DNA vaccines were used (Raynard *et al.*, 2002). For a review of DNA vaccines in aquaculture see Heppell and Davis (2000).

Encouragement for the development of a *L. salmonis* vaccine has come from the development of a vaccine for cattle against the Australian cattle tick *Boophilus microplus* (Andrade-Salas *et al.*, 1993; Jenkins *et al.*, 1993). Molecular components of the parasite, which do not usually come into contact with the host's immune system during an infection, can elicit a protective immunological response when incorporated into a vaccine. Vaccination of the cattle with the antibody derived from the membrane bound molecule Bm86 induced specific immunity that was successful in reducing parasite burden (Jenkins *et al.*, 1993).

Although the teleost immune system is less evolved when compared to that of mammals, the production of an effective vaccine against *L. salmonis* has been slow (Costello, 1993). Most of the work has attempted to determine whether or not vaccines are a feasible option rather than developing practical versions (Costello, 1993). The major focus of these studies has been on potential targets in the form of concealed antigens. The ideal way of delivering a potential vaccine to lice would be via the circulatory blood or mucus secretions of the host. Once ingested one of the first tissues that would come into contact with the antibodies would be the gut epithelium (Andrade-Salas *et al.*, 1993). An important consideration in the development of a vaccine is whether or not sea lice have a peritrophic membrane. This is formed from successive delaminations of material at the apices of the gut epithelial cells (Brunet *et al.*, 1994). They form envelopes that surround the luminal contents of the midgut or hepatopancreas and appear necessary to the digestive process. It is hypothesised that they: (i) protect the epithelium against mechanical abrasion, (ii) support enzymes, (iii) perform a filter function for osmotic regulation and (iv) act as a selectively permeable barrier against macromolecules (Brunet *et al.*, 1994; Raynard *et al.*, 2002). This may prevent an antibody binding to an antigen (Raynard *et al.*, 2002). Brunet *et al.* (1994) stated that all marine crustacea produce a periotrophic membrane however its

presence in *L. salmonis* is unclear. Neither Nylund *et al.* (1992 cited by Raynard *et al.*, 2002) or Bron *et al.* (1993b) found evidence in their respective studies on gut morphology, although Scott (1901 cited Raynard *et al.*, 2002) reported a thin layer of fragmented chitin lining the gut wall of *Lepeophtheirus pectoralis*. If it does exist in *L. salmonis* it may prevent antibodies from reaching their target antigen.

Grayson *et al.* (1991) analysed the serum antibody response of rainbow trout and rabbits immunised with whole body lice protein and Atlantic salmon naturally infected with *L. salmonis*. Immunohistochemical studies revealed that the sera bound to regions of the adult louse gut, suggesting that this serological recognition of louse antigens was a specific antibody response to antigens within the gut epithelium of the louse. Andrade-Salas *et al.* (1993) used monoclonal antibodies developed from mice immunised with louse extract to select individual antigens from louse recombinant DNA. They found that antigens bound to several areas but particularly the brush border of the gut epithelium and the cytoplasm of gut epithelial cells. This provided strong immunohistochemical support for the existence of potential target antigens in the gut. Grayson *et al.* (1995) immunised and then experimentally challenged Atlantic salmon with *L. salmonis*. They found that the response was variable and apparently directed only at adult females and the number of viable eggs they produced. Due to the presence of antigens in the brush border they believe that it is possible that the host response interferes with them, having a knock-on effect on feeding, which lowers the nutrition available for egg development. Although there were no obvious effects against the other stages Grayson *et al.* (1995) do not discount the possibility that there were sub-clinical effects.

Research carried out by Grayson *et al.* (1991; 1995) and Andrade-Salas *et al.* (1993) appears to contradict Brunet *et al.*'s findings that all crustacea have peritrophic

membranes. However, a substantial difference exists between finding potential targets and the development of a suitable vaccine. The underlying principle behind developing a vaccine similar to that used against *B. microplus* is based on the lice ingesting antibodies that then bind to the gut epithelium and impair its function. This approach requires a degree of similarity between the host-parasite interaction and digestive physiology of *L. salmonis* and *B. microplus* that may not exist. For example, although both parasites are haematophagous, the tick feeds exclusively on blood from its host whereas sea lice only take blood when they are in the adult stage and then only in small quantities relative to their size (Costello, 1993; Raynard *et al.*, 2002). This means that the potential for developing an antibody that targets the gut may be lower for *L. salmonis* than it was for *B. microplus* and that if a successful antigen is to be developed it must produce a significant amount of damage from a low exposure (Raynard *et al.*, 2002).

The morphology of the gut and the digestive processes of the sea lice are both important in the development of any vaccine. If the gut lumen contains proteolytic enzymes the half-life of any vaccine will be short. Further to this, the pH and osmolarity of the gut and its contents will have an effect on antibody function. Bricknell *et al.* (2002) found that salmon antibodies have a reduced binding ability when the osmolarity of the surrounding medium is greater than 500 mOsmol. The osmolarity of the gut contents of *L. salmonis* has not been measured but its haemolymph is iso-osmotic with seawater. As *L. salmonis* is an osmoconformer this indicates that the gut contents should be similar in osmolarity to seawater. If this is indeed the case, it is predicted that the efficacy of any vaccine would be significantly impaired (Bricknell *et al.*, 2002).

1.8 Research aims and objectives

The aim of the work presented in this thesis was to further our understanding of immunomodulation of Atlantic salmon by the copepodid and chalimus stages of the important parasitic ectoparasite, *Lepeophtheirus salmonis*. Butler (2001) did some preliminary investigations into the biochemical nature of the secretory/excretory product (SEP) of *L. salmonis* following settlement on an artificial salmon skin equivalent (ASSE) and reported a tangible product, most likely to be proteinaceous in nature. The present study investigated the proteomic nature of *L. salmonis* SEP further using a variety of biochemical techniques such as fast protein liquid chromatography (FPLC), 2-dimensional electrophoresis and zymography. Due to difficulties recreating the ASSE, however, an *in vitro* culture system was used to harvest the SEP prior to analysis on composition. This work is presented in Chapter 3. Chapter 4 examines the role that the chemokine interleukin-8 (IL-8) plays in the early stages of infection between *L. salmonis* and Atlantic salmon and Chapter 5 presents an analysis of the possible role that prostaglandin E_2 (PGE_2) plays in the settlement and early stages of *L. salmonis* infection.

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Chapter 2
General Materials & Methods

2.1 Sea lice collection

Eggstrings were collected from ovigenerous female lice on caged-salmon from fish farms on the West coast of Scotland and from wild salmon from Montrose Netting Station, Scotland using watchmaker's forceps and scalpels. Eggstrings were also supplied by the Marine Environmental Research Laboratory, Machrihanish and the FRS Marine Laboratory Fish Cultivation Unit, Aultbea.

2.2 Fish

The fish used in all experiments were supplied by either FRS Marine Laboratory Fish Cultivation Unit, Aultbea or the Marine Environmental Research Laboratory, University of Stirling, Machrihanish. Fish were maintained in aerated 1 m^2 pale circular tanks in seawater and fed *ad lib* twice a day.

2.3 Infection protocol

Copepodids were cultured in the apparatus shown in Figure 1. As live nauplii and copepodids usually aggregated within the upper third of the water column in response to light stimulus, the water was mixed thoroughly with air to obtain a uniform distribution. To determine the number of the different stages the air supply was switched off and an intense light source (fibre optic light) focused on the top third of the water column and left for 10 minutes. All lice around this area were collected into a 250 mL beaker using a sterile pastette. Five 5 mL sub-samples were taken from the beaker and placed in an open Petri dish and the number of live copepodids counted with the aid of a dissecting microscope and hand counter. The density of copepodids within the suspension was then calculated and the numbers required for infection determined.

2.3.1 Dip infection method

As low lice numbers were required experimental infection was carried out using a modified version of Sevatdal's (2001) tank method. This method gives a distribution over the surface of the fish similar to that seen in natural infections, and minimal gill settlement compared to the bath method (O'Shea, 2005).

Fish were anaesthetised using MS222 (Sigma) at 0.1 $g.L^{-1}$ seawater before being rinsed with clean seawater to prevent the anaesthetic contaminating the suspension of *L. salmonis*. Two fish were infected simultaneously by holding them for 60 seconds in 1 L seawater containing *L. salmonis* copepodids (less than 72 hours post hatching) at a concentration of 1.5 $lice.mL^{-1}$ seawater (starting concentration of approximately 1500 lice). Once infected, fish were transferred to full volume aerated tanks with the flow stopped for recovery. The inflow was restarted 18 hours post infection.

The appearance of small black spots, particularly on the surface of the fish, within 5 minutes of exposure to the *L. salmonis* copepodids indicated successful infection. The spots are thought to be an accumulation of melanocytes caused by a local cellular response to the copepodids (Grimnes and Jakobsen, 1996). Lice numbers were not limiting during the infection as actively swimming copepodids were observed in the infection chamber at the end of the treatment.

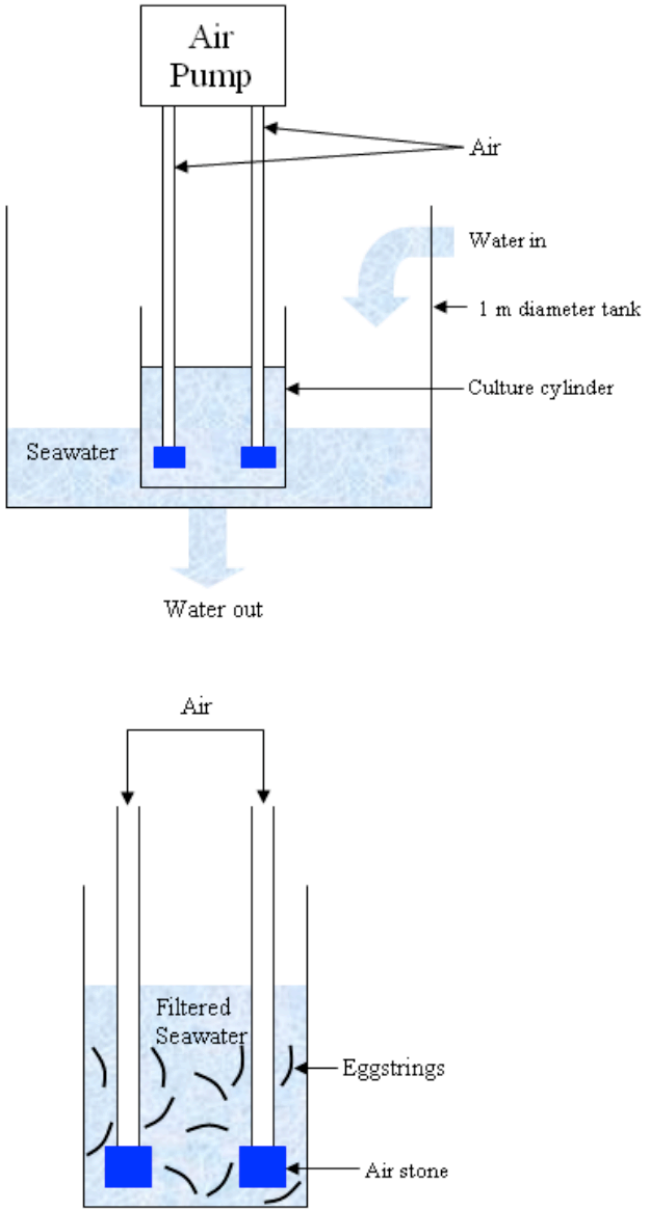

Figure 1: *In vitro L. salmonis* culture system. Eggstrings from ovigenerous females were removed and suspended in (0.2 µm) sterile-filtered seawater in the above apparatus at 10 °C until the eggs hatched into naupliar stages and then these moulted into copepodids.

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Chapter 3
The proteomics of *L. salmonis* secretory/excretory

3.1 Introduction

Weston-Davies and Nuttall (2002) reported that haematophagous parasites secrete a large number of molecules in their saliva, most of which are peptides and proteins. The authors go on to suggest that these substances have evolved to counteract the immune response of the host, as well as having other functions such as anticoagulation.

A tick bite should result in strong haemostatic, immune and inflammatory responses from the host defence system but tick saliva has evolved components to counter these responses (Steen *et al.*, 2006). In fact, the tick responses allow them to feed for days to weeks at one site (Ribeiro and Francischetti, 2003) compared to minutes to hours for other haematophagous parasites such as terrestrial lice and leeches. The added components have been found to exist in numerous forms including enzymes, enzyme inhibitors, Ig-binding proteins, amine-binding lipocalins and integrin inhibitors (Steen *et al.*, 2006).

The saliva of ticks has been shown to contain anti-clotting enzymes presumably that assist in maintaining the flow of blood. An example of such an enzyme is apyrase, which is almost ubiquitous in haematophagous arthropods. Apyrase hydrolyses adenosine diphosphate (ADP) released by damaged cells at the feeding site and thereby inhibits ADP-induced platelet aggregation (Ribeiro *et al.*, 1991, Ribeiro *et al.*, 1985, Mans *et al.*, 1998).

Furthermore, the physical presence of parasites can cause the host irritation thereby inducing grooming behaviour (Paesen *et al.*, 1999). Therefore it is very important that the parasite develop methods that enable them to overcome both responses. Depending on their environment, different types of parasite have evolved different methods for overcoming the host's immune system. These methods include the addition of

antihaemostatic (anti-clotting), vasoactive and immunomodulative substances in their saliva which either counteract the host's immune system or mask the parasite's presence (Volf *et al.*, 2000). Many of these compounds are thought to be proteinaceous in nature.

Butler (2001) studied the preliminary biochemistry of a substance he proposed *L. salmonis* produced to modulate its host's immune system. Working on the principle that some of the material produced will leach into the environment as the lice feed, Butler (2001) constructed an artificial salmon skin equivalent (ASSE) on which he settled *L. salmonis* copepodids and then collected the seawater for analysis. His analysis showed there was a tangible substance, which he called louse immunomodulatory factor (LIF), that warranted investigation. Butler (2001) found that heating LIF to 80 ºC had a significant negative effect on its activity/effectiveness, indicating it was not heat stable, and that the effect of LIF on macrophage chemotaxis could be diluted out. The inhibition of activity by heating and dilution are common properties of proteins. Butler (2001) also reported an increase in protein concentration when compared to control culture solutions. Further evidence of the proteinaceous nature of LIF was demonstrated by digesting it with the endopeptidase, proteinase K. Digested LIF-containing supernatants did not reduce the rate of macrophage chemotaxis, thereby indicating the inaction of the immunomodulatory component by the enzyme. Whilst proteinase K is a broad-spectrum protease with little cleave specificity, Worthington (1988, cited by Butler, 2001) reports no documented effect of proteinase K on non-proteins. Butler (2001) did, however, propose a second possibility as to the nature of LIF: it is only active in the presence of a protein or amino acid and that it is not a protein itself.

Chromatography has been used in many aspects of piscine and parasite research. For example, Abelseth *et al*. (2003) used anion exchange chromatography to isolate

complement component C3 from the serum of the spotted wolfish (*Anarhichas minor* Olafsen). Mañanós *et al*. (1997) used ion exchange chromatography in concert with FLPC to purify gonadotrophin from a hybrid striped bass (*Morone* sp.) and Neumann *et al*. (2000) used FPLC to characterise macrophage activating factor (MAF) from goldfish leucocytes.

Chromatography has previously been used to study *L. salmonis* and their infection of salmonids. Grayson *et al.* (1995) and Roper *et al*. (1995) looked for potential *L. salmonis* antigens with the possibility of using them to develop a vaccine, whilst Fast *et al*. (2004) used reverse phase high pressure liquid chromatography (RP-HPLC) and mass spectrometry to characterise prostaglandin E2 in the secretory products of *L. salmonis*. Firth *et al*. (2000) analysed the integumental biochemistry of salmon during an infection with *L. salmonis* using protein and enzymological techniques.

Avilan *et al*. (2000), Barbieri (1992) and Etges (1992) have all used zymography to study different aspects of *Leishmania* species. Ectoparasites studied using zymography include the mosquito *Anopheles stephensi* (Rosenfeld and Vanderberg, 1998) and *L. salmonis* (Firth *et al.*, 2000). Díaz-López *et al*. (1998) used zymography to characterise fish acid proteases and Lødemel and Olsen (2003) used the technique to study gelatinases in fish muscle.

The aim of this chapter was to identify and characterise the nature of the biomolecules present within the secretory/excretory product (SEP) produced by *L. salmonis* copepodids during settlement on Atlantic salmon. The SEP was cultured using Ussing's chambers (see section 3.2.1.1). Preliminary analysis measured the total protein content (section 3.2.2) whilst gel filtration was used to collect and characterise the size of proteins and peptides

(section 3.2.4). Two-dimensional electrophoresis (section 3.2.5) was used to compare SEP with controls whilst further analysis used zymography to examine the effect of various protease inhibitors (see section 3.2.6).

3.2 Materials and methods

3.2.1 *in vitro* supernatant culture

Ussing's chambers (Ussing *et al.*, 1950) were modified to allow the culture of copepodids *in vitro*.

3.2.1.1 Ussing's chambers

As is shown in Figure 2 the chamber is split into 2 by a 12 mm diameter disk of fish tail and 2 silicon washers. These form a watertight barrier between the chambers. The tail was taken from a freshly-euthanised Atlantic salmon. The lower chamber was supplied with circulating ×1 Hanks balanced salts solution (HBSS) to nourish the tissue whilst the upper chamber contains sterile seawater at 10 °C (collected from Montrose netting station and vacuum filtered across a 0.2 μm filter) plus the copepodids.

3.2.1.2 Production of supernatant

Twenty copepodids (all less than 72 hours post hatching) were added to the upper chamber of the Ussing's apparatus in 1 mL of sterile-filtered seawater (see section 3.2.1.1). The seawater had been vigorously aerated prior to use.

3.2.1.3 Collection of supernatant from Ussing's chambers

After the copepodids had been in the upper chamber of the Ussing's apparatus for 8 hours the supernatant was removed using a pastette. Samples from chambers using skin from the same fish and of the same type, i.e. with lice or without lice, were pooled and centrifuged at 20,000 *g* for 10 minutes to pellet particulates. Supernatant was aliquoted into 1 mL cryotubes, labelled and stored at -80 ºC.

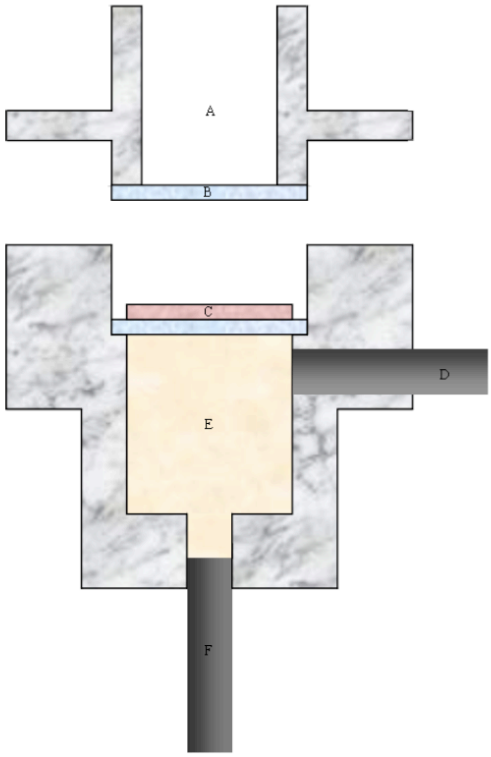


Figure 2: Ussing's chamber used to collect *L. salmonis* SEP. A = upper reservoir; B = silicon washer for watertight seal; C = fish tail; D = HBSS entrance; E = lower reservoir; F = HBSS exhaust

3.2.2 Total protein estimation of supernatant

The protein content of samples was determined using a commercially available bicinchoninic acid (BCA) kit following manufacturer's instructions (Pierce, Rockford IL USA). Briefly, 200 µL working reagent was added to diluted BSA standards, ddH_2O blanks and duplicate serial dilutions of supernatant made in ddH_2O in a 96-well flat bottomed microtitre plate (Nunc). All standards, samples and blanks were prepared in

triplicate. The plate was incubated for 30 minutes at 37 ºC. Following incubation plates were allowed to cool to room temperature and the absorbance measured at 562 nm using a Dynex Dias plate reader with Dynex Revelation V 3.1 analysis software.

3.2.3 Sodium disulphide polyacrylamide gel electrophoresis (SDS-PAGE)

The protein composition of supernatants was analysed using SDS-PAGE. A mini Protean 3 system (Bio-Rad) was used for all electrophoresis. Briefly, two 0.75 mm spacer plates and 2 short plates were cleaned with Virkon and scrubbed with ethanol. The clean plates were placed into the casting frame, short plate at the front, which in turn was placed in the casting stand. Double distilled water was added to test for leaks and then discarded.

The separating gel was prepared (see Appendix 1) and 3.7 mL added between the spacer and short plates. One millilitre water-saturated butanol (see Appendix 1) was slowly added to ensure the top of the gel set level and the solution was left for 60 minutes to polymerise. Once set, the water-saturated butanol was removed and the surface washed with ddH_2O. The electrophoresis stacking gel (see Appendix 1) was prepared and added along with an appropriate well comb. Gels were left for 30 minutes to polymerise. Once set the gels were transferred to the electrode assembly, locked into place and 500 mL ×1 SDS electrophoresis buffer (see Appendix 1) added.

Protein samples were prepared by mixing 12 μL reducing sample buffer (see Appendix 1) with 12 μL sample and boiled on a dri-block for 5 minutes (Techne Dri-block® DB-2A). Fifteen microlitres of sample-buffer mix was loaded into each well and run at 200 V for 5 minutes, or until the samples had passed through the stacking gel, and then run at 130 V until the dye front reached the bottom of the gel. Following electrophoresis gels were removed from the apparatus and stained as necessary.

3.2.3.1 Silver stain

Following electrophoresis, gels were incubated overnight in 100 mL silver stain fixative solution (see Appendix 1). The following day gels were rinsed for 10 minutes in ddH_2O and then soaked in silver stain fixative/sensitisation solution (see Appendix 1) for 5 minutes. The sensitised gels were rinsed for 20 minutes in 100 mL 40% ethanol followed by 20 minutes in ddH_2O. Gels were incubated for 1 minute in 100 mL ddH_2O + 20 mg sodium thiosulphate (Sigma) before being washed twice for 1 minute in ddH_2O. The rinsed gels were then soaked in 100 mL 0.1% silver nitrate solution (100 mg silver nitrate + 100 mL ddH_2O) for 20 minutes. Again gels were rinsed in ddH_2O for 1 minute then incubated in 100 mL silver stain sodium carbonate/formalin solution (see Appendix 1). Once the solution turned yellow it was immediately replaced with fresh solution and staining allowed to develop until the desired level attained. The reaction was stopped by adding 100 mL 5% acetic acid.

3.2.3.2 Coomassie blue stain

Following electrophoresis, gels were incubated overnight in 100 mL ddH_2O. The following day gels were soaked in Coomassie stain (see Appendix 1) for 30 minutes and then transferred to Coomassie destain (see Appendix 1) for 45 minutes. After this time the destain was replaced and gels left until the desired level of staining was attained.

3.2.4 Gel filtration chromatography

In gel filtration, samples are loaded into the top of columns packed with gels of cross-lined polymers with a known and controllable pore size, such as dextran. An elution buffer with pH and ionic strength suitable for sample preservation is pumped from the top of the column with large molecules being eluted first with smaller molecules hindered by the

pores. Therefore, molecules with a high molecular weigh are eluted first. The speed of elution directly affects the effectiveness of separation with slower speeds giving better resolution. An ÄKTA™ FPLC (fast performance liquid chromatography; Amersham Pharmacia Biotech) was used to simultaneously analyse the eluent for conductivity, pH and UV absorbance, plot the results on chromatograms and collect the eluent fractions.

One hundred microlitre samples of SEP/CSEP were loaded on a Superdex S200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) connected to an ÄKTA™ FPLC. Samples were eluted at 400 μL.minute^{-1} in 2 column volumes of eluent (~48 mL). Eluent was passed through a UV monitor at 254 nm and 500 μl fractions collected (Frac-901, Amersham Pharmacia Biotech). Changes in UV absorbance were plotted on a chromatogram (absorption (mAU) vs. Eluent.sample volume^{-1} (mL)).

Chromatographic peaks were discriminated using Unicorn™ version 3.0 software (Amersham Pharmacia Biotech). Peaks were defined as a minimum change in height of the absorbance curve of 0.01 mAU per 0.001 mL sample volume. As a result peaks may register on computer analysis that are undetectable by eye alone.

The column was calibrated by loading a 100 μL sample containing 0.7 mg.mL^{-1} ferritin (440 kilo Dalton, kDa), 2 mg.mL^{-1} aldolase (150 kDa), 2 mg.mL^{-1} albumin (66 kDa), 2 mg.mL^{-1} ovalbumin (43 kDa) and 2 mg.mL^{-1} ribonuclease A (13.7 kDa). The calibration curve was applied to the absorbance curve and Unicorn™ version 3.0 software used to determine the molecular weights of the peaks in the chromatogram. Block analysis of chromatographic data was done using Excel (Microsoft Corporation, USA).

3.2.5 2-D electrophoresis

Supernatants were analysed by 2-D electrophoresis in addition to 1-dimensional electrophoresis (see section 3.2.3). Prior to the first dimension the supernatants were concentrated using a ReadyPrep 2-D Cleanup Kit (Amersham Biosciences) according to manufacturer's instructions. Briefly, 300 µL precipitating agent 1 were added to 100 µL supernatant, mixed well and incubated on ice for 15 minutes. Following incubation 300 µL precipitating agent 2 were added and mixed well. The supernatant/precipitating agent mixture was centrifuged for 5 minutes at 29,000 *g* at 4 ºC after which the tube was promptly removed and the liquid phase was discarded. The tube was respun for 30 seconds and the liquid phase again discarded. Forty microlitres wash reagent 1 were added, the tube respun for 5 minutes at 29,000 *g* at 4 ºC and the liquid phase removed and discarded. Twenty-five microlitres ddH_2O were added and the tube mixed well. One millilitre (pre-chilled) wash reagent 2 and 5 µL wash 2 additive were added and the tubes mixed for 1 minute and then incubated at -20 ºC for 30 minutes. Tubes were vortexed for 30 seconds every 10 minutes during the incubation period. Following incubation tubes were centrifuged for 5 minutes at 29,000 g at 4 ºC. The liquid phase was removed and the pellet air-dried at room temperature until translucent.

Once dry the pellet was suspended in 125 µL ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer (Bio-Rad). The tube was vortexed for 30 seconds, incubated for 5 minutes at room temperature and vortexed again for 1 minute. The tubes were respun for 5 minutes at 29,000 g at room temperature to clarify the sample.

A ready-made 3 – 10 immobilised pH gradient (IPG) strip (Amersham Biosciences) was passively rehydrated overnight at 20 ºC using the clarified sample and a dedicated first dimension system (Protean IEF cell; Bio-Rad). The IPG strip was overlaid with 3 mL of

mineral oil (Bio-Rad) to minimize evaporation and urea crystallisation during rehydration. Temperatures lower than 20 ºC can cause the urea within the rehydration/sample buffer to crystallise whereas above 20 ºC can cause carbamoylation of proteins (transfer of a carbamoyl moiety, NH_2CO^-, to the amino group of an acceptor compound, Westermeier and Naven, 2002). Following rehydration of the strips and uptake of the sample, the first dimension was performed by running the IEF cell at 200 V for 60 seconds, 3,500 V for 90 minutes.

Following the first dimension IPG strips were soaked in SDS Equilibration buffer I (with DTT) (Bio-Rad) for 10 minutes followed by 10 minutes in SDS Equilibration buffer II (Bio-Rad). Once equilibrated IPG strips were dipped in ×1 SDS electrophoresis buffer (see Appendix 1) and transferred to a 10% SDS gel. IPG strips were sealed in place with agarose solution (see Appendix 1). Once sealed gels were run at 10 mA for 15 minutes and then 20 mA until the dye front was approximately 2 mm from end of the gel.

3.2.6 Zymography

Further assessment of the peaks of interest from the ÄKTA FPLC was performed using zymography. Protein content of peaks was estimated using a Pierce BCA kit (see section 3.2.2). Peaks were diluted with artificial seawater (Marin Tropic made up following manufacturer's instructions) to get 10 µL solution containing 4 µg protein. One microlitre of protease inhibitor (see Table 1) was added to each peak fraction, briefly vortexed and incubated at 30 ºC for 60 minutes. Following incubation 11 µL Å~2 sample buffer (see Appendix 1) was added and the mixture vortexed. Eleven microlitres of each mixture was loaded on a 12% SDS separating gel with added gelatine and 4% stacking gel (see Appendix 1). Gels were run for 120 minutes at 150 V.

The substrate used in the separating gel was dependent on the protease of interest: aspartic proteases exhibit high activity on β-casein (Sánchez-Chiang *et al*., 1997 cited by Castillo-Yáñez *et al*. 2004; Squires *et al*., 1986 cited by Castillo-Yáñez *et al*. 2004) whereas gelatinase proteases exhibit high activity on gelatine.

Table 1: Protease inhibitors using in zymography

Protease inhibitor	Concentration	Volume (µL)
Control (None)	-	-
Aprotinin	1 $mg.mL^{-1}$ ddH_2O	50
Pepstatin	1 $mg.mL^{-1}$ 9@1 v/v ethanol:acetic acid	50
O-phenanthroline	0.5 µ in methanol	100
Leupeptin	1 $mg.mL^{-1}$ ddH_2O	50
Iodoacetamide	0.5 M in ddH_2O	100
EDTA	0.5 M in ddH_2O	100

After electrophoresis, gels were removed from the cell and soaked in 25 mL wash buffer (see Appendix 1) for 10 minutes. This was repeated twice more. Following the final wash the well containing EDTA was separated from the other wells and soaked for 2 hours on a shaker at 30 ºC at 70 rpm (Unimax 100 with Inkubator 1000, Heidolph Instruments, Germany) in 25 mL incubation buffer (see Appendix 1). After the development of enzyme activity, gels were stained using Coomassie brilliant blue R-250 (Bio-Rad).

3.3 Results

3.3.1. Protein content

Protein concentration analysis of SEP and CSEP showed a significant increase in the total protein content of SEP compared to CSEP (ANOVA, $P<0.01$, $F = 2.61$, see Figure 3). Although the average protein content was higher at 10 ºC than at 4 ºC in both SEP and

CSEP, the observed differences were not sufficiently large to attain statistical significance at the 5% level (ANOVA, P>0.05). The concentration of protein in CSEP suggests that the excised disks of skin released protein into the surrounding environment. However, the increased protein concentration between SEP and CSEP suggests that the copepodids were also releasing protein into the surrounding environments.

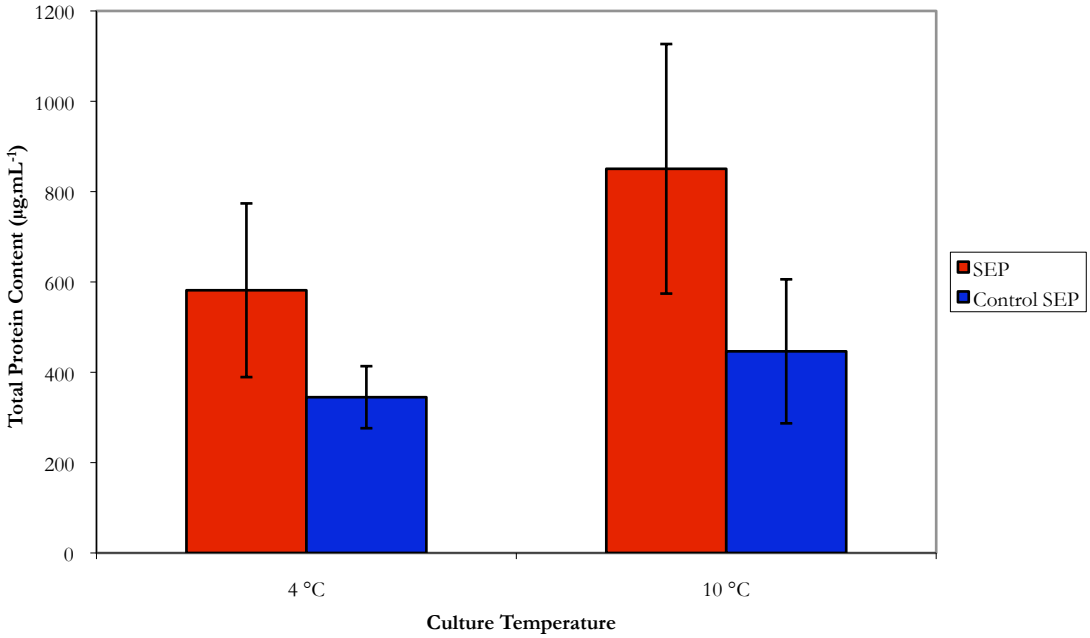


Figure 3: Protein content of SEP and CSEP plus standard deviation (n = 4) following analysis with BCA assay kit showing a significant increase in the total protein content. Although the mean protein content is higher at 10 °C than 4 °C the observed differences were not statistically significant.

3.3.2 2-D PAGE analysis of SEP and CSEP

As can be seen in Figure 4, there is a lot of noise in both the SEP and CSEP gels following 2-D analysis. The most likely source of noise is due to the non-sterile nature of the culture system, i.e. as well as proteins derived from *L. salmonis*, proteins from the degrading fish skin as well as air contaminants will also be have been detected. Due to the level of noise displayed in the gels it was decided not to pursue this approach further as it would have proved very time consuming and expensive to analyse every dot.

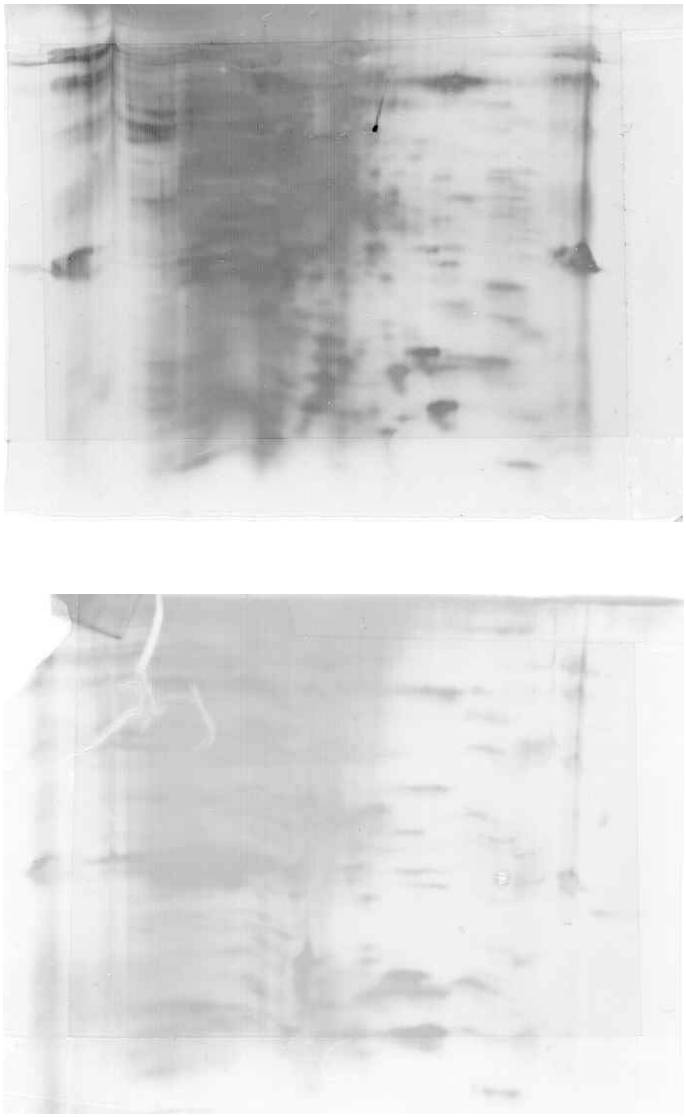


Figure 4: 2-D PAGE analysis of SEP (top picture) and CSEP (bottom picture). Due to background noise and non-sterile nature of the culture system there is a lot of contamination of both samples making it impossible to identify unique spots in the samples.

3.3.3 FPLC analysis of SEP

The analysis of SEP using FPLC is shown in Figure 5 peaks using the criteria defined in section 3.2.4 is displayed in Table 2. The table indicates the total number of peaks identified on the chromatogram and the volumes at which these peaks started, reached their maxima and ended. All SEP chromatograms analysed produced similar chromatographic outputs.

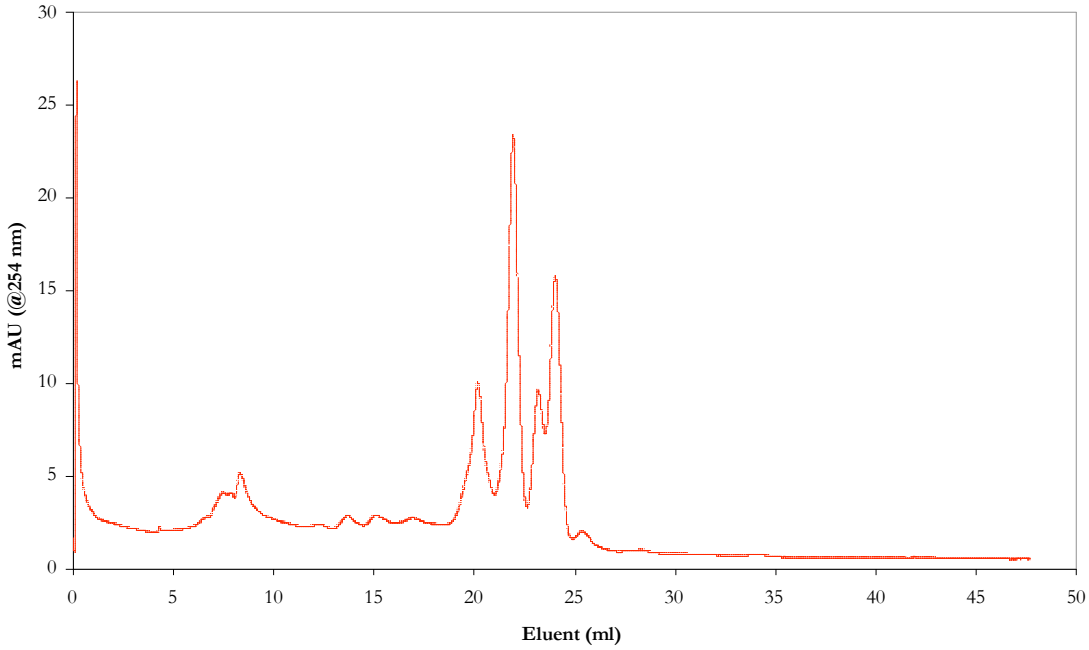


Figure 5: Typical chromatogram of SEP following analysis using FPLC. There are a total of 17 peaks defined using the criteria in section 3.2.4, the details of which are shown in the table below.

Table 2: Total number of peaks identified from SEP chromatograms and the volumes at which the peaks started, reached their maxima and ended.

Peak number	Peak start (mL)	Peak end (mL)	Peak max (mL)
1	0.08	1.31	.015
2	1.56	1.66	1.61
3	4.24	4.43	4.29
4	5.90	6.69	6.65
5	6.69	7.70	7.44
6	7.70	8.03	7.85
7	8.03	9.55	8.32
8	13.30	14.11	13.69
9	14.69	15.80	15.08
10	16.35	17.46	16.91
11	18.58	20.97	20.15
12	20.97	22.59	21.90
13	22.59	23.49	23.15
14	23.49	24.84	24.00
15	24.84	26.71	25.38
16	28.20	28.68	28.44
17	41.84	41.92	41.88

3.3.4 FPLC analysis of CSEP

The analysis of CSEP using FPLC is shown in Figure 6. A summary of all of the peaks using the criteria defined in section 3.2.4 is displayed in Table 3. The table indicates the total number of peaks identified on the chromatogram and the volumes at which these

peaks started, reached their maxima and ended. All CSEP samples analysed produced similar chromatographic output.

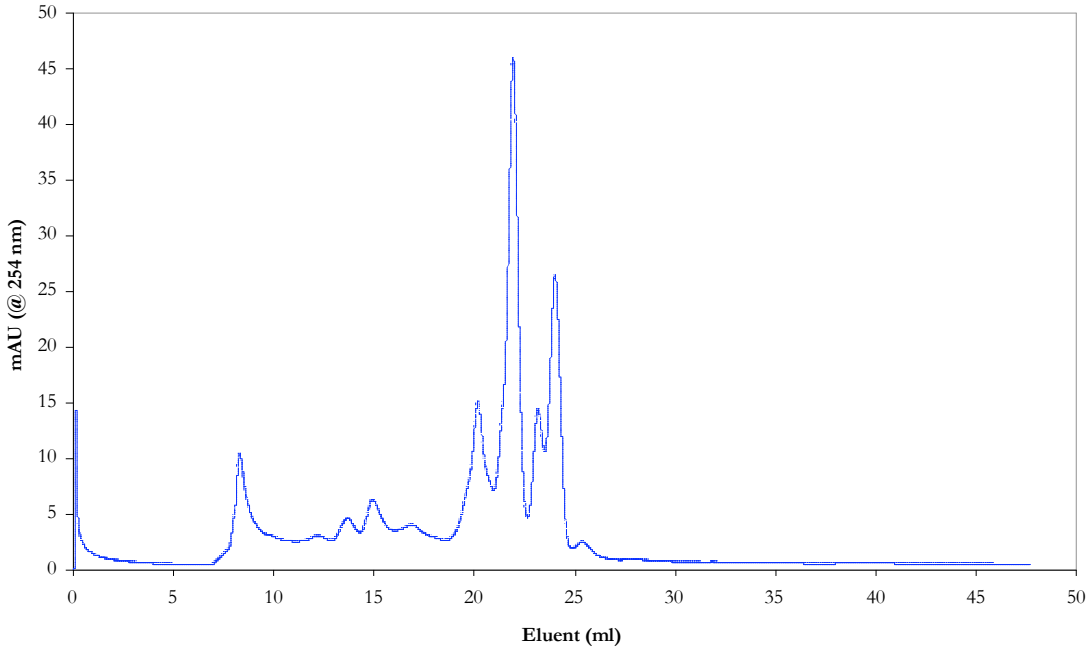


Figure 6: Typical chromatogram of CSEP following analysis using FPLC. There are a total of 13 peaks defined using the criteria in section 3.2.4, the details of which are shown in the table below.

Table 3: Total number of peaks identified from CSEP chromatograms and the volumes at which the peaks started, reached their maxima and ended.

Peak number	Peak start (mL)	Peak end (mL)	Peak max (mL)
1	0.07	0.98	0.13
2	7.82	9.56	8.29
3	11.26	12.83	12.19
4	12.83	12.93	12.9
5	12.93	14.26	13.67
6	14.26	15.97	14.92
7	15.97	18.48	16.86
8	18.48	20.92	20.15
9	20.92	22.62	21.9
10	22.62	23.47	23.15
11	23.47	24.84	23.99
12	24.84	26.33	25.38
13	31.78	31.84	31.8

3.3.5 FPLC analysis of sterile-filtered seawater (SW)

The analysis of sterile-filtered seawater using FPLC is shown in Figure 7. A summary of all of the peaks using the criteria defined in section 3.2.4 is displayed in Table 4. The table indicates the total number of peaks identified on the chromatogram and the volumes at which these peaks started, reached their maxima and ended. All SW samples analysed produced comparable chromatographic output.

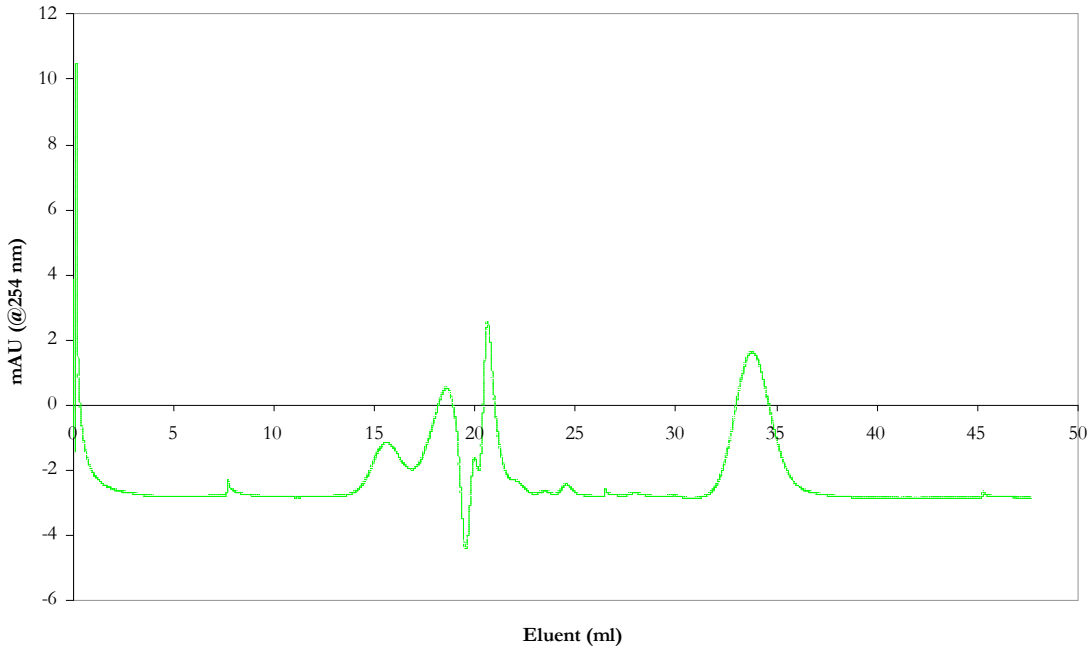


Figure 7: The molecular size of sterile filtered seawater (SW) following analysis using FPLC. There are a total of 15 peaks defined using the criteria in section 3.2.4, the details of which are shown in the table below.

Table 4: Total number of peaks identified from SW chromatograms and the volumes at which the peaks started, reached their maxima and ended.

Peak number	Peak start (mL)	Peak end (mL)	Peak max (mL)
1	0	0.06	0
2	0.06	1.94	0.14
3	7.6	8.27	7.69
4	13.73	16.81	15.56
5	16.81	19.28	18.54
6	19.75	20.16	19.96
7	20.16	22.92	20.61
8	22.92	23.91	23.43
9	23.91	25.23	24.55
10	26.4	27.09	26.47
11	27.5	28.39	27.95
12	29.81	29.96	29.89
13	31.57	36.64	33.74
14	45.19	45.51	45.27
15	47.53	47.62	47.62

3.3.6 Graphical representation of SEP chromatographic analysis

Chromatographic analysis of four separate SEP samples is shown in Figure 8. The blocks represent peaks that were identified using the criteria in section 3.2.4. There are nine volumes at which a peak is present in all four samples (or in adjacent fractions): 0, 8.0, 13.5, 16.5, 20.0, 21.5, 23.0, 23.5 and 25.0 mL. The other peaks do not appear in all of the samples (or in close proximity). Possible sources for these proteins are the same as those identified in section 3.3.2.

Figure 8: Graphical representation of SEP chromatographic analysis. The blocks of colour represent individual peaks. Orange blocks denote 2 peaks in adjacent fractions whilst pink blocks denote multiple peaks occurring within a single fraction. There are 9 volumes in which a peak is present in all samples (or in adjacent fractions)

3.3.7 Graphical representation of CSEP chromatographic analysis

Chromatographic analysis of four separate CSEP samples is shown in Figure 9**.** The individual blocks represent peaks identified using the criteria defined in section 3.2.4. There are nine volumes at which a peak is present in all four samples (or in adjacent fractions): 0.0, 8.0, 13.5 16.5, 20.0, 21.5, 22.5, 23.5, 24.5 mL. The other peaks do not appear in all of the samples (or in close proximity). Possible sources for these proteins are the same as those identified in section 3.3.2.

3.3.8 Graphical representation of SEP/CSEP chromatographic analysis

Comparative chromatographic analysis of SEP and CSEP samples is shown in Figure 10. Data is given for four separate pairs of samples. If a substance was present in all four SEP samples but not in the CSEP, this would suggest that *L. salmonis* was producing an immunomodulatory substance. Subtractive chromatographic analysis between SEP and CSEP demonstrates that there are no peaks present in SEP (blue blocks) that are not present in CSEP. However, there is no peak in SEP sample 2 at 24.5 mL but there is a peak at 25.0 mL. It is possible that the peak at 25.0 mL may have eluted slightly later than the rest and should be included in the earlier fraction. Should this be the case then all SEP samples contained peaks at 24.5 mL that were absent from the similar CSEP. Further analysis of the relevant fraction using 2-D electrophoresis is recommended.

If a compound was present in CSEP but not in SEP this would suggest that *L. salmonis* produce a substance that breaks down or inhibits a substance within the skin (hence it would not appear in SEP analysis). There were no peaks present in CSEP (red blocks) that was not present in SEP. However, should the SEP peak at 25.0 mL in sample 2 be recategorised as belonging in fraction 24.5 mL then all CSEP samples contained peaks at

25.0 mL that were absent from the similar SEP. Further analysis of the relevant fraction using 2-D electrophoresis is recommended.

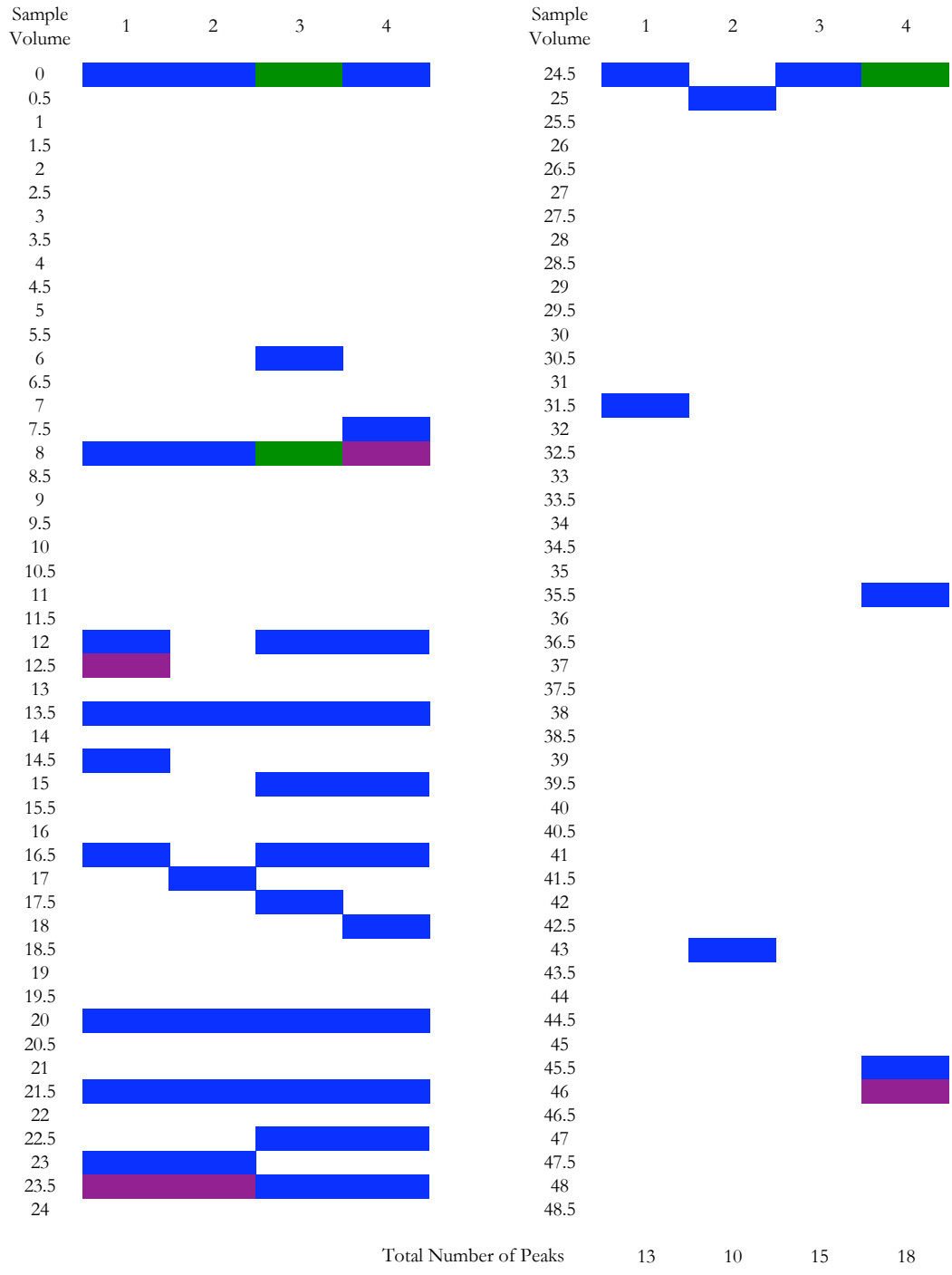


Figure 9: Graphical representation of CSEP chromatographic analysis. The blocks of colour represent individual peaks. Purple blocks denote 2 peaks in adjacent fractions whilst green blocks denote multiple peaks occurring within a single fraction. There are 9 volumes in which a peak is present in all samples (or adjacent fractions).

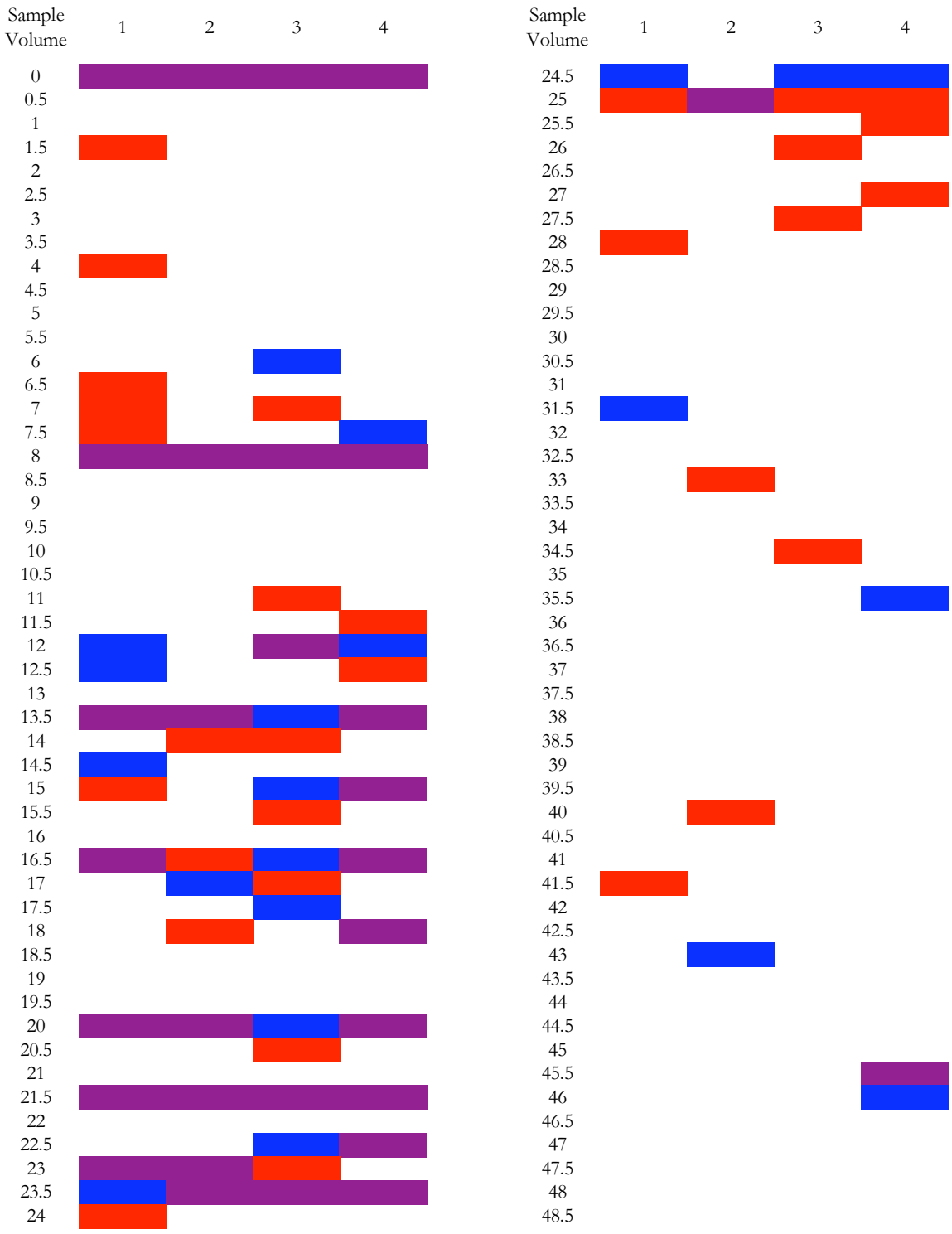


Figure 10: Graphical representation of SEP/CSEP chromatographic analysis. Red blocks denote fractions present only in SEP, blue blocks denote CSEP-only peaks and purple blocks denote peaks present in both SEP and CSEP. Subtractive chromatographic analysis shows no peaks consistently present in SEP or CSEP that is absent from CSEP or SEP respectively.

3.3.9 Chromatogram of fresh SEP and SEP stored -20 °C for 60 days

The analysis of freshly prepared SEP and SEP stored at -20 °C for 60 days is shown in Figure 11. A summary of all of the peaks using the criteria defined in section 3.2.4 is displayed in Table 5. The table indicates the total number of peaks identified on the chromatogram and the volumes at which these peaks started, reached their maxima and ended. All stored and fresh SEP chromatograms produced comparable chromatographic output.

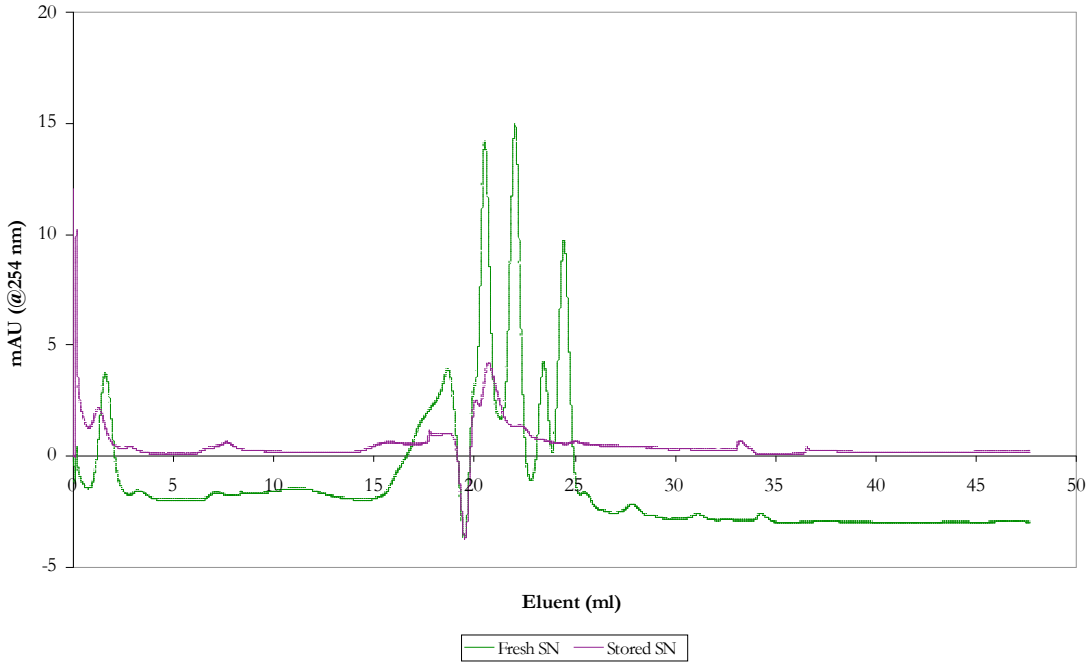


Figure 11: Chromatogram of fresh SEP and SEP stored at -20 °C for 60 days. The peaks present in the stored SEP very closely match those present in fresh SEP, however, they have a much reduced magnitude.

Table 5 shows that the pattern of peaks in fresh and frozen SEP is similar. Comparative analysis of the data from the chromatograms for fresh and frozen SEP shows a decrease in the magnitude of the peaks in the frozen SEP compared to the fresh SEP.

Table 5: Peak table comparing fresh SEP and stored SEP

Peak table from fresh SEP

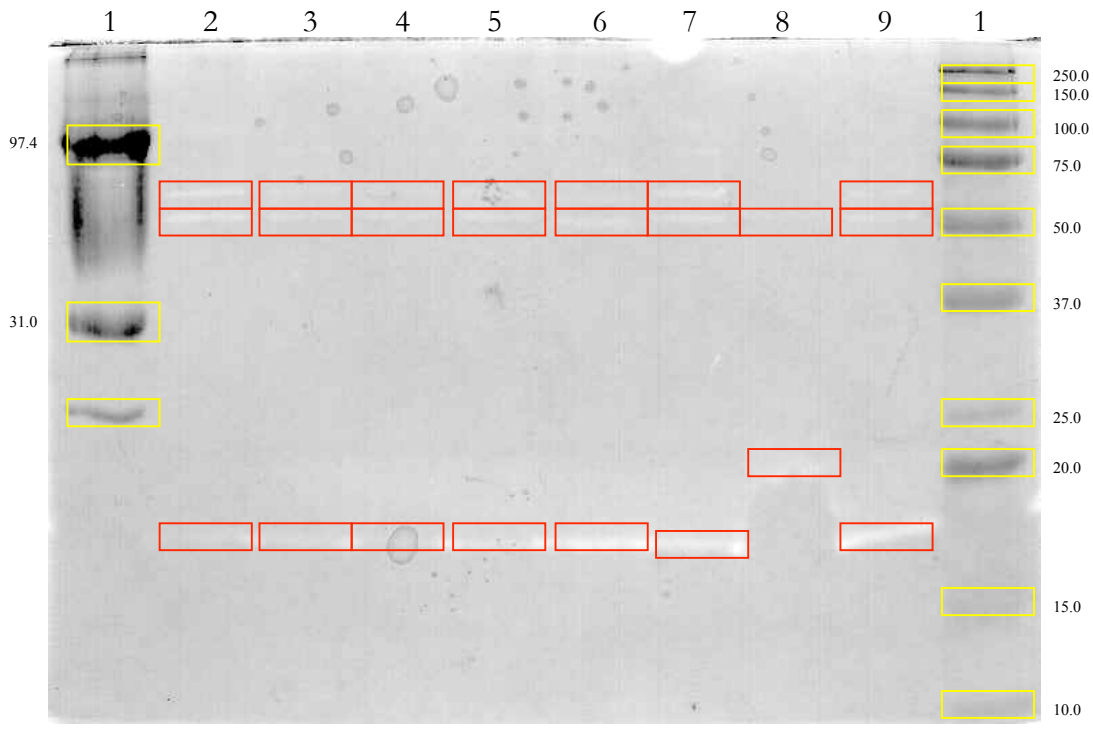
Peak Number	Peak Start (mL)	Peak End (mL)	Peak Max (mL)
1	0.00	0.09	0.00
2	0.09	0.74	0.16
3	0.74	2.78	1.61
4	2.78	4.24	3.25
5	6.61	7.84	7.07
6	8.30	8.67	8.37
7	14.83	19.30	18.66
8	19.63	21.29	20.49
9	21.29	22.78	21.99
10	22.78	23.85	23.42
11	23.85	25.22	24.42
12	25.22	26.66	25.48
13	27.10	27.22	27.16
14	27.22	28.49	27.85
15	29.86	31.70	31.10
16	33.93	34.88	34.27
17	45.90	45.98	45.94

Peak table from stored SEP

Peak Number	Peak Start (mL)	Peak End (mL)	Peak Max (mL)
1	0.00	0.01	0.00
2	0.07	0.77	0.14
3	0.77	2.41	1.27
4	2.41	3.66	2.87
5	6.27	6.82	6.73
6	6.82	8.60	7.67
7	17.18	18.09	17.75
8	18.09	19.02	18.59
9	19.81	20.22	20.03
10	20.22	21.97	20.67
11	21.97	23.14	22.27
12	24.62	25.42	25.03
13	32.95	33.95	33.23
14	36.48	36.98	36.54

3.3.10 Gelatin zymography

A typical result of zymograms using a gelatin substrate (at 2 $mg.mL^{-1}$) is shown in Figure 12 with bands of SEP and CSEP highlighted in red and the marker bands highlighted in yellow. The various inhibitors used are indicated in the table below the image. The results show there is no difference between CSEP and SEP when run on a gelatin-based zymogram, except when the inhibitor o-phenanthroline is used, in which case there is disappearance of gelatinase proteases approximately 60 kDa and 17 kDa in size and the appearance of a protease approximately 20 kDa.



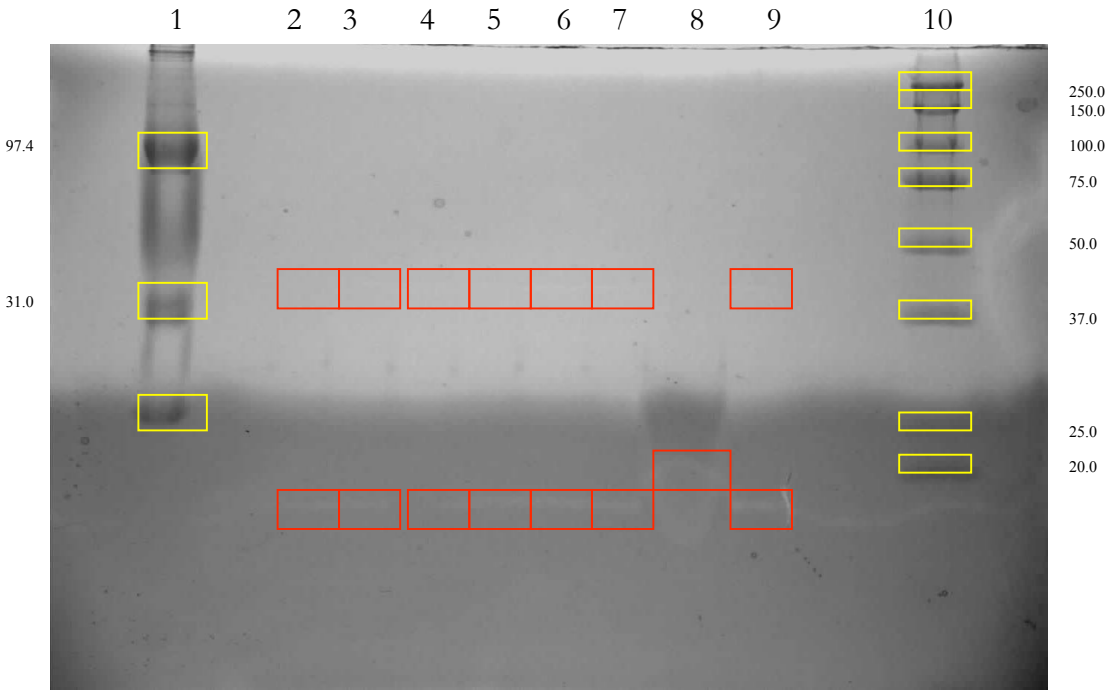
Number	Description
1	Low range marker
2	CSEP
3	SEP
4	SEP + aprotinin
5	SEP + iodoacetamide
6	SEP + EDTA
7	SEP + leupeptin
8	SEP + o-phenanthroline
9	SEP + pepstatin
10	Broad range marker

Figure 12: Gelatin zymogram of SEP and CSEP with bands highlighted. The table identifies the protease inhibitors used. When SEP is incubated with o-phenanthroline the proteases at approximately 60 kDa and 17 kDa disappear and a protease at approximately 20 kDa appears.

3.3.11 β-casein zymography

A typical result of zymograms using a β-casein substrate (at 2 $mg.mL^{-1}$) is shown in Figure 13 with bands of SEP and CSEP highlighted in red and the marker bands highlighted in yellow. The table identifies the protein inhibitors used. The results show there is no difference between CSEP and SEP when run on a β-casein-based zymogram, except when the inhibitor o-phenanthroline is used, in which case there is disappearance of proteases approximately 45 kDa in size disappear. However, there is the appearance of a protease

approximately 20 kDa in size when SEP is incubated with o-phenanthroline.



Number	Description
1	Low range marker
2	CSEP
3	SEP
4	SEP + aprotinin
5	SEP + iodoacetamide
6	SEP + EDTA
7	SEP + leupeptin
8	SEP + o-phenanthroline
9	SEP + pepstatin
10	Broad range marker

Figure 13: β-casein zymogram of SEP and CSEP with bands highlighted. The table identifies the protein inhibitors used. When the SEP is incubated with o-phenanthroline the protease at approximately 20 kDa appears.

3.4 Discussion

The results of the protein concentration analysis clearly show that SEP of *Lepeophtheirus salmonis* contains more protein than the CSEP at both 4 and 10 °C. The results from 2-D electrophoresis demonstrated that both SEP and CSEP are comprised of a complex mixture of proteinaceous material. However, due to the non-sterile nature of the Ussing's chamber culture system, it was not possible to identify which components were derived from the

lice and those derived from the environment or the fish skin. As a result the nature of the elevated protein is unknown and was, therefore, subjected to further analysis.

Comparison of the results for SEP, CSEP, SW and lice-containing water show no differences between protein profiles. Subtractive chromatographic analysis between SEP and CSEP demonstrated there are no peaks consistently present in either sample that are absent from CSEP and SEP respectively. This is not consistent with the results of Butler (2001) who used the ASSE to culture SEP as opposed to using freshly excised salmon skin. Butler (2001) found multiple unique peaks within SEP compared to control SEP, suggesting possible dimers, trimers and quadruples of a single molecule. The detection criteria used by Butler (2001) for identifying peaks were the same as those used in this study. Therefore, the peaks seen by Butler should also appear in this work. It is possible that the peaks seen by Butler were masked by background proteins derived from fish skin. Alternatively, there may have been an interaction between the ASSE and parasite leading to a compound being released from the skins. Butler (2001) noted that the breakdown of ASSE in seawater when incubated with copepodids liberated protein and, as the culture cycle progressed, the protein concentration of all samples (except seawater control) increased with time. An alternative hypothesis, as noted in section 3.3.8, is that there may be a unique peak around 24.5 mL in the SEP and at 25.0 in the CSEP but elution times from the FPLC may have partially hidden them. Whilst there does not appear to be the dimers, trimers or quadruples as reported by Butler (2001), further investigation of these fractions is recommended. Additionally, analysis of SEP and CSEP at different wavelengths may highlight substances that were undetectable at 254 nm due to different sensitivities.

Proteins in the SEP of haematophagous parasites are not uncommon. When they start

feeding the parasite triggers an immune response in their host. Don *et al*. (2004) reported the common dog hookworm (*Ancylostoma caninum*) expresses a detergent soluble, haemolytic factor to lyse ingested erythrocytes. The activity of the substance was heat-stable and unaffected by the addition of protease inhibitors, metal ions, chelators and reducing agents. Trypsin ablated lysis indicating that the haemolysin is a protein (Don *et al.*, 2004).

Enzymes that may have evolved to modulate other host responses include the tick salivary kininases, which hydrolyse circulating kinins such as bradykinin, resulting in smooth muscle contraction (Steen *et al.*, 2006). An example is the angiotensin-converting enzyme like protein (ACE-like protein) found in the salivary glands of the hard tick *Rhipicephalus (Boophilus) microplus* (Riding *et al.*, 1994, Jarmey *et al.*, 1995). Steen *et al*. (2006) suggest that ACE-like protein is produced by *Rhipicephalus (Boophilus) microplus* as a host cardiovascular system modulator or anti-inflammatory agent through non-specific kininases activity.

Work by Ribero and Mather (1998) demonstrated that saliva and salivary gland extract of *Ixodes scapularis* had activity suggestive of a metalloprotease with kininase activity. Steen *et al*. (2006) suggest that ticks might secrete enzymes like this to reduce host inflammatory response.

The vertebrate coagulation system involves a series of enzyme-catalysed zymogen activation steps. Therefore, a common finding amongst haematophagous parasites is saliva with coagulation inhibiting properties (Valenzuela, 2004). Hirudin (found in the saliva of leeches) is known to inhibit host anticoagulant enzymes of the serine endoproteinase family, particularly thrombin, by a mechanism including competitive substrate inhibition

(Naski *et al.*, 1990). Serine proteinase inhibitors are also produced in the salivary glands of several tick species and in most cases are anticoagulants and/or immunomodulators (Steen *et al.*, 2006).

Tick host homologues, i.e. proteins produced by ticks that mimic host proteins, have been shown to be modulators of the host immune and inflammatory responses. Tick macrophage inhibitor factor is a peptide produced by the salivary glands of *Amblyomma americanum* and has the ability to inhibit the migration of macrophages, thereby protecting the tick from macrophage attack (Jaworski *et al.*, 2001).

Ticks have also been shown to produce amine-binding proteins in their saliva. Steen *et al*. (2006) propose that the role of such proteins is to counter the inflammatory and vasoactive amines produced during the host's response to parasitism.

Paesen *et al*. (1999) reported the presence of a histamine-binding protein (Ra-HBP3) in the saliva of nymphs and larvae of the tick *Rhipicephalus appendicularus*. The authors propose that Ra-HBP3 subverts the host's system by sequestering histamine. Histamine is crucial in the inflammatory response. Weston-Davies and Nuttall (2002) believe that histamine may be used by mammals as a response to ectoparasites, aiding detection and removal.

Should a protein be present in the 24.5 mL fraction of the SEP it may play a role similar to that found by Don *et al*. (2004) in *A. caninum* or by Naski *et al.* (1990) in leeches as *L. salmonis* are known to feed opportunistically on blood (Bricknell *et al*., 2003). Alternatively, the limited immune response shown by *S. salar* in response to *L. salmonis* infection could be explained if a protein in the SEP fraction inhibits salmon macrophage

migration, as found in ticks by Jaworski *et al*. (2001) or ameliorates inflammation in response to ticks (Steen *et al.* 2006).

The decrease in magnitude of the peaks in SEP samples stored at -20 ºC for 60 days in the current study suggest that protein degradation has occurred. It is proposed that proteases from either the lice or the fish skin were contained in the supernatants; however, the source cannot be ascertained from these results.

From the results of this study, SEP run on a zymogram with gelatine substrate showed no difference from CSEP, except when the inhibitor o-phenanthroline was present. Likewise, the zymogram run on a β-casein substrate found no differences between the SEP and CSEP except when o-phenanthroline was present. When run on a gelatine substrate and treated with o-phenanthroline there was a disappearance of gelatinase proteases approximately 60 kDa and 17 kDa in size. However, a protease approximately 20 kDa appeared when SEP was incubated with o-phenanthroline. On the β-casein substrate a protease approximately 45 kDa disappears but a band at 20 kDa appeared.

O-phenanthroline is a metalloprotease inhibitor, chelating the divalent metal atom of the protease. The disappearance of bands suggests that the proteases are being inhibited. The appearance of a band, however, suggests that the large protease may have been cleaved to produce a new protease that is not inhibited by o-phenanthroline, and hence not a metalloprotease. As the results for SEP and CSEP on both substrates were the same, the appearance of a new protease was not investigated further. However, future work could involve combining protease inhibitors to determine the nature of the new protease as well as repeating the experiment using fresh solutions of o-phenanthroline.

It is possible that the potential protein seen in the 24.5 mL SEP fraction from the FPLC could be a protease, possibly a metalloprotease. Williamson, *et al*. (2003) note that parasite peptidases facilitate the invasion of host tissues, aid in the digestion of host proteins and play a role in host immune system evasion. In hypodermosis (the disease associated with the cattle parasite *Hypoderma lineatum* (warble fly)) the adult flies pass through the skin of the cattle's back, lay eggs and become bee-like flies after pupation. For the 8 months prior to emergence, the parasite migrates through the host's tissues (myiasis) causing no inflammation (Chabaudie and Boulard, 1992). Bouldard (1970 cited by Wikel and Alarcon-Chaidez, 2001) proposed that the effect of hypodermosis was linked to the host's humoral immune response but subsequent work by Bouldard and Bencharif (1984) demonstrated that serine proteases of *H. lineatum* deplete activity of both alternative complement pathway and classical complement pathway of the bovine host. Taylor *et al*. (1998) found that targeting the C3 inhibits the common activation point where the alternative and classical complement pathways converge.

The A1 family of aspartic proteases includes the lysosomal processing enzyme cathepsin D and digestive enzymes from blood-feeding parasites including *Plasmodium falciparum* plasmepsins and schistosome cathepsins D (Banerjee *et al.*, 2002, Brindley *et al.*, 2001, Williamson *et al.*, 2003). The aspartic proteases are thought to be responsible for the first step in degradation of host haemoglobin. Another group of proteases, the cysteine proteases, are involved in haemoglobin digestion in *S. mansoni* (Wasilewski *et al.*, 1996), *Plasmodium falciparum, Haemonchus contortus* (Knox *et al.*, 1993) and *N. americanus* (Brown *et al.*, 1995).

A second family of aspartic proteases has been identified from the intestines of the haematophagous strongyle nematodes, the nemepsins (Williamson, 2003). This second

family includes *Na*-APR-2 from adult *Necator americanus*, *Haemonchus* Pep1 and a similar protease from the infective larvae of *Strongyloides stercoralis* (Williamson, 2003, Longbottom *et al.*, 1997, Gallego *et al.*, 1998).

It should be noted that the suite of behaviours the lice use to find a suitable attachment site might cause damage to the host epidermis thereby eliciting protein release. Once the copepodid makes initial contact with the skin it undergoes a period of close searching for a suitable attachment site. During this phase it uses its maxillipeds to grip the fish's surface (Bron *et al.*, 1991). During the next phase of attachment the louse drives its hooked second antennae into the epidermis, usually with repeated stabbing action. The anterior edge of the dorsal cephalic shield is drawn down and forwards causing the epithelium to lift andaggregate in front of it. During this process, the epidermal cells are compressed (Bron *et al.*, 1991). Tully *et al*. (1993) and Dawson (1998) both report aggregations of copepodids around the base of the dorsal fin of sea trout (*Salmo trutta*) causing epidermal erosion sufficient to expose the fin rays. It is possible that the increased protein levels in SEP compared to CSEP seen in the present study could be attributed to the burrowing behaviours of the settling copepodids rather than as a result of secretions by the lice.

The final stage of copepodid attachment is the formation of the frontal filament (Bron *et al.*, 1991). The filament anchors the larvae to the hose prior to moulting to the chalimus I stage. Bron *et al*. (1991) reported that the parasite adheres to the basement membrane of the host's epidermis using a glue-like secretion. The secretion is injected under the epithelium where it spreads along the basement membrane with the epidermal cells overlaying to form a 'basal plate' (Bron *et al.*, 1991). The authors found that this structure contains proteinaceous components. Further analysis of the formed frontal filament demonstrated that it also contains proteins, with the internal structure appearing to be

composed of densely-packed protein fibres (Bron *et al.*, 1991). It is possible that the increase in total protein of SEP seen in the present study is the result of the beginnings of frontal filament formation. Using ASSE, Butler (2001) was unable to get *L. salmonis* copepodids to extrude the frontal filament, although they did demonstrate the other settlement behaviours. Therefore it is proposed that a potential reason for the difference between Butler's results and those obtained in this research may be the lack of frontal filament formation in the former study.

From the results obtained in this part of the study it would appear that proteins can be discounted as being part of the SEP of *L. salmonis*. It should be noted, however, that further analysis of the eluent from the FPLC column at 24.5 – 25.0 mL may provide contrary information. Furthermore, that should there be a protein with a very high efficacy for modulating the host's immune system, it could be presents in amounts undetectable using FPLC. Biological assays are more sensitive than chemical assays (Dr. Tony Ellis, Pers. Comm.).

3.5 References for Chapter 3

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Chapter 4
The role of interleukin-8 in *L. salmonis* infections

4.1 Introduction

Cytokines are low molecular weight, cell-derived glycoproteins that control inter-cellular communication of a variety of target cells responsible for immune responses (Savan and Sakai, 2006). The term cytokine is frequently used to refer to interleukins, tumour necrosis factors, interferons, colony stimulating factors and chemotactic cytokines (chemokines). The genes responsible for cytokines act locally in an autocrine (the target and secretory cell are the same) and paracrine (the target cells are in the vicinity of the secretory cell) manner (Savan and Sakai, 2006).

Chemokines are important mediators of innate immunity; they attract, recruit and activate a variety of leukocyte types towards inflammatory foci (Baggiolini, 1998, Chen *et al.*, 2005, Savan and Sakai, 2006; Gonzalez *et al.* 2007). Chemokines are categorised into one of four groups based on the arrangement of two invariant cysteine residues: CXC, C, CC and CX3C (Bacon *et al.*, 2002). CXC chemokines can be further classified into two sub-groups based on the presence of a glutamate-leucine-arginine (ELR) motif preceding the CXC sequence (Chen *et al.*, 2005). Although there is an abundance of work done on mammalian cytokines, there is a dearth of equivalent piscine work (Chen *et al.*, 2005) and as such only CC and CXC chemokines have been isolated from fish to date (Savan and Sakai, 2006).

The first CXC chemokine that has been isolated and cloned from fish is interleukin-8 (IL-8), also known as CXCL8 (Najakshin *et al.*, 1999, Savan and Sakai, 2006). IL-8 is an ELR-containing chemokine that is produced by numerous cell types. In mammals it is produced by macrophages/monocytes, epithelial cells, neutrophils, fibroblasts and endothelial cells upon infection or stimulation by cytokines such as IL-1β and TNFα (Jimenez *et al.*, 2006). Being a CXC chemokine, IL-8 predominantly promotes the

recruitment of neutrophils (Jimenez *et al.*, 2006, Sigh *et al.*, 2004), inducing their activation of the leukotriene pathway by releasing their granular content and by their increased adherence to endothelial cells and nitric oxide production (Jimenez *et al.*, 2006). In mammals IL-8 is also a chemoattractant for other cell types such as basophils, T lymphocytes and NK cells, as well as enhancing the permeability of endothelial cells (Jimenez *et al.*, 2006).

Table 6 shows species of fish where IL-8 has been successfully characterised. It must be noted, however, that an understanding of the biological role of IL-8 has not been achieved in any of these species.

Table 6: IL-8 cloned species

Species/Phyla	References
Japanese flounder (*Paralichthys olivaceous*)	Lee *et al.*, 2001
Rainbow trout (*Oncorhynchus mykiss*)	Laing *et al.* , 2002, Fujiki *et al.*, 2003
Lamprey (*Lampetra fluviatilis*)	Najakshin *et al.*, 1999
Banded dogfish (*Triakis scyllia*)	Inoue *et al.*, 2003b
Channel catfish (*Ictalurus punctatus*)	Chen *et al.*, 2005
Silver chimaera (*Chimaera phantasma*)	Inoue, *et al*., 2003a

Fish and humans share a degree of synteny (being on the same chromosome) in genomes and Savan and Sakai (2006) state that by phylogenetic analyses, piscine IL-8 genes closely resemble mammalian CXCL8 genes and cluster in a single monophyletic group.

Amoebic gill disease (AGD) is a protozoan ectoparasitic disease associated with infection by *Neoparamoeba* species. It is characterised by epithelial hyperplasia that manifests as gill lesions. Bridle *et al*. (2006) report that besides the epithelial hyperplasia that presents as grossly visible, pale, multifocal gill lesions, there is a less apparent infiltration of leukocytes and oedema associated with a local inflammatory response. The lesion-

associated leukocytes are predominantly found in the central venous system (CVS) where they extravasate into the lesion and are thought to participate in lesion repair.

Bridle *et al*. (2006) found that following infection with *Neoparamoeba*, the expression levels of IL-8 in the liver of rainbow trout were significantly increased at 7 days post infection (PI) relative to controls, but there was no difference between groups by 14 days PI. In the anterior kidney, however, the authors reported no AGD-induced up-regulation of IL-8; indeed they reported a (statistically non-significant) down-regulation of IL-8 at 7 days PI that was absent by 14 days PI.

Histological examination of AGD gill lesions by Bridle *et al.* (2006) revealed a lack of any great leukocyte infiltration to the infected gills. This, coupled with the significant increase in liver IL-8 gene expression, as well as apparent up-regulation of iNOS (inducible nitric oxide synthase) and IL-1α1 gene expression at 7 days PI suggests the involvement of a systemic response to the AGD infection (Bridle *et al.*, 2006). Likewise, the authors attribute the (non-significant) down-regulation of immune-regulatory gene expression levels in the anterior kidney at 7 days PI to a possible migration of leukocytes from the kidney, thus suggesting the involvement of a systemic response.

Following infection of rainbow trout (*Onchorhynchus mykiss*) with *Gyrodactylus derjavini*, a monogenean ectoparasite, Lindenstrøm *et al*. (2004) reported no changes in the expression patterns of IL- 8. The authors found the lack of IL-8 induction surprising taking into account the clear indication of other pro-inflammatory mediators in the study. They do acknowledge, however, that it cannot be precluded that other chemokines could be involved or that IL-8 may be induced at time points other than those sampled (0, 4 and 8 days PI). The latter possibility is supported by the results of Bridle *et al*. (2006).

The aim of this chapter was to investigate whether or not the chemokine interleukin-8 was one of the secreted substances hypothesised by Jones *et al*. (2007) and Johnson *et al*. (2002) as being involved in the observations of limited tissue responses to *L. salmonis* in Atlantic salmon during the early stages of infection.

4.2 Materials and methods

Twelve Atlantic salmon post-smolts were infected with *L. salmonis* copepodids following the protocol defined in section 2.3. The lice were left to develop on the fish until they reached the chalimus I stage. At an ambient water temperature of 12 ºC this took approximately 5 days. Following infection, the fish were examined every 2 days for the presence of the parasite. The chalimus stages were found around the base of the fins and on the tail. Twelve Atlantic salmon that were not exposed to *L. salmonis* were used as control fish.

Once the lice were at the appropriate stage the fish were euthanised by MS222 overdose and the areas of louse attachment excised using a 5 mm cork borer. Heart, liver, kidney, spleen and pyloric cæca were sampled aseptically and all samples were transferred to pre-labelled microcentrifuge tubes (Eppendorf) containing RNALater for qPCR analysis (see section 4.2.1).

4.2.1 Real-time (quantitative) polymerase chain reaction (qPCR)

The polymerase chain reaction (PCR) has revolutionised the detection of DNA and RNA. A single copy of a particular sequence can be specifically amplified and detected. Theoretically there is a quantitative relationship between the amount of starting target sequence and the amount of PCR product at any given cycle. In practice, however, it is

common for replicate reactions to yield different amounts of PCR products. The development of real-time (quantitative) PCR (qPCR) has eliminated the variability traditionally associated with quantitative PCR thereby allowing routine and reliable quantification of PCR products.

4.2.2 qPCR chemistry

qPCR is the most sensitive and reproducible method for quantifying DNA target concentration in biological solutions by means of the polymerase chain reaction. Fluorescent probes (fluorescent dyes with quenchers in close proximity; see Figure 14) are used preferentially to intercalating agents. The principal drawback to intercalator-based detection is that both specific and non-specific products generate signal. Whilst the probe is intact, the proximity of the quencher reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space. The probe is sited between forward and reverse primers, with the fluorescence dye at the 5' end and the quencher at the 3' end. During amplification the dye and quencher are physically separated resulting in 'de-quenching' and an increase in the reporter dye signal. Cleavage of the probe removes it from the target strand allowing primer extension to continue to the end of the template strand. Therefore, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, effecting an increase in fluorescence intensity proportional to the amount of amplicon produced. The advantage of fluorogenic probes over DNA binding dyes is that specific hybridisation between probe and target is required to generate the fluorescent signal. As all three primers must bind correctly to produce an amplification plot, signals are highly unlikely to result from mispriming events.

4.2.3 qPCR quantitation

qPCR reactions are characterised by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of target, the sooner a significant increase in fluorescence is observed. An amplification plot is the plot of fluorescence signal versus cycle number and in the initial cycles there is little change in signal. This defines the baseline for the plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. The parameter cycle threshold (C_t) is defined as the factional cycle number at which the fluorescence passes the fixed threshold with a plot of the log of initial target copy number for a set of standards versus C_t being a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring C_t and using the standard curve to determine starting copy number.

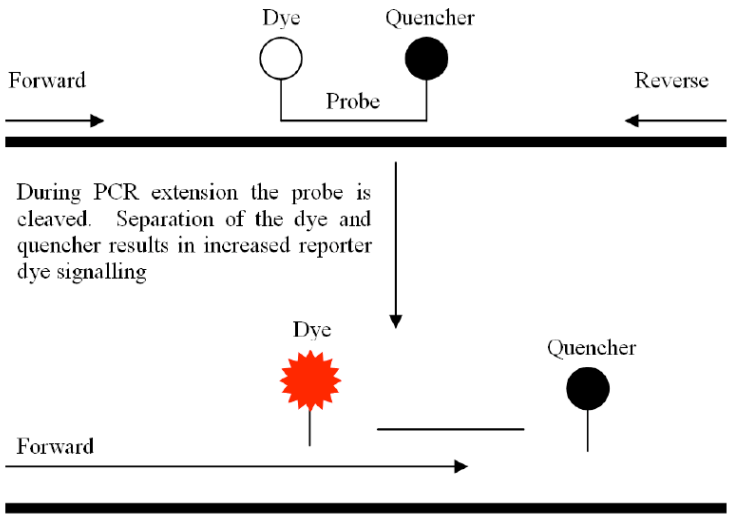

Figure 14: Principles of qPCR (modified from Powell, 2003)

4.2.4 RNA extraction

The RNA of samples was extracted using a commercially available kit (RNeasy® Mini,

Qiagen) and following the manufacturer's instructions. All tissue samples were stored at -20 ºC in RNAlater® (Sigma) prior to RNA extraction. Briefly, 10 µL β-mercaptoethanol (Sigma) was added to 1 mL RLT buffer and thoroughly vortexed. One stainless steel tissue lyser bead (5 mm, Qiagen) and 600 µL of RLT/β-mercaptoethanol were added to the required number of 2 mL Safelock tubes followed by the tissue sample. The tissue was disrupted using a Qiagen Tissue Lyser (60 seconds at 30 1/S) and the lysate centrifuged at 12,000 *g* for 2 minutes. Following centrifugation the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube, 600 µL 70% ethanol added and the solution aspirated with a pipette. Rapidly, 600 µL of sample was added to an RNeasy column fitted in a 2 mL collection tube and centrifuged for 15 seconds at 12,000 *g*. The flow-through was discarded and the process repeated with the remaining sample. Once all of the sample had been passed through RNeasy column 700 µL RW1 was added to the column and centrifuged for 15 seconds at 12,000 *g*. The flow-through was discarded and the column transferred to a fresh 2 mL collection tube. Five hundred microlitres RPE were added to the column and centrifuged for 15 seconds at 12,000 *g*. The flow-through was discarded and a further 500 µL RPE added to the column and centrifuged for 2 minutes at 12,000 *g*. To elute the RNA the column was transferred to a fresh 1.5 mL microcentrifuge tube (with lid removed) and 200 µL DEPC-dH_2O added directly on to the silica membrane and centrifuged for 120 seconds at 12,000 *g*. The eluate was transferred to a fully labelled 1.5 mL tube for storage at -80 ºC. Negative extraction controls (designated A control) were conducted by performing a blank extraction. Controls were taken through subsequent RT and PCR steps.

4.2.5 Reverse transcription

Complementary DNA (cDNA) was synthesised using the Taqman® RT kit (Applied Biosystems) in a final reaction volume of 25 µL. Firstly, 9.625 µL extracted RNA (see

section 4.2.4) + 1.25 µL 50 µM oligo $d(T)_{16}$ were mixed in an RNase-free tube, denatured at 70 ºC for 10 minutes before being chilled on ice. In a separate tube 2.5 µL ×10 reverse transcriptase (RT) buffer + 5.5 µL magnesium chloride ($MgCl_2$) + 5.0 µL deoxynucleotide triphosphates (DNTPs) + 0.5 µL Rnase inhibitor and 0.625 µL Multiscribe reverse transcriptase were mixed. To the RNA/oligo dT mix, 14.125 µL of the mix were added and incubated at 48 ºC for 90 minutes followed by 95 ºC for 5 minutes. Negative RT controls were conducted by performing reactions containing no target (designated B control). Controls were taken through subsequent PCR steps.

4.2.6 qPCR

qPCR assays were performed on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems) running Sequence Detection System (SDS) software Version 1.1 (Applied Biosystems). Primer and probe sets were obtained for Atlantic salmon translation elongation factor 1α (α-elf; Magnus Devold, Pers. Comm.) or designed using ABI Primer Express software (see Table 7). All primers and probes were specifically designed and tested to sit at a splicing site (see Figure 15). This prevented amplification of genomic DNA (gDNA) from complementary DNA (cDNA) made from RNA contaminated with gDNA. The efficiency of the primers and probes were tested using a 10-fold serial dilution of known template to produce a standard curve. The critical threshold (C_t) values were determined and expression values normalised against the reference gene, ELF, were calculated using standard curve method (AMI User Manual).

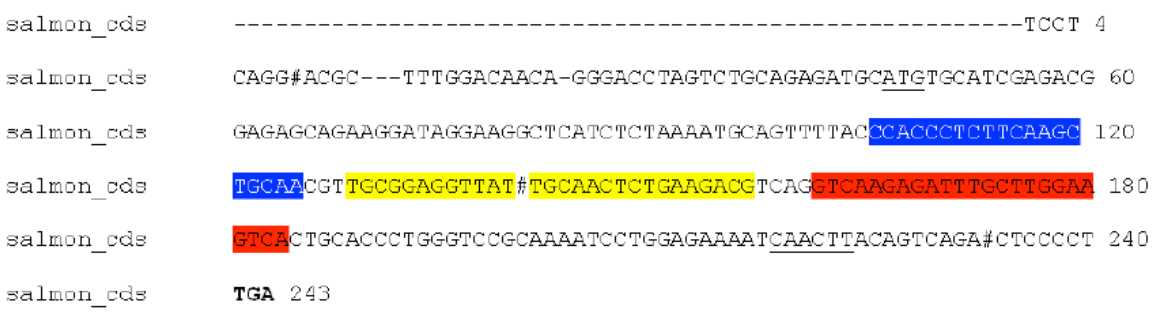

Figure 15: Primer and probe sites for IL-8 in Atlantic salmon (Blue = forward primer, yellow = probe, red = reverse primer)

In an RNAse-free tube, the reaction mix was prepared by mixing 10 µL of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (×2) (Applied Biosystems) with 1 µL ×20 assay mix + 8 µL DEPC-dH_2O. Nineteen microlitres of reaction mix was added to each well of a 96-well plate (ABI). One microlitre of cDNA (see section 4.2.5) was added to each well, the plate covered with an adhesive lid (ABI) and centrifuged at 20,000 *g* for 30 seconds to pellet well contents. Following centrifugation plates were transferred to the detection system and processed using the cycling conditions shown Table 8. Controls were conducted for each primer and probe set containing no target (designated C control).

Table 7: Primers and probes used for real-time PCR analysis

Atlantic salmon gene target	Upstream primer	Downstream primer	Taqman® probe	Amplicon size	Genbank accession number
α-elf-1α	CCCCTCCAGGA CGTTTACAAA	CACACGGCCCA CAGGTACA	FAM-ATCGGT# GGTATTGGAA C	57	AF321836
IL-8	CCACCCTCTTCA AGCTGCAATGC AA	TGACTTCCAAGC AAATCTCTTGAC	FAM-TGCGGAGGTTATTGCA ACTCTGAAGACG	79	CA061522

Table 8: Thermal cycler conditions for qPCR

Initial setup	Each of 45 cycles	
	Denature	Anneal/Extend
HOLD	CYCLE	
2 minuate @ 50 °C (AmpErase uracil N-clycosylase incubation)		
10 minutes @ 95 °C (AmpliTaq Gold polymerase activation)	15 seconds @ 95 °C	60 seconds @ 60 °C

4.2.7 Analysis of qPCR results

The results of qPCR were analysed according to manufacturers' instructions (AMI Manual). However, before analysis could take place the efficiency of the probes had to be determined (see section 4.2.8).

4.2.8 Validation of reaction efficiencies and relative quantification

In order to determine if the salmon host cell endogenous sELF-1α assay control might permit the relative quantification of IL-8 as well as ensure efficient performance of each assay, the Molecular Genetics department, FRS Marine Laboratory conducted a validation experiment (see section 4.2.6 for method). The results of the validation experiment have been published by Snow *et al*. (2006).

For each assay triplicate reactions were conducted on each dilution of a 10-fold serial dilution of cDNA prepared from concentrated standards. Standard curves were generated by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold of sELF assays Figure 16). The equations of the best-fit lines were derived from linear regression and the values for sELF and IL-8 (Table 9) applied to Equation 1 for comparison of results. Reaction conditions with respect to probe and primer concentrations were independently evaluated as described in the Applied Biosystems literature.

Equation 1: Calculations for qPCR. q_t = converted number equivalent; b = y-intercept; c_t = critical threshold; a = PCR efficiency (from gradient of slope))

$$q_t = 10^{\frac{(b - c_t)}{a}}$$

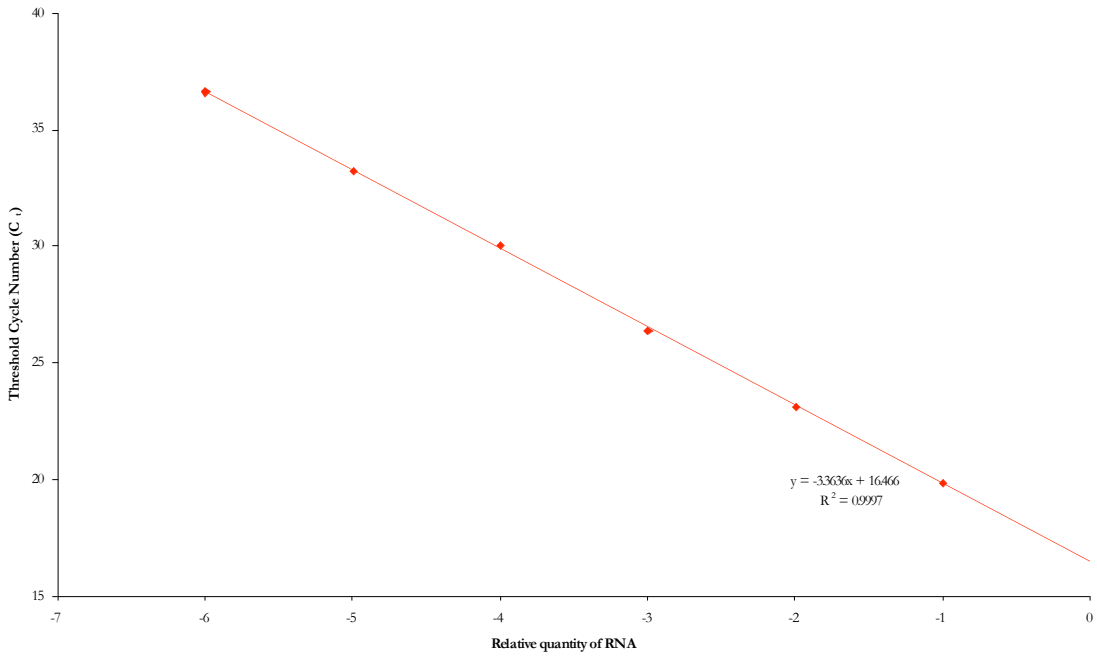

Figure 16: Standard curve of relative RNA dilution required to elevate fluorescence above threshold. Equations were derived from linear regression (data courtesy of M. Snow FRS Marine Laboratory, Aberdeen).

Table 9: Values for qPCR equation taken from Figure 15

Probe	a (PCR efficiency)	b	Reference
sELF	3.3636	16.466	Snow *et al.*, 2006

4.3 Results

The results of the assays are shown in Figure 17 through to Figure 22. This study has demonstrated that during infection with *L. salmonis* there is a change in the expression levels of the chemokine interleukin-8 in the tissues of Atlantic salmon: heart (Figure 17) and spleen tissue (Figure 18) both show a statistically significant increase in expression levels relative to controls (two-tailed T-test, T = 2.31, $p = 0.015$, DF = 21; two-tailed T-test, T = 4.47, $p < 0.001$, DF = 22 respectively). Tissue from the head kidney (Figure 19) show a statistically significant decrease in expression levels relative to controls (two-tailed T-test, T = -2.37, $p = 0.032$, DF = 15). The results for fins (Figure 20), liver (Figure 21) and pyloric cæca (Figure 22) do not show a significant difference in expression levels

(two-tailed T-test, $p>0.05$).

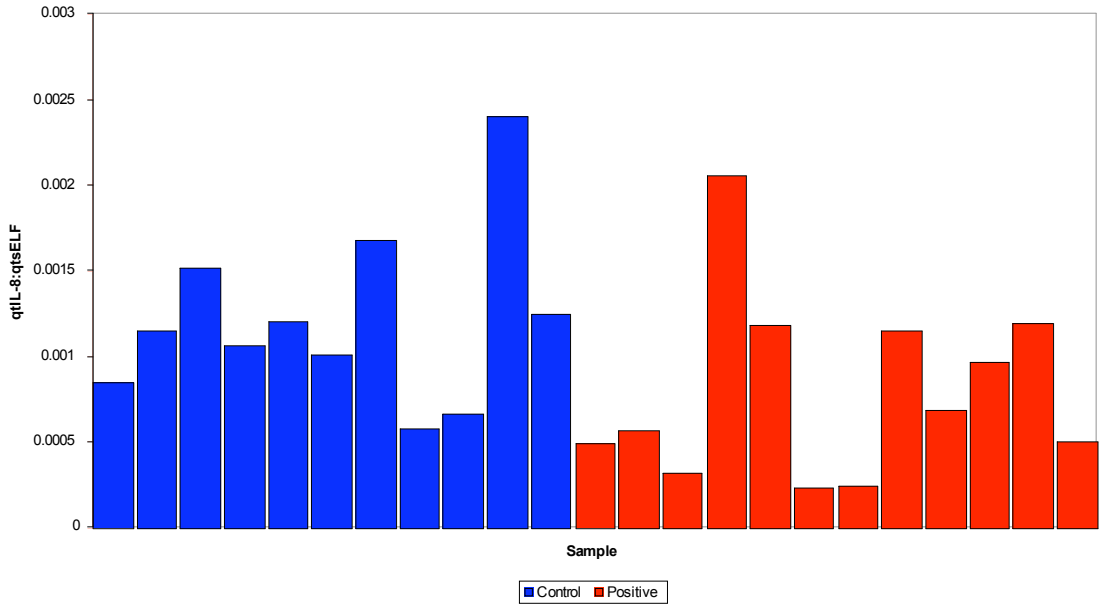

Figure 17: IL-8 gene expression in heart tissue samples from 12 uninfected (control) fish compared to 11 infected (positive) fish that survived long enough to be sampled. There is a statistically significant increase in expression levels in tissues from fish infected with *L. salmonis* relative to controls (two-tailed T-test T = 2.31, $p = 0.015$, DF = 21)

Hearts from fish infected with *L. salmonis* show increased levels of IL-8 expression relative to controls. Without the assumption of equal variances, the value of the test statistic comparing control and sample hearts is 2.56 (with 22 degrees of freedom). The *p* value given by MINITAB is 0.009. Therefore there is very strong evidence against the null hypothesis that the levels of IL-8 gene expression are equal.

Spleens from fish infected with *L. salmonis* show increased levels of IL-8 expression relative to controls. Without the assumption of equal variances, the value of the test statistic comparing control and sample spleens is 5.86 (with 22 degrees of freedom). The *p* value given by MINITAB is less than 0.001. Therefore there is very strong evidence against the null hypothesis that the levels of IL-8 gene expression are not equal.

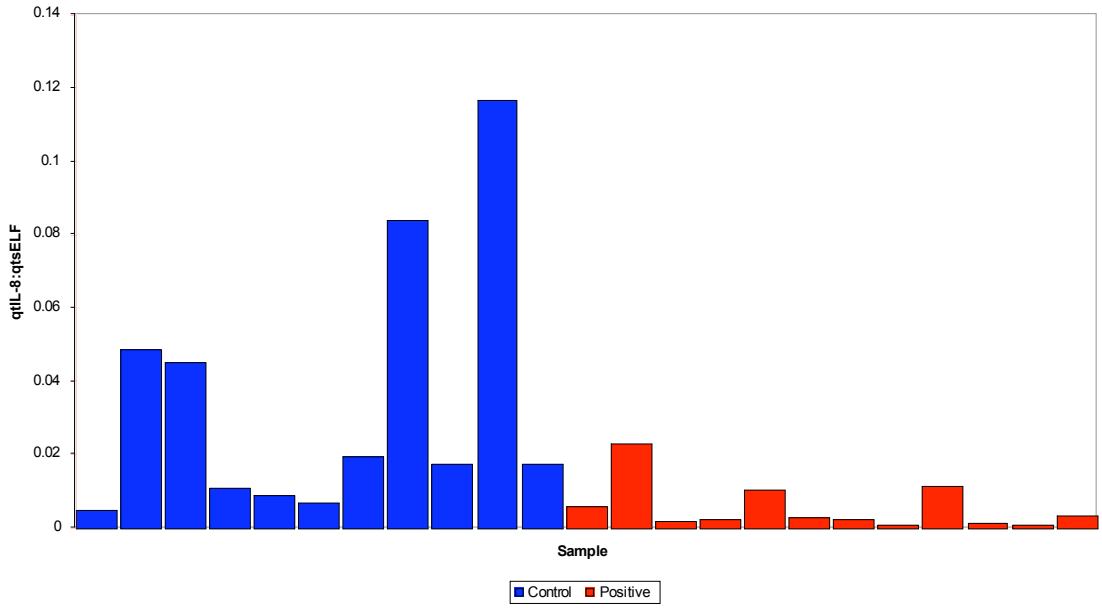

Figure 18: IL-8 gene expression in spleen tissue samples from 12 uninfected (control) fish compared to 11 infected (positive) fish that survived long enough to be sampled. There is a statistically significant increase in expression levels in tissues from fish infected with *L. salmonis* relative to controls (two-tailed T-test, T = 4.47, $p < 0.001$, DF = 22).

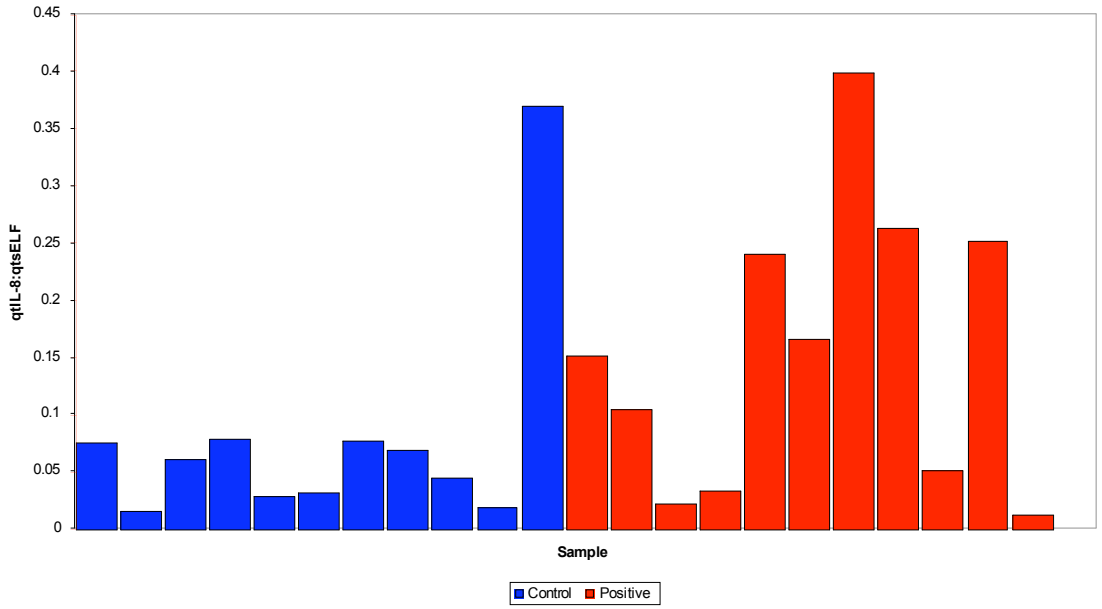

Figure 19: IL-8 gene expression in head-kidney tissue samples from 12 uninfected (control) fish compared to 11 positive (infected) fish that survived long enough to be sampled. There is a statistically significant decrease in expression levels in tissues from fish infected with *L. salmonis* relative to controls (two-tailed T-test, T = -2.37 $p = 0.032$, DF = 15).

Anterior kidney from fish infected with *L. salmonis* show decreased levels of IL-8 expression relative to controls. Without the assumption of equal variances, the value of the

test statistic comparing control and sample anterior kidney is -2.37 (with 15 degrees of freedom). The *p* value given by MINITAB is 0.032. Therefore there is moderate evidence against the null hypothesis that the levels of IL-8 gene expression are not equal.

Fins from fish infected with *L. salmonis* do not show altered levels of IL-8 expression relative to controls. Without the assumption of equal variances, the value of the test statistic comparing control and sample fins is -0.79 (with 21 degrees of freedom). The *p* value given by MINITAB is 0.780. Therefore there is little evidence against the null hypothesis that the levels of IL-8 gene expression are not equal.

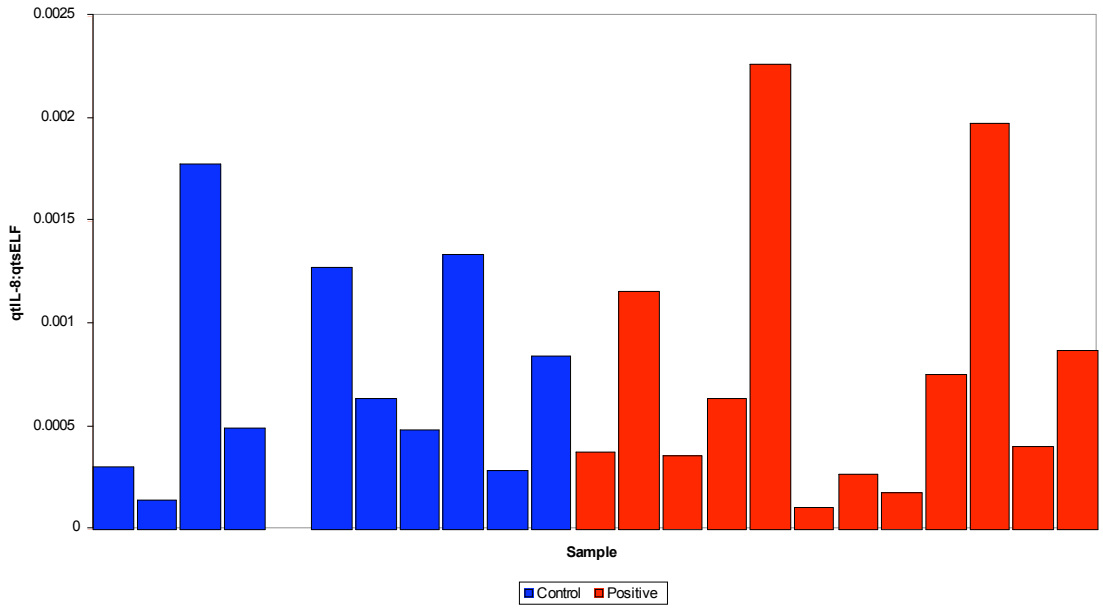

Figure 20: IL-8 gene expression in fin tissue samples from the 12 uninfected (control) fish compared to the 10 samples from infected (positive) fish that survived long enough to be sampled. There is no significant difference between infected and uninfected samples (two-tailed T-test, $p > 0.05$).

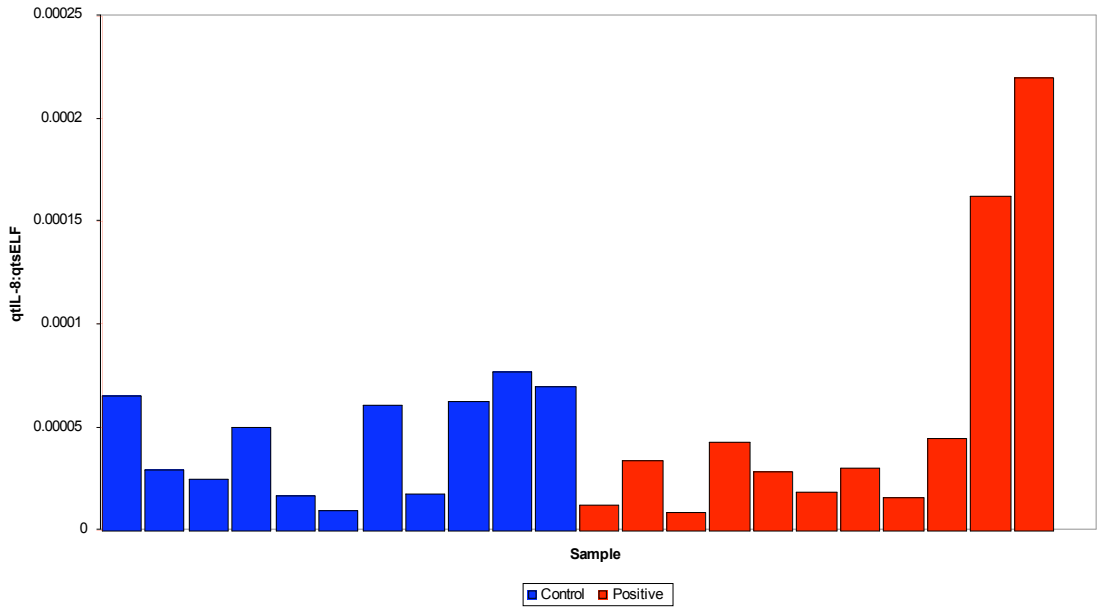

Figure 21: IL-8 gene expression in liver tissue samples from 12 uninfected (control) fish compared to 11 infected (positive) fish that survived long enough to be sampled. There is no significant difference between infected and uninfected samples (two-tailed T-test, $p > 0.05$).

Livers from fish infected with *L. salmonis* do not show altered levels of IL-8 expression relative to controls. Without the assumption of equal variances, the value of the test statistic comparing control and sample livers is 0.36 (with 22 degrees of freedom). The *p* value given by MINITAB is 0.360. Therefore there is little evidence against the null hypothesis that the levels of IL-8 gene expression are not equal.

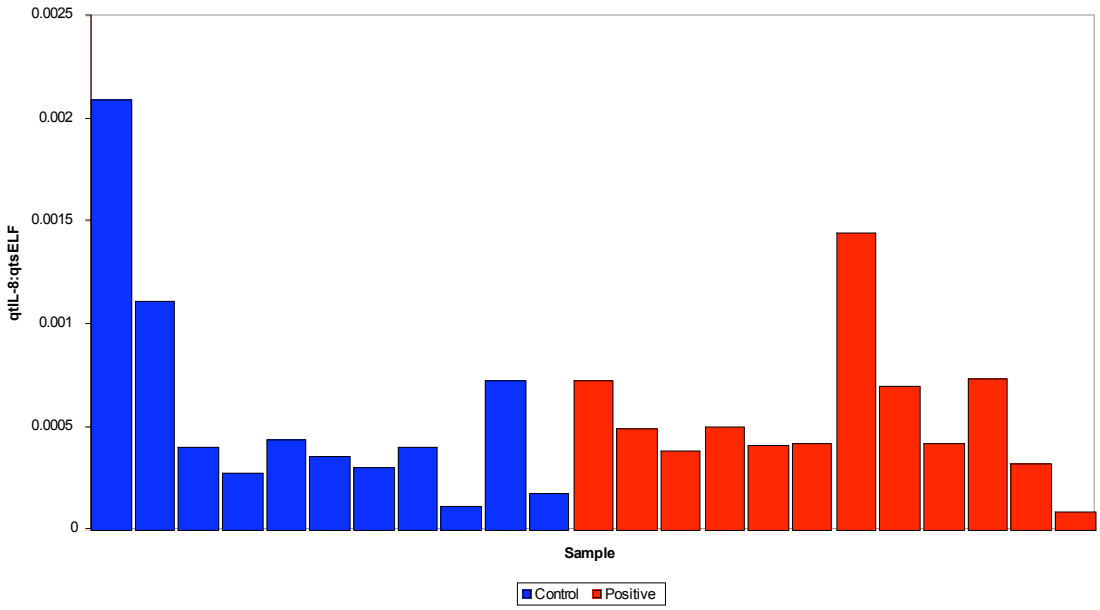

Figure 22: IL-8 gene expression levels in pyloric cæca tissue samples from 12 uninfected (control) fish compared to 11 infected (positive) fish that survived long enough to be sampled. There is no significant difference between infected and uninfected samples (two-tailed T-test, $p > 0.05$).

Pyloric cæca from fish infected with *L. salmonis* do not show altered levels of IL-8 expression relative to controls. Without the assumption of equal variances, the value of the test statistic comparing control and sample pyloric cæca is -0.36 (with 22 degrees of freedom). The *p* value given by MINITAB is 0.638. Therefore there is little evidence against the null hypothesis that the levels of IL-8 gene expression are not equal.

4.4 Discussion

Interleukin-8 is a chemokine that directs inflammatory and immune cells to sites of injury and infection in mammals. The principal role of IL-8 is to control the movement and activity of neutrophils (Hajnická *et al.*, 2001). In Atlantic salmon the major secondary lymphoid organs are the spleen and anterior kidney. Therefore it is not unreasonable to have assumed that both organs would respond to the sea lice in the same manner. This is not the case as there is a significant difference between the spleens of infected and non-

infected but not in kidney expression levels. Differences in the tissue of the spleen and kidney may account for the differences in IL-8 expression. Leknes (2001b) reports free, single macrophages and large accumulations of macrophages, i.e. melanomacrophage centres (MMCs), occur regularly in the spleen, trunk kidney and liver of platies (*Xiphophorus maculates* L.). However, when stained with Mayer's haemalum-eosin these cells in the spleen and liver are packed with a dense, yellow material whereas the corresponding cells in the trunk kidney were filled with a dense, grey material (Leknes, 2001b).

The MMCs have been also observed in the spleen, kidney or liver of several teleostean species but, with the exception of platies, they have not been studied and compared intraspecifically (Tsujii and Seno, 1990, Meseguer *et al.*, 1994, Leknes, 2001b). Furthermore, the morphology of the teleostean MMCs and the intracellular structure differ between individuals, species and organs (Agius, 1985, Haaparanta *et al.*, 1996). For example the MMCs within the kidney of goldfish (*Carassius auratus* L.) contain much more melanin than spleen MMCs of the same fish (Herraez and Zapata, 1991). Work by Leknes (2001b) revealed that macrophages in the MMCs of platy kidney often contain melanin-like granules that are lacking in splenic MMCs.

The MMCs in teleosts contain lipofuschin (aka lipofuscin, a polymer of lipids and proteins) and some haemosiderin, a protein that stores iron (Herraez and Zapata, 1991, Meseguer *et al.*, 1994, Press and Evensen, 1999). Herraez and Zapata (1991) and Meseguer *et al*. (1994) both report that splenic MMCs in goldfish contain more haemosiderin than those found in the kidney. Leknes' (2001b) study showed that the MMCs of platy spleen and liver normally contain large quantities of iron(III) ions, whereas the MMCs of kidney do not contain this type of iron ion.

As well as structural differences, there is evidence from other studies that the kidney and spleen respond differently when challenged with pathogens. Falk *et al.* (1995) investigated the distribution and phenotype of splenic- and anterior kidney-derived leukocytes from Atlantic salmon that were developing infectious salmon anaemia (ISA). The authors concluded that the earliest changes associated with experimental ISA infection could be identified in the leukocyte populations of the spleen rather than the head kidney. Work by Irwin and Kaattari (1986) indicated that the spleen is an important immune responsive organ, whilst Zapata and Cooper (1990) noted that the head kidney is important for the elaboration of an immune response. Thus, an interpretation of the findings of the present study is that macrophages activated in the spleen during the early immune response (hence the increased IL-8 expression levels) migrate to the head kidney to elaborate an immune response. Ackermann *et al*. (2004) report the expression of IL-8 mRNA in buffy coat cells, suggesting that infiltrating leukocytes may contribute significantly to IL-8 levels detected in other tissue samples. This could, therefore, explain the results of the tissues that did not show a significant change in levels of IL-8 expression. An alternative explanation for the decrease in IL-8 expression levels in the kidney may be due to a restriction in the time points selected for sampling. It is unlikely that sampling occurred too early, however it is possible that samples were taken too late in the infection. Therefore, a down regulation in head kidney expression levels may be due to the migration of leukocytes out of the kidney.

The heart tissue of Atlantic salmon infected with *L. salmonis* showed increased expression of the IL-8 gene. In Atlantic salmon foreign organic substances are mainly taken up by the kidney (Smedsrød *et al.*, 1993, Dalmo *et al.*, 1996). However, in cod (*Gadus morhua* L.) such substances are cleared from the circulation mainly by the heart endothelium whilst the kidney plays a relatively small role (Sørensen *et al.*, 1998, Dalmo *et al.*, 1996, Smedsrød *et al.*, 1995). In the platy, it has been shown that the heart endothelium, and in particular the

atrium, is able to take up and store large quantities of foreign ferritin from the circulation and thus have a similarly important role in clearance of the circulation as the corresponding cells in cod (Leknes, 2001a). If the heart muscle of the Atlantic salmon is removing components of the lice secretions from the blood it may be stimulating IL-8 production in a role similar to those cells in the heart of the cod and platy.

Whilst this is the first record of a general, systemic response in IL-8 expression in response to infection with *L. salmonis*, the presence of anti-IL-8 substances has previously been recorded in other animals. Hajnická *et al.* (2001) showed that salivary gland extracts from several ixodid tick species (*Dermacentor reticulates, Amblyomma variegatum, Rhipicephalus appendiculatus, Haemaphysalis inermis* and *Ixodes ricinus*) possess anti-IL-8 activity. The activity appears to be mediated by one or more molecules present in salivary glands. By binding IL-8, these molecules inhibit binding of the chemokine to its receptors thereby inhibiting chemotaxis of neutrophils (Hajnická *et al.*, 2001). Infiltrating neutrophils release chemokines, such as IL-8, which serve to reinforce the recruitment of additional neutrophils to the developing inflammation (Fast *et al.*). Therefore anti-IL-8 activity may also facilitate bloodfeeding, which forms a minor component of *L. salmonis*' diet (Bricknell *et al.*, 2003). Furthermore, Hajnická *et al.* (2001) propose that by producing IL-8 binders as opposed to IL-8 receptor antagonists the ticks may have found the most efficient means of controlling IL-8 activity in a range of different host species. This mechanism may also explain the ability of *L. salmonis* to parasitise more species than just Atlantic salmon (see Pike and Wadsworth, 1999 for a more extensive list).

It should be noted that whilst it is possible to say that *L. salmonis* leads to a significant increase in IL-8 gene expression in heart and spleen tissues, and a significant decrease of expression in anterior kidney it cannot be concluded that those tissues that do not show a

significant change relative to controls are unaffected. It was predicted that the local site (i.e. fins) would have shown increased expression even if none of the others did. If there is no significant difference between positive (infected) samples and negative (uninfected) controls this could suggest an immunosuppressive effect, but not one severe enough to show a statistically significant effect. In order to test this hypothesis a known positive sample is required. To this author's knowledge no known sample currently exists.

In summary, I propose that structural differences in the tissue of the spleen and kidney, as described by Leknes (2001b), and the movement of activated macrophages from the spleen to the head kidney, account for the differences in IL-8 expression levels. Furthermore, it is proposed that the heart muscle of the Atlantic salmon removes components of the lice secretions from the circulating blood, thereby stimulating IL-8 production in a role similar to that seen in the hearts of cod and platys. Clearly further investigations into the expression of the IL-8 gene are needed. Not only would it be beneficial to chart the changes in IL-8 expression over a defined period of time, but it would also be interesting to sample circulating macrophages at these time points. This would allow testing of Ackermann *et al.*'s (2004) proposition that the levels of IL-8 expression detected in tissues are as a result of infiltrating buffy coat cells.

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Chapter 5
The role of prostaglandin E_2 in *L. salmonis* infections

5.1 Introduction

Eicosanoids comprising mainly of prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), are lipid based mediators with a short half-life that act in an autocrine and paracrine manner (Smith, 1989, Daugschies and Joachim, 2000). They are synthesised *de novo* from polyunsaturated fatty acids (PUFAs; see Figure 23) and share a chain length of 20 carbon atoms (C-20) as a common feature (Cayman Chemical Company, 2005). The prefix *eicosa*- or icosa- (from the Greek for 20) denotes the C-20 (Beare-Rogers *et al.*, 2001). According to their biochemical properties and biosynthetic pathways eicosanoids are classified into epoxids, hydroxyeicosatetraenic acids (HETEs), hydroxyoctadecadienic acids (HODEs), hydroxyperoxyeicosatetraenic acids (HPETEs), lipoxins, prostaglandins (PGs), thromboxanes (TXs) and hepoxilins (Smith, 1989, Kühn and Borngräber, 1998). PGs have a cyclopentane structure whereas TXs are characterised by an oxane ring; both are collectively named prostanoids (Slater and McDonald-Gibson, 1987).

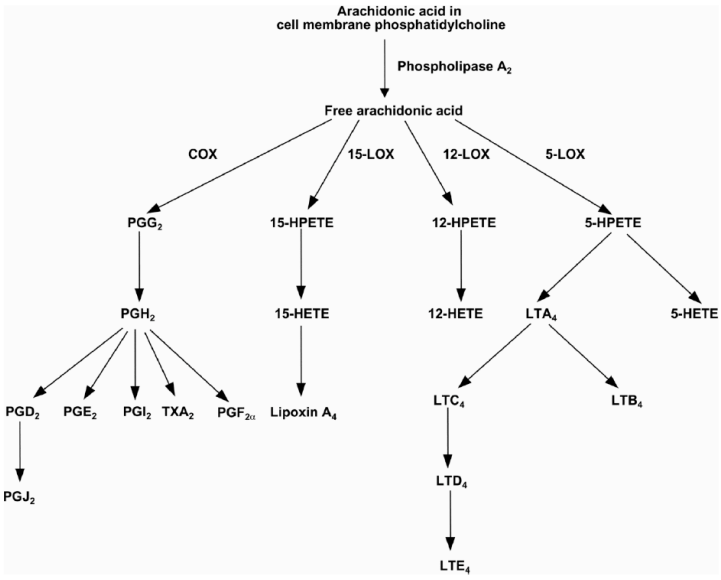

Figure 23: Simplified scheme of pathways of eicosanoid synthesis (Calder, 2005)

In vertebrates cyclooxygenase (COX) is the key enzyme for prostanoid synthesis (see

Figure 23), catalysing the conversion of arachidonic acid first to the intermediate PGG_2, which is subsequently peroxidised to PGH_2 (Belley and Chadee, 1995). This is then enzymatically converted to the various bioactive prostanoids (Smith, 1989).

In mammals there are 2 isoforms of COX: COX-I, which is expressed constitutively (synthesised in the absence of any particular stimulus) and is slightly upregulated by hormones, and COX-II, which is highly inducible (Belley and Chadee, 1995, Kühn and Borngräber, 1998). COX-I is a glycoprotein complex of two identical haem-containing sub units of 70 kDa and is a monotopic membrane protein found primarily in the endoplasmic reticulum of mammalian cells. In contrast, COX-II is believed to be a glycoprotein doublet containing several *N*-linked glycosylation sites with a molecular mass of up to 74 kDa (Belley and Chadee, 1995). *N*-linked glycosylation is required for maximum activity of both isoforms (Belley and Chadee, 1995). Belley and Chadee (1995) report that arthropod-derived COX acts primarily in a constitutive manner (COX-I), however, the discovery of COX-II in mammals has led to a renewed interest in the possibility that to whether parasites may produce a similar enzyme that can be used in pathogenesis and/or immunoregulation (Belley and Chadee, 1995).

Weinheimer and Spraggins (1969) were the first to discover eicosanoids in an invertebrate animal, the octocoral *Plexaura homomella*. Since that time eicosanoid synthesis has been demonstrated to occur in other invertebrate species (Daugschies and Joachim, 2000), playing important roles in ion transport, neurobiology and reproduction (Smith, 1989, Stanley-Samuelson, 1991, De Petrocellis and Di Marzo, 1994, Stanley and Miller, 1998, Ogg and Stanley-Samuelson, 1992). Whilst mammalian eicosanoids are synthesised *de novo*, it is unknown whether this is true for invertebrates. De Petrocellis and Di Marzo (1994) reported that non-parasitic invertebrates are able to store eicosanoids as lipoxin

derivates in certain tissues.

Prostaglandins are very important in the biology of invertebrate animals, regulating events within tissues and cells (Stanley-Samuelson, 1994b). The presence and significance of eicosanoids in invertebrates has been presented from various perspectives in several reviews (Brady, 1983; 1985; Stanley-Samuelson and Loher, 1986; Stanley-Samuelson, 1987; 1993; 1994a; 1994b, Sauer *et al.*, 1993; Lamacka and Sajbidor, 1995; Stanley-Samuelson and Pedibhotla, 1996; De Petrocellis and Di Marzo, 1994). Most of the work focusing on COX-II expression in parasites has concentrated on those parasites of public importance such as *Schistosoma mansoni* (Salafsky and Fusco, 1985; Fusco *et al.*, 1993; Salafsky *et al.*, 1984; Salafsky and Fusco, 1987b;a), *Entamoeba histolytica* (Belley and Chadee, 1995), *Trichobilharzia ocellata* (Nevhutalu *et al.*, 1993) and *Taenia taeniaeformis* (Leid and McConnell, 1983). Recently, however, more resources have gone into studying haematophagous arthropods such as ticks and the effects that PGs can have on their feeding.

The saliva of many tick species has been shown to contain a complex cocktail of pharmacologically active compounds such as immunosuppressants, analgesics, anticoagulants and anti-platelet aggregatory compounds that facilitate feeding (Bowman *et al.*, 1996). Normally a host's haemostatic processes would stop leakage from a blood vessel damaged through haematophagous parasite feeding. This would involve circulating platelets adhering to the damaged vessel wall, being activated and then aggregating to form a plug in the gap and to provide a scaffold for the coagulation process and fibrin clot. Most of the haematophagous arthropods studied to date, however, inhibit platelet aggregation by secreting the enzyme apyrase (Bowman *et al.*, 1996). Those arthropods that do not secrete apyrase instead secreted other anti-platelet aggregatory compounds such as PGI_2 and PGD_2

(Bowman *et al.*, 1996).

The PGs have been detected in the saliva of the cattle tick, *Boophilus microplus*, and the dog tick *Amblyomma americanum* by bioassay and chromatography techniques (Stanley-Samuelson, 1994a) and in the saliva of *L. salmonis* (Fast *et al.*, 2004). There are reports of PGs in other tick tissues; however, the physiological roles of these compounds remains to be elucidated (Stanley-Samuelson, 1994a). Kemp and Bourne (1980) report that histamine causes ticks to detach from their host but other mediators such as bradykinin, PGE_2 and 5-HT (serotonin) have no behavioural effect. The authors conclude that PGs alter the behaviour of the tick to increase the likelihood of finding a suitable host.

The prostaglandins PGE_2 and PGI_2, and to a lesser extent PGD_2, are potent vasodilators that cause dilation of the vascular smooth muscle thereby increasing blood flow (Bowman *et al.*, 1996). In general PGs are able to induce vasodilation without increasing plasma leakage and the associated pain by preventing mast cell degranulation. Furthermore, potent vasoconstriction peptides that are released by the vascular endothelium in response to mechanical injury, shear, stretch, turbulent flow or inflammatory mediators at the site of insult are countered by PGs and in particular PGE_2. The saliva of the tick *Ixodes dammini* (*Ixodes scapularis*) contains substantial quantities of 6-keto-PGF1α, the stable degradation product of PGI_2 (Ribeiro *et al.*, 1988). Fezza *et al*. (2003) report that PGE_2 within the salivary gland of ixodid ticks can act in either an autocrine or paracrine manner through its interactions with PGE_2 receptors. This induces exocytosis (secretion) of bioactive proteins.

Despite prolonged and continuous attachment of many haematophagous arthropods natural hosts mount immune responses that are ineffective (Bowman *et al.*, 1996). Experimental evidence from Wikel (1996) has suggested that rather than evading the host's immune

system, haematophagous arthropods suppress it via components of their saliva. The salivary homogenates of several tick species have been shown to impair T-cell function, possibly due to a reduction in the production of cytokines that are vital for ontogeny of the immune response, including recruitment, activation and proliferation of immune cells and also the inflammatory response (Bowman *et al.*, 1996). Ribeiro *et al.* (1985) report the PGE_2 content of *I. dammini* saliva has an inhibitory effect on IL-2 production by T-cells. Further experiments by Inokuma *et al*. (1994) indicate that that PGE_2 content of *Boophilus microplus* saliva was responsible for the inhibition of T-cell proliferation. A conflicting report from Urioste *et al*. (1994), however, reported a similar T-cell suppression from *I. dammini* saliva which lacked PGE_2. This led the authors to conclude that salivary PGE_2 plays a minor role in the immunosuppressive activity of tick saliva. Bowman (1996) proposes that factors other than PGE_2 are potentially immunosuppressive but PGs may exert a limited immunosuppressive effect.

The PGs are intricately involved with pain and inflammation (Bowman *et al.*, 1996). It would be logical to assume that during feeding haematophagous arthropods cause pain to the host thereby increasing the likelihood that grooming will dislodge the parasite. Examination of the process of pain and inflammatory events, however, suggests that ticks not only render the pro-inflammatory properties of PGs ineffective but saliva-derived PGs exhibit anti-inflammatory actions at the feeding site (Bowman *et al.*, 1996).

Lepeophtheirus salmonis infections of susceptible hosts, e.g. Atlantic salmon, are notable in that no significant inflammatory response is elicited (Johnson and Albright, 1992a). However, a well-developed inflammatory response is associated with resistance to infection in coho salmon *(Oncorhynchus kisutch*) (Johnson and Albright, 1992a). Fast *et al*. (2004) and Johnson and Fast (2004) both propose that immunomodulation of the host in

the absence of high cortisol levels is responsible for the lack of response in Atlantic salmon. The identification of trypsin and PGE_2 in adult *L. salmonis* secretions by Fast *et al*. (2004, 2003) and Firth *et al*. (2000) have given validity to this hypothesis.

The aim of this study was to determine if secretions of *L. salmonis* chalimus stages contain the prostanoid PGE_2. This chapter describes the commercial enzyme-linked immunoassay (EIA) technique used to measure PGE_2 levels in *Lepeophtheirus salmonis*.

5.2 Materials and methods

Once the lice had developed to the first chalimus stage fish were euthanised and samples taken (see section 5.2.1) and analysed using a commercially available PGE_2 assay kit (see section 5.2.4)

5.2.1 Infection of fish

Twelve Atlantic salmon were infected following Sevatdal's 2001 method (see section 2.3). The lice were left to develop on the fish until they reached the chalimus I stage. At an ambient water temperature of 10 ºC this took 10 days. Following infection the fish were examined every 2 days for the presence of the parasite. The chalimus stages were found around the base of the pectoral, pelvic, anal and dorsal fins as well as on the tail (see Figure 24).

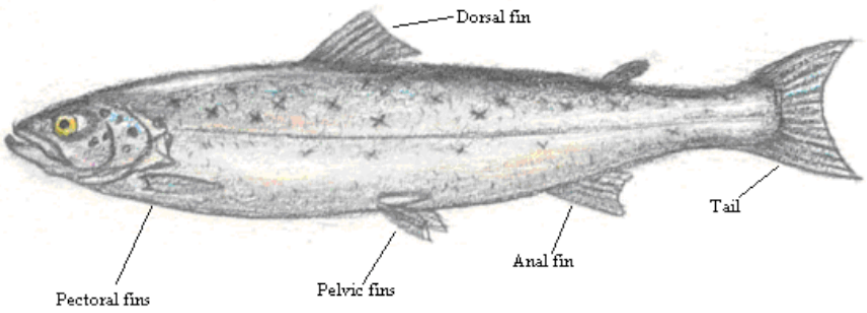

Figure 24: Sampling areas on salmon

5.2.1 Stimulation of PGE_2 in chalimus secretory products

The chalimus were stimulated to produce PGE_2 using a modified version of the protocol used by Fast *et al.* (2004). Briefly, fish were euthanised using MS-222-overdose once the lice were at the appropriate stage and placed under a dissecting microscope (Wyld). Discs of skin containing the attached lice were removed using a gauge 6 needle (Biomark) and transferred to sterile filtered (0.22 μm) seawater. Discs of skin that did not contain lice were taken as controls.

Once in the laboratory the discs containing lice were separated into groups of 5 and allocated to either a treatment or control group using a random number table (see Table 10). The groups were incubated in either 1 mL of 0.05 mM dopamine solution (Pers. Comm. Mark Fast; Sigma dissolved in 0.22 μm sterile-filtered seawater) or 1 mL sterile-filtered seawater for 60 minutes at 10 ºC.

Table 10: Treatment groups for lice from PGE_2 assay experiment, based on a predicted total of 105 discs of skin. Groups of 5 discs of skin were incubated in either 1 mL of 0.05 mM dopamine solution (DA) or 1 mL sterile-filtered seawater (C)

Group 1	DA	Group 7	C	Group 13	DA	Group 19	DA
Group 2	DA	Group 8	DA	Group 14	C	Group 20	DA
Group 3	DA	Group 9	DA	Group 15	DA	Group 21	DA
Group 4	DA	Group 10	C	Group 16	C		
Group 5	DA	Group 11	C	Group 17	C		
Group 6	C	Group 12	C	Group 18	DA		

5.2.2 Stabilisation and acidification of samples

Following incubation, tubes were centrifuged briefly to pellet the lice and the liquid phase harvested into fresh microcentrifuge tubes (Scientific Laboratory Supplies). To prevent metabolism or breakdown of the eicosanoids, 5% (50 μL) formic acid were added per millilitre to reduce the pH to approximately 3. One hundred and fifty microlitres of absolute ethanol was added to solubilise the eicosanoids and allow for easier extraction.

5.2.3 Solid phase extraction

In the laboratory samples were centrifuged for 5 minutes to pellet debris. The eicosanoids were then extracted as follows. A Sep-pak C18 cartridge was pre-washed with 5 mL methanol followed by 10 mL ddH_2O. The sample was loaded onto the cartridge and washed drip-wise with 10 mL ddH_2O followed by 5 mL 15% ethanol and then 5 mL hexane:chloroform (65:35 v/v). The eicosanoids were eluted into a stoppered test tube by pushing through 10 mL ethyl acetate, evaporated to dryness under nitrogen (Nevap) and re-suspended in 1 mL 100% methanol. Samples were stored at -20 ºC until assayed (see section 5.2.4). Used cartridges were regenerated by washing with 10 mL methanol and 10 mL ddH_2O.

5.2.4 PGE_2 assay

Prostaglandin E_2 concentration was measured using a commercially available kit following the manufacturer's instructions (Cayman Chemicals). The assay works on the principle that *in vivo* PGE_2 is rapidly converted into an inactive metabolite (13,14-dihydro-15-keto PGE_2) by the prostaglandin 15-dehydrogenase pathway (see Figure 25) (Hamberg and Samuelsson, 1971; Granström *et al.*, 1980).

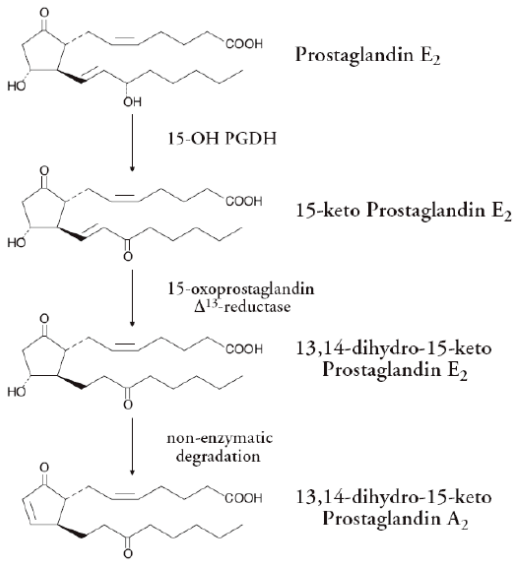

Figure 25: The metabolism of PGE_2 through the prostaglandin 15-dehydrogenase pathway

The plate layout for the ELISA is shown in Figure 26. Throughout the procedure different tips were used to pipette the buffer, standards, samples, tracers and antibodies. Furthermore, each pipette tip was equilibrated prior to use by slowly filling the tip and gently expelling the contents several times.

	1	2	3	4	5	6	7	8	9	10	11	12
A			ST7	ST7	S	S	S	S	S	S	S	S
B	NSB	NSB	ST8	ST8	S	S	S	S	S	S	S	S
C	ST1	ST1	S	S	S	S	S	S	S	S	S	S
D	ST2	ST2	S	S	S	S	S	S	S	S	S	S
E	ST3	ST3	S	S	S	S	BLK	BLK	S	S	S	S
F	ST4	ST4	S	S	S	S	S	S	S	S	S	S
G	ST5	ST5	S	S	S	S	S	S	S	S	S	
H	ST6	ST6	S	S	S	S	S	S	S		B_0	B_0

Figure 26: Prostaglandin E_2 assay plate layout. NSB = non-specific binding; ST = standard; S = sample; BLK = blank

Briefly, the contents of one vial of EIA Buffer Concentrate were diluted with 90 mL ddH_2O. Care was taken to rinse the vial to remove any salts that may have precipitated. The contents of a 5 mL vial of Wash Buffer Concentrate were diluted to a total volume of 2 litres with ddH_2O and then 1 mL Tween 20 added.

The contents of the PGE_2 standard were reconstituted with 1 mL EIA buffer (bulk standard) and stored at 4 ºC until used. To prepare the standard for use in EIA 8 clean test tubes were labelled 1 through 8 and 900 µL EIA buffer added to the first test tube and 500 µL each of the others. One hundred microlitres (100 µL) of the bulk standard was added to tube 1 and vortexed thoroughly. The standard was then serially diluted by transferring 500 µL from tube 1 to tube 2 and mixing thoroughly. This process was repeated for tubes 3 – 8.

The PGE_2 acetylcholine esterase (AChE) tracer was reconstituted with 6 mL EIA buffer to give 100 determinations (dtns). A red tracer dye was included at 1 part per 100 to aid visualisation of the tracercontaining wells. Similarly, 100 dtns PGE_2 antibody were reconstituted in 6 mL EIA buffer with a blue tracer dye added at a final dilution of 1:100 to aid visualisation of anti-serum containing wells.

One hundred microlitres of EIA buffer was added to each of the NSB wells and 50 µL added to the maximum binding wells (B0). Fifty microlitres from standard tube 8 were added to both of the lowest standard wells (S8) and then 50 µL from tube 7 added to each of the next two standard wells (S7). This was repeated until all the standards were aliquoted. Next, 5 µL of sample and 45 µL EIA buffer were added per well. Fifty microlitres of PGE_2 AChE tracer were added to each well except the blank (BLK) wells and finally 50 µL PGE_2 monoclonal antibody added to each well except the non-specific binding (NSB) and BLK wells. A quick reference pipetting summary is shown in Table 11.

Table 11: Quick reference pipetting summary for PGE_2 assay

Well	EIA Buffer	Standard/Sample	Tracer	Antibody
BLK	-	-	-	-
NSB	100 µL	-	50 µL	-
B_0	50 µL	-	50 µL	50 µL
Standard/Sample	-	50 µL	50 µL	50 µL

Once all reagents had been added the plate was covered with a plastic film lid and incubated for 18 hours at 4 ºC. When the plate was ready to develop 100 dtns Ellman's Reagent were reconstituted in 20 mL ddH_2O. The wells of the plate were emptied over a sink, and rinsed 5 times with wash buffer before adding 200 µL Ellman's Reagent to each well. The plate was re-covered with its plastic film lid, placed in a dark box and incubated at room temperature for 80 minutes on an orbital shaker. Once incubated the plate was read at 405 nm on a plate reader and the percent bound for each standard and sample was calculated using Equation 2:

Equation 2: Calculating percentage bound to non-bound in PGE_2 assay

$$\%B/B_0 = \frac{(\text{standard or sample OD} - \text{NSB OD})}{(B_o \text{ OD} - \text{NSB OD})} \times \frac{100}{1}$$

5.3 Results

The results from the EIA used to measure PGE_2 levels from salmon skins infected with lice incubated in dopamine show a wide spread of concentrations ranging from 411.883 $pg.mL^{-1}$ to 9085.962 $pg.mL^{-1}$. The mean concentration was 3023 ± 2987.417 $pg.mL^{-1}$. This was based on a total of 11 samples, each of 5 skins, after 2 anomalous results were discounted. The anomalous results were discounted as one was a negative value (-902.896 $pg.mL^{-1}$) and the second was almost fivefold the next highest concentration (45996.948 $pg.mL^{-1}$ compared to 9085.962 $pg.mL^{-1}$). Graphical representation of the results for these 11 samples and their mean is shown in Figure 27. The results from the EIA used to measure PGE_2 levels from salmon skins not infected with lice incubated in dopamine also show a

wide spread of concentrations ranging from 4275.590 $pg.mL^{-1}$ to 5783.053 $pg.mL^{-1}$. The mean concentration was 5090 ± 761.123 $pg.mL^{-1}$. This was based on a total of 3 samples, each of 5 skins, after 1 anomalous result was discounted. The result that was discounted was over 150 times the concentration of the next highest value (1025331.007 $pg.mL^{-1}$ compared to 5783.052 $pg.mL^{-1}$). Graphical representation of the results for these 3 samples and their mean is shown in Figure 28. Comparing the mean results from both assays suggest that the presence of *L. salmonis* chalimus on the skin reduces the production of PGE_2.

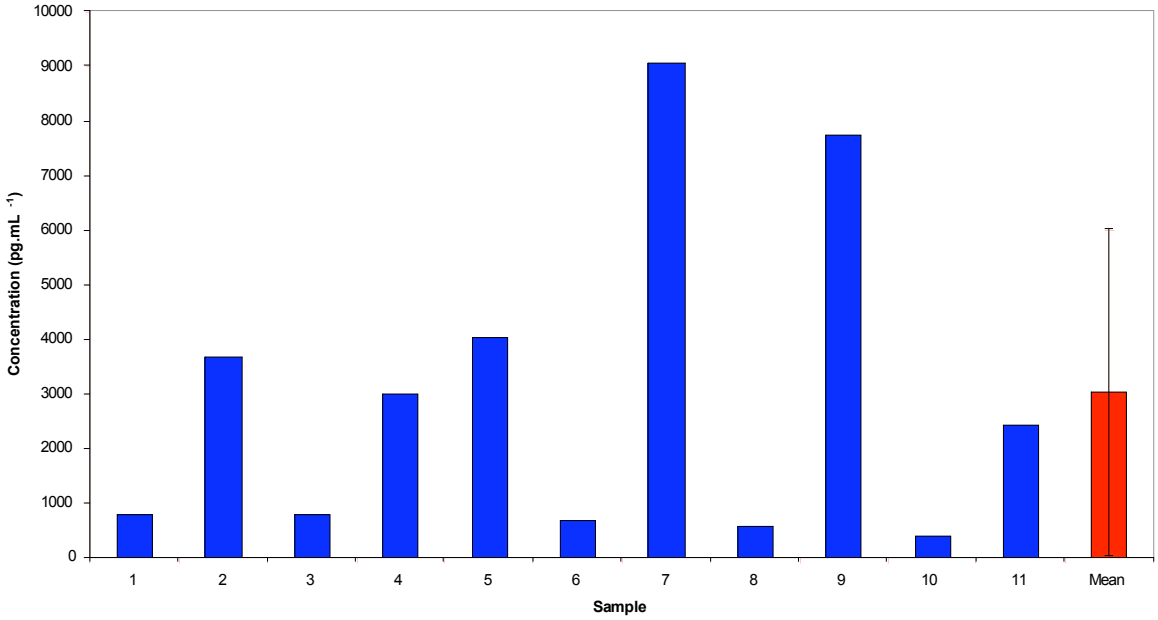

Figure 27: Concentration of PGE_2 from 11 groups of 5 skins, each with lice attached, after incubation for 60 minutes at 10 °C in 0.05 mM dopamine. There is a wide range of concentrations ranging from 411.8 $pg.mL^{-1}$. The mean concentration across all samples taken was 3023 ± 2987.4 $pg.mL^{-1}$.

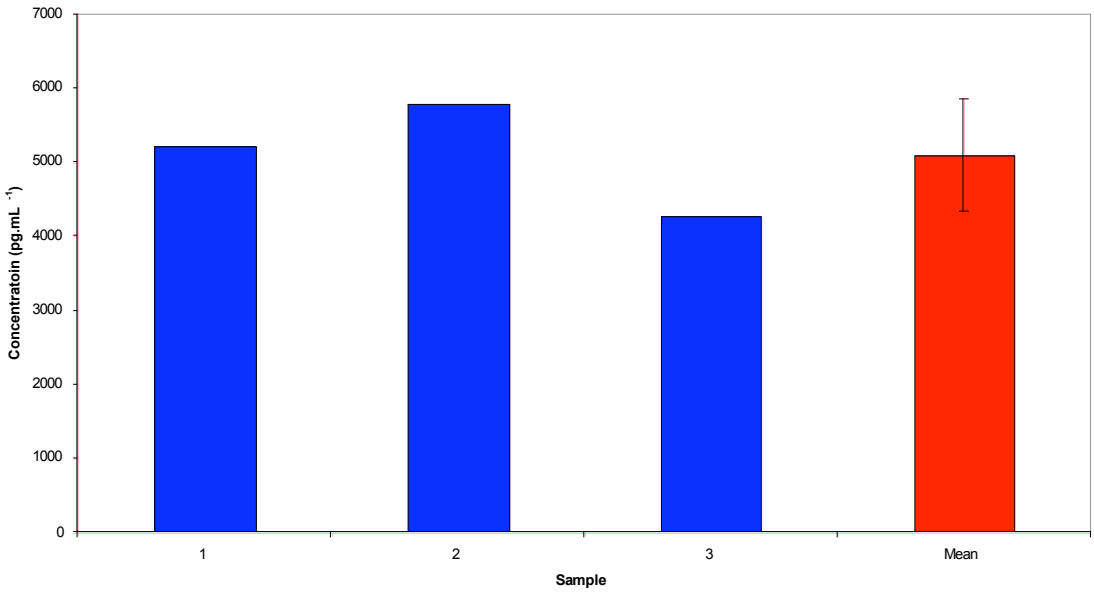

Figure 28: Concentration of PGE_2 from 3 groups of 5 skins, each without lice attached, after incubation for 60 minutes at 10 °C in 0.05 mM dopamine. The concentrations range from 4275.6 to 5783.1 $pg.mL^{-1}$. The mean concentration across all samples taken was 5090 ± 761.1 $pg.mL^{-1}$.

The results from the EIA used to measure PGE_2 levels from salmon skins infected with lice incubated in sterile-filtered seawater show a wide spread of concentrations ranging from 692.948 $pg.mL^{-1}$ to 2988.791 $pg.mL^{-1}$. The mean concentration was 1419.946 ± 972.592 $pg.mL^{-1}$. This was based on a total of 5 samples, each of 5 skins, after 3 anomalous results were discounted. The three discounted values ranged from between 5 to 12 times the concentration of the other samples. Graphical representation of the results for these 5 samples and their mean shown in Figure 29. Likewise, the results from the EIA used to measure PGE_2 levels from salmon skins not infected with lice incubated in sterile-filtered seawater show a wide spread of concentrations ranging from 4345.105 $pg.mL^{-1}$ to 7434.833 $pg.mL^{-1}$. The mean concentration was 4543 ± 1453.959 $pg.mL^{-1}$. This was based on a total of 4 samples, each of 5 skins. Graphical representation of the results for the samples and their mean is shown in Figure 30.

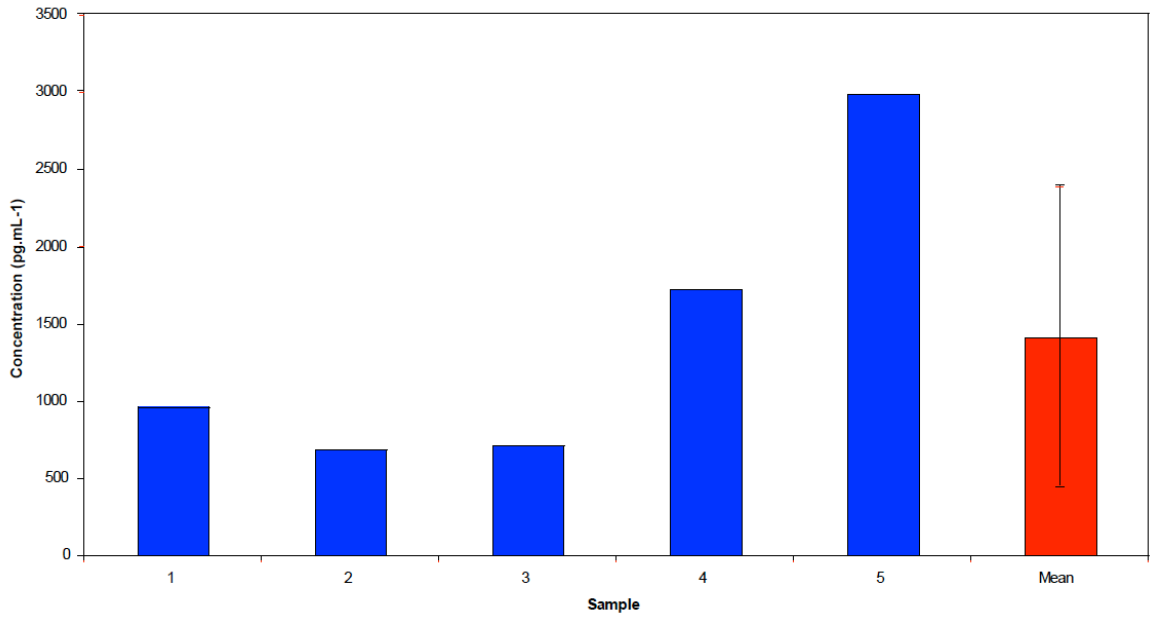

Figure 29: Concentration of PGE_2 from 5 groups of 5 skins, each with lice attached, after incubation for 60 minutes at 10 °C in 0.22 µm sterile-filtered seawater. The concentrations range from 692.948 $pg.mL^{-1}$ to 2988.791 $pg.mL^{-1}$. The mean concentration across all samples taken was 1419.946 ± 972.592 $pg.mL^{-1}$.

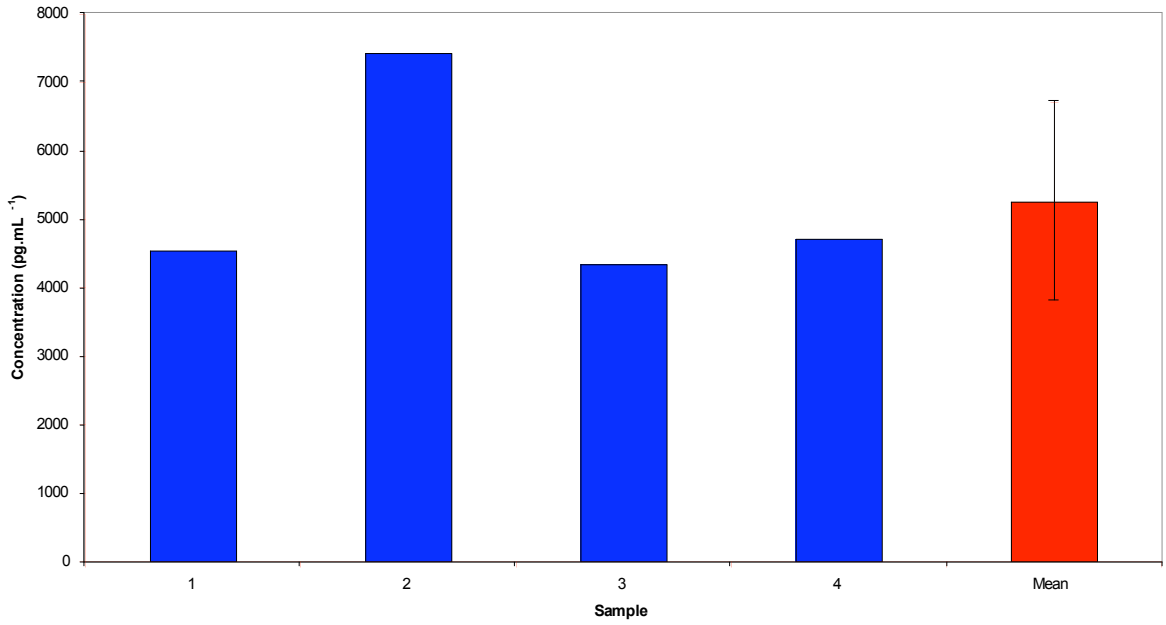

Figure 30: Concentration of PGE_2 from 4 groups of 5 skins each without lice attached, after incubation for 60 minutes at 10 °C in 0.22 µm sterile-filtered seawater. The concentrations range from 4345.1 to 7434.8 $pg.mL^{-1}$. The mean concentration across all samples taken was 4543.105 ± 1453.959 $pg.mL^{-1}$.

The results from the EIA used to measure PGE_2 levels from lice incubated in dopamine show a wide spread of concentrations ranging from 8.221 $pg.mL^{-1}$ to 19.373 $pg.mL^{-1}$. The mean concentration was 13.873 ± 5.578 $pg.mL^{-1}$. This was based on a total of 3 samples,

each of 5 lice, after 1 anomalous result was discounted for concentration levels that suggested contamination. Graphical representation of the results for these 3 samples and their mean is shown in Figure 31. The results from the EIA used to measure PGE_2 levels from lice incubated in sterile-filtered seawater show a spread of concentrations ranging from 8.525 $pg.mL^{-1}$ to 12.788 $pg.mL^{-1}$. The mean concentration was 10.657 ± 3.014 $pg.mL^{-1}$. This was based on a total of 2 samples, each of 5 lice, after 2 anomalous results were discounted due to concentrations ranging from 12 to 200 times the concentration of the other results. Graphical representation of the results for these 2 samples and their mean is shown in Figure 32.

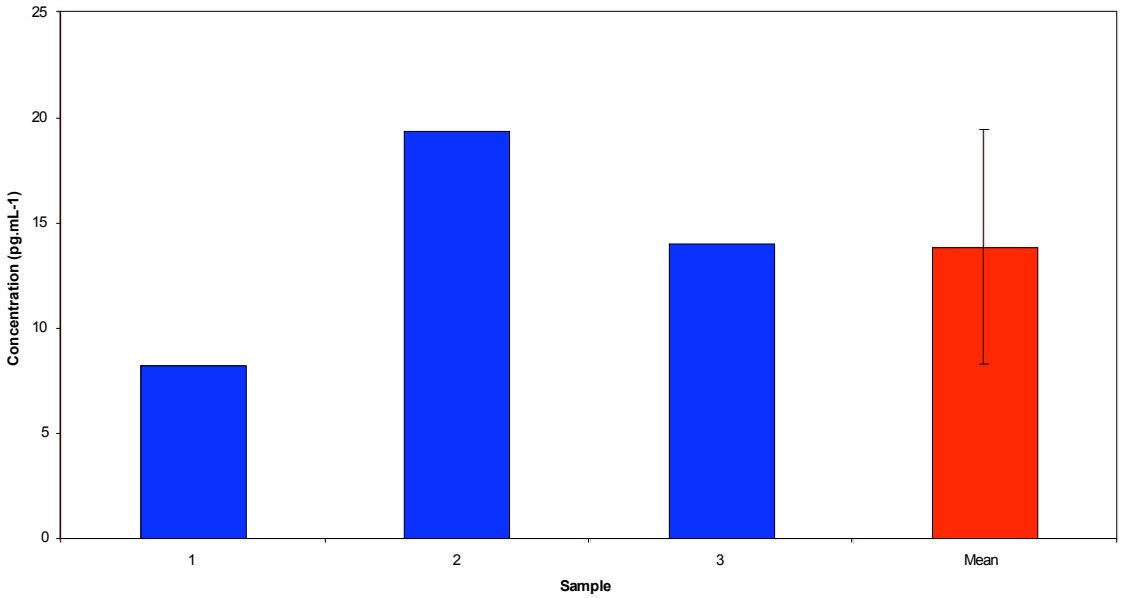

Figure 31: Concentration of PGE_2 from 3 groups of 5 lice, after incubation for 60 minutes at 10 °C in 0.05 mM dopamine. The concentrations range from 8.2 to 19.4 $pg.mL^{-1}$. The mean concentration across all samples taken was 13.9 ± 5.6 $pg.mL^{-1}$.

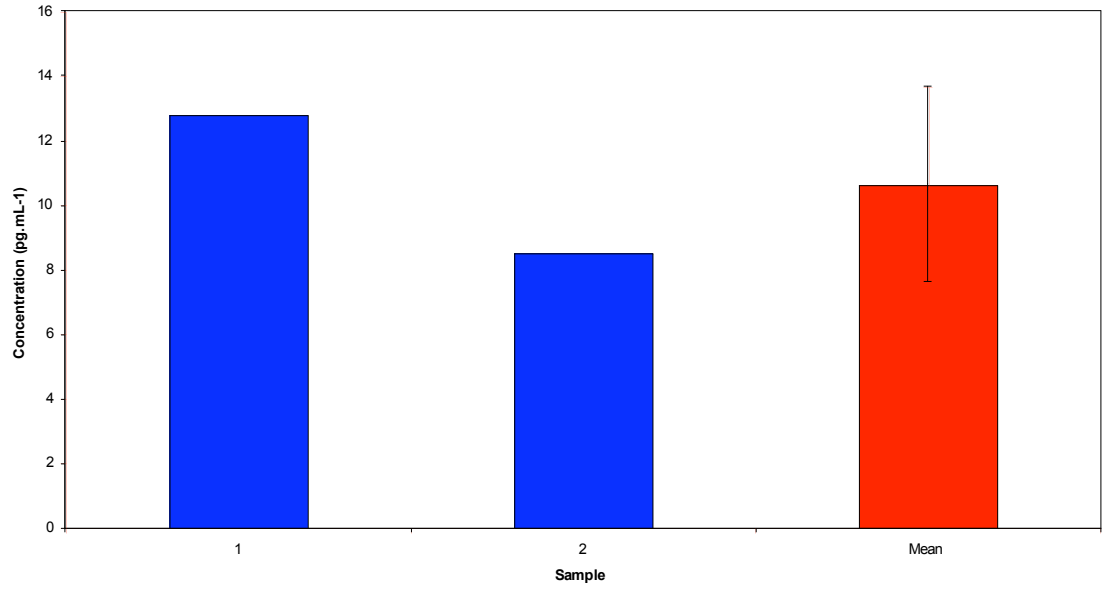

Figure 32: Concentration of PGE_2 from 2 groups of 5 lice, after incubation for 60 minutes at 10 °C in 0.22 µm sterile-filtered seawater. The concentration range from 8.5 to 12.7 $pg.mL^{-1}$. The mean concentration across all samples taken was 10.7 ± 3.0 $pg.mL^{-1}$.

A summary of all of the results from the EIA assay is shown in Table 12.

Table 12: Summary of results for all PGE_2 assays

Sample	PGE_2 Mean Concentration (± SD)
Skin with lice incubated in dopamine	3023.76 ± 2987.42 $pg.mL^{-1}$.louse
Skin without lice incubated in dopamine	5090.44 ± 761.13 $pg.mL^{-1}$
Skin with lice incubated in seawater	1419.95 ± 972.59 $pg.mL^{-1}$.louse
Skin without lice incubated in seawater	4543.95 ± 1453.96 $pg.mL^{-1}$
Lice incubated in dopamine	13.873 ± 5.578 $pg.mL^{-1}$.louse
Lice incubated in seawater	10.657 ± 3.014 $pg.mL^{-1}$.louse

5.4 Discussion

Prostaglandin E_2 (PGE_2) is an eicosanoid that is known to play a variety of roles in the feeding and avoidance of host immune responses in arthropod parasites. The present study has demonstrated that *L. salmonis* chalimus stages do indeed produce quantifiable levels of PGE_2.

Fast *et al*. (2004) confirmed the presence of PGE_2 in dopamine-induced secretions of *L.*

salmonis at concentrations in the same range as those in the saliva of several other arthropod parasites. The authors also recorded the absence of any other type of PG, which correlates with the results for most the saliva of most tick species (Aljamali *et al.*, 2002). During the present study, no type of PG other than PGE_2 was detected, which agrees with the findings of Aljamali *et al*. (2002) and Fast *et al.* (2004). However, it must be noted that EIA kits used in this study have a high specificity for PGE_2. Therefore, the likelihood of detecting non- PGE_2 prostaglandins was minimal.

This study also found a high level of variation in PGE_2 production by *L. salmonis* even within groups that had been off the host for the same amount of time. This concurs with the findings of Fast *et al*. (2004), who reported similar variation in adult lice. Aljamali *et al*. (2002) reported a fivefold variability in levels of prostaglandin in the saliva of *Amblyomma americanum* from the same population following dopamine stimulation. The authors also noted that prostaglandin production can also vary depending on the length of time since the most recent feeding. Based on the results of this study it is proposed that the variation seen in the levels of PGE_2 production of *L. salmonis* chalimus stages are the result of minor ontogenetic differences between the chalimus on the samples. Furthermore, as chalimus stages feed a minimal amount compared to the later developmental stages, this suggests that length of time since feeding can be discounted as a possible explanation.

Fast *et al*. (2005) demonstrated that PGE_2 at physiologically meaningful levels, and in the absence of a stress response, was able to inhibit expression of IL-1β, COX-II and MH class I and II genes and Pinge-Filho *et al*. (1999) reported PGE_2 down-regulates the pro-inflammatory cytokines IL-1β and TNFα. Furthermore, Fast *et al*. (2006) concluded that, as blood constitutes a (minor) component of the sea louse's diet (Brandal *et al.*, 1976; Bricknell *et al.*, 2003; Haji Hamid *et al.*, 1998) PGE_2 could be used by *L. salmonis* to

increase blood to the feeding site but also to prevent leukocyte recruitment and presentation of parasitic antigens to T lymphocytes. This concurs with the findings of Papadogiannakis *et al*. (1984), Papadogiannakis and Johnsen (1987) and To and Schrieber (1990) who all propose that PGE_2 may also adversely affect site-specific leukocyte recruitment and function. Fast *et al*. (2004) propose that the role of PGE_2 as a systemic modulator is unlikely, citing its high instability, which leads to it losing its activity following one passage through the circulatory system in mammalian models. The findings of Fast *et al.* (2004) and the findings of the present study may explain Jónsdóttir *et al*.'s 1992 and Johnson and Albright's 1992b findings that there is minimal tissue response in Atlantic salmon to *L. salmonis* beneath the site of active feeding and attachment yet an inflammatory response in tissues surrounding the lesion.

Down-regulation of host inflammatory cytokines has been observed in several other host-parasite relationships such as *Rhipicephalus sanguineus* (Ferreira and Silva, 2001), ixodid ticks (Fuchsberger *et al.*, 1995), *Rhipicephalus appendiculatus* (Gwakiska *et al.*, 2001), *Ixodes ricinus* (Kopecky *et al.*, 1999) and *Ixodes scapularis* (Schoeler *et al.*, 1999; 2000). Ferreira and Silva (1998) report that the saliva from *Rhipicephalus sanguineus* ticks also impairs T cell proliferation and IFN-γ-induced macrophage microbial activity. Prostaglandin E_2 can cause polarisation towards a Th_2 lymphocyte response by downregulating pro-inflammatory cytokines (Betz and Fox, 1991), which has also been observed in other arthropod parasites hosts (Ramachandra and Wikel, 1992; Schoeler and Wikel, 2001) and can delay the clearance of secondary bacterial infections (Dalton and O'Neill, 2002).

It cannot be discounted that the production of host mucus may also be increased due to the presence of PGE_2 (Fast *et al.*, 2004). This is of particular importance in *L. salmonis*

infections as it forms part of their diet. Nettesheim and Bader (1996) and Tani *et al*. (2002) report that PGE_2 encourages mucin secretion from rat tracheal and gastric epithelial cells. Furthermore, Nolan *et al*. (1999) observed increased mucus production by Atlantic salmon skin epithelia following infection with low numbers of *L. salmonis* adults. However, the potential role of PGE_2 in this observation has yet to be elucidated.

In artificial infections in the laboratory, infection with high numbers of *L. salmonis* commonly results in host mortality at the moult from chalimus to pre-adult stage without the development of lesions (Bjorn and Finstad, 1997; Grimnes and Jakobsen, 1996; Ross *et al.*, 2000). Additionally, Fast *et al.* (2002) and Mustafa *et al.* (2000b) both report reduced macrophage function and increased susceptibility to secondary infection in infected fish without the presence of a cortisol response. PGE_2 or other *L. salmonis*-derived compounds may be responsible at least in part for these observations (Fast *et al.*, 2004). For example the sudden and high level of host mortalities reported at the moult from chalimus to the pre-adult stage has parallels with a toxic shock response. Whilst the role of PGE_2 in toxic shock is unknown, prostanoids release has been demonstrated to occur early in the course of shock. The inhibition of such a release has been shown to significantly increase survival in mammalian toxic shock models (Ball *et al.*, 1986; Lefer, 1983).

In conclusion there is a good body of evidence to support the hypothesis that *L. salmonis* immunomodulates its hosts by producing substances such as prostaglandin E_2. Fast *et al*. (2004) reported adult *L. salmonis* are capable of producing PGE_2, which they propose modulates the host's immune system. The present study has demonstrated that the chalimus stages of *L. salmonis* are also capable of producing PGE_2 and whilst its exact purpose has not been investigated, many of the observations reported during the early stages of *L. salmonis* can be attributed to PGE_2.

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Chapter 6
General conclusions

6.1 General conclusions

The aims of this study were to further our understanding of the immunomodulation of Atlantic salmon by the copepodid and chalimus stages of *Lepeophtheirus salmonis*, focusing on three specific areas: i) investigating the proteomic nature of copepodid secretory/excretory product, ii) examining the role of the chemokine interleukin-8 (IL-8) and iii) analysing the possible role of prostaglandin E_2 (PGE_2) in the settlement and early stages of *L. salmonis* infection.

The skin of fish acts as the first line of defence, protecting the animal against both the environment and infectious pathogens. Therefore, before an ectoparasite can establish itself on a host it must first disrupt the skin (Tosi, 2005). *L. salmonis* produce physical and enzymatic damage at the site of their attachment and feeding to which Atlantic salmon hosts appear not to mount an immune response (for a summary of the pathological effects of *L. salmonis* attachment and feeding see the review by Johnson & Fast, 2004). Several studies have suggested that various stages of the *L. salmonis* life cycle secrete and/or excrete immunomodulatory compounds that are at least in part responsible for the lack of the host response seen at their sites of attachment and feeding (Bell *et al.,* 2000, Fast *et al*., 2002, 2003, Johnson *et al*., 2002, Ross *et al*., 2000). The findings of the present research suggest that any such compounds are not proteinaceous in nature. However, further investigation is recommended of the FPLC fractions eluted at between 24.0 and 25.5 mL.

The present study also found that IL-8 expression levels in tissues change following infection with *L. salmonis*. Both the heart and spleen showed significant increases in IL-8 gene expression whilst the anterior (head) kidney, fins, liver and pyloric cæci showed no significant increase. As the spleen is one of the major secondary lymphoid organs in

Atlantic salmon it would not be unreasonable to have predicted this result. However, the author cannot find any record of the heart playing a similar role in Atlantic salmon. Gonzalez *et al*. (2007c) believe that molecules secreted by parasites could alter the expression of immune-related genes in fish, although the authors do concede that there is a dearth of current knowledge in this area. The first response of the innate immune system to infection or tissue damage is inflammation and it is characterised by an increased blood supply to the affected area. This is followed by the extravasation of leukocytes from capillaries into the affected tissue (Gonzalez *et al*., 2007a). It is proposed that molecules within the SEP of *L. salmonis* enter the circulatory system of the host and are transported through the heart and spleen, stimulating an increase in IL-8 gene expression. This would agree with the theories of Gonzalez *et al*. (2007a) who suggest that the presence of high numbers of leukocytes, most probably neutrophilic granulocytes, at sites of mechanically induced skin damage are directly related to the up-regulation of gene expression similar to those shown in this study. Based on this observation it is concluded that *L. salmonis* is capable of modulating the immune response of Atlantic salmon with regards to IL-8.

The full extent of how much modulation takes place remains unclear, however. Whilst it is possible to investigate the up-regulation of IL-8 gene expression, it is not currently possible to investigate if there is a concurrent down-regulation of IL-8 gene expression. Gonzalez *et al*. (2007c) hypothesise that down-regulation of immune-related genes could be related with host signals released after the disruption of epithelial cells caused by parasites and Hajnická *et al*. (2001) reported several ixodid tick species produce compounds with anti-IL-activity, which the authors propose may facilitate feeding. Furthermore, Gonzalez *et al*. (2007b) found, amongst other things, that there was a significant down-regulation in C-lection (CL) expression in tissues including the liver.

The authors propose that this pronounced down-regulation could be due to the fast migration of CL-expressing cells, probably neutrophilic granulocytes from the blood to the site of inflammation. Furthermore, the authors propose that the systemic down-regulation of the CL molecule is probably a control mechanism related to the acute phase response (a systemic reaction to tissue injury and/or infection).

In order to test the hypothesis that IL-8 expression levels are suppressed during *L. salmonis* infection it is necessary to have a positive sample – a compound that is known to suppress IL-8 production. At present this author cannot find a record of such a sample. Any future work in this area should investigate whether or not there is anti-IL-8 modulation of Atlantic salmon by *L. salmonis*. Further investigation should also be carried out as to the role and function of the heart in *L. salmonis* infections.

Determining the levels of prostaglandin E_2 produced by chalimus stages of *L. salmonis* demonstrated that quantifiable amounts were produced, albeit in highly variable in concentrations. This concurs with the findings of Fast *et al.* (2004) who reported similar findings in adult *L. salmonis*. There is a substantial body of evidence to support the hypothesis that *L. salmonis* modulates its hosts at the site of attachment and feeding. Fast *et al*. (2004) propose that immunomodulation is most likely achieved by compounds secreted by the parasite including PGE_2, trypsin and as yet unidentified substances.

Although the aims of this investigation have been met, an extensive list of further work has been noted throughout. The main findings can be summarised as follows:

- Any compounds secreted by the lice are unlikely to be proteinaceous in nature;

however, further investigation of FPLC eluent at 24 – 26 mL is recommended.

- *L. salmonis* are capable of modulating the immune response of Atlantic salmon with regards to IL-8, however, the full extent to which this is possible is not currently known.
- Chalimus *L. salmonis* are capable of producing quantifiable levels of prostaglandin E_2 and this can be further used to modulate the hosts' immune response at the site of attachment and feeding.

6.2 References for Chapter 6

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Appendices

Appendix 1 Solutions

Agarose sealing solution

Reagent	Quantity
SDS electrophoresis buffer	100 mL
Agarose	0.5 g
Bromophenol blue	200 μL of a 1% solution

Ammonium persulphate (APS, 10%)

Reagent	Quantity
Ammonium persulphate	0.1 g
ddH_2O	Make up to 1 mL

Electrophoresis separating gel (10%)

Reagent	Quantity
ddH_2O	4.1 mL
Tris buffer (1.5 M, pH 8.8)	2.5 mL
10% SDS	100 μL
30% acrylamide/bis solution	3.33 mL
10% APS	50 μL
TEMED	10 μL

Electrophoresis separating gel with substrate (12%)

Reagent	Quantity
ddH_2O	2.35 mL
Tris buffer (1.5 M, pH 8.8)	2.5 mL
10% SDS	100 μL
30 % acrylamide/bis solution	4.0 mL
Substrate solution	1 mL
10% APS	50 μL
TEMED	5 μL

*The substrate can be either gelatine, β-casein or haemoglobin

Electrophoresis stacking gel (4%)

Reagent	Quantity
ddH_2O	6.1 mL
Tris buffer (0.5 M, pH 6.8)	2.5 mL
10% SDS	100 μL
30% acrylamide/bis solution	1.3 mL
10% APS	50 μL
TEMED	10 μL

Gelatine solution

Reagent	Quantity
Gelatine	50 mg
ddH_2O	5 mL

RTQ-PCR Mystery Mix

Reagent	Quantity
×10 RT buffer	2.5 μL
Magnesium chloride	5.5 μL
DNTPs	5.0 μL
RNase inhibitor	0.5 μL
Multiscribe RT	0.625 μL

SDS (10%)

Reagent	Quantity
Sodium dodecyl sulphate (SDS)	5 g
ddH_2O	Make up to 50 mL

SDS electrophoresis buffer

Reagent	Quantity
Tris base	30.3 g
Glycine	144 g
SDS	10 g
ddH_2O	Make up to 10 L

SDS equilibration buffer (stock solution)

Reagent	Quantity
Tris buffer (1.5 M, pH 8.8)	10 mL
Urea	72.07 g
Glycerol	69 mL
SDS	4 g
Bromophenol blue	400 μL of a 1% solution
ddH_2O	Make up to 200 mL

Silver stain fixative solution

Reagent	Quantity
Ethanol	40 mL
Acetic acid	10 mL
ddH_2O	50 mL

Silver stain fixative/sensitisation solution

Reagent	Quantity
Gluteraldehyde	0.05%
Formalin	0.01%
Ethanol	40 mL
ddH_2O	60 mL

Silver stain sodium carbonate/formalin solution

Reagent	Quantity
Sodium carbonate	2.5 g
ddH_2O	100 mL
Formalin	0.04%
ddH_2O	100 mL

The sodium carbonate solution is make up separately from the formalin solution. Just before use the two solutions should be mixed together.

Tris buffer (0.5 M, pH 6.8)

Reagent	Quantity
Tris base	6 g
ddH_2O	80 mL
ddH_2O	Make up to 100 mL

pH to 6.8 with NaOH

Tris buffer (1.5 M, pH 8.8)

Reagent	Quantity
Tris base	27.23 g
ddH_2O	100 mL
ddH_2O	Make up to 150 mL

pH to 8.8 with HCl

Triton X-100 (10%)

Reagent	Quantity
Triton X-100	10 mL
ddH_2O	90 mL

Water saturated butanol

Reagent	Quantity
Butan-2-ol	100 mL
ddH_2O	100 mL

Allow to settle into 2 layers. Use from the top layer and add more butan-2-ol when necessary.

Zymography ×2 sample buffer

Reagent	Quantity
Tris buffer (0.5 M, pH 6.8)	1 mL
ddH_2O	400 μL
Glycerol	800 μL
10% SDS	1.6 mL
0.2% Bromophenol blue	200 μL

Zymography incubation buffer

Reagent	Quantity
Zymography wash buffer	25 mL
Magnesium chloride ($MgCl_2$, FW = 95.21)	0.12 g
Calcium chloride ($CaCl_2$, FW = 111.0)*	0.18 g

*$CaCl_2$ inhibits EDTA. Incubation buffer should be made without $MgCl_2$ for EDTA gels

Zymography wash buffer

Reagent	Quantity
50 mM Tris base, pH 7.5	0.61 g in 50 mL ddH_2O
ddH_2O	25 mL
10% Triton X-100	25 mL

Appendix 2 Fish species

Latin binomial	Common name
Anguilla Anguilla	European eel
Atheresthes stomias	Pacific arrowtooth flounder
Chimaera phantasma	Silver chimaera
Clupea harengus	Atlantic herring
Ctenolabrus rupestris	Goldsinny wrasse
Cyprinus carpio	Common carp
Danio rerio	Zebrafish
Dicentrarchus labrax	European sea bass
Helostoma temmincki	Kissing gourami
Ictalurus punctatus	Channel catfish
Lampetra fluviatilis	Lamprey
Leiostomus xanthurus	Spot croaker
Oncohynchus mykiss	Rainbow trout
Oryzias latipes	Japanese medaka (rice) fish
Paralichthys olivaceous	Japanese flounder
Pimephales promaelas	Fathead minnow
Salmo salar	Atlantic salmon
Salvelinus alpinus	Arctic charr
Sparus aurata	Gilthead sea bream
Trachinotus marginatus	Plata pompano
Triakis scyllia	Banded dogfish