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Comparative susceptibilities of salmonids to *Lepeophtheirus salmonis* infections: biochemical and physiological studies

A Thesis

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ABSTRACT

The objective of the present study was to investigate possible mechanisms behind the differential susceptibility of coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*) and Atlantic salmon (*Salmo salar*) to sea lice (*Lepeophtheirus salmonis*) infection. Baseline levels of a number of innate immune factors in fish mucus and plasma were examined to establish a foundation from which to examine immunological, biochemical and physiological changes associated with lice infection. Finally, the response of lice to mucus of different species was examined.

Rainbow trout exhibited the highest activities of baseline mucous lysozyme and proteases of all three species in seawater (SW). Seawater reared rainbow trout and Atlantic salmon had a higher ratio of serine to metalloproteases than coho salmon. Coho salmon also exhibited a high percentage of calcium-dependent proteases, which were not observed in the other two species. Interestingly, all three species exhibited higher protease and alkaline phosphatase activities in the mucus of SW reared fish as compared to those reared in fresh water (FW). Mucous lysozyme activity was higher in Atlantic and coho salmon reared in FW as compared to SW. Differences in innate factors of fish reared in FW versus SW may be an evolutionary adaptation related to the pathogens these fish encounter in differing environments (eg. lysozyme vs. bacteria in FW, proteases vs. parasites in SW).

For the sea lice challenge experiment, rainbow trout, coho and Atlantic salmon were cohabited to ensure equal sea lice infection levels. Despite the same

initial intensity of infection, a significant decrease in lice density over time was only observed on coho salmon. Lice development was slowest on coho salmon followed by rainbow trout and then Atlantic salmon. Both infected Atlantic salmon and rainbow trout exhibited increases in mucous lysozyme at 1 day post infection (dpi), possibly as a result of a combination of initial lice infection and crowding. Mucous lysozyme also increased in infected coho salmon at 21 dpi, possibly in response to the presence of pre-adult sea lice. Multiple bands of low molecular weight (LMW) proteases increased in infected rainbow trout (at 21 dpi) and Atlantic salmon (at 14, 21 dpi), possibly due to the increase in lice biomass over time. Increases in LMW proteases were accompanied with decreases in phagocytic capacity and respiratory burst of macrophages in both infected rainbow trout (at 21 dpi) and Atlantic salmon (at 14, 21 dpi). The rate of lice development, host immunosuppression and lice infection densities suggest that susceptibility towards lice infection follows the order Atlantic salmon > rainbow trout >> coho salmon. The lack of differences in blood physiological parameters (plasma cortisol, glucose, electrolyte, protein and hematocrit) between susceptible and resistant species and the lack of any response to lice infection suggests that a unique systemic response is not associated with the differences in susceptibility between these species. However, the differences in mucous enzyme activities between species suggest there may be biochemical factors in the mucus of coho salmon that are responsible for successful rejection of sea lice.

To determine the response of lice to the mucus of different species, live lice were incubated in the presence of fish skin mucus or seawater. Alkaline phosphatase levels increased in Atlantic salmon mucus and protease activities

increased in rainbow trout mucus following incubation with *L. salmonis*. Multiple bands of LMW proteases were secreted by lice more consistently in the presence of rainbow trout and Atlantic salmon mucus as compared to coho salmon mucus, winter flounder (*Pseudopleuronectes americanus*) mucus and seawater. The resistance of coho salmon to lice infection may be related to factors in the mucus that inhibit LMW protease secretions from the louse. Further work must be done to determine a factor in coho salmon mucus that blocks these secretions, or possibly, a factor in susceptible species that stimulates lice secretions.

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LIST OF ABBREVIATIONS

ACP	**Alternative complement pathway**
ACTH	**Adrenocorticotrophic hormone**
AP	**Alkaline phosphatase**
APS	**Ammonium persulfate**
AS	**Atlantic salmon**
CRH	**Corticotrophin releasing hormone**
CS	**Coho salmon**
DTT	**DL-Dithiothreitol**
DFO	**Department of Fisheries and Oceans**
dpi	**Days post infection**
EDTA	**Ethylenedinitrilo tetraacetic acid**
EGC	**Eosinophilic granule cells**
FCS	**Fetal calf serum**
FWR	**Freshwater-reared**
HPI-axis	**Hypothalamus-pituitary-interrenal cells**
IFN	**Interferon**
ISA	**Infectious salmon anemia**
LMW	**Low molecular weight**
MAC	**Membrane attack complex**
NBT	**Nitroblue tetrazolium**
OD	**Optical density**
PBS	**Phosphate buffered saline**
PMA	**Phorbol myristate acetate**
POMC	**Proopiomelanocortin**
RT	**Rainbow trout**
SDS-PAGE	**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**
SWR	**Seawater-reared**
TEMED	**Tetramethylethylenediamine**

LIST OF TABLES AND FIGURES

1.0 GENERAL INTRODUCTION

1.1 Introduction to salmon aquaculture

Salmon aquaculture is an extremely large industry on both the east and west coasts of Canada, as well as in countries such as Norway, Scotland, Chile and the United States. According to the Department of Fisheries and Oceans – Canadian Stock Assessment Secretariat, between 60,000 – 70,000 tonnes of salmonids (including trout and steelhead salmon) were produced from the aquaculture industry in Canada in 1998 [1]. The gross revenues of this production were worth nearly $ 0.5 billion [1] and the industry is still growing. Of Canada's total tonnage, approximately 80 % is made up of Atlantic salmon (*Salmo salar*), 10 % chinook salmon (*Oncorhynchus tshawytscha*), and the rest is made up of coho salmon (*O. kisutch*), steelhead salmon and rainbow trout (*O. mykiss*). The farming of salmon is mainly carried out at netpen grow out sites in the coastal waters off the Atlantic and Pacific coasts of Canada. At these sites, new smolts are stocked at anywhere from 5,000 to 15,000 fish per pen (15 m diameter or length X 12 m deep) [2]. Stocking densities are generally kept below maximum levels during smolt introduction in order to prevent death and disease as this is a stressful time for the fish. Stocking densities at cage sites are species dependent. Atlantic salmon can be stocked at densities from 15-30 kg/m^3, whereas species with more inter-fish aggression, such as chinook, are stocked at 4-8 kg/m^3 [2]. At high densities, relative to other species (15-30 kg/m^3), most

Atlantic salmon display schooling behaviour and individual territoriality is depressed [3].

Once brought onto feed in seawater, Atlantic salmon are easy to grow to a large size [3]. They can be handled with less scale loss, are more docile and less easily stressed under culture conditions than other salmonid species. Atlantic salmon also maintain a higher quality carcass when taken to market, in comparison to chinook [3]. However, despite the culture success of Atlantic salmon there are still available markets for other cultured salmonids such as rainbow trout, chinook and coho salmon. Each species has its own positive and negative aspects for farming. Coho and chinook salmon require a shorter freshwater rearing period, but have a high risk of early sexual maturation compared to other species [2]. Conversely, rainbow trout are a hardy species with well understood biology and low risk of early maturation. The reason why early maturation is a problem is that once fish mature they begin to allocate more energy towards reproduction and the quality of their meat decreases [3]. Hence, age and size of maturity are some of the important factors in salmonid culture. As well, site conditions, species' disease resistance and species' market stability all have effects on the profitability of a cage site operation.

Selection of quality species and strains is only the beginning of the culture process. Proper production strategies ensure that the fish selected for culture are grown at minimal costs for maximum profits. A proper understanding of the biology of each species is required to best manage aquaculture operations.

1.2 Salmon biology and aquaculture production

Salmon aquaculture production is closely linked to the natural life cycle of the cultured fish. Most salmonids, and more specifically the ones grown in the culture industry, are anadromous fishes, which means they are born in fresh water, migrate to sea where they spend most of their life, before returning to spawn in the same rivers they were hatched. Unlike many salmonids, such as chinook, coho, sockeye (*O. nerka*), and pink salmon (*O. gorbuscha*), Atlantic salmon and rainbow trout are repeat spawners and do not die shortly after their first spawn [2].

Anadromous salmonids spawn between summer and fall producing millions of eggs, which hatch into alevin the following spring [2]. Depending on the species of fish and their individual growth rates, anadromous salmonids may migrate to seawater prior to their first winter after hatching [2]. These fish are known as S0 salmon, while those that spend their first winter after hatching in freshwater are known as S1 salmon. Most coho and chinook salmon will undergo their seawater migration as S0 salmon (5-25 g), much smaller than Atlantic salmon and steelhead that usually undergo the migration as S1 salmon (50-65 g) [2]. The fish that do not reach the respective size ranges needed for seawater migration remain in fresh water for another year [3]. Therefore, some chinook and coho salmon migrate to sea as S1 salmon and some Atlantic salmon and steelhead migrate to sea as S2 salmon (migrate after second winter). Size is an important pre-selection for seawater life as larger smolts are better swimmers and have less trouble adapting to the changing osmoregulatory environment [2].

To ready themselves for life at sea, most salmonids undergo a process known as smoltification. Smoltification occurs prior to saltwater introduction and is an evolutionary process that prepares fish for a saltwater existence and makes the transition from fresh water to seawater much easier [2,3]. Smoltification involves several physiological changes in the fish such as decreased prolactin and increased growth hormone and cortisol levels [4,5]. Smoltification also involves behavioural and morphological changes such as the fish swimming downstream, losing their parr markings and becoming more streamlined [2]. Seawater adapted rainbow trout, however, are not true anadromous salmon like their steelhead cousins and do not undergo the smoltification process [3]. Seawater adapted rainbow trout react in response to increasing salinity rather than prepare themselves in advance for these changes.

Anadromous salmonids will stay in the sea for 1.5 - 3 years until mature (species-dependent) and then return to fresh water to spawn, with the exception of grilse (early maturing salmon), which mature and return to fresh water to spawn earlier than the rest of their cohorts [2].

In aquaculture, fish are grown in a freshwater hatchery until they have smolted and are then transferred to seawater grow out sites. Through manipulations of temperature and photoperiod at the hatchery [6] and netpen feeding regime to maximize growth rates [2], salmonids take less time to reach market size in culture than in the wild.

Similar to all farming, incidence of disease is a concern at every level of production in aquaculture. To combat this, there are several health management

measures at all hatchery and grow out sites. Fish vaccination and antibiotic treatments are used to prevent and treat diseased fish in fresh and saltwater [7-9]. Separating different year classes of fish and allowing diseased sites to fallow for 1-3 months before re-stocking are other management techniques all used to reduce the risk of disease outbreaks [7,8].

As fish farming expands throughout Canada and the world, concern for its effects on natural ecosystems increases [10,11]. More recently the concern for natural ecosystems has focussed on the prevalence of disease in farming sites and implications for dwindling wild fish populations. Epizootic infections of sea lice, *Lepeophtheirus salmonis*, a common marine ectoparasitic copepod of salmonids, have resulted in heavy losses to sea-farmed trout in the past [12,13] and have more recently cost millions of dollars worth of damage to aquaculture in the waters of Canada, Norway and the United States. Although *L. salmonis* is not found in great abundance in wild salmonid populations [14-24], it has been suggested that fish farms act as a vector for louse infections of wild populations, and proximity of a wild population to farmed sites can be correlated to the degree of infection [25]. Whether or not *L. salmonis* is a factor in the decline of wild populations, it remains a constant environmental concern on both sides of the Atlantic and Pacific Oceans.

1.3 Sea Lice

The salmon louse, *L. salmonis*, is a saltwater ectoparasitic copepod of fish of the genera *Salmo*, *Oncorhynchus* and *Salvelinus* [26,27]. The life cycle of *L. salmonis* consists of 2 free-swimming nauplius stages, 1 free swimming infective copepodid stage, 4 attached chalimus stages, 2 mobile pre-adult stages and 1 mobile adult stage [28,29]. The time from hatching to the infective copepodid stage can take anywhere from 1 – 2 wks (usually 10-14 days) depending on temperature and salinity. High temperatures allow faster development [28]. The naupliar and copepodid stages are planktonic. Infective copepodids remain free-swimming until they find a salmonid host. Initial attachment to the host involves the use of the second antennae and maxillipeds [29]. It is not known exactly how copepodids find their hosts but it is hypothesized that this is done though a combination of mechano- and chemoreception and vision (S. Johnson, personal communication) [30]. The louse holds onto the salmon by the second antennae and maxillipeds for 4 -5 days (10^0C) until it produces a frontal filament by which it adheres itself to the host and moults to the chalimus I stage [12,13,24,28,29,31]. The frontal filament is a secreted matrix of unknown composition that forms a flexible attachment between the louse and the fish. On the host, sea lice larvae are distributed mainly around the areas of low vascularization [13,30]. The nauplius and free-swimming copepodids are non-feeding stages, but after attachment, *L. salmonis* feeds on host skin, mucus and blood [32]. Sea lice are only able to move in a circular motion around the frontal filament attachment site during the chalimus stages. During moults between chalimus stages, however, the lice are temporarily

able to move across the host before re-attaching with the frontal filament. After moulting to a pre-adult, the frontal filament is detached and the louse is able to move freely across the surface of its host [28,29,31,32]. Free-moving pre-adults and adults can be found anywhere on the fish surface but mainly congregate near the anus, behind the dorsal fin and on the head [12,13].

The feeding activity of *L. salmonis* at more mature stages can be detrimental to the host, causing noticeable lesions and in extreme cases causing exposure of the cranial cavity, osmoregulatory failure and death [12,13,33-39]. *Lepeophtheirus salmonis* breaks down the physical barrier of mucus and epidermal cell layers causing diffusion of ions and loss of plasma and proteins, thus disrupting the host's ability to maintain a homeostatic internal environment. Not only does lice feeding result in osmoregulatory problems, but the breakdown of the physical barrier between the fish and its external environment may contribute to secondary consequences, such as bacterial, parasitic and/or viral and fungi occurring infections [12,13,33-39]. Low level *L. salmonis* infection can also predispose fish to other parasitic infections [40]. A decrease in immune function and a significant increase in the abundance of *Loma salmonae* lesions was observed in rainbow trout, previously infected with *L. salmonis*, compared to naïve rainbow trout [40].

1.4 Current techniques in controlling sea lice

To deal with salmon lice epizootics, the fish farming industry uses mass treatments at caged sites. Chemotherapeutants such as organophosphates

(dichlorvos and azamethiphos), teflubenzuron, pyrethroids, avermectins and hydrogen peroxide [7,41] have been used in Norway and Scotland where *L. salmonis* has been a serious problem over the last decade. Treatments, specifically organophosphates and hydrogen peroxide, while toxic to the sea lice, are also quite toxic to fish [42-44]. Fish hyperactivity is usually observed during treatments and high mortality rates can result if treatment indices are not followed properly [42] or if compounds do not dissolve evenly throughout the bath [45]. Severely immunocompromised fish may have higher mortality rates following treatment than if treatment is avoided. Potential damage to local biota is also a major concern following treatment. Teflubenzuron is a chitin-synthesis inhibitor that is effective against all moulting stages of sea lice, but also has high tissue residence times and may be harmful to local crustacean populations. Ivermectin has been shown experimentally to bio-accumulate in the tissues of bivalves following 6-day exposure [47-48]. These problems, coupled with the fact that most treatments other than chitin-synthesis inhibitors and avermectins [49] are ineffective against early life stages of lice (copepodids and chalimus I -IV), make current treatment methods somewhat inadequate.

Hydrogen peroxide, azamethiphos, dichlovos and pyrethroids are administered in a bath in which the fish are crowded together in a reduced amount of water closed off from the outside water flow by a tarp and kept for ca. 30 min with supplemental oxygen [3,7,8]. Upon completion of the bath, the tarp is removed and the contents of the bath are released to the local environment. It is thought that release of organophosphates into the surrounding aquatic environment can endanger indigenous sea life [8,11]. Bath treatments do not just have environmental implications but are also

very labour and cost intensive. Since these compounds are only effective against the mobile life stages and given the short time over which it is effective, it can become necessary to treat the fish repeatedly over a short period. Oral treatments of avermectins and chitin-synthesis inhibitors have a much higher bioavailability and protect the fish for a longer duration having increased tissue residence times [46,48].

All of the compounds mentioned above have their pros and cons. These depend on the life stage of the fish, how long before it is brought to market and how often a farmer can afford to treat. Organophosphates can be deleterious to humans yet they are eliminated relatively rapidly from the tissues of fish when administered via bath treatments. Oral treatments however (avermectins and chitin-sythesis inhibitors), remain in the fish tissues longer and farmers have to allow for enough time for the compounds to be eliminated from the fish body before harvesting to meet withdrawal times established by regulatory authorities [2,46,48].

Aside from chemical treatment, salmon farm management has enlisted strategies such as site fallowing and use of a single year class [7,8]. Fallowing involves leaving a site without fish for up to 2-3 months to allow resident lice populations to die off before re-stocking the cages with new fish. This can be very effective in the first year after re-stocking, but the lice eventually return. Keeping year classes separate is another technique to prevent lice and other diseases spreading to new arrivals (smolts) from older fish at the same location. Both of these strategies can lower the number of lice in a given area; however they depend on the cooperation of all farmers within the area. In small bays filled with different fish farmers it can be difficult for everyone to come to agreement on fallowing and year class strategies.

The Bay of Fundy, for example, has many small bays in which there are several cage operations with many different owners. In this case a unified management agreement which is followed to the letter by all operators is difficult to enforce.

With the emergence of vaccination as an effective technique to reduce disease outbreaks in aquaculture [9], there has been research into vaccines against sea lice. Attempts have been made to innoculate susceptible salmonid species against sea lice using crude extracts of *L. salmonis* to elicit an antibody response [49]. However, experimental vaccines have, as yet, to show any protection against sea lice.

1.5 Stress in fish

When the internal homeostasis of a fish is threatened by external stimuli, the fish is termed under 'stress' [50]. Like all living things, fish must be able to adjust to changes in their everyday environment and have developed mechanisms for dealing with stress. Stress can result from daily fluctuations in water temperature, salinity, dissolved oxygen and photoperiod [51-58] or develop from complex interactions with pathogens such as *Aeromonas salmonicida,* the causative agent of furunculosis, or *L. salmonis*. For farmed fish, stressors can also include sorting, weighing, handling, tagging and transportation.

Fish have an integrated stress response in which the adrenergic response complements the hypothalamic-pituitary-interrenal (HPI-axis) response releasing corticosteroids [59]. Cortisol and epinephrine are released in an attempt to re-establish homeostasis [60] through their affects on hydromineral balance, increasing oxygen uptake, circulating glucose, cardiac output and gill blood flow [61,62]. While it

may at first appear that the adrenergic and HPI-axis pathways are distinct, they can interact with one another. Administration of adrenaline in fish has been shown to induce plasma cortisol increases and administration of cortisol has been linked to increased sensitivity of ß -adrenergic signal transduction in red blood cells and hepatocytes [59]. There are many peripheral factors that will also affect the stress response. These may include but are not limited to, species and individual fish differences, and pre-existing environmental conditions such as time of day, water temperature and pH.

The adrenergic response may act independently or in concert with the HPI-axis response. Observing the adrenergic response, through the measurement of catecholamines is difficult since they have a very short half-life in the blood. Cortisol, however, remains in the system much longer and since it is much easier and more accurate to measure it is commonly used in the laboratory to assess the stress response in fish [59]. For this reason, and previous work showing cortisol's immunosuppressive activities [63], we chose to assess the HPI-axis rather than the adrenergic response in this study.

The HPI-axis response can be summed up as a cascade of hormones [64]. External stressors stimulating the hypothalamus result in the release of corticotrophin-releasing hormone (CRH). The main role of CRH is in triggering the pituitary gland to release adrenocorticotrophic hormone (ACTH) [59]. As seen in mammals, other neuropeptides can interact with CRH affecting the secretion of ACTH, such as vasopressin [59]. Other peptides also interact with CRH in controlling ACTH release; however these relationships are unclear. Adrenocorticotrophic hormone is cleaved

from proopiomelanocortin (POMC), along with ß-endorphin and melanotrophins [59]. Although the roles played by ß-endorphin and melanotrophins are unclear in the stress response, it has been suggested that, under some forms of stress in tilapia, they may act with ACTH to co-ordinate cortisol release [65]. A rapid release of ACTH within 3-4 min of exposure to acute stress stimulates the release of cortisol [59]. Unrelated peptides such as angiotensin and urotensin I and II can also directly stimulate cortisol release from interrenal tissue but are not considered part of the primary pathway [59].

The stress response can have different effects on an individual depending on the type of stressor. Stressors are either acute or chronic. Acute stressors are those that occur for a brief period of time, such as removing a fish from water for 30 sec. Chronic stressors cause low levels of stress on the fish, but this occurs continuously over time. Chronic stressors would include fish living at sub-optimal pH or temperature for an extended period of time (i.e. weeks) [62]. These types of stress can have different overall effects on the stress response. After exposure to an acute stressor, plasma cortisol levels rise very quickly (ie. within 5-8 min). These cortisol levels may return to normal resting levels in the plasma within 1-2 h as cortisol is rapidly metabolized by the liver and then excreted [66]. However, under chronic stress conditions, cortisol levels may remain elevated although below peak levels. It can take weeks to months for the cortisol levels to return to normal. Under these conditions cortisol levels may not decrease to previous normal levels but result in a higher resting level of cortisol in the blood [66].

Once cortisol is released it has short-term beneficial effects on carbohydrate metabolism and hydromineral balance. Unfortunately, chronic stress and its associated maintenance of high cortisol levels depresses growth, reproduction and immune functions [64]. Growth is depressed through the allocation of energy to other sources, reducing the energy available for somatic growth [67]. Depressed gonadal steroid levels in the plasma also occurs due to chronic stress resulting in ovarian atresia [67]. As well, immune function is decreased through a reduction in the numbers and functions of lymphocytes and thus limits the fish's ability to mount an immune response [68,69].

Chronically elevated levels of cortisol can also dull the ACTH response when the fish are acutely stressed [70,71]. This can also have an inhibitory effect on interrenal cells of the head kidney, which prevents the secretion of additional cortisol [70]. Persistently elevated cortisol levels can therefore dampen the further production of cortisol in stress situations.

The release and flux of cortisol in the manner described above and the immediate release of adrenaline through the sympathetic nervous system is part of the primary response to stressful stimuli. The primary response, in turn, causes blood and tissue alterations, which are termed the secondary response. An individual with chronically elevated cortisol can become hyperglycemic through cortisol-stimulated gluconeogenesis, utilizing proteins and lipids as substrates. Therefore, elevated cortisol levels can result in elevated blood glucose levels [67]. In freshwater fishes, chronic and acute stressors can cause hemodilution of ions (sodium, chloride, potassium) through the osmotic influx of water [72]. Adrenaline, for example, enhances

gill circulation and gas exchange which augments water uptake, resulting in ion loss through amplified urine production [72]. In saltwater fishes the opposite occurs and hemoconcentration of ions is the problem [72,73]. Impaired water balance not only results in high ion concentrations in saltwater fish, but high hematocrit and blood protein concentration as well.

1.6 Fish immune system

The immune response of all vertebrates consists of two general systems. The first is non-specific immunity, also known as innate immunity, in which preformed or induced molecules or mechanisms eliminate pathogens in a nonspecific fashion [74]. Induced mechanisms in the non-specific response are carried out by phagocytic cells. The second system of the immune response is known as the specific immune response and involves lymphocytes [74]. This response is induced, specific, and can be referred to as acquired immunity. Specific or acquired immunity requires a very complex network of cells and proteins that allows its host to respond specifically to antigens from many potential pathogens. The host response is primarily carried out using immunoglobulins [75]. Despite the usefulness of acquired immunity, immunoglobulins in fish show less diversity of type and a longer delay in response time than in "higher" vertebrates [76]. Fish, therefore, depend more heavily on the non-specific immune response until an adaptive response can be established. Cellular activities of the non-specific immune response have been previously studied, including fish infected with sea lice [40,68]. There have also been no specific responses

described for fish infected with sea lice [49]. The present study, therefore, focused on the non-cellular, non-specific immune response of host fish.

1.6.1 Non-specific immune function of fish mucus

Various antimicrobial agents can be found in the serum, mucus, skin, gills and intestines of fish to prevent colonization by microorganisms [69,77]. The first obstacle a potential pathogen must overcome is the mucus secreted by the fish epidermis. Fish skin mucus is a heterogenous layer of mucins and glycoproteins affixed to microridges on the epidermal surface [78]. The mucous layer has several functions, including but not limited to, providing a lubricant for more efficient locomotion [79], contributing to osmoregulation control [80] and preventing microorganism colonization of the epidermis [81]. Mucus creates a boundary layer between the epidermal surface and the external environment that microorganisms must cross. This layer is continuously being sloughed off and regenerated by goblet cells [81-84]. This defense mechanism has a two-pronged effect; 1) it prevents attachment by potential pathogens and (2) if microorganisms are able to attach they are released through sloughing of the mucous layer [69,77,85].

The mucous layer contains bactericidal agents secreted from the epidermis that are effective in conjunction with its physical properties [69,77,78,81-87]. Examples of factors of the innate immune response found in epidermal mucus are several types of proteases, complement proteins, antimicrobial proteins, lysozyme and other enzymes. Lysozyme is distributed through various tissues, in the blood and

within the mucous layer. It occurs mainly in the neutrophils and monocytes and can be found to a lesser degree in macrophages [85]. In rainbow trout, lysozyme has been observed to occur in tissues rich in leukocytes and at sites where the risk of bacterial infection is high, such as in the mucous layer of the epidermis and gills [88]. Lysozyme cleaves glycosidic bonds in the peptidoglycan layer of Gram positive bacteria and has been shown to have antimicrobial properties against Gram negative bacteria as well [69,77]. Most bacterial cell walls contain a glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine, which is cleaved by lysozyme [89]. Lysozyme isolated from rainbow trout has exhibited the ability to kill prominent fish pathogens at levels expected to be encountered in the aquatic environment [90]. Two types of lysozyme have been isolated from rainbow trout serum and skin mucus [91]. One type of lysozyme (type II) is able to kill Gram negative bacteria associated with Hitra disease, furunculosis, vibriosis and enteric redmouth [90,92], while the other is yet to be characterized. Lysozyme is also found in yolk sacs of salmonid eggs and has been linked to prevention of vertical disease transmission [89,93].

Several of the enzymes within the mucus, however, do not have well understood purposes and functions. An example is alkaline phosphatase which is found in fish mucus and serum. It operates maximally at alkaline pH to catalyze the hydrolysis of phosphate compounds as well as transfer phosphoryl groups to acceptor molecules [94]. Although alkaline phosphatase has been studied in humans [95] and other organisms [96-98], its biological functions remain relatively unknown. There are 4 distinct classes of this enzyme with functions ranging from bone mineralization to

inorganic phosphate transport. It is found in macrophages [99,100] and has been linked to cell growth, apoptosis, cell migration and protein phosphorylation [96-98]. Alkaline phosphatase is a lysosomal enzyme suggested to have a possible protective role in fish during the first stages of wound healing [101]. In carp and Atlantic salmon, elevated levels of alkaline phosphatase in mucus or mucus secretory cells have been shown to increase under parasitic infection [87] and stressful living conditions [102] and may have importance in either stress or immune responses, or both.

Complement proteins are also found in mucus and are capable of lysing microorganisms through the membrane attack complex (MAC) [44,85]. There are two main complement pathways known as the classic and alternative complement (ACP) pathways [44,85]. The ACP is more prominent in fish. In the ACP, lipopolysaccharide cell walls of Gram negative bacteria activate the complement protein C3 and lead to the formation of the MAC [44,85]. Activation results in the production of many biologically important peptides involved in inflammatory responses [44,85]. Ultimately the opsonized bacteria are phagocytized and lysed. Complement proteins are involved in a range of biological activities, such as enhancing phagocytosis, anaphylactic activity and chemotactic activity for leukocytes [85]. C-reactive protein also enhances phagocytosis and respiratory burst and can act as an opsonin in fish serum and mucus by binding to C-polysaccharide and phosphatidylcholine [85]. Agglutinins, including lectins and other receptor specific substances, and immunoglobulins are present in fish serum, mucus and bile [85]. Agglutinins act as opsonins and bind to carbohydrates and proteins that are constituents of microbes [69,76]. Complement proteins and agglutinins are an integral

part of the innate immune systems in the binding and recognition of pathogenic organisms.

Several antimicrobial proteins have also been isolated from fish skin mucus. These include small molecular weight proteins (<10 kDa), such as paradaxin in Moses sole (*Pardachirus marmoratus*) [103] and pleurocidin in winter flounder (*Pseudopleuronectes americanus*) [104], as well as higher molecular weight proteins. Proteins of molecular weight 27, 31 and 70 kDa have been isolated from the skin of the common carp (*Cyprinus carpio*) [105] and 15, 15.5 and 30 kDa proteins have been isolated from the channel catfish (*Ictalurus punctatus*) [106], all with the ability to lyse Gram negative and Gram positive bacteria.

Another important group of substances found in fish mucus are proteases. They are involved in many biological functions including the non-specific immune response. Proteases are found in the cells of most tissues and the types of protease present depend on the function of the particular cell in question. Metallo- and serine proteases have been observed in the mucus of Atlantic salmon [107]. In mammals and birds, those useful in wound repair and anticoagulation are found in the azurophil granules of leukocytes [108], while those important in digestion, like trypsin, are found in the acinar cells of the pancreas [108]. Trypsin has also been isolated in the pancreas of Atlantic salmon and rainbow trout [109-111]. Most proteases are secreted in an inactive or latent form and are activated by enzymatic cleavage of peptide bonds [108].

Proteases are classified by the chemical residue, or nucleophile, at the active site of the enzyme [108]. The four different types of proteases include: 1)

cysteine, which use a sulfhydryl side chain of cysteine as a nucleophile, 2) serine, which use an activated hydroxyl side chain of serine as a nucleophile, 3) aspartic, which always contain aspartic acid as part of the two to three amino acids comprising the active site residues and 4) metalloproteases, which have the divalent cation zinc bound to two or three of the amino acids in the residues of the active site [108].

Cysteine proteases are found primarily in lysosomes and, in some cases, function as anchors for endogenous protease inhibitors along the cell surface [112-114]. This group of proteases degrade intracellular proteins [115] and promote cell proliferation [116]. Serine proteases are found throughout animal tissues but are highly concentrated in the pancreas (trypsin), stomach, intestine [117] and in immune cells [118]. They are employed in bacterial digestion [119], anticoagulation [120], wound repair [121] and inflammation [122]. Metalloproteases have similar functions to serine proteases in inflammation [123] and wound repair [124]. These proteases, however, are mainly located in cells of the kidney, brain and liver [125,126]. The least studied group of proteases, aspartic, are involved in antigen processing [127] and bioactive peptide formation [128]. Similar to cysteine proteases, aspartic proteases are found in lysosomes of cells throughout most tissues [129].

Proteases with trypsin activity have been discovered in the mucus of rainbow trout [110] and in the mucus secreting cells of Atlantic salmon [109]. Trypsin, isolated from rainbow trout mucus, has demonstrated the ability to lyse Gram negative bacteria such as *Vibrio anguillarum* [110]. In the European eel, *Anguilla anguilla*, epidermal cells secrete proteases with antibacterial activity [130]. Under thermal stress, eel epidermis has shown increased lysis of three bacterial pathogens

by cathepsins L and B [131]. Cathepsins L and B, as well as a serine protease and aminopeptidase, have also been discovered in the epidermis of American (*A. rostrata*) and Japanese (*A. japonica*) eels [132-134]. To date, serine, metallo- and cysteine proteases have been characterized in fish mucus and serum [69,76,107]. In general, proteases catalyze the hydrolysis of a variety of polypeptide substrates and perform integral functions in protein turnover in organisms. Proteases break down structural proteins (e.g. collagens) and hence are involved in tissue resorption and remodelling [135]. Macrophage and monocytes release a variety of serine and metalloproteases in order to assist them in migration to the site of infection. Through removal of the amino-terminal propeptide, serine proteases can also activate latent matrix metallo-proteases[136]. This interaction is crucial in proteolytic inflammatory cascades and can overcome high levels of endogenous protease inhibitors affecting a net degradation of proteins [137]. Since proteases are effective at binding and breaking down proteins they may be proficient in non-self protein elimination [138]. Because proteases break down large peptides into smaller peptides or amino acids for digestion, they can be deleterious in high concentrations in tissues. In humans, mutations in genes responsible for aspartic proteases and their inhibitors can result in muscular dystrophy and the consumption of healthy tissues [139]. For this reason, proteases are closely regulated in tissues by their inhibitors. A major function of protease inhibitors in blood is to regulate coagulation and complement cascades and maintain homeostasis of body fluids [69]. In interstitial tissues, metalloprotease inhibitors are expressed in high concentrations to prevent widespread destruction during an acute inflammatory reaction. In chronic inflammatory situations (e.g.

arthritis), protease levels exceed the inhibitor concentrations and are therefore destructive in nature. There are inhibitors for all four types of proteases, but α-2 macroglobulin, a soluble blood protein, is an inhibitor that binds to all proteases [69,76]. Protease inhibitors can be used to combat extracellular digestion of fish tissues by microorganisms and parasites [69,76]. The lack of α-2 macroglobulin has been linked to furunculosis susceptibility in brook trout (*Salvelinus fontinalis*) [140] and the development of anemia following *Cryptobia salmositica* infection in rainbow trout [141]. In the eastern oyster, *Crassostrea virginica*, protease inhibitory capacity has been positively correlated with resistance to Dermo, a disease caused by the protozoan *Perkinsus marinus* [142]. The last two cases are examples of the lack of a particular non-specific immune factor being linked to species-specific susceptibility to a particular disease. Closely related species such as coho salmon, chinook salmon, rainbow trout and Atlantic salmon can show differences in susceptibility to diseases such as infectious hematopoetic necrosis virus [143], sea lice [144], bacterial kidney disease [145] and furunculosis [9]. It may be possible that species differences in the presence of certain non-specific immune factors may also cause the differences in relative susceptibility of closely related salmonids to these diseases.

1.7 Parasitic mechanisms for feeding and evading host defenses.

Many parasites, including parasitic arthropods, also effectively utilize proteases for digestion of host tissues [146-148]. They may serve the purposes of clot dissolution [149], immunosuppression and host blood digestion [146-152]. Several different types of serine and cysteine proteases and other enzymes have been discovered in salivary extracts and gut homogenates of parasitic arthropods. Hematophagous arthropods salivate into the wounds they create in their host. It is proposed that the saliva permits optimal availability of blood by decreasing the effectiveness of the localized hemostatic and inflammatory responses of the host [153]. Digestion of host blood is mediated by a wide range of proteases and other enzymes in the cattle tick and mosquito. Salivary extracts from the cattle tick, *Boophilus microplus*, and the mosquito, *Aedes aegypti*, have shown the presence of valine aminopeptidases and multiple types of phosphatases and esterases [146]. Extracts from the blood-feeding copepod, *Phrixocephalus cincinnatus*, have shown the presence of cysteine endopeptidases similar to mammal cathepsins L, B and H [148]. These extracts have also shown the presence of serine proteases, plasmin and thrombin [148]. It is hypothesized that these proteases may work against host coagulation and assist in blood digestion [148]. Specifically in fish, aspartic protease activity in fillets of Pacific hake (*Merluccius productus*) have been shown to increase considerably once infected with the myxosporidians *Kudoa thyrsitis* and *K. paniformis* [154]. Metalloproteases are used by nematode and trematode pathogens to invade fish tissues [155], and by the fish parasite *Cryptobia salmositica* to lyse red blood cells [156]. High blood concentrations

of the protease inhibitor, α 2-macroglobulin, have also been linked to resistance by brook trout to the development of anemia following *C. salmositica* infection [141]. *Vibrio anguillarum* and *V. vulnificus* produce metalloproteases that activate pre-kallikrein to kallikrein, in host tissues. This conversion results in severe edema at the site of infection [157]. Other *Vibrio* spp., such as *V. cholera*, show a 100-fold decrease in virulence in protease deficient strains compared to the wild type [158].

Parasites not only secrete enzymes and other compounds into host tissues and blood to feed, but also to decrease the host's ability to mount a substantial immune response. Several parasites use cysteine proteases to degrade host immunoglobulins [159-161]. The trematode parasite, *Fasciola hepatica*, secretes two cysteine proteases into its host, capable of degrading extracellular matrices and basement membrane molecules. *Fasciola* spp. may therefore use these proteases to assist in penetration of the host and migration to preferable tissues, essential in growth[162]. *Entamoeba histolytica*, the protozoan responsible for amoebic dysentery, uses cysteine proteases to degrade host extracellular matrices and complement components [163]. Saliva from *Rhipicephalus sanguineus*, a tick of mice and dogs, impairs T-cell proliferation and interferon (IFN)-γ -induced macrophage microbicidal activity [152]. The saliva of the cattle tick, *Ixodes dammini,* inhibits host platelet aggregation through the presence of apyrase, and mast cell degranulation by prostaglandin E_2 [164].

Trypsin, in particular, is a digestive enzyme secreted in the guts of many animals to digest consumed proteins [165]. Trypsin is essential to the mosquito, *Anopheles gambiae*, in breaking down blood components for digestion following a

blood meal [165]. Trypsin has been isolated from *L. salmonis* [107,166] and shown to be secreted in the presence of Atlantic salmon mucus [166]. Other enzymes such as glycosidases and alkaline phosphatases have also been isolated from arthropod parasite salivary glands [146] and may all have functions in host tissue digestion, immune system evasion and/or immuno-suppression.

There are several mechanisms that parasitic organisms enlist to enable them to stay one step ahead of the host immune response. Whether it it is to increase blood flow for feeding, break down meal components or to suppress the immune response of the host, parasitic secretions into the host are as important in the host-parasite relationship as the host immune response.

1.8 Current investigation

1.8.1 Problem

The susceptibility of salmonids to sea lice infestation is well documented [12-23,25,33-39,63,167]. The heterogeneity of susceptibility between species has also been observed in a few studies [33,63,144]. From these studies it appears that coho salmon are the most resistant species of all salmonids, rejecting the ectoparasite within 10 days post infection [144]. Coho salmon have shown a well-developed epithelial hyperplasia and inflammatory response [144] to *L. salmonis* infection, while more susceptible salmonid hosts have not. Atlantic salmon appear to be the most susceptible of the North American species studied and shows osmoregulatory

problems often leading to death when infection numbers are high [36,39]. Not only has a difference been shown in total numbers of lice between salmonid species in co-infection studies, but chinook salmon have demonstrated an ability to slow the development of *L. salmonis* and delay the moulting process to the more detrimental mobile stages, relative to Atlantic salmon [144].

1.8.2 Objective of the current investigation

It is the objective of this study to quantify the differences in susceptibility and resistance to *L. salmonis* infection between Atlantic salmon, coho salmon and rainbow trout. This study will attempt to elucidate some of the different biochemical and physiological responses of host fish to lice infection and possibly uncover the mechanisms by which some salmonids can repel the lice and some cannot. The species were chosen based on the findings that salmonids exhibit differences in their ability to handle sea lice infection and the relative absence of data from rainbow trout in the literature on sea lice related stress. The stress response of rainbow trout under other stressful stimuli, however, is well characterized and allows for useful comparisons [102,168-174]. Rainbow trout is also becoming a more important fish for saltwater culture due to its reduced susceptibility to infectious salmon anemia (ISA) compared to Atlantic salmon. Thus, understanding the ability of this species to deal with sea lice may facilitate the prevention of subsequent problems in rainbow trout and steelhead aquaculture.

Carrying out this comparative study will build upon our current understanding of *L. salmonis* interactions within the family Salmonidae. There are two main questions driving this research project: (1) What are the differences in host specie's responses to lice infection? (2) What are the differences in lice responses to different host species? A better understanding of host-parasite interactions will help develop more effective measures for controlling sea lice.

1.8.3 Specific aims

1) This investigation will compare factors that may influence innate immunity in rainbow trout, Atlantic salmon and coho salmon. Mucus and blood samples will be collected from each species and baseline enzyme and protein profiles of each species will be compared. Lysozyme and alkaline phosphatase activities will be measured to determine whether baseline levels of these enzymes are similar between species. Protein and protease profiles will be determined using zymography and SDS-PAGE analysis, also to observe species- specific differences. The first aim of this research is to develop the basic knowledge required to better understand innate responses of these species to different diseases.

2) This investigation will compare the stress responses of rainbow trout, Atlantic and coho salmon to infection with *L. salmonis*. Salmon will be cohabited in the same tanks and infected with *L. salmonis*. Based on previous work showing coho expulsion of sea lice within 10 days [144], there will be 3 sampling days

within the first 10 dpi (1, 3, 7) and two sampling days after, based on expected sea lice life stages (14, 21 dpi) [33-37,39,144]. Over the course of the infection blood cortisol, glucose, protein and electrolyte concentrations (sodium, potassium and chloride) and hematocrit will be measured. Previous work has shown elevated cortisol levels, decreased hematocrit, and electrolyte loss following sea lice infection in Atlantic salmon [36,39]. Since the stress response of Atlantic salmon to lice infection is well documented, it will operate as a useful standard for comparison of the stress responses of rainbow trout and coho salmon to infection with *L. salmonis*. Effects of low level *L. salmonis* infections in Atlantic salmon have been studied and will also serve as useful standards of comparison with this study [38,107]. Comparison of stress responses between susceptible and resistant salmonid species to lice infection should help elucidate whether the presence or severity of the stress response is associated with susceptibility to sea lice. The total number and stages of lice on each fish will be recorded and correlated with blood stress indicators to demonstrate their interrelationship for each species. The first hypothesis is that rainbow trout and Atlantic salmon will show similar susceptibility to lice infection, while coho salmon will reject the lice soon after infection. The second hypothesis is that Atlantic salmon and rainbow trout will show signs of stress in the latter days of infection, while coho salmon will not.

3) The determination of protein profiles and enzyme activities of mucus of rainbow trout, Atlantic and coho salmon following infection with *L. salmonis* will elucidate species-specific changes in mucosal composition subsequent

to infection. Since Atlantic salmon surface mucus has shown different protein profiles between infected and non-infected individuals (Ross and Johnson, personal communication), it is reasonable to assume that different protein profiles will occur within coho salmon and rainbow trout and between all 3 species. Alkaline phosphatase and lysozyme assays will be performed to demonstrate and quantify the presence of these enzymes in the mucus of each species. Mucous protease profile differences between species will also be determined. Trypsin, for instance, has been observed in epidermal goblet cells of Atlantic salmon [109] and skin mucus of rainbow trout [110] and zymography will be used to detect this protease's activity. These enzymes can be an integral part of host non-specific defenses and possibly, in certain cases, parasitic feeding. For this reason it will be interesting to observe the relationship between enzymes in different species following sea lice infection and whether they are involved in both susceptible and resistant species' responses to infection. The hypothesis is that coho salmon will show an enzymatic response to infection within the first 10 days as a means of providing protection against lice infection. However, it is hypothesized that any enzymatic response by rainbow trout and Atlantic salmon will decline later in the infection (14 and 21 dpi), as lice infections have previously shown immunosuppressive effects in susceptible species [40,68].

4) To observe the louse response to different host species, mucus obtained prior to infection from the 3 species discussed, as well as mucus from a fourth species (one unaffected by sea lice such as flounder), will be incubated with

live sea lice. These incubations will then be tested for enzyme and protein profiles to determine the parasitic induced alterations in the mucus of different host and non-host species. The ability of lice to secrete different proteins or enzymes or different concentrations of these proteins/enzymes in response to different host and non-host species will be quantified. The hypothesis of this fourth aim is that sea lice will show greater enzyme and protease secretions in response to mucus from susceptible species (Atlantic salmon and rainbow trout) than to a non-host (flounder) or a resistant host (coho salmon) species.

2.0 COMPARISON OF BASELINE BIOCHEMICAL IMMUNE FACTORS OF PLASMA AND SKIN MUCUS IN RAINBOW TROUT, ATLANTIC AND COHO SALMON

2.1 Abstract

Susceptibility to different diseases among closely related species, such as coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*) and Atlantic salmon (*Salmo salar*), is variable. The innate immune system plays a major role in disease resistance. Hence, biochemical parameters related to innate immunity were characterized and compared for three salmonids; rainbow trout, Atlantic salmon and coho salmon in seawater and freshwater. Mucous protein profiles, proteases, alkaline phosphatase and lysozyme as well as plasma lysozyme activities were analyzed. Plasma protein and hematocrit were also measured.

Rainbow trout had significantly higher mucous protease activity than Atlantic salmon and significantly higher lysozyme activities than coho and Atlantic salmon, in seawater. Atlantic salmon, on the other hand, had the lowest activities of lysozyme and mucous proteases, in comparison to the two other salmonids in seawater. Atlantic and coho salmon had higher mucous lysozyme activities in freshwater as compared to seawater. There was no significant difference between mucous lysozyme activities in any of the three species reared in freshwater; however, rainbow trout still had a significantly higher plasma lysozyme activity in comparison to the other two species. All three species exhibited significantly lower mucous alkaline phosphatase and protease activities in freshwater than in seawater. Despite the differences in the levels of protease activity, rainbow trout and Atlantic salmon

skin mucus consisted of similar relative proportions of serine- and metalloproteases (75% and 25%, respectively). These differed from the skin mucus of coho salmon (65% serine- and 35% metalloproteases), many of which were calcium-dependant proteases (60%). Protein profiles showed rainbow trout and coho salmon mucus to be more similar to each other than to Atlantic salmon mucus in both freshwater and seawater. All three species demonstrated a great deal of variation in comparison to one another. However, the mucus of rainbow trout and coho salmon had a higher relative abundance of low molecular weight proteins in comparison to Atlantic salmon mucus.

2.2 Introduction

Teleost fish have evolved innumerable host-pathogen relationships some of which are unique to just a few or even a single species. Interestingly, even closely related species can show differences in susceptibility towards the same pathogen [140,141,144]. In salmonids, coho salmon (*O. kisutch*) is less susceptible to diseases like infectious hematopoetic necrosis virus [143] and ectoparasitic infection with sea lice, *Lepeophtheirus salmonis*, while species such as chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), steelhead/rainbow trout (*O. mykiss*) and Atlantic salmon (*S. salar*) are susceptible [144,175]. Relative susceptibilities of Atlantic salmon, rainbow trout and coho salmon to sea lice infection will be compared in Chapter 3. Different genus' show variation in susceptibitlity to other diseases. Bacterial kidney disease is observed to be more of a problem with *Oncorhynchus*

species than *Salmo* species [145], while *Salmo* species appear to be more susceptible to furunculosis than *Oncorhynchus* species [9]. The immunological mechanisms for these differences are as yet not understood. It is important to identify potential biochemical parameters that may explain these susceptibility differences among closely related species.

The aquatic environment poses a unique problem to fish in that they are in constant interaction with possible sources of pathogens throughout their habitat. Mucus covering the fish's epidermal surface therefore becomes the first line of defense against invading pathogens. The epidermal layer of the skin is also important in this aspect as it secretes the layers of mucus integral in host defense mechanisms. Enzymes and other proteins located within the mucus, such as lysozyme, have bactericidal activities [86] and may serve to combat certain types of infection.

Biochemical and physiological data were collected from rainbow trout, Atlantic and coho salmon maintained in fresh water and seawater to observe potential differences in biochemical immune factors between these fish in fresh and seawater. The possibility remained that since seawater adapted rainbow trout do not undergo the smoltification process, unlike coho and Atlantic salmon, that some differences between these species may be attributed to life history variation.

The purpose of this study was to quantify baseline biochemical parameters of the plasma and skin mucus in rainbow trout, coho and Atlantic salmon in order to develop the basic knowledge required to better understand the response and resistance of these species to different diseases.

2.3 Materials and methods

2.3.1 Fish and their maintenance

2.3.1.1 Seawater-reared (SWR) fish

The fish used for this study were yearling (S1) Atlantic salmon, seawater-adapted rainbow trout, and coho salmon. Atlantic salmon (St. John River strain) and rainbow trout (Kamloops strain) were obtained from Department of Fisheries and Oceans (DFO) certified hatcheries in Pictou, NS, and Murray River, PEI, respectively. Coho salmon were obtained through the research facilities of Aqua Health Limited, Charlottetown, PEI, originally received from DFO's Big Quallicum hatchery, Vancouver, BC. On arrival, fish of each species were placed in separate 1500 L tanks initially in fresh water and then smoltified by gradually increasing the salinity with artificial seawater (Instant Ocean,® Aquarium Systems, Mentor, OH) over a two week period. The fish were maintained in 30±2 ppt (salinity) at 10 ± 1°C for at least 2 wk. After acclimation, 80 fish of each species were randomly distributed to two different tanks. Forty fish of each species were cohabited in each tank (1500 L) and further acclimated for a 2 wk period to allow species to adapt to one another. Stocking densities were approximately 10 kg/$m^3$for each tank. Dissolved oxygen levels were constantly monitored and maintained at ca. 8 ppm. The photoperiod was maintained at 14 h light: 10 h dark and fish were fed (feed mixture of equal parts: 2.0 Vextra ™, 3.0 Hipro ™ and 4.0 Vigor ™, Corey Feed Mills, Fredericton, NB) twice daily (0900 and 1300) to satiation. At the onset of the

experiment, Atlantic salmon and rainbow trout had a mean (±SD) weight of 113.1 ± 22.8 g and 187.5 ± 40.2 g respectively, while coho salmon weighed 39.5 ± 9.6 g.

As part of another study, the fish underwent crowding for a period of 12 h by decreasing the volume of the tanks to 1/3 capacity (ca. 30 kg/m^3), with reduced flow and supplemental oxygen. This was carried out under decreased light to minimize fish activity. This was only carried out once and fish were returned to ambient conditions following the 12 h crowding event to minimize stress. The fish were sampled prior to and 49 days after the crowding event (dpc). The 49 dpc sampling date was chosen to allow for any effects of crowding to diminsh.

2.3.1.2 Freshwater-reared (FWR) fish

Fish were obtained from the same sources and were the same strains as the fish kept in seawater (Section 2.3.1.1). Atlantic salmon and rainbow trout were approximately the same age as the fish in seawater (18 - 21 months), while the freshwater coho salmon were one year older (30 months). These fish were maintained and fed under the same conditions as the fish in seawater, with the following exceptions: All fish were maintained in freshwater and were not cohabited. Freshwater Atlantic and coho salmon were stocked at 200 fish per tank (1500 L), while the rainbow trout were stocked at 20 fish per tank (150 L). Stocking densities of freshwater tanks were approximately 10 kg/m^3. On sampling day, the mean (± SD) weights for the freshwater Atlantic salmon, rainbow trout and coho salmon

were 124.7 ± 54.3 g, 231.7 ± 44.1 g, and 198.2 ± 84.1 g, respectively. Freshwater-reared fish did not undergo a crowding event and were only sampled on one day.

2.3.2 Sampling

2.3.2.1 SWR fish

Prior to the crowding event and 49 days post-crowding, 5 fish of each species from each tank were randomly sampled. Fish were kept off-feed for 24 h prior to sampling to reduce feeding stress and minimize debris at the bottom of the tank. Each fish was placed into individual buckets containing a lethal dose of anesthetic (200 mg/L MS-222, Syndel Laboratories, Vancouver, BC). Length, breadth, and weight were measured and blood and mucous samples were obtained from each fish. On each fish, breadth was measured dorso-ventrally from the anterior end of the dorsal fin to the belly. Mucous samples were collected in bags containing 100 mM ammonium bicarbonate (SIGMA, St. Louis, MO) buffer (pH 7.8), as described by Ross *et al.* [74]. The fish were then gently rolled from side to side in the bag to allow for mucus to adhere to the sides of the bags and shed into the buffer solution. Mucous samples were centrifuged at 1500 x *g* for 10 min at 4^0C. Following centrifugation, mucous samples were aliquoted into 1.5 mL microfuge tubes and stored at -80^0C until analyzed. Blood was extracted from the caudal vein (ca. 1-3 mL) with 1 or 5 cc syringes and 21 or 25 G-1 needles flushed with heparin (10 i.u./mL)(SIGMA).

2.3.2.2 FWR fish

Sampling procedure for fish in freshwater was the same as the procedure described in Section 2.3.2.1, with the following exceptions. Ten fish were sampled from each tank on one sampling day.

2.3.3 Analysis of blood samples

Blood samples were spun for 5 min at 4000 x *g* to separate plasma using an Micromax Model CL Centrifuge (International Equipment Company, Needham Heights, MA). Hematocrit and plasma protein levels were obtained using capillary tubes and refractometer respectively [39]. Hematocrit and plasma protein concentrations were compared between FWR species and SWR species to ensure that all fish were at similar physiological states. Lysozyme levels in the blood plasma were determined using a lysozyme turbidometric assay as previously described by Ross *et al.* [87].

2.3.3.1 Plasma lysozyme assay [87]

Rainbow trout, coho salmon and Atlantic salmon plasma samples were diluted to 1/200, 1/100, 1/50, respectively, with sodium phosphate buffer. The sodium phosphate buffer was made up of 40 mM NaH_2PO_4, pH 6.2 (2.76 g of $NaH_2PO_4 \cdot H_2O$ dissolved in 500 mL deionized water, and pH adjusted to 6.2 with 1M NaOH) ($NaH_2PO_4 \cdot H_20$ obtained from Caledon, Georgetown, ON; MW = 137) and stored at

4 ^{0}C. Each diluted sample (25 µL) was added to 50 µL of buffer and pre-incubated in a microplate reader for 15 min at 30^{0}C. Substrate was made up of 0.6 mg/mL suspension of lyophilized *Micrococcus lysodeikticus* (SIGMA) in 40 mM NaH_2PO_4 buffer, pH 6.2 (12 mg of *M. lysodeikticus* dissolved in 20 mL of buffer) prepared fresh every day. To assure that the substrate was also at the assay temperature, substrate was also maintained at 30^{0}C for 15 min prior to incubation. Substrate (25 µL) was then added to the mucus/buffer mixture in a microplate and the ODs read as quickly as possible.

The assay was performed in 96-well plates (Costar, Cambridge, MA) and optical densities (OD) read by a microplate reader (Thermomax Microplate Reader, Molecular Devices, Sunnyvale, CA). The microplate was incubated at 30^{0}C and OD was measured continuously (1 reading every 15 sec) over 1 h at 450 nm. The initial rate of reaction was used to calculate the activity, with one unit of activity being defined as the amount of enzyme that catalyzed a decrease in absorbance at 450 nm of 0.001/min. Data was analyzed using Soft Max Pro software (Version 1.2, Molecular Devices)

Controls were included to subtract background OD of the buffer and substrate (negative control) and to ensure the assay was working (positive control). The negative control contained 50 µL of buffer and 50 µL of substrate, while the positive control contained 50 uL of buffer, 25 µL of substrate and 25 µL (2500 units/mL) of chicken egg white lysozyme (Chicken Egg White, SIGMA, 53,200 units/mg solid used). Stock lysozyme solution was made up at 2500 units/mL in 40 mM NaH_2PO_4, pH 6.2 (4.699 mg of chicken egg white lysozyme in 100 mL of

buffer). This solution was aliquoted in 1.5 mL microcentrifuge tubes and stored at -20^0C.

2.3.4 Analysis of mucous samples

Prior to analysis, mucous samples were thawed on ice and centrifuged at 9,300 x *g* for 2 min and the supernatant used for each assay. Protein concentrations of mucous samples were determined to enable enzyme activities to be standardized.

Protease activities (azocasein hydrolysis and zymography), lysozyme (lysozyme turbidometric assay) and alkaline phosphatase (alkaline phosphatase assay), were measured from the mucus of sampled fish as described by Ross *et al.*[87]. Protease zymography was determined using a modification of Hassel *et al.* (1996) [176].

All assays were analyzed using the same software and hardware as Section 2.3.3.1. Individual assay conditions are provided below.

2.3.4.1 Determination of protein concentrations

Protein concentrations of mucous samples were determined using a dye binding method [177]. Five µL of mucous sample were added to 250 µL of a dilute commercial Bradford solution (1 vol of BioRad protein assay dye reagent in 4 vol of deionized water) and immediately read at 590 nm on a microplate reader. Prior to sample reading, a standard curve for the Bradford solution was developed using dilutions (0.1 - 1.4 mg/mL) of bovine γ-globulin (BioRad, Richmond, CA).

2.3.4.2 Mucous protein composition (SDS-PAGE)

Protein composition in the mucus was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [178]. Samples were diluted 1:1 with SDS-PAGE sample buffer containing 5% (v/v) 2 M DL-dithiothreitol (DTT) (SIGMA). Samples were then boiled for 5 min prior to loading. Between 0.5 – 1 μg of protein from each mucous sample was added to each well. Proteins were electrophoresed on a 12% acrylamide gel running at 150 V at room temperature for approximately 1 h and silver stained (Appendix A) [179]. Low range unstained markers (BioRad) were also loaded. The molecular weights of the markers were 97.4 kDa, 66 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14.5 kDa.

The first step in the silver staining method [179] involved placing the gels in fixative 1 for 10 min (40% (v/v) ethanol and 10% (v/v) acetic acid). This was followed by a 10 min rinse (ddH_2O) and a step to cross link proteins for 5 min in fixative 2 solution. This solution contained 0.05% glutaraldehyde (50% v/v, Fisher, Nepean, ON), 0.01% (v/v) formalin (BDH, Toronto, ON), and 40% (v/v) ethanol. Next the gels were rinsed in 40% (v/v) ethanol for 20 min and then ddH_2O for a further 20 min. Gels were then transferred to sodium thiosulphate solution (0.2 g/L) (Anachemia, Montreal, PQ) for 1 min and rinsed twice in ddH_2O for 1 min. Incubation of gels followed in 0.1% (w/v) silver nitrate (BDH) for 20 min, then washed in ddH_2O for 1 min and finally developed in 2.5% (w/v) sodium carbonate (Fisher) and 0.04% (v/v) formalin solution. When desired contrast was obtained gels were placed in 5% AcOH (v/v).

2.3.4.3 Azocasein assay procedure [87]

Substrate was made fresh daily by dissolving 25 mg of azocasein (SIGMA) in 5 mL of 100 mM ammonium bicarbonate (SIGMA) (3.95 g of ammonium bicarbonate dissolved in 500 mL of deionized water and adjusted to pH 7.8, if necessary, with either 1 M HCl or 1 M NaOH).

Supernatants (50 µL) from original mucous samples (Section 2.3.4) were incubated with 125 µL substrate at 30^{0}C. After 19 h of incubation, the reaction was stopped with 50 µL of 20% trichloroacetic acid (TCA, Fisher). Samples were centrifuged at 17,000 x *g* for 5 min at room temperature and 100 µL of supernatant was added to microplate wells. 100 µL of 0.5 NaOH was added to the same wells and the plate read at 450 nm in a microplate reader. One unit of activity was defined as the amount of enzyme causing an increase in absorbance of 0.001 OD units per 19 h.

The negative controls contained 50 µL of ammonium bicarbonate and 125 µL of substrate, while the positive controls contained 50 µL of bovine trypsin (SIGMA) (10 µg of bovine trypsin prepared in 1 mL of 1 mM HCl) and 125 µL of substrate.

2.3.4.4 Protease characterization by specific inhibitors (azocasein assay) [176]

To characterize the types of proteases present in the fish mucus, the previous method (Section 2.3.4.3) was followed with one addition. Replicates containing different protease inhibitors were included. Individual tubes containing 1.6 µL (of a 1.0 mg/mL stock in H_2O) leupeptin, 1.0 µL (of a 1.0 mg/mL stock in H_2O) pepstatin , 1.0 µL (of a 2.0 mg/mL stock in H_2O) aprotinin, 4.0 µL (of a 500 mM stock) *o*-phenanthroline, 4.0 µL (of a 500 mM stock) iodoacetamide and 3.0 µL (of a 500 mM stock) ethylenediamine tetraacetic acid (EDTA), were incubated with substrate and mucus samples as described above (All protease inhibitors were obtained from Boerhinger Mannheim, Indianapolis, IN).

Mucous samples were taken from five representative fish of each species to determine the types of protease present in the mucus of each species. Inhibition was characterized as a percentage of the total protease activity in each mucous sample according to the following equation:

$$1 - \frac{\text{(Activity with inhibitor)}}{\text{(Activity without inhibitor)}} \times 100\%$$

2.3.4.5 Protease zymography [87]

An equal amount of mucous sample (3 µg protein) was added 1:1 with 2X SDS-PAGE sample buffer (Appendix A). This mixture was loaded onto a 12% SDS-PAGE gel containing 0.1% gelatin. Pre-stained low range molecular weight markers (BioRad) of the following weights 106 kDa, 77 kDa, 50.6 kDa, 35.5 kDa,

29.1 kDa and 20.9 kDa were also loaded onto each gel. Gels were run at 4^0C and 150 V for approximately 1 h. Following electrophoresis, gels were washed 3 times also at 4^0C with wash buffer (Appendix A), at 4^0C, for 10 min each. The gels were then incubated in the same buffer, containing 50 mM $MgCl_2$ (Caledon) and 6.25 mM $CaCl_2$ (Caledon), on a shaker for 19 h at 30^0C. Gels were stained in 0.1% amido black (BDH) in MeOH/H_2O/AcOH (45:45:10) for 1 h and destained with MeOH/H_2O/AcOH (50:48:2) until good contrast was obtained. Protease zymography was completed for each fish, however, only representative fish are shown in results.

2.3.4.6 Protease characterization by specific inhibitors (protease zymography method) [176]

The same procedure, as described in Section 2.3.4.5, was followed with one exception. Following the 3rd wash, the gel was cut into strips and each strip incubated in incubation buffer (25 mL) with or without one of six protease inhibitors: 50 µL (of a 1.0 mg/mL stock in H_2O) leupeptin giving a final concentration of 0.002%, 50 µL (of a 1.0 mg/mL stock in H_2O) pepstatin giving a final concentration of 0.002% , 25 µL (of a 2.0 mg/mL stock in H_2O) aprotinin giving a final concentration of 0.002%, 100 µL (of a 500 mM stock) *o*-phenanthroline giving a final concentration of 2 mM, 100 µL (of a 500 mM stock) iodoacetamide and 100 µL (of a 500 mM stock) EDTA both giving final concentrations of 2 mM . The gel slices being incubated with EDTA, did not include calcium chloride in the incubation buffer. Zymography gels for all the fish sampled were analyzed to determine the

most common mucous protease patterns for each species, from which representative samples were chosen. Mucous samples were taken from five representative fish of each species, to determine the types of proteases present in the mucus of each species.

2.3.4.7 Mucous lysozyme assay

The lysozyme assay for mucous samples was the same as the procedure used for blood samples (Section 2.3.3.1), with one exception. Frozen samples were sublimated to dryness in a vacuum centrifuge overnight to remove ammonium bicarbonate buffer and re-dissolved in an equal volume of 40 mM NaH_2PO_4 (Caledon), pH 6.2. Samples were then centrifuged at 9,300 x *g* for 2 min and supernatants used for assay.

2.3.4.8 Alkaline phosphatase assay [87]

Assay buffer consisted of 100 mM ammonium bicarbonate (SIGMA), 1 mM $MgCl_2$ (BDH, Montreal, PQ.) pH 7.8 (3.95 g of ammonium bicarbonate and 0.102 g of $MgCl_2 \cdot 6H_2O$ dissolved in 500 mL of deionized water and adjusted to pH 7.8, if necessary, with either 1 M HCl or 1 M NaOH), and was prepared fresh daily.

Substrate was made up of 4 mM p-nitrophenyl phosphate in 100 mM ammonium bicarbonate, 1 mM $MgCl_2$, pH 7.8 (74.22 mg of *p*-nitrophenyl phosphate, disodium, hexahydrate (substrate 104, FW=371.1, SIGMA) dissolved in 10 mL of

buffer). The stock solution was aliquoted to 1.5 mL microcentrifuge tubes and stored at -20^0C.

Mucous sample (25 µL) was added to 55 µL of buffer and pre-incubated in a microplate reader for 15 min at 30^0C. To assure that the substrate was also at the assay temperature prior to incubation, substrate was maintained at 30^0C for 15 min. Substrate (20 µL) was then added to the mucus/buffer mixture and the assay read on microplate as quickly as possible. The plate was incubated at 30^0C and OD was measured continuously (1 reading every 15 sec) over 1 h at 405 nm in a Thermomax Microplate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 pM of *p*-nitrophenol product in 1 min. The extinction coefficient of *p*-nitrophenol in the microplate wells was experimentally determined.

Negative and positive controls were run to allow comparison of different plates on different days. Negative controls contained 80 µL of buffer and 20 µL of substrate, while positive controls contained 55 µL of buffer, 20 µL of substrate and 25 µL of alkaline phosphatase enzyme (Bovine Intestinal Mucosa, SIGMA) (0.5 mg/mL).

2.3.5 Statistical analysis

All statistical analysis was performed using SigmaStat for Windows Version 2.0 (SPSS Inc. Chicago, IL). To determine whether there was a tank or day effect in seawater-reared fish, 2 - way analysis of variance (ANOVA) was used to

compare parameters. The model used was: $y_{ij} = \mu + \lambda_i + \gamma_j + (\lambda\gamma)_{ij} + \xi_{ij}$. Where, i = tank #, j = day #, μ=overall effect, λ= sum of tank effects, γ= sum of day effects, $(\lambda\gamma)$= interaction and ξ = error. No tank or day effect was found and data was pooled to give a larger sample size for statistical testing. One-way ANOVA were used to compare parameters between species followed by multiple comparison Tukey's tests (Bartlett statistic corrected). Differences were significant when $p<0.05$. All data was checked for normality and non-normal data was transformed using ($\sqrt{x}$) or log (x+1) transformations. All values shown in this investigation, are mean ± SEM, except in the case of population weights where SD were used.

Spearman rank order correlations were used to determine whether there was a correlation between the levels of different enzymes.

2.4 Results

There were no significant differences in hematocrit or plasma protein levels within or between species in freshwater or seawater. (Table 2 I).

Mucous protein profiles showed a high degree of variation between SWR individuals as well as between SWR species. In general, SWR rainbow trout and SWR coho salmon had more similar mucous protein profiles to each other than when compared with protein profiles of SWR Atlantic salmon. A 31 kDa protein appeared to have a higher intensity relative to other proteins in SWR Atlantic salmon

Table 2 I: Comparison of biochemical and physiological parameters in freshwater (FW, n=10) and seawater-reared (SW, n=20) rainbow trout, coho salmon and Atlantic salmon. Data are expressed as means (± SEM). * Indicates significant differences between SW and FW ($p<0.05$). ** Indicates significant differences between SW and FW ($p<0.01$). (a) Indicates significant differences from coho salmon; (b) indicates significant differences from Atlantic salmon; (c) indicates significant differences from rainbow trout.

Parameter	Rainbow trout		Coho salmon		Atlantic salmon	
	FW	SW	FW	SW	FW	SW
Hematocrit (%)	**46.7 ± 1.4**	**45.6 ± 1.7**	**51.1 ± 1.5**	**52.5± 1.8**	**46.3 ± 1.6**	**47.1 ± 1.5**
Plasma Protein (g/100 mL)	**4.4 ± 0.4**	**5.4 ± 0.5**	**6.3 ± 0.6**	**6.8 ± 0.6**	**6.3 ± 0.3**	**6.0 ± 0.4**
Mucous Lysozyme (U/mg protein)	**185.6 ± 78.1**	**65.0 ± 13.6** a,b	**110.8 ± 50.3**	**19.1 ± 7.1** c	**200.4 ± 63.6**	**13.6 ± 5.1*** c
Plasma Lysozyme (U/mg protein)	**256.7 ± 36.0** a,b	**195.8 ± 37.8** a,b	**33.3 ± 26.2** c	**25.7 ± 10.4** c	**14.6 ± 8.3** c	**34.4 ± 8.4** c
Mucous Protease (U/mg protein)	**0.013 ± 0.001**	**0.051 ± 0.005*** b	**0.011 ± 0.001**	**0.036 ± 0.010**	**0.006 ± 0.001**	**0.026 ± 0.005*** c
Mucous Alkaline Phosphatase (U/mg protein)	**0.292 ± 0.041**	**1.04 ± 0.30****	**0.237 ± 0.023**	**0.75 ± 0.12****	**0.082 ± 0.019**	**0.45 ± 0.18****

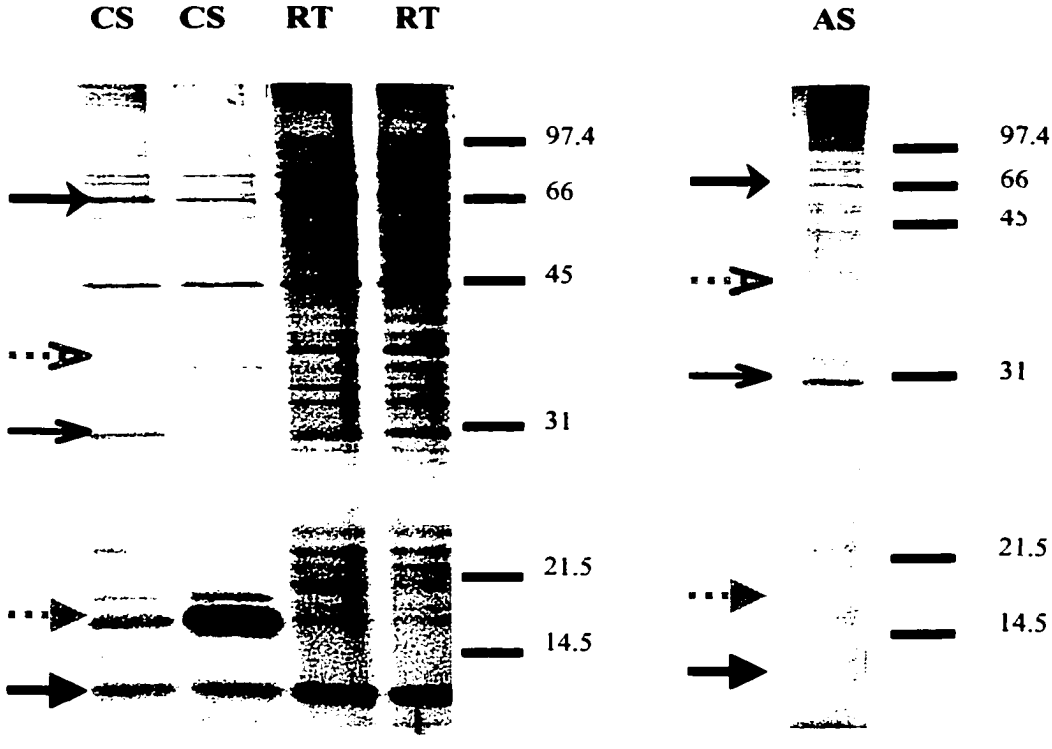
mucus than in the mucus of SWR rainbow trout and SWR coho salmon (Figure 2.1). Low molecular weight proteins were either at very low concentrations and intensity following silver stain or were not present at all in the mucus of SWR Atlantic salmon. In contrast, there were two bands of high relative intensity (<14 kDa and 18 kDa) in SWR coho salmon and one distinct band of high relative intensity (<14 kDa) in SWR rainbow trout (Figure 2.1). The same 18 kDa protein, however, was observed to have high relative intensities in a number of the FWR rainbow trout. There was also a 38 kDa protein with greater abundance in SWR rainbow trout than in SWR Atlantic and SWR coho salmon mucus. A protein in the vicinity of the 66 kDa marker appeared to have relatively high abundance in the mucus of all three species (Figure 2.1). The banding intensity was not quantified for any proteins.

Mucous protein profiles showed a similar degree of variation within all FWR species as seen in SWR species. Variation between protein profiles of SWR rainbow trout, coho salmon and Atlantic salmon and their freshwater counterparts appeared no greater than the individual variation observed within each species (Figure 8.1, Appendix B).

Seawater-reared rainbow trout had significantly higher mucous protease activity than SWR Atlantic salmon ($p<0.05$) (Table 2 I). All three species had significantly higher protease activity in SW than in FW. Most FWR rainbow trout (7/10), FWR Atlantic (9/10) and FWR coho salmon (7/10) had undetectable amounts of mucous protease activity.

To determine the types of protease present in the mucus SWR fish, specific inhibitors were added to the azocasein hydrolysis assay. Rainbow trout

Figure 2.1: Mucous protein profiles of three seawater-reared salmonid species. Representative samples of rainbow trout (RT), coho salmon (CS), Atlantic salmon (AS). Molecular weights (kDa) are along the right hand side of the gel. Each lane contains 1 µg of mucous protein. (➡) indicates <14.5 kDa protein, (⇢) indicates 18 kDa protein, (➡) indicates 31 kDa protein, (⇢) indicates 38 kDa protein, (➡) indicates ca. 66 kDa protein. Representative samples expressed here were chosen based on the most common protein banding patterns observed for each species.



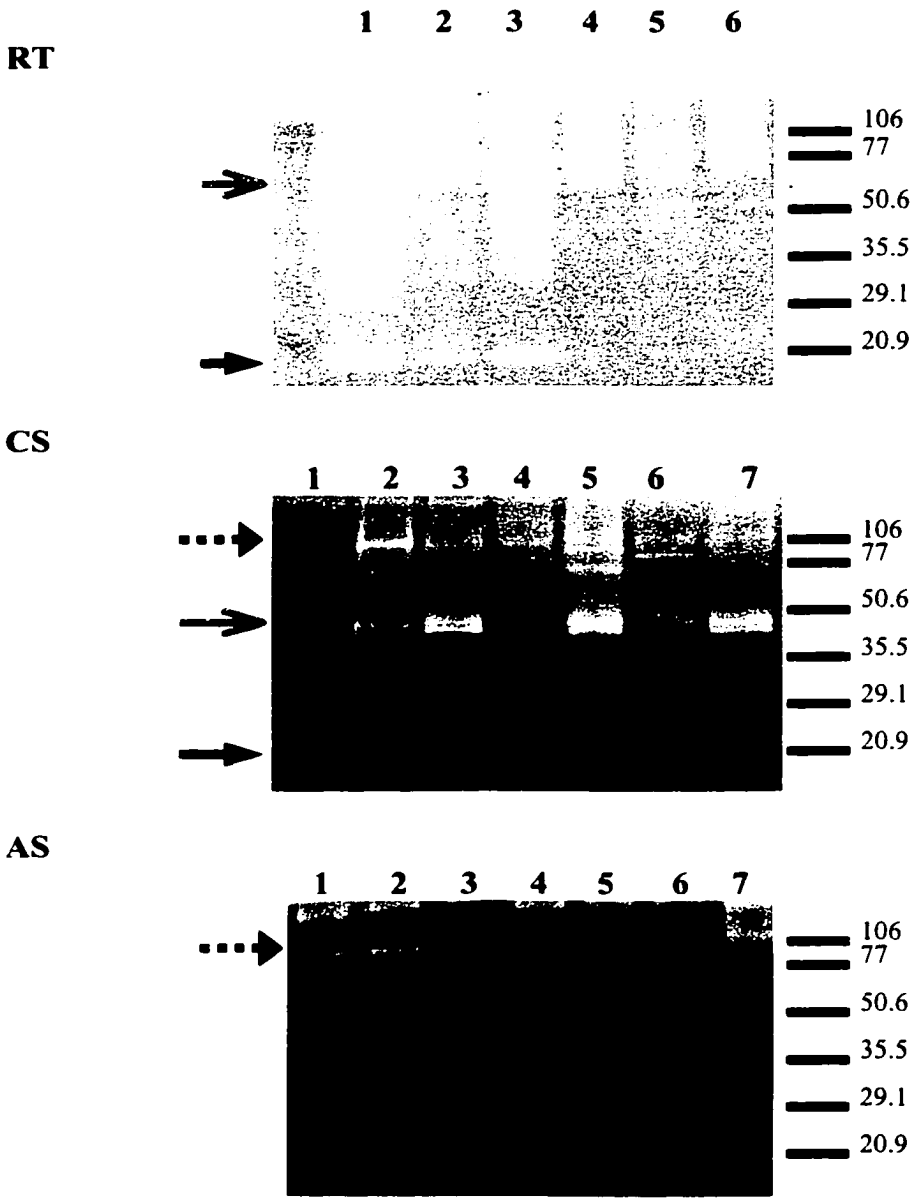
mucous protease activity was inhibited 70% by aprotinin, 15-20% by leupeptin and 25% by *o*-phenanthroline (Table 2 II). Protease activity in coho salmon was inhibited 60% by leupeptin, 80% by aprotinin, 40% by *o*-phenanthroline and 60% by EDTA (Table 2 II). In Atlantic salmon mucus, protease activity was inhibited 60% by aprotinin, 20% by pepstatin (in 3 out of 10 samples), 30% by leupeptin, and 25% by *o*-phenanthroline (Table 2 II). Mucous protease activity increased slightly in all three species in the presence of iodoacetamide and was inhibited 100% in all three species in the presence of both aprotinin and *o*-phenanthroline (Table 8 I, Appendix B).

Protease zymography showed that when equal amounts of mucous protein were loaded SWR rainbow trout appeared to have much higher intensity in protease banding than SWR coho and SWR Atlantic salmon, particularly in the medium to high molecular weight range (Figure 2.2). A low molecular weight protease band (18 kDa) was observed in the mucus of approximately 50% of the SWR rainbow trout sampled. Seawater-reared coho salmon mucus had protease bands present in the mid- to high molecular weight range, similar to SWR rainbow trout but at lower intensities (Figures 2.2). Two distinct protease bands (42-45 kDa) were observed in the mucus of SWR coho salmon but not in the other two species (Figure 2.2). Seawater-reared coho salmon also had a low molecular weight protease present in the mucus (18 kDa) of 60% of the fish sampled, similar to SWR rainbow trout, but at lower intensity. Seawater-reared Atlantic salmon mucus had very little protease banding at all, and those present were all in the high molecular weight range (60-100 kDa) (Figure 2.2).

Table 2 II: Effect of specific protease inhibitors on protease activity in the mucus of three seawater-reared salmonid species, using the azocasein assay. Individual tubes containing specific inhibitors were incubated with mucous samples and azocasein substrate for 19 h. Representative fish (n=5) were chosen for each species and mean ± SEM is displayed. Values are expressed as a percent inhibition of the total (100 %) control protease activity. Control protease activity was defined as the total protease activity in samples without protease inhibitors. Azocasein assays were analyzed for all the fish sampled and the samples with the most common protease activities determined. Five of these samples were then chosen as representative samples for each species.

Inhibitor	Rainbow trout	Coho salmon	Atlantic salmon
Aprotinin (0.011 mg/mL)	**73.5±11.2**	**80.0±7.1**	**60.0±6.1**
Leupeptin (0.006 mg/mL)	**17.5±12.5**	**60.0±7.1**	**30.0±4.5**
Pepstatin (0.006 mg/mL)	**0**	**0**	**20.0±15.0**
Iodoacetamide (10 mM)	**0**	**0**	**0**
***o*-phenanthroline** (10 mM)	**20.0±6.5**	**35.8±9.6**	**25.0±6.7**
EDTA (8 mM)	**0**	**60.0±5.0**	**0**

Figure 2.2: Protease zymography gel of seawater-reared rainbow trout (RT), coho salmon (CS) and Atlantic salmon (AS) mucus. Molecular weights (kDa) are indicated along the right hand side of the gel. Representative samples of fish mucus are numbered at the top of the gel. Each well contains 3 µg of mucous protein. (➡) indicates low molecular weight protease, (➡) indicates mid- to high molecular weight proteases (40-80 kDa), (▪▪▪➡) indicates high molecular weight protease bands. Representative samples expressed here were chosen based on the most common protease banding patterns observed for this species.



Specific inhibitors were added to zymography experiments to characterize the individual proteases present in the mucus of these three fish species. In rainbow trout mucus the addition of aprotinin removed protease bands of 18, 33 and 45 kDa (Figure 2.3 lane ii). Dense banding in the range of 48-55 kDa was eliminated as well as banding between 55-73 kDa (Figure 2.3 lane ii). The 73-74 kDa protease band remained, while the 80 kDa band intensity was decreased along with all protease banding above 80 kDa (Figure 2.3 lane ii). Seawater-reared rainbow trout mucus incubated with pepstatin, iodoacetamide and EDTA showed no observable differences from mucus incubated without inhibitors (Figure 2.3 lanes i, iv, v, vii). The addition of leupeptin to SWR rainbow trout mucus resulted in a decrease in the intensity of the 33 and 80 kDa bands (Figure 2.3 lane iii). *o*-Phenathroline incubation, similar to aprotinin, resulted in the removal of the 33 kDa band, and a reduction in the intensity of bands ranging from 48-53 and 55-73 kDa (Figure 2.3 lane vi). When using a combination of aprotinin and *o*-phenanthroline most of the SWR rainbow trout mucous proteases were inhibited, leaving only residual protease activity in the 77-83 kDa range (Figure 2.4).

In the mucus of SWR coho salmon, the two mid-molecular weight range proteases (42-45 kDa) were eliminated following incubation with *o*-phenanthroline (Figure 2.3 lane vi). The 65 and 85 kDa proteases were also eliminated when *o*-phenanthroline was incubated with coho salmon mucus (Figure 2.3 lane vi). Incubation with aprotinin decreased the intensity of the two medium molecular weight proteases (42-45 kDa) as well as the 65 kDa protease in SWR coho salmon mucus (Figure 2.3 lane ii). No significant decreases in SWR coho salmon

Figure 2.3: Protease zymography gels of mucus with and without protease inhibitors for seawater-reared rainbow trout (RT), coho salmon (CS) and Atlantic salmon (AS). All samples were incubated in 25 mL incubation buffer + specific inhibitors for 19 h. (i) Control (ii) aprotinin (2 µg/mL), (iii) leupeptin (2 µg/mL), (iv) pepstatin (2 µg/mL), (v) iodoacetamide (2 mM), (vi) *o*-phenanthroline (2 mM), (vii) EDTA (2 mM). Molecular weights (kDa) are along the right side of the gels. Each lane contains 3 µg of mucous protein. Samples expressed here represent the most common protease banding patterns observed for each species.

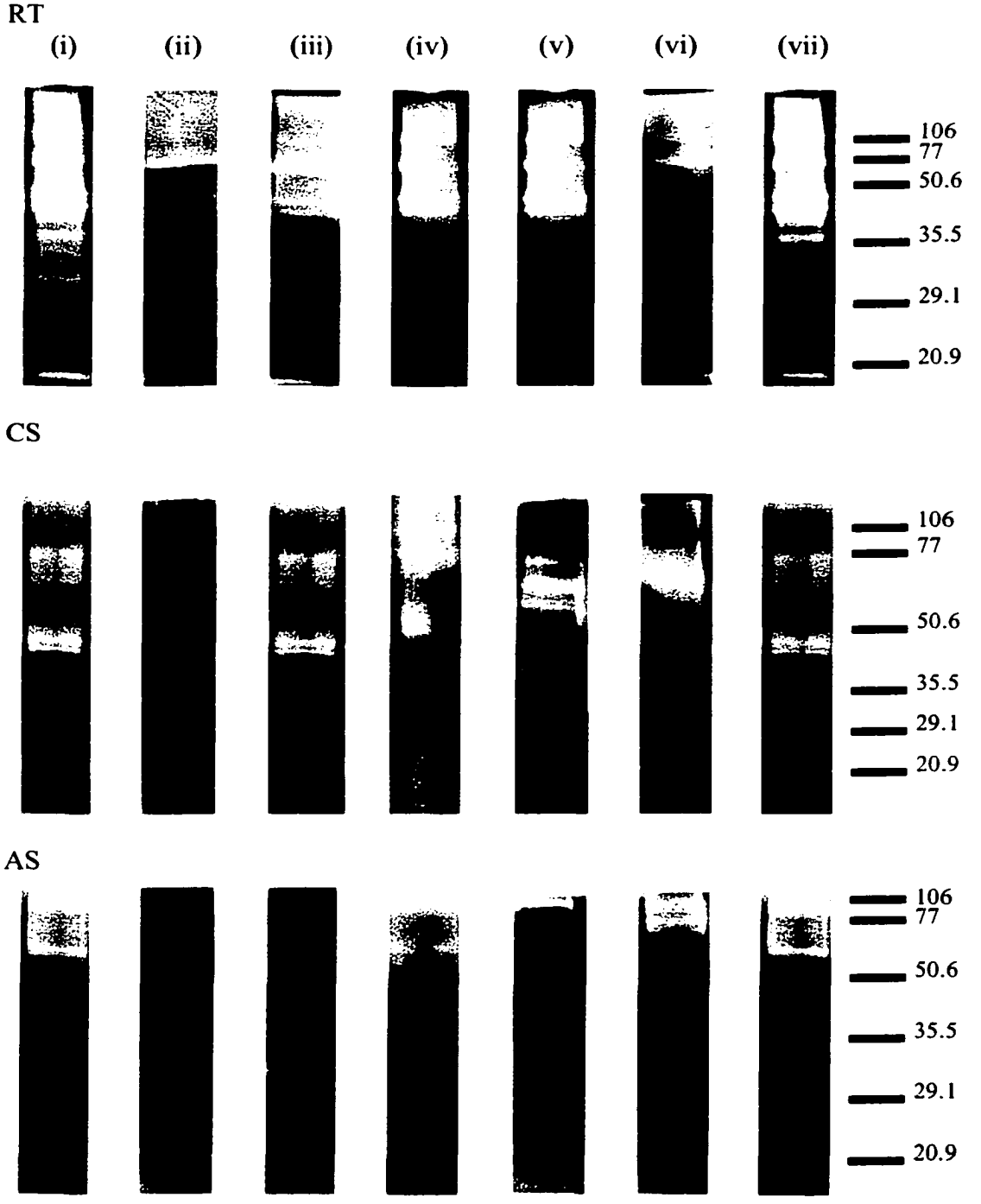
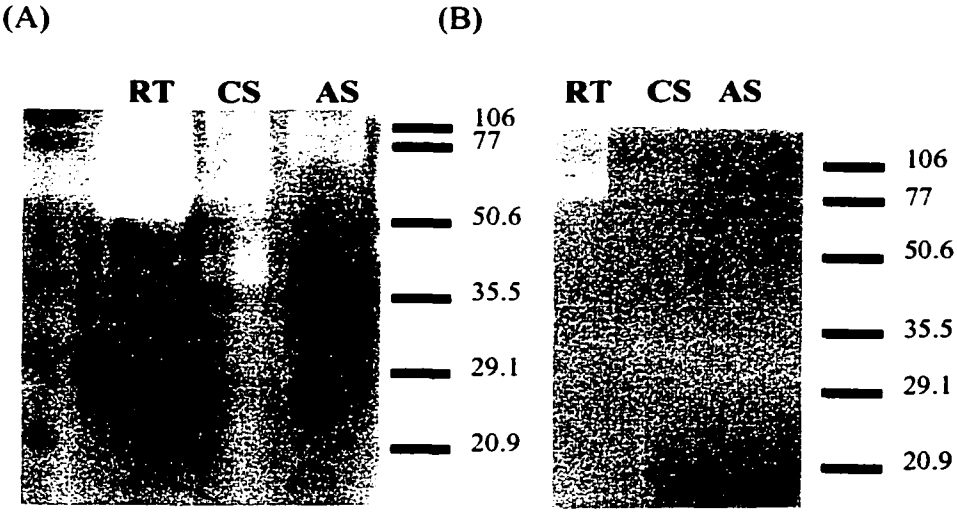


Figure 2.4: Protease zymography gel of protease inhibition of mucus from three seawater-reared salmonid species. (A) Protease activity prior to addition of inhibitors (B) protease activity after 19 h incubation with aprotinin (2 µg/mL) and *o*-phenanthroline (2 mM). Representative samples of (RT) rainbow trout, (CS) coho salmon, (AS) Atlantic salmon. Molecular weights (kDa) are along the right hand side of the gel. Each lane contains 3 µg of mucous protein. Representative samples expressed here were chosen based on the most common protease banding patterns observed for each species.



mucus protease activity was observed in incubations with leupeptin, pepstatin, iodoacetamide or EDTA. Mucous proteases were mostly inhibited using a combination of aprotinin and *o*-phenanthroline (Figure 2.4).

Seawater-reared Atlantic salmon mucous proteases above 80 kDa were eliminated by addition of aprotinin to the incubation buffer (Figure 2.3 lane ii). Two distinct bands remained, one at 45-47 kDa and another at 55-60 kDa (Figure 2.3 lane ii). Addition of leupeptin decreased the intensity of the 45-47 kDa band (Figure 2.3 lane iii). *o*-Phenanthroline eliminated bands at 45-47 and 55-60 kDa when incubated with SWR Atlantic salmon mucus (Figure 2.3 lane vi). No inhibition was observed when EDTA, iodoacetamide or pepstatin were added to the incubation buffer. Similar to SWR coho salmon, mucous proteases of SWR Atlantic salmon were mostly inhibited using a combination of aprotinin and *o*-phenanthroline (Figure 2.4).

Mucous lysozyme activities were significantly higher ($p<0.05$) in SWR rainbow trout compared to SWR Atlantic salmon and SWR coho salmon. Mean lysozyme activity in the mucus of SWR rainbow trout were 65.0 ± 13.6 U/mg protein, while in SWR coho salmon and SWR Atlantic salmon mucus, mean lysozyme activities were 19.1 ± 7.1 U/mg protein and 13.6 ± 5.1 U/mg protein, respectively (Table 2 I). Plasma lysozyme levels were also significantly higher in SWR rainbow trout compared to SWR coho and SWR Atlantic salmon ($p<0.01$). Seawater-reared rainbow trout plasma lysozyme levels were 195.8 ± 37.8 U/mg protein, while SWR coho and SWR Atlantic salmon plasma lysozyme levels were much closer to mucous lysozyme activities at 25.7 ± 10.4 U/mg protein and 34.4 ± 8.4 U/mg protein, respectively (Table 2 I).

There were no significant differences in mucous lysozyme levels between FWR species. However, Atlantic salmon and coho salmon had much higher mean specific mucous lysozyme activities when reared in freshwater than in seawater. There was a great deal of variation within individuals of both FWR species: some fish had lysozyme activities below the detectable limit (Atlantic salmon, n=3, coho salmon, n=4) while others had very high specific activities in the range of 200-300 U/mg protein. Despite the large variation in mucous lysozyme activities, Atlantic salmon ($p<0.05$) had significantly higher lysozyme activities in the mucus of FWR fish than SWR fish. Rainbow trout and coho salmon mucous lysozyme levels were not significantly different between FWR and SWR fish. Interestingly, there was no significant difference in plasma lysozyme activity between FWR and SWR fish (Table 2 I). Mean plasma lysozyme levels in freshwater were 256.7 ± 36.0 U/mg protein, 33.3 ± 26.2 U/mg protein and 14.6 ± 8.3 U/mg protein for rainbow trout, coho and Atlantic salmon, respectively (Table 2 I).

There were no significant differences in mucous alkaline phosphatase levels between the three species in seawater. There were very low specific activities of alkaline phosphatase in SWR rainbow trout (1.04 ± 0.30 U/mg protein), SWR coho salmon (0.75 ± 0.12 U/mg protein) and SWR Atlantic salmon mucus (0.45 ± 0.18 U/mg protein)(Table 2 I). All three species had significantly higher mucous alkaline phosphatase levels in SWR fish as compared to FWR fish. Most fish of all three FWR species had undetectable limits of this enzyme. Only three of ten FWR rainbow trout, two of ten FWR Atlantic salmon and two of ten FWR coho salmon showed detectable levels of alkaline phosphatase activity (Table 2 I).

2.5 Discussion

The skin mucous layer and epidermis are important in fish immunity because they are the first sites of interaction between the host and potential pathogens. Within these layers are many enzymes and antimicrobial proteins which are thought to be involved in innate immunity of the fish. Profiles of proteins and proteases in the mucus are important as their presence, or absence, may assist in resistance to particular pathogens. Differences in activities of antimicrobial enzymes, such as lysozyme, and how they relate to the structure of skin mucus and epidermal layers may also relate to the differences observed in disease resistance.

All three SWR species showed similar plasma protein and hematocrit levels. The physiological resting ranges overlap in these three species [168] suggesting that they were all in similar physiological condition during the experiment. Differences between species were, however, observed in the mucus. Using protein bands that consistently had high intensities across species (following silver stain) as references for comparison, mucous protein profiles in general were more similar between SWR coho salmon and SWR rainbow trout than SWR Atlantic salmon. Running samples side by side on the same gel demonstrated that the mucus of SWR rainbow trout and SWR coho salmon both contained several LMW protein bands of high intensity that were not present in SWR Atlantic salmon. Several of the medium and high range MW protein bands were also of similar intensity between SWR rainbow trout and SWR coho salmon but dissimilar to SWR Atlantic salmon. It is possible that some of these proteins represent antibacterial peptides.

Antibacterial peptides and proteins are very important in prevention of bacterial colonization. Several antibacterial proteins have been isolated from the mucosal epithelia of fish showing strong pore forming capacities in the bacterial cell membrane [103-106]. Antibacterial proteins with a molecular weight of 30, 31 and 70 kDa have been isolated from the common carp (*Cyprinus carpio*) [105] and catfish (*Ictalurus punctatus*) [106]. All three species in this study exhibited strong banding in these size ranges as well. Antibacterial peptides have previously been isolated from salmonids such as coho salmon [180] and rainbow trout [91]. A 65 kDa mucous protein isolated from FWR rainbow trout has exhibited the ability to form ion channels and lyse bacteria, suggesting a role in limiting microorganism colonization [104]. Interestingly, all three species in this investigation showed a mucous protein of similar intensity in this size range as determined by SDS-PAGE analysis.

The azocasein method of protease detection provides information on total protease activity while zymography allows us to observe the molecular weights and relative abundances of different proteases present [87]. Differences were observed in the specificity of mucous proteases for the substrates, gelatin (in the zymography gels) and azocasein. Pepstatin inhibited acid proteases in SWR Atlantic salmon mucus (ca. 20 %) from hydrolyzing azocasein in 3 out of 10 samples; however, there was no inhibition of any proteases hydrolizing gelatin. The acid proteases may more easily hydrolyze azocasein as compared to gelatin. The addition of pepstatin therefore resulted in a measureable inhibition of protease activity in the presence of azocasein substrate. The acid proteases may not have been active under conditions

necessary for zymography analysis (denatured by SDS) and thus inhibition could not be observed. For these reasons, it still remains uncertain as to whether SWR Atlantic salmon mucus contains acid (aspartic) proteases. Seawater-reared coho salmon mucous protease hydrolysis of azocasein was significantly inhibited with EDTA, but gelatin hydrolysis was not. In this case, EDTA may not have been able to properly remove or chelate Ca^{++} from the zymography gels (Ca^{++} binding with sulphate ions) and therefore was unable to inhibit calcium-dependant proteases in protease zymography. Dodecyl sulfate ions may bind to the Ca^{++} interfering with EDTA activity.

Proteases in the mucus of all three SWR species showed a high degree of inhibition in the presence of aprotinin, an inhibitor of serine proteases, and *o*-phenanthroline, an inhibitor of metalloproteases in both zymography and azocasein assays. Leupeptin, another serine protease as well as a thiol inhibitor, also inhibited proteases in the mucus of all three SWR species (azocasein assay), but to a lesser degree than aprotinin and *o*-phenanthroline. Using the azocasein assay, SWR Atlantic salmon and SWR rainbow trout mucous proteases appeared to consist mainly of serine (70-80%) or aprotinin inhibited proteases and metallo- (20-25%) or *o*-phenanthroline inhibited proteases. Protease inhibition using the azocasein assay did not add up to 100 % for the three species studied. This is due to the fact that many of the inhibitors used inhibit similar proteases. Aprotinin and leupeptin, for instance, both inhibit serine proteases. Proteases present in the mucus may also fall under more than one category: calcium-dependent serine proteases, calcium-dependent metalloproteases, serine activated metalloproteases, etc.

The results of protease zymography for both SWR Atlantic salmon and SWR rainbow trout were consistent with the results from azocasein analysis. Inhibition by aprotinin of protease bands at 18, 45 and >80 kDa in the mucus of SWR rainbow trout suggests that these bands are serine proteases. The 33 and 48-73 kDa protease bands in these species were inhibited by both aprotinin and *o*-phenanthroline suggesting that these may be either metalloproteases that are activated by serine proteases or possibly metalloproteases that are inhibited by aprotinin. Inflammatory serine proteases in humans have been shown to activate latent matrix metalloproteases through the removal of the amino terminal propeptide [136]. Proteolytic modifications can occur at other sites of proteins and proteases as well, since most proteases have more than one potential substrate [137].

In SWR Atlantic salmon mucus, the inhibition of a 45-47 and a 55-60 kDa protease bands by *o*-phenanthroline suggests the presence of two metalloproteases. Firth *et al.* also showed inhibition of a 60 kDa protease, in the mucus of Atlantic salmon controls, with *o*-phenanthroline [107]. Similar to SWR rainbow trout, all SWR Atlantic salmon mucous protease bands > 80 kDa were at least partially inhibited following incubation with aprotinin, suggesting that they are serine proteases. This is again consistent with Firth *et al.* who demonstrated inhibition of a 80 kDa protease, in the mucous of Atlantic salmon controls, with aprotinin [107].

Seawater-reared coho salmon mucous protease contained more metalloproteases than the other two SWR species (ca. 35% inhibition with *o*-phenanthroline using the azocasein assay). In comparison to SWR rainbow trout

and SWR Atlantic salmon, proteolytic activity in SWR coho salmon mucus appears to be made up of a higher proportion of metallo- (35%) to serine proteases (65-80%) using the azocasein assay. In addition, SWR coho salmon was the only species in which protease activity was inhibited by EDTA (ca. 60% using the azocasein assay), a known calcium chelator, although there was no observed reduction in protease band intensity or number in zymograms following incubation with EDTA. Calcium plays a stabilizing role in protease tertiary structure. In the denaturation/renaturation process during protease zymography the detergents Triton X-100/SDS may interact with and stabilize the calcium binding domains. Alternatively, calcium-dependent proteases may not be detectable by protease zymography. Either way this would not permit observations on EDTA inhibition in the gels. Bands of 65 and 85 kDa in coho salmon mucus were inhibited by *o*-phenanthroline (metalloproteases) and two bands at 42-45 kDa and a band at 65 kDa were inhibited by both aprotinin and *o*-phenanthroline. The presence of aprotinin- and *o*-phenanthroline-inhibited proteases in coho salmon mucus was similar to protease bands in rainbow trout, which may mean that these are closely related proteases.

Seawater-reared rainbow trout and SWR coho salmon mucus showed the presence of a LMW serine protease band (18 kDa) that was not observed in SWR Atlantic salmon mucus. Trypsin is a LMW serine protease (18 kDa), normally produced by the acinar cells of the pancreas [108], but has also previously been isolated from the mucus of rainbow trout [110]. It is possible that the 18 kDa protease observed in SWR rainbow trout and SWR coho salmon mucus may also be trypsin. Trypsin has not previously been described in coho salmon mucus. Trypsin is normally a digestive

enzyme but studies have shown trypsin, isolated from rainbow trout mucus, to have microbiocidal activity [110].

The proteases present in fish mucus may have a variety of functions. Metalloproteases are important in wound healing [124] and inflammatory responses [123], as are serine proteases [121,122] which are also involved in bacterial digestion [119]. These proteases may diffuse into the mucus through epithelial cell damage, but may also have functions in the defense against potential pathogens. Aspartic proteases, as found in the mucous samples of three out of ten SWR Atlantic salmon, are usually found in lysosomes (eg. Cathepsin D) and have been linked to antigen processing in other animals [181,182]. Aranishi and Nakane also observed cathepsins with antibacterial properties in the epidermis of eels [132,134]. It is possible that some acid protease activity results from the release of lysosomes in the normal turnover of epidermal cells. In general, mucous proteases may not be as detectable across the mucous surface of the entire animal as they are at localized sites.

Lysozyme, an enzyme that cleaves the glycosidic bonds of the peptidoglycan layer of bacteria, was present in the mucus of all three species. Rainbow trout had a very high activity of lysozyme in the mucus as compared to the other two species when fish were maintained in seawater. The lysozyme specific activity in SWR rainbow trout mucus was lower than the levels found in the plasma. Plasma lysozyme activity was significantly higher in SWR rainbow trout (ca. 5-7 X) as compared to the other two SWR species. Both SWR Atlantic salmon and SWR coho salmon had very low mucous and plasma lysozyme levels. Lysozyme specific activities were similar in the mucus and plasma of both SWR coho salmon and SWR

Atlantic salmon. Lysozyme levels in kidneys of FWR Atlantic salmon have also been previously reported as being approximately 15 fold lower than FWR rainbow trout [183].

While it is uncertain how enzymes such as lysozyme, alkaline phosphatase and proteases arrive in the mucus, there are two debatable hypotheses: (1) the enzymes are secreted into the mucous layer from epidermal cells or (2) they are transported from the serum to target cells and tissues and diffuse into the mucous layer. Modes of entry into the mucous layer have been argued for several other immune factors as well. Smith and Ramos reported free hemoglobin release from fish erythrocytes into surrounding tissues, which then infiltrated into the mucus from the skin [184]. Brokken *et al.* suggested that peroxidase entered the mucus of the common carp also through erythrocyte release [185]. In contrast, Iger and Abraham observed a large increase in the production of alkaline phosphatase in pavement and mucous cells in the epidermis of carp following wounding [101]. They also observed phagocytosis occurring in mucous and pavement cells for 12 h after wounding before leucocytic phagocytosis began [101], suggesting that mucous cells may play an important role in early phagocytosis and prevention of bacterial colonization. Buchman also demonstrated partial correlation between mucous cell density and resistance to the monogenean parasite, *Gyrodactylus derjavini,* in rainbow trout [186]. Mucous cells may therefore provide a significant source of alkaline phosphatase, lysozyme and proteases in the mucous layer. Further research using histochemical and immunohistochemical analysis would be useful in localizing the sources of these enzymes.

Although all three SWR species showed similar levels of plasma protein and hematocrit, Atlantic salmon and coho salmon had undergone a physiological change that the rainbow trout had not, namely smoltification. For this reason some parameters observed in SWR fish were also measured for all three species in FW. Fish reared in FW had not undergone smoltification. There did not appear to be any significant differences in plasma and mucous lysozyme, hematocrit, plasma protein or mucous protein profiles between rainbow trout in FW and rainbow trout following adaptation to SW. However, there did appear to be differences in mucous lysozyme levels in both Atlantic salmon and coho salmon in FW as compared to SW. This occurred without a significant change in plasma lysozyme activity. All three species also showed significantly lower mucous protease and alkaline phosphatase activities in FW as compared to SW. It has not been determined whether there are different rates of mucus turnover between SWR and FWR fish and whether this might have an impact on mucous enzyme levels. Changes in enzyme activity could be due to changes in expression and/or cell turnover. The differences between activities of these enzymes in FW and SW may represent part of the evolutionary adaptation permitting these fish to live within a saltwater environment. Greater levels of lysozyme in the mucus may provide better protection against particular bacterial pathogens in FW (eg. *Aeromonas salmonicida*), while the presence of alkaline phosphatase and proteases may afford better protection against particular viral (eg. infectious salmon anemia) and invertebrate pathogens in SW (eg. sea lice). Due to the sample size and specific conditions of this study further investigation of this change in enzyme composition is warranted.

The differences in disease resistance among salmonid species is easily comprehended when observing differences in immune factors of fish from the same subfamily with close phylogenetic relationships [187]. For instance, coho salmon have exhibited a well-developed inflammatory response to sea lice infections, which are not observed in Atlantic and chinook salmon [144]. Lower levels of blood α–2 macroglobulin in rainbow trout as compared to brook char (*Salvelinus fontinalus*) have been linked to a greater susceptibility of rainbow trout to *Cryptobia salmositica* in comparison to brook char [141]. The differences that the species in this study displayed in their mucous layer may be directly linked to their immune response to potential pathogens. This association is based on the fact that the mucous layer is the first line of defense against potential pathogens in the aquatic environment. All three species exhibit bacteriocidal enzymes and proteins within these layers. Rainbow trout, however, exhibit higher protease and lysozyme activities than the other two species. This may help explain the hardiness of rainbow trout as a species that has been readily introduced and grown as a cultured species throughout the world [2]. Coho salmon had very low lysozyme activity in comparison to rainbow trout. Atlantic salmon, on the other hand had the lowest activities of lysozyme and proteases, in comparison to the other two seawater salmonids. Atlantic salmon mucus also lacked LMW proteins and particular protease bands observed in rainbow trout and coho salmon. The bacteriocidal properties of mucous lysozyme and particular mucous proteins and proteases may have a direct effect on the innate immune response of these species toward certain pathogens. The differences in mucous and plasma biochemical parameters of rainbow trout, coho salmon and Atlantic salmon,

therefore, reflect the variations these species exhibit in disease resistance and susceptibility. Further examination and comparison of proteins and enzymes in mucus could lead to more definitive roles for these various factors.

3.0 PLASMA AND MUCOSAL DIFFERENCES IN RAINBOW TROUT, COHO AND ATLANTIC SALMON, EXPERIMENTALLY INFECTED WITH *LEPEOPHTHEIRUS SALMONIS*

3.1 Abstract

Physiological, immunological and biochemical parameters of blood and mucus were compared in three salmonid species (rainbow trout [*Oncorhynchus mykiss*], Atlantic [*Salmo salar*] and coho salmon [*Oncorhynchus kisutch*]) following infection with sea lice (*Lepeophtheirus salmonis).* The three salmonid species were cohabited in order to achieve similar initial infection conditions. Initial lice intensity was not significantly different between species when corrected for size of hosts. However, the lice density was effectively reduced on coho salmon within 7 – 14 days while lice persisted at roughly equal numbers on Atlantic salmon and rainbow trout. Lice also matured more slowly through these early stages on coho salmon than on the other 2 species. There was a difference in maturation of lice on rainbow trout and Atlantic salmon with slower development of the lice on rainbow trout. At 21 dpi there was an increase in the number of lice on coho salmon. Respiratory burst and phagocytosis assays were run on head kidney macrophages to assess immunological status of the fish. Macrophages from infected Atlantic salmon had a significantly lower respiratory burst ($p < 0.05$) and phagocytic capacity ($p< 0.05$) at 14 and 21 dpi, while infected rainbow trout macrophages had significantly lower respiratory burst and phagocytic capacities ($p < 0.05$) at 21 dpi, compared to uninfected fish at the same time. The slower development of lice, coupled with delayed suppression of immune parameters, suggests

that rainbow trout are slightly more resistant to lice than Atlantic salmon. Lysozyme, alkaline phosphatase and protease activities were examined in the mucus of each fish. Infected Atlantic salmon and rainbow trout showed immediate increases in mucous lysozyme activites at 1 dpi ($p< 0.05$), which decreased over the course of the rest of the study. Mucous lysozyme levels of infected rainbow trout, however, remained significantly higher than controls over the entire period. Coho salmon lysozyme activities did not increase in infected fish until 21 dpi ($p < 0.05$), possibly as part of a response to the presence of pre-adult sea lice at that time. Mucous alkaline phosphatase levels were significantly higher in infected Atlantic salmon as compared to controls at 3 and 21 dpi ($p < 0.05$) but were not different between conditions in the other two species. Low molecular weight proteases increased in infected rainbow trout and Atlantic salmon on the last days of the study (14, 21 dpi), possibly due to the increase in lice biomass. Plasma cortisol, glucose, electrolyte, protein and hematocrit levels all remained within physiological limits for each species, with no overall differences occurring between infected and control fish. The lack of differences in blood physiology between susceptible and resistant species suggests that these parameters are not associated with the difference in susceptibility between coho salmon and Atlantic salmon or rainbow trout. However, the differences in mucous enzyme activities between species suggest that resistance of coho salmon to lice may be mediated by factors in the mucus.

3.2 Introduction

In recent years, epizootic infections of sea lice, *Lepeophtheirus salmonis,* a common marine ectoparasitic copepod of salmonids, have resulted in heavy losses and millions of dollars worth of damage to aquaculture throughout the Northern Hemisphere [7,12]. Sea lice feed on mucus, skin and blood of salmonids causing osmoregulatory failure and death in extreme cases [36,37]. Attempts at controlling this parasite with chemotherapeutants have yet to produce long term protection for the fish without also showing toxicity towards fish or extended tissue residence times. It is our expectation that through a better understanding of host-parasite relationships we may develop more effective control measures for the future.

Host species differences in susceptibility towards sea lice infection has previously been reported [35,144]. While sea trout, *Salmo trutta*, can be observed to carry high infection levels [35], Johnson and Albright observed a resistance towards lice infection in coho salmon, *Oncorhynchus kisutch* [144]. However the mechanism for coho resistance remains undetermined. Atlantic salmon, a very important commercial species on both coasts of Canada, is extremely susceptible and often exhibits lesions and decreased immune function due to lice infection [36,39,68]. Atlantic salmon have shown changes in blood physiology such as cortisol, hematocrit and protein levels [39] and mucus biochemistry, such as alkaline phosphatase and protease activities [87], during lice infection. Lice infection has also induced decreased macrophage function [68] and obvious epidermal changes such as mucous cell exhaustion [36] in infected Atlantic salmon.

A cohabitation study was conducted to directly compare the differences in susceptibility to *L. salmonis* infection of different host species and to determine physiological and immunological changes in blood and biochemical changes in the skin mucus between infected and control fish of different species. The differences in the response of resistant species and that of relatively susceptible hosts could give insight into the mechanisms by which coho salmon reject *L. salmonis* shortly after infection.

3.3 Materials and methods

3.3.1 Fish and their maintenance

The fish used for this study were yearling (S1) Atlantic salmon, seawater-adapted rainbow trout, and coho salmon. Atlantic salmon (St. John River strain) and rainbow trout (Kamloops strain) were obtained from the Department of Fisheries and Oceans (DFO) certified hatcheries in Pictou, NS, and Murray River, PEI, respectively. Coho salmon were obtained through the research facilities of Aqua Health Limited, Charlottetown, PEI, originally from DFO's Big Qualicum hatchery, Vancouver, BC. On arrival, fish of each species were placed in separate 1500 L tanks and smolted by gradually increasing the salinity with artificial seawater (Instant Ocean,® Aquarium Systems, Mentor, OH) over a two week period. The fish were maintained in 30 ± 2 ppt (salinity) at 10 ± 1°C for at least two weeks, prior to cohabitation. After the above acclimation, 160 fish of each species were randomly distributed to two different groups (controls and tests) in four different tanks (two tanks for control fish and two tanks for

test fish). There were 40 fish of each species cohabited in each tank (1500 L) and re-acclimated for another two-week period. Dissolved oxygen levels were constantly monitored and maintained at ca. 8 ppm. The photoperiod was maintained at 14 h light: 10 h dark and fish were fed (same mixture as in Chapter 2) twice daily (0900 and 1300) to satiation. At the onset of the experiment, Atlantic salmon and rainbow trout had average weights (± SD) of 113.1 ± 22.8 g and 187.5 ± 40.2 g respectively, while coho salmon weighed an average 39.5 ± 9.6 g. There were 160 fish for each species used in this experiment. Densities of each tank were chosen (ca. 10 kg/m^3) to reflect aquaculture density standards (10-15 kg/m^3, for Atlantic salmon) [2], while at the same time allowing for growth of the fish over the course of the experiment.

In an attempt to compensate for stocking density differences between the beginning and end of the experiment, an excess number of fish (10 for each species) were added to the tanks at the beginning of the study that would not be used in sampling. Therefore, the number of fish withdrawn for sampling from each tank would be partially compensated for, in terms of density, by the growth of the fish remaining.

3.3.2 Sea lice culture and laboratory infection

The infective copepodids were grown in the laboratory and used for infecting fish following the methods described by Mustafa *et al.* [68]. Ovigerous sea lice were collected from Atlantic salmon in aquaculture sites in the lower Bay of Fundy and transported to the laboratory at the Atlantic Veterinary College. Lice were kept in 4-5 L

buckets on ice with constant aeration during their transport. Egg strings from these lice were removed and maintained in large plastic buckets (10 L) with sieved aerated seawater (27 ppt) collected from the same locality of the sea lice collection and at approximately the same temperature (10 ± 1^0C). The culture was maintained for 10 days by which time the optimum number of eggs had hatched and developed into the infective copepodid stage (approx. 75% copepodid and 25% nauplius).

Approximately 100-150 infective copepodids/fish were added to the designated test tanks under reduced water volume (ca. 40-60 kg/m^3) and flow for 12 h. Supplemental oxygen was added to maintain tanks at ca. 8 ppm, and 70 μm mesh was placed over outflows to prevent loss of copepodids. Control tanks were subjected to the same reduced volume and flow as in test tanks except without addition of sea lice.

3.3.3 Sampling

On days 0 (prior to infection), 1, 3, 7, 14, and 21 post-infection, 5 fish of each species from each tank were randomly sampled. Fish were kept off feed for 24 h prior to sampling to reduce feeding stress and minimize debris at the bottom of the tank. The tank order was chosen randomly every day to prevent effects between tanks from sampling order. Each fish was placed into individual 10 L buckets containing a lethal dose of anesthetic (200 mg/L; MS-222). Length, breadth, and weight were measured immediately. Breadth was measured in the same manner as in Section 2.3.2. Each fish was placed in individual bags containing 10 mL of 100 mM ammonium bicarbonate (pH 7.8) buffer for the collection of mucous samples, using the same method laid out in

Section 2.3.2.1.

Blood was extracted from the caudal vein (ca. 3-5 mL from rainbow trout and Atlantic salmon, <1 mL from coho salmon), less than 3 min after death, with 1 or 5 cc syringes and 16 or 21 G needles flushed with heparin (10 i.u./mL) (SIGMA). Head kidneys from each fish were then removed aseptically for macrophage assays. Finally, fish were individually bagged and sea lice were counted and staged from each. A minimum of 30 lice were staged for each species on each day to provide a sufficient sample size unless there were not enough lice available on the fish (see coho salmon results). These counts were also supplemented by net and bucket counts from the corresponding fish. Contents of each bucket were passed through individual 70μm Nytex mesh-nets.

3.3.4 Analysis of blood samples

Blood samples were spun for 5 min at 4000 x *g* to separate plasma using an IEC Model CL Centrifuge. Plasma was decanted and stored at -80^0C until further use. Blood samples obtained from the sampled fish were used to determine plasma levels of cortisol (Coat-A-Count ® RIA, Inter Medico, Markham, ON), glucose (colorimetric glucose oxidase method), sodium, potassium, and chloride (indirect ion selective electrode [ISE] method) [39]. Both glucose and electrolyte levels were measured on the Hitachi 911 autoanalyzer (Hitachi Corporation, Missasauga, ON). The detection limit for the Coat-A-Count ® RIA assay is 2 µg/L and the kit's cortisol antiserum is extremely specific for cortisol (Coat-A-Count ® RIA, Inter Medico). For most compounds cross

reactivity is less than 1%. Although some crossreactivity occurs with other steroids, their physiological concentrations are low in comparison to cortisol and therefore would not interfere significantly with this method's cortisol measurements (as per manufacturer's guidelines). Hematocrit and blood protein levels were determined as in Section 2.3.3 [39].

3.3.5 Analysis of kidney samples for macrophage function

Isolation of head kidney macrophages and detection of their functions (respiratory burst activity and phagocytosis) were performed following the methods described by Mustafa *et al.* [68].

The surfaces of euthanized fish were sterilized with 70% ethanol prior to exposing the anterior kidney through peritoneal cavity incision and viscera removal. Head kidneys were aseptically removed (2-3 min after death) and placed in 2 mL of suspension buffer (L-15 medium containing 2% fetal calf serum (FCS), Appendix A). Tissues were macerated with sterile sieve and pestle and suspended in 2 mL of suspension buffer prior to centrifugation at 400 x *g* for 15 min. Cells were re-suspended in 1 mL buffer and counted using a hemocytometer. Trypan blue exclusion test was used to determine viability. Trypan blue (SIGMA) was added to the suspension (0.5 mL of 0.4% Trypan blue + 0.3 mL of L-15 + 0.2 mL of cell suspension, giving a dilution factor of 5) and non-blue viable cells were counted. The suspension was placed on a hemocytometer and viable cells counted in four large squares. Number of viable cells counted per volume was used to calculate the cells in suspension (# counted x dilution factor (5) x 10^4)/mL.

The suspension was then centrifuged again at 400 x *g* for 15 min and the pellet re-suspended in L-15 with 0.1% FCS (1 mL); the cell concentration was adjusted to approximately 10^6 viable cells/mL. This cell suspension was then aliquotted (100 µL per well) onto double etched microscope slides and pre-incubated for 2 h at 18^0C in a plastic container with 2 damp sheets of paper towel covering the bottom. The container was closed to maintain moisture within.

3.3.5.1 Respiratory burst assay

Following pre-incubation, slides were washed with ca. 5 mL of phosphate buffered saline (PBS, Appendix A). 100 µL of nitroblue tetrazolium (NBT)/phorbol myristate acetate (PMA) solution (NBT dissolved in L-15 medium with 0.1% FCS at 1 mg/mL + PMA at 1 µg/mL) was added to each well and incubated for 45 min. Slides were then washed again with ca. 5 mL of PBS and the proportion of active cells (stained purple) to non-active cells was determined. First the double etched slide was placed on a light microscope stage and the stage maneuvered until the objective lens was situated over the top left corner of one of the wells. Next, by moving the stage left to right and top to bottom, the proportion of active: non-active cells was systematically determined in each well using the first 100 cells encountered in the field of view. This was carried out at 100 X total magnification and using a tally counter.

3.3.5.2 Phagocytosis

Following pre-incubation in the moist container, 100 µL of formalin/heat-killed bacteria, *Yersinia ruckeri,* (ca. 10^7 cells/mL) were added to each well and incubated for a further 2 hours. Slides were then washed with ca. 5 mL PBS, fixed with methanol and stained with Diff-Quick ® (Leukostat Stain kit, Fisher) [68]. The phagocytosis assay determined the proportion of macrophage cells that were able to take up five or more formalin/heat-killed bacteria. The same counting method as that described in Section 3.3.5.1 was followed for this procedure, with the exception that the proportion of cells that had taken up >5 bacteria were compared with those that had taken up <5 bacteria.

For both the respiratory burst and phagocytosis assays a macrophage-enriched environment was created through the washing step. Most other cells in the population were removed and the macrophage cells remained due to their adherance to the glass slide.

3.3.6 Analysis of mucous samples

Mucous samples obtained from fish were used to determine mucous alkaline phosphatase (alkaline phosphatase assay), lysozyme (lysozyme turbidometric assay) and protease activities (azocasein hydrolysis and zymography) as described in Section 2.3.4. All fish samples were analyzed using protease zymography and representative samples chosen from the samples that exhibited the most common mucous

protease patterns for each species.

3.3.6.1 Protease inhibition procedure

Testing for protease inhibitory activity in mucus was carried out by examining whether mucus could inhibit bovine trypsin (ICN Biomedicals, Aurora, OH) activity as follows: 10 µg of bovine trypsin prepared in 1 mL of 1mM HCl was added (5 µL) to mucous sample + azocasein substrate and incubated, as per azocasein assay procedure (Section 2.3.4.4) . The reaction was stopped after 19 h by the addition of 20% TCA (Fisher) (50 µL) and centrifuged at 17,000 x *g* for 5 min. The supernatant (100 µL) was then transferred to wells containing 0.5 M NaOH (100 µL) and read at 450 nm by a Thermomax microplate reader (Molecular Devices).

Negative controls were included to subtract background OD of the buffer and substrate. The negative control contained 5 µL of trypsin (as prepared above), 45 µL of buffer and 50 µL of substrate.

3.3.7 Statistical analysis

The same software as in Section 2.3.6 was used for statistical analysis. To test for tank effect, 1-way Analysis of Variance (ANOVA) was used to compare test tank 1 with test tank 2 and control tank 1 with control tank 2. No significant differences were observed between tanks and data was pooled into two groups (control and infected). Two-way ANOVA was used to compare infected and control fish over time within a

species. Three-way ANOVA was used to test for significant differences among condition (infected and control fish), species and time. All ANOVAs were followed by multiple comparison Tukey's tests (Bartlett statistic corrected). The same statistical model used in Section 2.3.6 was used in the studies described in this chapter, with the exception of using species, day and treatment as parameters. Differences were considered significant when $p<0.05$. Spearman rank order correlations were used to determine whether there were correlations between lice counts and enzyme activities. The same correlation statistics were employed to observe whether or not there was a correlation between any of the following: lysozyme activity, alkaline phosphatase activity, protease activity and cortisol. All data was checked for normality and non-normal data was transformed using ($\sqrt{x}$) or log (x+1) transformations. All values shown, in this investigation, are mean ± standard error of means, except in the case of population weights where standard deviations were used.

Zymography measurements of protease activity were assessed as +/- through the presence or absence of bands. A positive response was designated when the series of low-molecular weight protease bands were present in fish skin mucus [74]. Results were analyzed using Chi-squared analysis. Increases in protease activity (or lack thereof) between controls and infected fish were compared.

In all cases, samples from the same fish were run, in triplicate, more than once on the same assays to ensure results were repeatable. In the case of zymography, all samples were run 3-4 times to ensure that the bands present were not artifactual.

3.4 Results

3.4.1 Lice counts and life stages

The total number of lice was significantly lower on coho salmon than the two other salmonid species on all days. There were no significant differences in lice load on any day between rainbow trout and Atlantic salmon (Figure 3.1). However, due to the significant size differential between the different fish species, length and breadth were used to calculate an estimate of available surface area for the sea lice (Length X Breadth X 2). When the number of lice per fish was corrected to account for the calculated available surface area for the lice, no significant differences in lice abundance were observed per square area in any of the three species at 1 and 3 dpi. Coho salmon had significantly lower lice/surface area than both rainbow trout and Atlantic salmon at 7, 14 and 21dpi (Figure 3.2). The number of lice on coho salmon decreased significantly from 1 to 14 dpi with only 3 lice found on a total of 10 fish by 14 dpi. At 21 dpi the mean number of lice increased to about 1 louse per fish on coho salmon. The number of lice decreased on rainbow trout and Atlantic salmon from 14 to 21 dpi. However, rainbow trout had significantly lower lice/surface area than Atlantic salmon at 14 and 21 dpi.

At 1 and 3 dpi, all sea lice found on infected fish were in the infective copepodid stage (n=99), with one exception; one pre-adult male was found on a coho salmon from an infected tank at 1 dpi. At 7 dpi the sea lice were 100% chalimus I stage larvae (n=20) on coho salmon (Figure 3.3). Rainbow trout had 60% chalimus I and 40% chalimus II stage larvae (n=30), while Atlantic salmon had 30% chalimus I and 70%

Figure 3.1: Mean (±SEM) intensity of *L. salmonis* infection on rainbow trout (-◆-), Atlantic (--■--) and coho salmon (--▲--) at various days post-infection (dpi). (a) Lice counts significantly lower than rainbow trout and Atlantic salmon, (b) Lice counts significantly higher than at 1, 3, 7 and 21 dpi within a species, (c) Lice counts significantly higher than at 3, 7 and 21 dpi within a species.

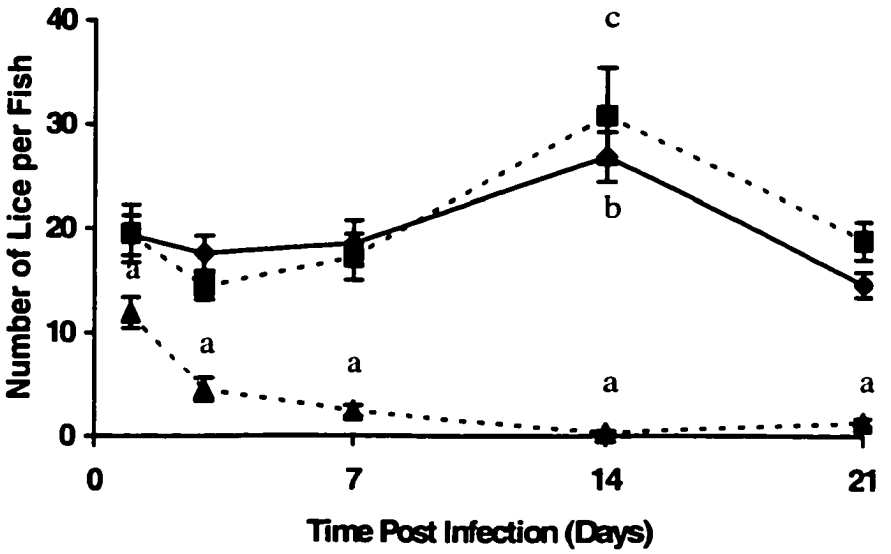


Figure 3.2: Mean (±SEM) intensity of *L. salmonis* infection on rainbow trout (-◆-), Atlantic (--■--) and coho salmon (--▲--) at various days post-infection corrected for surface area of fish available to louse (breadth X fork length X 2).
(a) Lice counts significantly lower than on Atlantic salmon for that particular day, (b) lice counts significantly lower than on rainbow trout on that particular day.

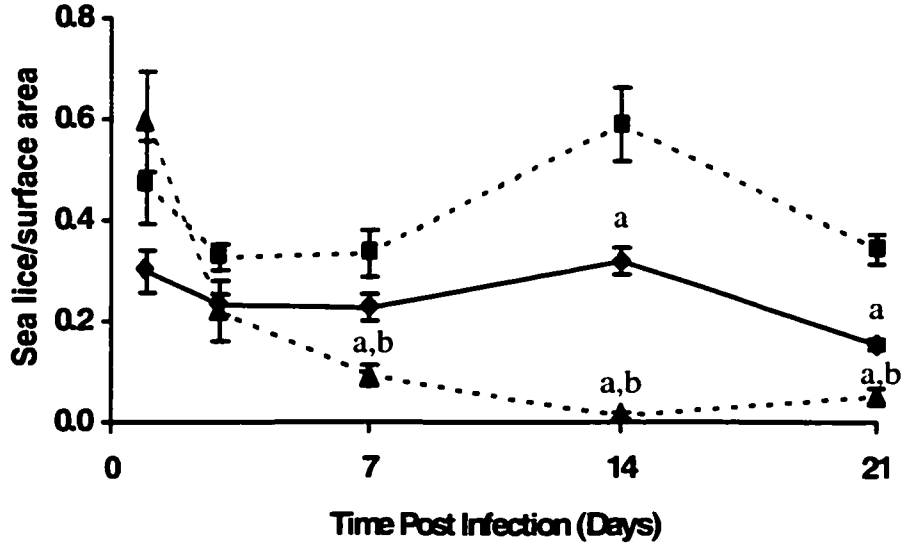
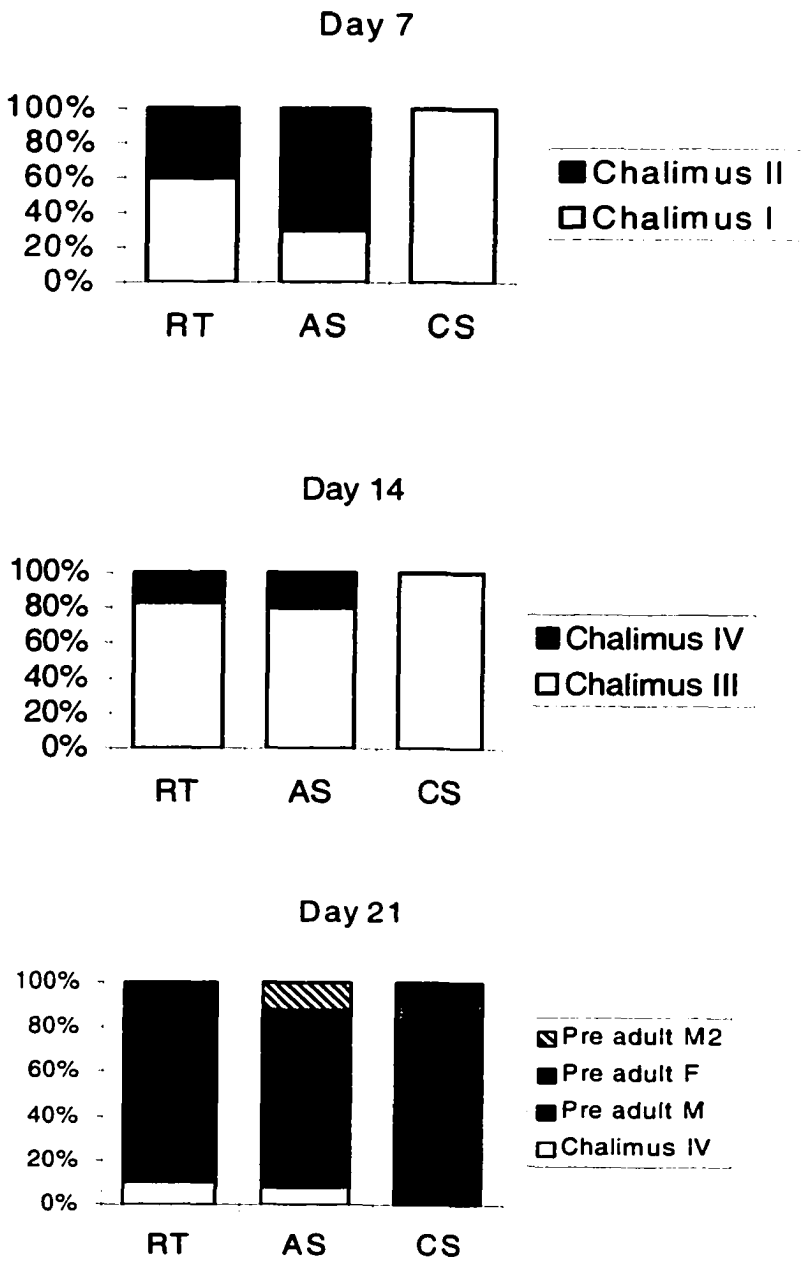


Figure 3.3: Life stages of *L. salmonis* on rainbow trout (RT), Atlantic (AS) and coho salmon (CS) on 7, 14 and 21 dpi. The number of lice staged for each day is as follows: n=50 for RT, n=30 for AS and n=20 for CS (except on the day when there were too few lice on the sampled CS; ie. 14 dpi, n=3 and 21 dpi, n=10).



chalimus II stage larvae (n=30) (Figure 3.3). By 14 and 21 dpi the species differences were less obvious. Lice were 80% chalimus III stage larvae on rainbow trout (n=50) and Atlantic salmon (n=30), and 100% on coho salmon (n=3) on day 14. At 21 dpi, lice were mostly pre-adults (ca. 90%) on all species and only male pre-adults (n=10) were found on coho salmon. There were still approximately 10% chalimus IV stage larvae on rainbow trout (n=50) and Atlantic salmon (n=30) (Figure 3.3).

3.4.2 Blood physiology

Due to small volumes of blood obtained for coho salmon not all of the physiological parameters could be recorded from the blood, hence the incomplete data obtained for coho salmon blood electrolytes, glucose and osmolality. Plasma sodium, potassium and chloride concentrations were not significantly different between infected and control fish or between Atlantic salmon and rainbow trout. Mean sodium concentrations for both Atlantic salmon and rainbow trout varied between 150-165 mmol/L over the course of the experiment (Table 3 IA,B). Mean concentrations of chloride were between 120-135 mmol/L in Atlantic salmon and 120-130 mmol/L in rainbow trout (Table 3 1A,B). Glucose concentrations and osmolality were also not significantly different between infected and control fish or between these two species. Mean glucose concentrations were between 2-8 mmol/L in both Atlantic salmon and rainbow trout, while mean osmolality concentrations were between 310-340 mosmol/L (Table 3 IA,B). Mean cortisol concentrations did not show any significant differences within species between infected and control fish, except in rainbow trout at 1 dpi. At 1

Table 3 I: Physiological parameters in the blood of three salmonid species over time, following *L. salmonis* infection. Data is presented by means (± SEM). (A) rainbow trout, (B) Atlantic salmon and (C) coho salmon. (c) indicates controls fish (n=10 on each sampling day), (i) indicates infected fish (n=10 on each sampling day).

A: Rainbow trout

Time Post Infection (Days)

Parameters		0	1	3	7	14	21
Hematocrit (%)	c	53.0±1.8	46.1±1.1	46.6±1.5	44.9±1.8	44.3±1.6	43.0±1.3
	i	49.2±1.5	49.4±1.7	45.6±1.6	45.8±1.1	44.7±1.3	44.6±0.9
Protein (g/100mL)	c	7.27±0.31	5.74±0.32	5.82±0.23	6.26±0.38	6.41±0.42	7.18±0.45
	i	6.95±0.28	6.30±0.37	6.10±0.27	6.06±0.26	6.55±0.32	6.49±0.31
Sodium (mmol/L)	c	161.8±3.2	157.2±1.2	158.6±1.2	162.8±0.7	157.3±1.1	160.1±0.9
	i	161.9±1.3	158.6±1.8	155.6±4.4	165.2±0.8	161.0±0.8	160.8±1.1
Potassium (mmol/L)	c	0.70±0.03	0.90±0.07	1.32±0.20	0.78±0.03	0.89±0.07	0.79±0.05
	i	0.81±0.03	0.96±0.10	1.16±0.22	0.83±0.05	0.80±0.04	0.65±0.02
Chloride (mmol/L)	c	125.7±3.8	125.9±1.2	124.9±1.6	125.9±0.7	121.6±0.9	122.0±1.1
	i	126.0±1.1	127.9±2.4	120.4±3.9	130.2±1.0	126.8±0.8	125.3±1.2
Glucose (mmol/L)	c	4.65±0.32	6.10±0.41	6.63±0.35	4.53±0.44	6.62±0.61	4.51±0.41
	i	4.44±0.37	5.32±0.48	6.52±0.42	5.28±0.36	7.30±0.58	6.54±0.67
Osmolality (mosm/L)	c	337.4±4.4	326.7±2.9	329.5±2.8	331.2 ±1.3	327.0±2.6	340.6±1.7
	i	336.7±2.1	328.1±4.0	329.1±8.9	332.2±2.4	332.8±1.2	342.2±3.0

B: Atlantic salmon

Time Post Infection (Days)

Parameters		0	1	3	7	14	21
Hematocrit (%)	c	58.9±2.4	48.25±1.1	57.6±1.3	46.8±1.6	50.1±1.2	47.6±1.4
	i	56.3±2.0	45.3±1.4	51.9±1.3	50.8 ± 1.2	47.6±0.9	46.6±1.5
Protein (g/100mL)	c	6.14±0.32	4.40±0.42	5.29±0.34	4.59±0.51	5.13±0.28	5.03±0.48
	i	4.50±0.43	3.85±0.40	4.65±0.30	5.01±0.27	4.75±0.33	5.01±0.18
Sodium (mmol/L)	c	158.5±3.4	156.6±0.6	157.6±0.7	163.7±1.0	159.8±1.5	159.3±1.1
	i	155.6±2.5	152.8±1.0	156.3±1.7	163±0.6	159.7±0.7	162.5±1.0
Potassium (mmol/L)	c	1.13±0.30	0.89±0.04	1.06±0.05	0.97±0.05	0.98±0.05	1.02±0.07
	i	0.80±0.06	0.88±0.04	1.26±0.13	0.83±0.04	1.10±0.06	0.84±0.06
Chloride (mmol/L)	c	128.2±2.3	126.4±0.9	123.3±0.7	131.2±1.4	127.0±1.5	126.9±1.8
	i	128.6±2.8	122.8±1.0	121±1.5	132.5±0.8	129.9±1.3	129.6±0.9
Glucose (mmol/L)	c	4.83±0.36	4.50±0.17	7.13±0.41	5.47±0.29	7.93±0.92	7.59±0.72
	i	4.65±0.32	4.78±0.29	7.16±1.01	5.43±0.24	7.24±0.65	7.76±0.39
Osmolality (mosm/L)	c	327.8±2.3	319.2±1.6	322.4±1.8	317.8±1.3	323.5±3.1	328.0±2.5
	i	319.3±4.2	312.7±3.0	326.3±4.1	324.1±1.3	325.1±2.4	339.3±2.0

C: Coho salmon

Time Post Infection (Days)

Parameters		0	1	3	7	14	21
Hematocrit (%)	c	59.9±2.5	55.1±2.1	62.7±1.7	59.9±1.6	50.6±2.4	56.0±1.7
	i	61.1±2.6	55.3±2.8	58.1±2.8	55.3±1.6	51.2±1.5	48.9±2.3
Protein (g/100mL)	c	6.12±0.32	5.01±0.36	5.42±0.36	4.95±0.35	4.14±0.61	5.16±0.49
	i	5.77±0.18	5.08±0.52	4.73±0.43	4.91±0.32	5.31±0.46	5.27±0.43
Cortisol (nmol/L)	c	23.8±3.3	41.1±6.2	26.2±3.3	32.7±7.7	26.5 ±3.5	31.1 ±4.6
	i	32.5±5.1	51.2±4.2	47.4±8.3	37.0±3.8	16.3±2.3	38.6±8.9

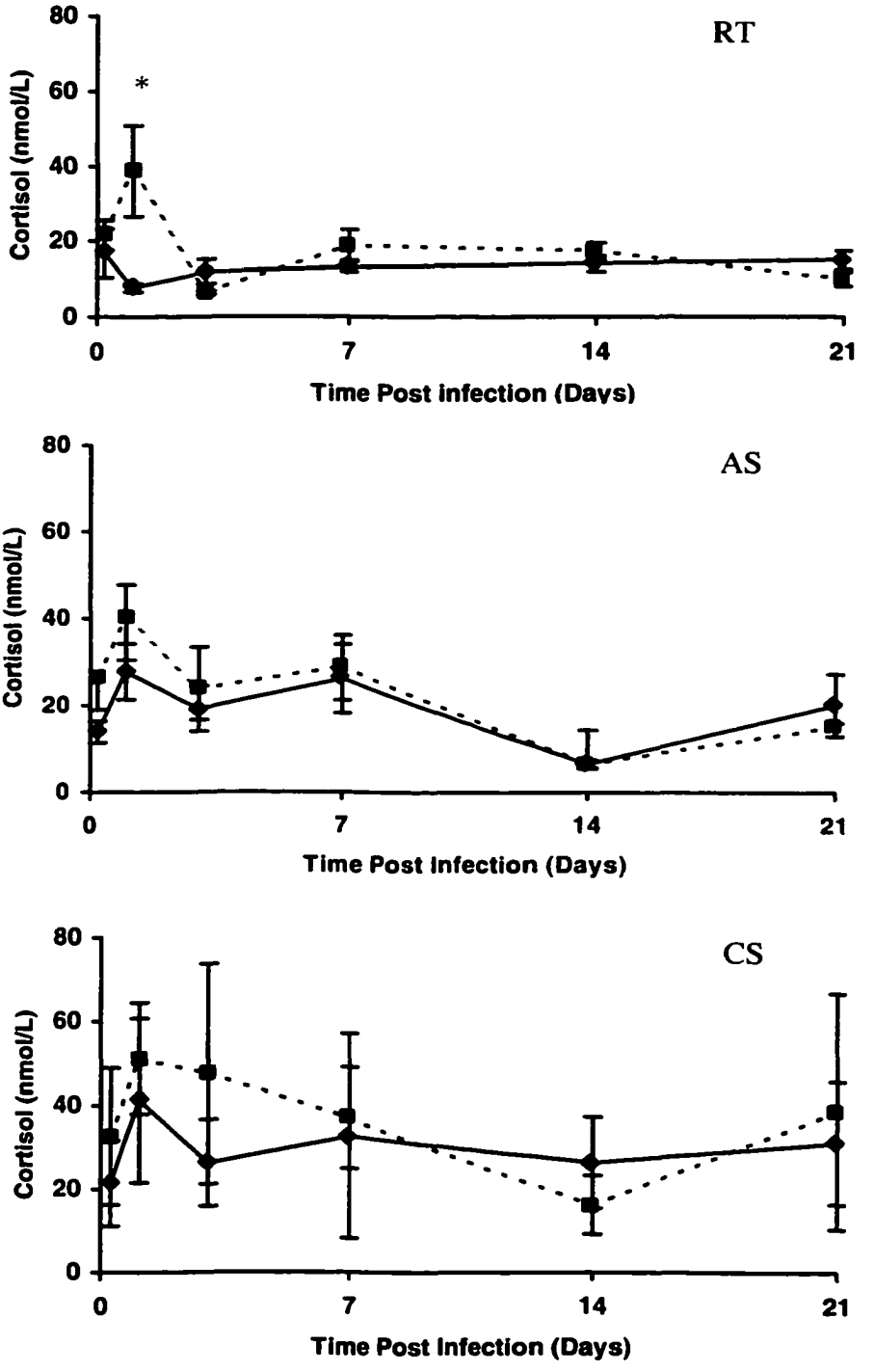
dpi, infected rainbow trout had significantly higher mean cortisol concentrations when compared to controls ($p<0.05$) (Figure 3.4). Mean cortisol concentrations were significantly higher in infected fish of all species at 1 dpi in comparison to the infected fish on all subsequent sampling days ($p<0.05$). There were no significant differences in mean cortisol concentrations between species.

Mean blood hematocrit and plasma protein concentrations were not significantly different between infected and control fish for any species but species differences were observed. Both infected and control coho salmon had higher hematocrit levels than Atlantic salmon and rainbow trout (Table 3 I). The general trend in all three species was a decrease in hematocrit levels over the course of the experiment. Throughout the experiment rainbow trout mean blood hematocrit ranged from 40 – 47%, while Atlantic salmon mean blood hemtocrit ranged from 45 – 55% and coho salmon from 48 – 60% (Table 3 I). Mean plasma protein concentrations were significantly higher in rainbow trout than Atlantic and coho salmon. In rainbow trout (infected and controls combined), blood protein concentrations peaked at 0 and 21 dpi at 7.1 and 6.8 g/100mL, respectively. In coho and Atlantic salmon (infected and controls combined) mean blood concentrations of protein peaked on day 0 at 6.0 g/100mL and 5.3 g/100mL, respectively (Table 3 I).

3.4.3 Respiratory burst activity and phagocytic capacity

Viability of macrophage cells was high (ca. 90 %) for control and test fish of all three species. There were no significant differences in macrophage viability

Figure 3.4: Comparison of mean (±SEM) plasma cortisol concentrations between *L. salmonis* infected (-■-) and non-infected fish (-◆-) in three salmonid species over time: (RT) rainbow trout, (AS) Atlantic salmon and (CS) coho salmon. * Denotes significantly different from control within the day ($p<0.05$).



between control and infected fish or between species. The respiratory burst activity (Figure 3.5) and phagocytic capacity (Figure 3.6) of head kidney macrophages from rainbow trout, were not significantly different between infected and control groups until 21 dpi, when a significant suppression ($p<0.05$) was observed in the infected group. Infected Atlantic salmon demonstrated a significant suppression ($p< 0.05$) of respiratory burst (Figure 3.5) activity and of phagocytic capacity (Figure 3.6) in the head kidney macrophages collected at 14 and 21 dpi. There was no significant difference in respiratory burst activities (Figure 3.5) and phagocytic capacities (Figure 3.6) between control and sea lice infected coho salmon.

3.4.4 Mucous biochemistry

In rainbow trout, alkaline phosphatase activities increased at 1 dpi in controls and 3 dpi in infected fish and then decreased over the rest of the experiment (Figure 3.7). Alkaline phosphatase activities were significantly higher in infected Atlantic salmon mucus at 3 and 21 dpi in comparison to controls (Figure 3.7). Although alkaline phosphatase activities appeared higher in the mucus of infected fish compared to controls at 7 and 14 dpi, the data was not significantly different ($p=0.09$ and $p=0.1$ respectively). Rainbow trout mucus had significantly higher alkaline phosphatase activities than the other two species throughout the experiment, except when compared to control Atlantic salmon on day 0. Coho salmon mucus showed no significant differences in alkaline phosphatase activities between infected and control fish throughout the experiment (Figure 3.7).

Figure 3.5: Mean (±SEM) respiratory burst activity in the macrophages from the head kidney of *L. salmonis* infected (-■-) and non-infected fish (-◆-) over time. (RT) rainbow trout, (AS) Atlantic salmon, (CS) coho salmon. * Denotes significantly different from control within the day ($p<0.05$).

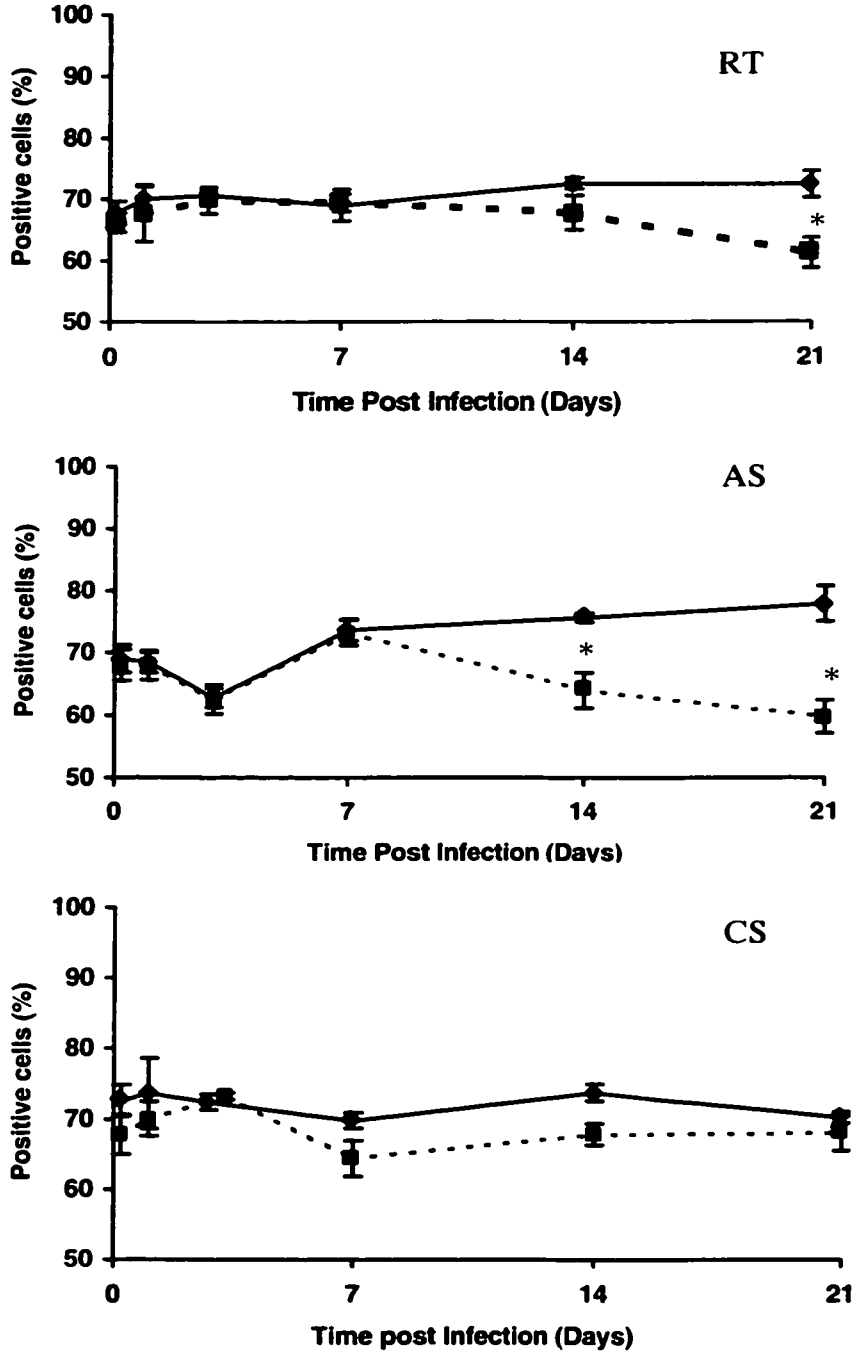


Figure 3.6: Mean (±SEM) phagocytic activity in the macrophages from the head kidney of *L. salmonis* infected (-■-) and non-infected fish (-◆-) over time. (RT) rainbow trout, (AS) Atlantic salmon, (CS) coho salmon. * Denotes significantly lower than control within the day ($p<0.05$).

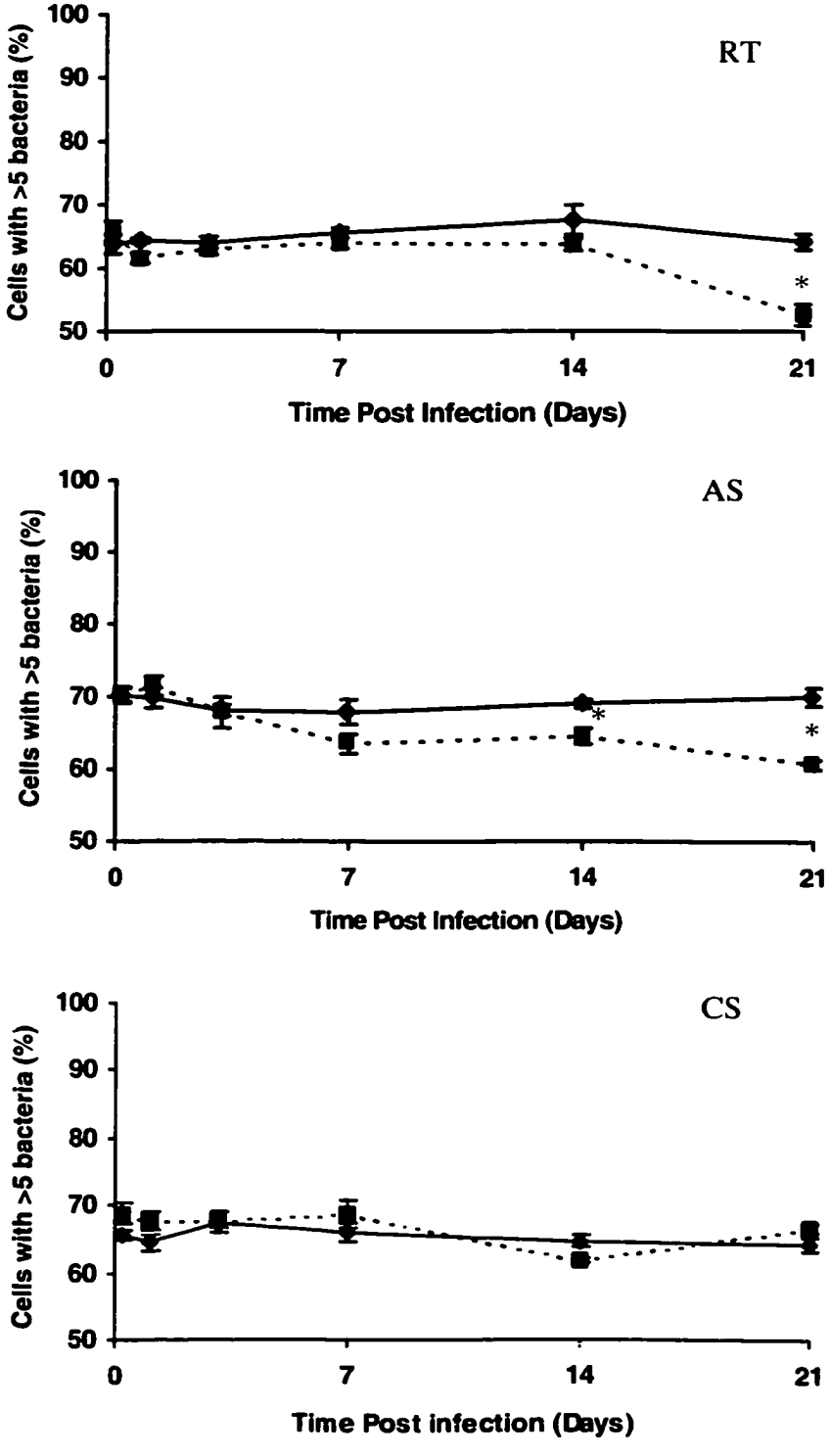
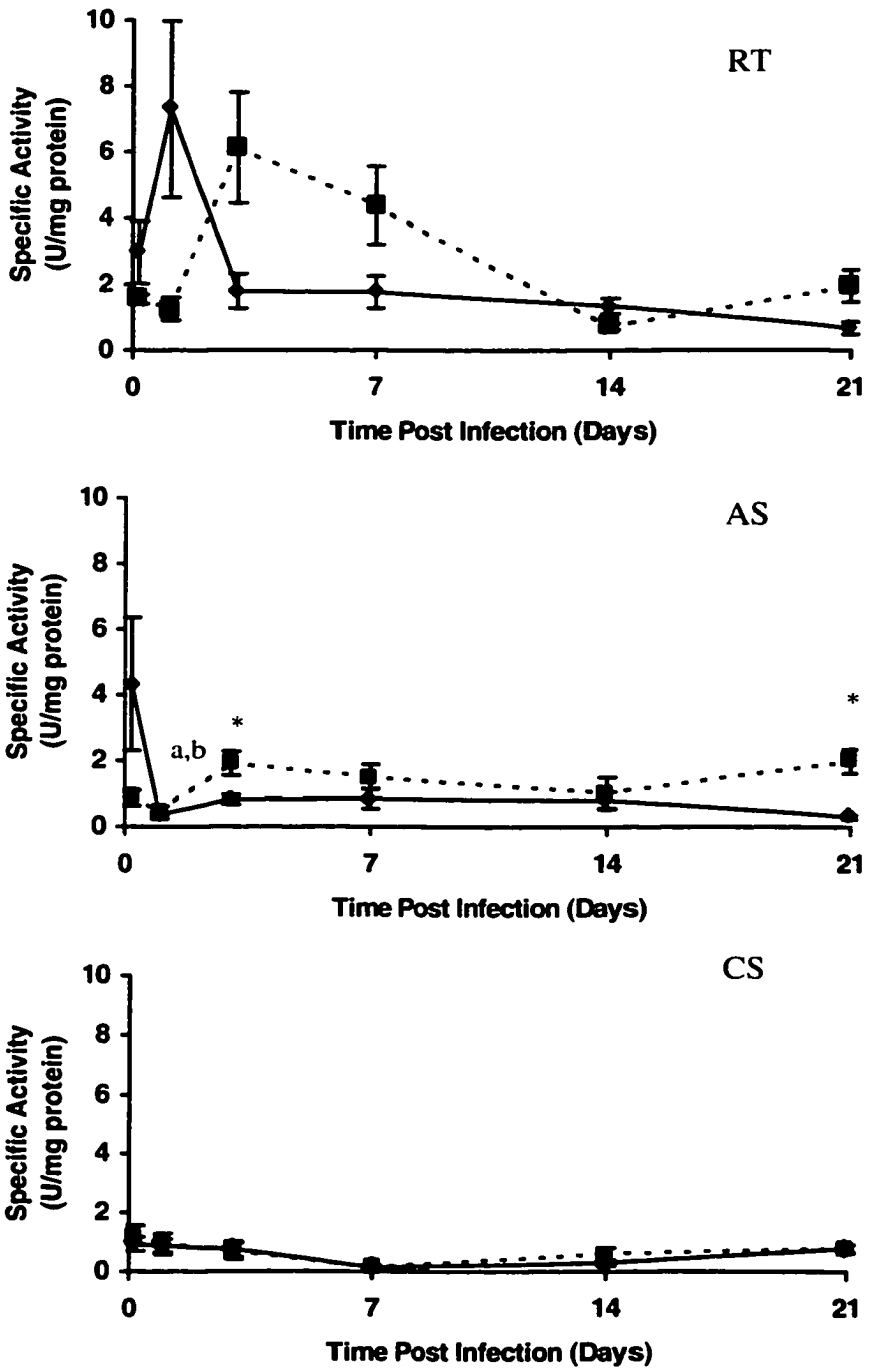


Figure 3.7: Mean (±SEM) specific activity of alkaline phosphatase in mucus of *L. salmonis* infected (-■-)and non-infected fish (-◆-) of three salmonid species over time. (RT) rainbow trout (AS) Atlantic salmon (CS) coho salmon. * Indicates significant differences from control within the day ($p<0.05$).
(a) Indicates 1 day post infection (dpi) levels are significantly lower in controls than 3 dpi, (b) indicates 1 dpi are significantly lower in infected than at 7 and 21 dpi.



Lysozyme activities in the mucus of infected Atlantic salmon and rainbow trout peaked at 1 dpi. Rainbow trout controls showed maximum activity at day 0 in mucous lysozyme activity (Figure 3.8). Rainbow trout also showed significantly higher mucous lysozyme activities in infected fish compared to controls on all days post-infection (Figure 3.8). Lysozyme activities were significantly higher in mucus of infected Atlantic salmon compared to uninfected fish at 1 dpi but there were no significant differences and activity remained low on all other days (Figure 3.8). Lysozyme activities were significantly higher in infected coho salmon mucus at 21 dpi (Figure 3.8). On this day lysozyme activity increased by tenfold from the rest of the days in the experiment. There was a high degree of variation since lysozyme activity was high in 6 of 10 fish, while 4 fish showed very little lysozyme activity. In general, rainbow trout mucus had significantly higher lysozyme activities than the other 2 species throughout the study, except when compared with infected coho salmon at 21 dpi.

Quantitative assessment of protease activity, as determined with azocasein, showed no significant difference between infected and control rainbow trout and Atlantic salmon or significant differences between species. In rainbow trout, mucous protease activity rose almost sixfold in both infected and control fish from day 0 to I dpi and then began to decline in control fish. Infected fish, however, continued to show elevated mucous protease activity peaking at 7 dpi before decreasing at 14 and 21 dpi (Figure 3.9). Mean mucous protease activity appeared to be higher in infected Atlantic salmon than in controls on all days after lice infection, however the differences were not significant (Figure 3.9). Only at 21 dpi did coho salmon show significant differences between mucous protease activity in control and infected fish with control coho salmon

Figure 3.8: Mean (±SEM) specific activity of lysozyme in mucus of *L. salmonis* infected (-■-) and non-infected fish (-◆-) of (RT) rainbow trout, (AS) Atlantic salmon and (CS) coho salmon. * Indicates significant differences from control within the day ($p<0.05$). (a) at 0 dpi mucous lysozyme activities are significantly higher in controls than on all other days. (b) Activities at 1 dpi are significantly higher in infected fish than at 3, 7, 14 and 21 dpi. (c) Activities at 21 dpi are significantly higher in infected fish than on all other days.

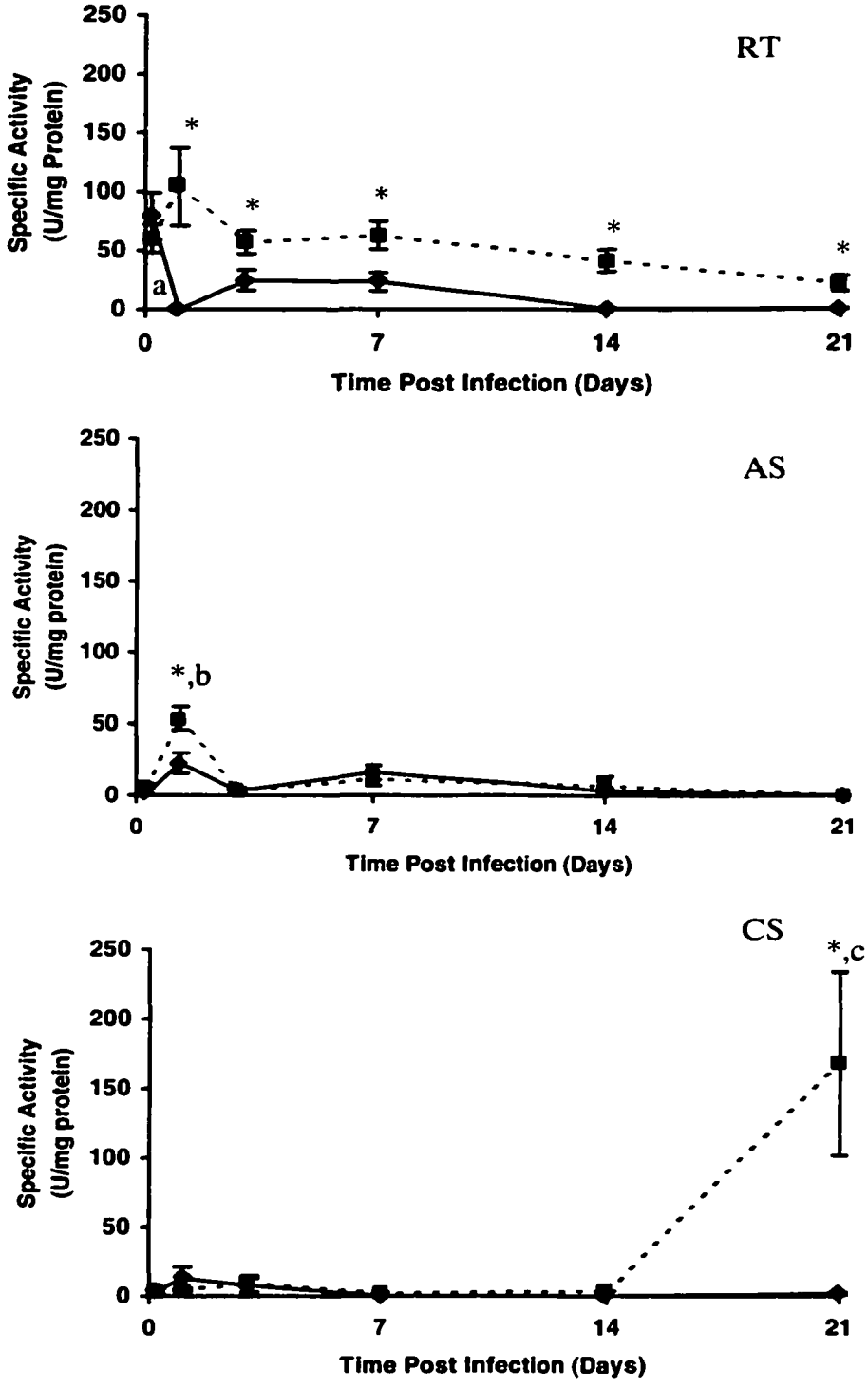
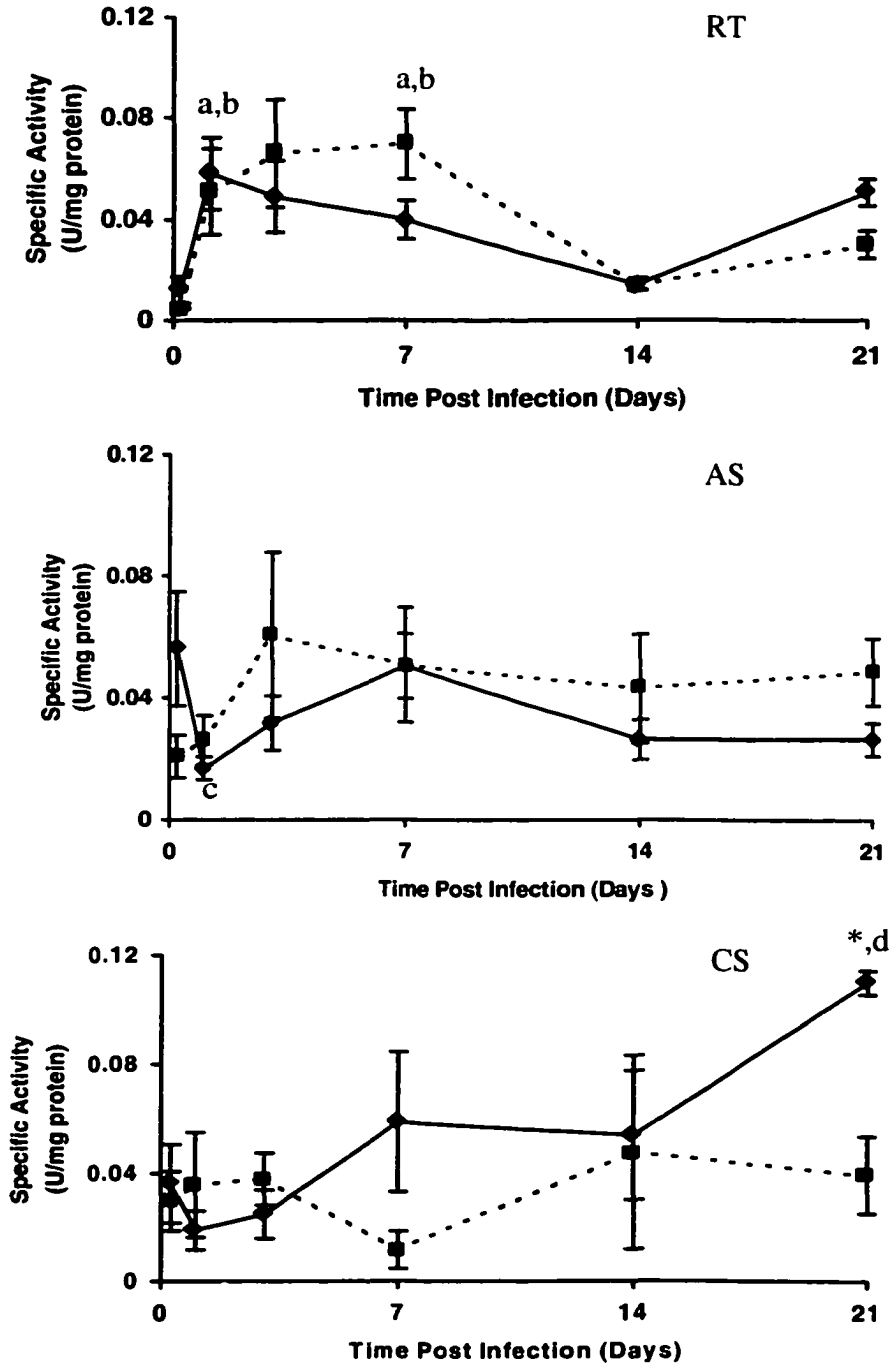


Figure 3.9: Mean (±SEM) protease activity in the mucus of *L. salmonis* infected (-■-) and non-infected fish (-◆-) of (RT) rainbow trout, (AS) Atlantic salmon and (CS) coho salmon. Activity determined by azocasein hydrolysis at 30°C. * Indicates significant differences from control within the day ($p<0.05$). (a) activities are significantly higher in controls and infected fish on these days than prior to infection (day 0), (b) activities are significantly higher in controls and infected fish than at 14 dpi. (c) activity at 1 dpi are significantly lower in controls than at 7 dpi. (d) activities at 21 dpi are significantly higher in controls than on all other days.



having significantly higher protease activity in their mucus compared to mucus from infected coho salmon (Figure 3.9).

Infected rainbow trout and Atlantic salmon both showed the presence of a series of low molecular weight proteases (15-20 kDa) by the later days of the trial (Figure 3.10). In rainbow trout, the series of low molecular weight (LMW) protease bands differed from the single band observed in controls, previously (Section 2.4). The number of infected rainbow trout having these bands of LMW proteases significantly increased at 21 dpi ($p<0.01$). Atlantic salmon showed a similar increase in the number of infected fish having the same low-molecular weight proteases in infected fish at both 14 ($p<0.05$) and 21 dpi ($p<0.01$). Ten out of ten infected rainbow trout (21 dpi) and Atlantic salmon (14 and 21 dpi) showed the presence of multiple bands in the LMW range, while no control fish in either species showed the presence of multiple bands in this range.

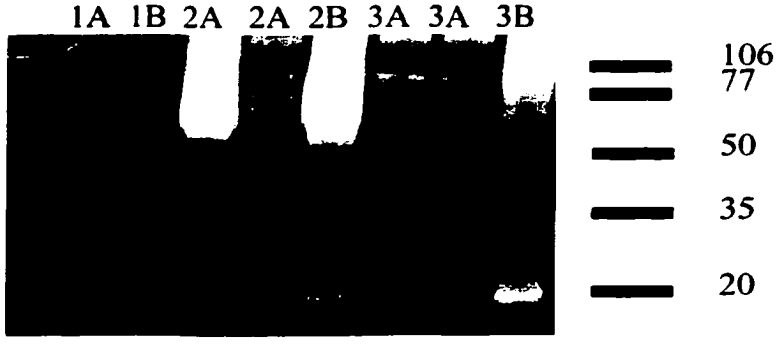
The mucus from infected and control rainbow trout, coho and Atlantic salmon were assessed for their ability to inhibit the activity of bovine trypsin. No inhibition of trypsin activity was observed in any mucous samples (Table 8 II, Appendix B).

3.4.5 Correlation data

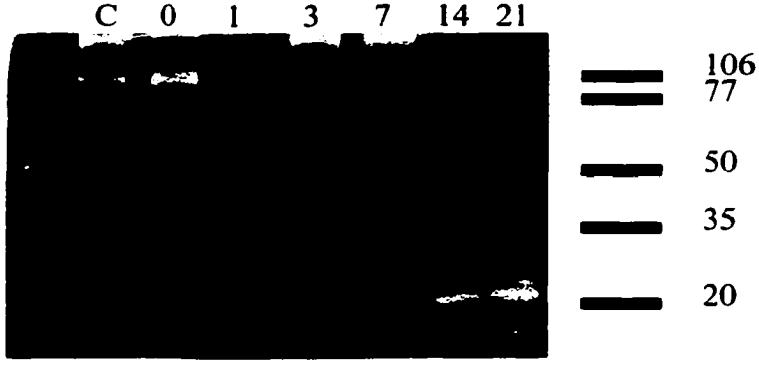
There were no correlations between lice densities and mucous enzyme activities. There were also no correlations between lysozyme, protease or alkaline phosphatase activities in the three species studied. There was a strong positive correlation between cortisol and mucous lysozyme activity in rainbow trout (Spearman

Figure 3.10: Zymogram of protease activity in (i) the mucus of infected and non-infected coho salmon (1), rainbow trout (2) and Atlantic salmon (3) at 21 dpi; each lane contains 3µg of mucous protein and samples are representative of each species; (A) controls and (B) *L. salmonis* infected. (ii) the mucus of control (C) and *L. salmonis* infected (0, 1, 3, 7, 14, 21 dpi) Atlantic salmon. Each lane contains 3µg of mucous protein and samples are representative of Atlantic salmon samples for each day. The control sample was also representative of control samples throughout the study. Numbers along the side of each gel indicate molecular weights (kDa). Representative samples expressed here were chosen based on the most common protease banding patterns observed for each species.

(i)



(ii)



r=0.634, p=0.03). Similar to rainbow trout, mucous lysozyme levels had a positive correlation with plasma cortisol in Atlantic salmon (Spearman r=0.6550, p=0.02).

3.5 Discussion

This study compared physiological, immunological, and biochemical responses of three salmonid species to sea lice infection. Sampling days were based on previous reports to observe the rejection of sea lice by coho salmon (within the first 10 days) [144] and the immunological effects of lice infection on rainbow trout and Atlantic salmon (starting at 14 dpi) [68].

To obtain similar lice infection densities, all three species were cohabited for this study. Initial infection rates appeared similar when corrected for fish size. It is recognized that rainbow trout has a slightly different body shape than the other two species, being more rotund and less streamlined. The calculations employed in this study would lead to an underestimation of rainbow trout available surface area in comparison to the more streamlined Atlantic and coho salmon. This should slightly overestimate the number of lice pre surface area. This would only increase the significant difference between rainbow trout and Atlantic salmon, and should not significantly effect the differences between rainbow trout and coho salmon. Lice numbers dropped dramatically over the course of the experiment on coho salmon. This decrease in lice counts on coho salmon is in agreement with previous observations [63,144]. Sea lice numbers remained relatively high on rainbow trout and Atlantic salmon throughout the experiment as has been previously reported for Atlantic salmon [37,144]. By 14 and 21 dpi all species had

significantly different levels of lice infection per available surface area (Atlantic salmon>rainbow trout>>coho salmon). The sea lice also matured at a slower rate on coho salmon than on Atlantic salmon and rainbow trout. Johnson and Albright demonstrated that *L. salmonis* on chinook salmon took longer to mature than on Atlantic salmon [144]. The significantly lower lice prevalence/available surface area, at 14 and 21 dpi, and the lower percentage of chalimus II larvae at 7 dpi, on rainbow trout as compared to Atlantic salmon, leads to the suggestion that rainbow trout may not be as favourable a host as Atlantic salmon. The increase in numbers of lice at 14 dpi in both rainbow trout and Atlantic salmon may, however, be attributable to the difficulty in counting lice during their early life stages thereby increasing the potential to underestimate lice counts at these early sampling intervals. Indeed, Bjorn and Finstad demonstrated an approximate 10% increase in total lice counted from 7 to 14 dpi and a 1% decrease in total lice counts from 14 – 19 dpi on sea trout [37]. At 21 dpi, the decrease in lice numbers on both rainbow trout and Atlantic salmon may be attributed to lice mobility once reaching the pre-adult stage. The increase of lice on coho salmon occuring at 21 dpi (Figure 1a) probably resulted from the migration of mobile pre-adults from the Atlantic salmon and rainbow trout cohorts. Movement of lice at this pre-adult life stage has previously been observed to occur between different hosts [188] and different host species [189]. Interestingly, only male pre-adults were found on coho salmon at 21 dpi. This may be due to the small number of lice staged (n=10), or may have implications in sea lice biology. Pre-adult males mature earlier than females giving them more time and possibly more opportunity to migrate to coho salmon hosts. More work is required to elucidate any relationship between gender, maturation and migration of sea lice among

hosts.

The presence of one pre-adult louse on one of the coho salmon at 1 dpi raises some questions. The lice were added to the tank at the start of the trial in the form of copepodids. Therefore, it is not possible for the pre-adult louse to have been the result of the infection. All filter systems were clean and no contamination of lice in control tanks occurred. Since all experimental tanks used in this experiment were empty of fish until 15 days prior to the experiment it is not possible that lice were in the system previously. All fish, having been smoltified at our facilities, were naïve and had no previous exposure to sea lice. It is also impossible for the coho salmon to have been contaminated prior to this point since all fish were kept under quarantine conditions until cohabitation. It was therefore concluded that the pre-adult louse came from splashing of fish in an adjacent tank, not involved in this experiment which contained salmon infected with lice at approximately 3-4 weeks post infection. This was considered to be an isolated incident, which had a negligible effect on the outcome of this study.

No differences in blood chemistry were observed between infected and control fish in any species following lice infection. Electrolyte, cortisol, glucose and protein concentrations and hematocrit readings all remained within established physiological resting ranges for infected and control fish throughout the study [168]. There was, however, a decrease in hematocrit in both infected and control Atlantic salmon and rainbow trout over the course of the experiment. Even though cortisol levels were within resting physiological ranges significantly higher cortisol concentrations were observed in infected rainbow trout at 1 dpi. The higher cortisol concentrations at 1 dpi were most likely a result of fish crowding, as the tank volumes were reduced during the 12-hour

infection period for sea lice attachment. Rainbow trout may be more sensitive to the double stressor under cohabitation than the other two species studied. The overall lack of changes in physiological parameters between infected and control fish follows the observations made by others that until sea lice reach the pre-adult life stages, lice induce very little apparent physiological stress response in the salmonid hosts [33,36,37,144].

Infected Atlantic salmon showed decreases in respiratory burst and phagocytic capacities at 14 and 21 dpi. The decreased macrophage activities are consistent with the observations of Mustafa *et al.* for Atlantic salmon experimentally infected with sea lice [68]. Infected rainbow trout showed a similar decrease in immune function at 21 dpi. It has been suggested that mobile stages of sea lice have a greater impact on the physiology and immune function of fish than earlier stages [36,37,39]. Transformation of lice from immobile to mobile stages results in significant changes in lice activities and distribution on the host. The lice had reached a mobile stage by 21 dpi but were still chalimus III – IV on day 14 (immobile). The decrease in macrophage function appeared to begin prior to the pre-adult stage and may be due to increased damage of feeding as the lice get larger or may be due to immunosuppressive secretions from the lice. Salivary gland extracts from other arthropod parasites, such as Ixodid ticks, have shown immunomodulatory effects on host macrophage and lymphocyte cytokine production [190,191].

Since the physiological parameters studied were within normal ranges for coho salmon it was determined that these fish were not influenced by stressful stimuli. However, macrophage function was depressed at later stages of infection in infected rainbow trout and Atlantic salmon. This effect may be the result of a chronic stress

response or due to unknown immuno-suppressive factors released by the sea lice. Cortisol levels were not at chronically high levels, which is the typical indication of chronic stress. The decreased macrophage function is the only systemic difference identified between these species associated with sea lice infection.

Differences in mucous biochemistry were observed between species and between infected and non-infected fish within species. Mucous lysozyme levels were significantly higher in infected coho salmon at 21 dpi as compared to controls. This could be attributed to the fish responding to the mobility of pre-adults, as previously mentioned. Since the early life stages of sea lice are easily sloughed off by coho salmon, the occurrence of pre-adults may induce a higher level of response involving lysozyme secretion into the mucous layer.

In infected rainbow trout, mucous lysozyme increased at 1 dpi and remained significantly higher than controls throughout the experiment. Increases in blood lysozyme levels have also been reported in rainbow trout and Atlantic salmon as a result of increased cortisol concentrations [169]. Infected rainbow trout had significantly higher cortisol concentrations than controls at 1 dpi and may have contributed to the maximum mucous lysozyme activity achieved on this day, since a strong positive correlation between cortisol and mucous lysozyme was observed. Work by Demers and Bayne linked plasma lysozyme levels and cortisol in rainbow trout, but did not correlate these parameters [172]. It is unlikely, however, that the increased cortisol concentrations were the sole reason for the mucous lysozyme increase in our study since the cortisol concentrations were within the physiological resting range. The effect of stress on lysozyme activity is still unclear and further study is warranted. Demers and Bayne

suggested lysozyme increases as an adaptation to compensate for an increased susceptibility to pathogens following acute cortisol elevation[172]. Conversely, Fevolden *et al.* suggest that increases in blood lysozyme following acute stress exposure may reflect a greater susceptibility to disease [192].

Despite evidence that plasma and mucous lysozyme levels have no correlation between seawater-reared fish and freshwater-reared fish (Section 2.4), other stimuli may have similar effects on plasma and mucous lysozyme levels. The observed decrease in mucous lysozyme activity in control rainbow trout from 0 dpi to 1 dpi and increase by 3 dpi is similar to findings by Roed *et al.*, who observed a decrease in rainbow trout plasma lysozyme after initial crowding stress [170]. This decrease was observed 30-35 h after the crowding event, with plasma lysozyme levels recovering over the next 3-4 days. Jeney *et al.* also observed a decrease in rainbow trout lysozyme 2 h after stress, followed by recovery to pre-stress levels after 1 week [171].

Infected Atlantic salmon exhibited significantly higher ($p<0.05$) mucous lysozyme levels than controls at 1 dpi. The observed increase in lysozyme activity in Atlantic salmon mucus appeared to be cumulatively affected by crowding and exposure to lice. Although increases in lysozyme activity in both control and infected fish were observed, the increase in infected fish was significantly higher than the non-infected fish.

Over the course of the experiment mucous lysozyme activity, as well as blood hematocrit readings, decreased in rainbow trout and to some degree in Atlantic salmon. Pickering and Macey showed that repeated handling (ie. every 2 days) of char (*Salvelinus alpinus*) increased the incidence of mucous cells in the skin epithelium with recovery to normal levels requiring a period of one month [83]. If mucous lysozyme

activities are similarly affected, it is possible that the two wk period of acclimation at the start of the trial may account for the high lysozyme levels in rainbow trout in the first days of the experiment as being a consequence of slow acclimation. It is also possible that a hierarchal structure between different host species caused the reductions in mucous lysozyme activities and blood hematocrit levels over time. Caruso and Lazard showed decreases in tilapia blood lysozyme levels in subordinate fish compared to the dominant ones [193]. It is possible that this occurred in both rainbow trout and Atlantic salmon due to the presence of coho salmon. Coho salmon are aggressive fish as both juveniles and adults in the wild defending certain areas of the stream [3]. Probably the most likely reason for blood hematocrit and mucous lysozyme levels dropping after 1 dpi in both species is an acclimatization of the fish to cohabitation and sampling stress.

No significant differences were observed between infected and non-infected fish protease activities using azocasein hydrolysis in Atlantic salmon and rainbow trout. However, protease activity in control coho salmon mucus 21 dpi was significantly higher than infected fish. The reason for this is unclear. Pickering and Macey found that changes in skin mucus of char can take up to one month to return to normal levels [83]. In this study, 21 dpi was approximately 36 days after first cohabiting all three species. Resting ranges for coho salmon mucous protease activity observed in Section 2.4 were also very low, strongly suggesting high levels in 21 dpi control fish were anomolous.

It was also hypothesized that the lack of an increase in protease activity in the mucus of infected fish at 21dpi could be the result of lice infection and the possible presence of protease inhibitors in infected fish mucus. However, subsequent examination

of protease inhibitors in mucus failed to demonstrate protease inhibition activity.

Qualitative measurements of protease activity using zymography showed similar increases in low-molecular weight (LMW) proteases over time in infected Atlantic salmon, as previously demonstrated by Ross *et al* [87]. Low molecular weight proteases in the mucus of *L. salmonis* infected Atlantic salmon was reported as early as 3 dpi by Ross *et al* [87]. Although lice densities on Atlantic salmon here are similar to those used by Ross *et al.* the Atlantic salmon used in that study had a mean weight of 56.9 g and were kept at a temperature ranging up to 14^0C [87]. Similar *L. salmonis* densities on smaller fish would increase the ratio of *L. salmonis* secretory volume to host mucus volume. Younger stages of lice may therefore not produce enough LMW proteases to be observed in the greater quantity of host mucus in the present study. This would explain why infected Atlantic salmon did not show significantly higher levels of LMW proteases until 14 and 21 dpi.

As in Atlantic salmon, rainbow trout mucus also showed significantly higher levels of LMW proteases in infected fish at 21 dpi and peak lysozyme activities at 1 dpi. Firth *et al.* determined that these LMW proteases were actually trypsin or trypsin-like proteases secreted by the louse [107]. The increase of mucous lysozyme early in the infection is probably a host response by Atlantic salmon and rainbow trout to first contact with the lice, whereas the presence of LMW protease results from production by the louse itself, perhaps to assist in blood feeding [107]. Trypsin has also been linked to feeding in other arthropod parasites [128]. There was no increase in LMW proteases in coho salmon over the course of the experiment.

Alkaline phosphatase activities also appear to be linked with interactions

between Atlantic salmon mucus and sea lice. Iger and Abraham observed strong alkaline phosphatase reactions in mucous and pavement cells of carp 30 min after wounding [101]. Mucous cells showed increased production of alkaline phosphatase up until 6 h after wounding and then returned to control levels 24 h later [101]. Atlantic salmon infected with sea lice had a significantly higher mucous alkaline phosphatase activity at 3 and 21 dpi, as compared to controls, and this may be due to wounding caused by the lice. The time of the increases are interesting in that early in the infection the lice are preparing for their first moult on the host (3 dpi). Later increases in alkaline phosphatase activity coincide with the first appearance of pre-adults (21 dpi). However no similar relationship was found in rainbow trout or coho salmon. Buchmann and Bresciani failed to demonstrate a significant change in alkaline phosphatase activity in the mucus or epidermis of rainbow trout following infection with the monogenean parasite, *Gyrodactylus derjavini* [195]. The source of increased alkaline phosphatase activity is still uncertain and studies involving incubations of sea lice in salmon mucus may help to identify the source.

Early attachment by sea lice did not appear to elicit an enzymatic response in coho salmon mucus, at least in terms of lysozyme, alkaline phosphatase and protease activities. Coho salmon must therefore rely on other means of defense against lice. Johnson and Albright observed a well-developed inflammatory response of coho salmon to *L. salmonis* infection as early as 1 dpi [144]. Later in the infection they also observed epithileal hyperplasia in infected coho salmon [144]. Physiological levels of the blood parameters studied (cortisol, glucose, indicators of the stress response) did not differ significantly between infected and control fish of any species. For this reason it does not appear that stimulation of the HPI-axis is involved in the rejection of sea lice by coho

salmon. However, increased susceptibility to *L. salmonis* infection in coho salmon has been observed following cortisol implantation [63], suggesting chronically elevated cortisol levels results in immunosuppression.

Rainbow trout and Atlantic salmon both exhibited a decrease in head kidney macrophage function in the absence of increased cortisol. Coho salmon, on the other hand, had very low numbers of sea lice and did not show a similar decrease in macrophage function. It is interesting that despite the many differences between Atlantic salmon and saltwater adapted rainbow trout, they both respond in a similar manner to lice infection. Both show increases in mucous lysozyme, early in infection, while both show decreased macrophage function and increased LMW protease secretions from the louse late in the infection.

As was noted in lice counts and stages, the lice appeared to mature at a slightly slower rate and were less abundant/available surface area on rainbow trout than Atlantic salmon. This coupled with the observations that the depressed immune function and presence of multiple bands of LMW proteases occurred later in rainbow trout (21 dpi) as compared to Atlantic salmon (14 dpi), suggests that rainbow trout may be a less favourable host than Atlantic salmon. This has not been shown previously under laboratory conditions, however, Jackson *et al.* observed lower numbers of *L. salmonis* on rainbow trout than on Atlantic salmon kept in adjacent cages, along the west coast of Ireland [167,196].

It appears that initial lice infection elicits an enzymatic response in the skin mucus of rainbow trout and Atlantic salmon. However, the response does not appear to be capable of preventing the infection. This could be due to lice secretions,

which assist in breaking down this barrier [107]. Once lice biomass increases to a critical level on a fish, or the lice reach the pre-adult stage, the lice secretions may also have an immunosuppressive effect, which explains the observations of a depression in immune function in these fish [68]. Since there were no significant differences between resistant and susceptible species' blood physiology it seems likely that the differences are occurring in the mucus and epithelial layers of the host fish. Furthermore, the rapidity with which coho salmon rejected the lice infection leads to the hypothesis that the resistant factors are part of the innate immune response of coho salmon. There may also be factors in the skin mucus of rainbow trout and Atlantic salmon (as well as other susceptible hosts) that facilitates lice infection and growth rather than potential inhibitory factors in coho salmon mucus that discourage lice infection. Factors present in the integumental mucous layers of these fish may determine whether hosts are susceptible or resistant to sea lice infection.

4.0 BIOCHEMICAL STUDIES OF SEA LICE SECRETIONS FROM ADULT *LEPEOPHTHEIRUS SALMONIS* INCUBATED IN FISH SKIN MUCUS

4.1 Abstract

Pre-adult and adult sea lice (*Lepeophtheirus salmonis*) were incubated with seawater and mucous samples from rainbow trout (*Oncorhynchus mykiss*), coho salmon (*O. kisutch*), Atlantic salmon (*Salmo salar*) and winter flounder (*Pseudopleuronectes americanus*), to determine enzyme activity in lice secretions in the presence of fish skin mucus. Mucous samples from six fish were combined to obtain pooled samples for each species. Mucous samples from 10 individual rainbow trout, coho salmon and Atlantic salmon were also obtained to observe lice responses to individual hosts of each species. Alkaline phosphatase levels were significantly increased ($p<0.05$) in Atlantic salmon mucus and protease activities were significantly increased ($p<0.05$) in rainbow trout mucus following incubation with *L. salmonis*. Fish mucus and seawater samples were also analyzed using protease gel zymography to observe changes in low molecular weight (LMW) protease banding following lice incubation. There were significantly higher proportions of samples containing multiple bands of LMW proteases following lice incubation in rainbow trout and Atlantic salmon mucus compared to incubation in seawater. Following lice incubation, coho salmon and flounder mucus, however, showed no significant increases in the number of samples containing multiple bands of LMW proteases in comparison to seawater. The resistance of coho salmon to lice infection may be related to the lack of LMW protease secretions

from the louse into coho salmon mucus. Further work must be done to determine a factor in coho salmon mucus that blocks these secretions, or possibly, a factor in susceptible species that stimulates lice secretions.

4.2 Introduction

Although salmon lice, *Lepeophtheirus salmonis*, are infective towards most seawater salmonids, they are found more frequently on certain speices. As has been shown in Section 3.4, lice develop at a faster rate on Atlantic salmon compared to coho salmon. This infection can cause a decrease in the immune response of Atlantic salmon. Whether host preference is due to 1) the nutritional content of the mucus, skin and blood on which lice feed, 2) easier evasion of host defences in certain species, or 3) a combination of both of these factors, is not yet understood. Coho salmon have a documented resistance to lice and have been shown to shed lice relatively quickly (Section 3.4.1) [144]. It is hypothesized that this resistance towards infection is due to innate resistance or a non-specific immune response. The observation that there is little difference in blood physiology between resistant and susceptible species, even during rejection of lice by coho salmon (Section 3.4.2), leads to a hypothesis that the differential resistance is due to factors in the mucus and epithelial layers of these host species. Lice spend the majority of their life cycle in the skin mucus of their fish host [28].

The mucous layer is the first site of interaction between host and pathogen and the first line of defence for the fish host. *Lepeophtheirus salmonis* has previously been observed to release enzymes in the presence of Atlantic salmon mucus [107]. It is

suggested that these enzymes are secreted into host mucus but may also constitute louse excretions. Since these enzymes are released in response to host mucus they will be referred to throughout as secretions.

It is important to study lice interaction with host defences in the absence of confounding host variables to prevent obscuring *L. salmonis* secretions. By incubating live lice with samples of fish mucus it becomes possible to observe lice responses to the substrates they inhabit in the absence of active host cells.

The purpose of this study was to observe sea lice responses to mucus from different salmonid host species, as well as that of a non-host, non-salmonid species (flounder), in an attempt to observe distinctions in lice interactions with different hosts. The observed increases in fish mucous alkaline phosphatase and lysozyme activities following infection in previous studies (Section 3.4.4) led to one of two possible conclusions: (1) the enzymes are being produced by the fish, or (2) the enzymes are being produced by the louse. It was hypothesized that live lice incubations would allow us to determine the source of this enzyme activity. Incubating lice from different sources (Brown's Bay, BC and St. George, NB) in mucus under similar conditions would allow us to observe possible population differences in *L. salmonis* responses.

4.3 Materials and methods

4.3.1 Mucus collection

The salmonids used for this study were from the same cohort as in Section 3.3. The fish were maintained under the same conditions. Following smoltification or transfer to seawater the fish underwent a minimum two-week acclimation process prior to mucus sampling. Six fish of each species (rainbow trout, coho and Atlantic salmon) were anesthetized with tricaine methanesulfonate (MS-222 (100 mg/L)) and gently placed in bags filled with 10 mL of sterile seawater (28 ppt, salinity). Mucus was collected with the same bag technique as previously described [87]. All mucus was then pooled for each species prior to storage at –80^{0}C. Approximately 7 wks later, a further 10 fish of each species were anesthetized and mucus collected in a similar fashion. These mucous samples were not pooled to allow for observations of individual fish differences.

Mucus was also obtained from juvenile (ca. 150-200 g) winter flounder (n=6) to compare lice interactions with non-host species' mucus. Flounder were obtained from the Ocean Sciences Centre, Memorial University, Newfoundland and maintained in 500 L tanks at 16^{0}C and 23 ppt (salinity). The flounder were fed a commercial feed and kept at 14 h light: 10 h dark photoperiod. Flounder were anesthetized and mucus sampled using the same procedure as for the salmonid species. Mucous samples from flounder were also pooled.

4.3.2 Lice collection and maintenance

4.3.2.1 Atlantic coast lice

Atlantic salmon (St. John River strain maintained at 10^0C and 30 ppt [salinity] in 40 L tanks) were infected with *L. salmonis* copepodids obtained from the Bay of Fundy. Lice were grown for 38 days on Atlantic salmon under laboratory conditions as previously described [39]. Following removal, pre-adult and adult lice were kept in aerated seawater (10 L) of the same temperature and salinity for 24 h prior to incubation. It was hoped that 24 h would allow enough time for the lice to digest the contents of their last meal but not cause a decrease in condition due to starvation. A longer period between host removal and incubation was discounted because lice can become moribund when deprived of a host for greater than one week.

4.3.2.2 Pacific coast lice

Pre-adult and adult lice were also collected from Atlantic salmon located at one site in Brown's Bay, Vancouver Island, BC. Lice sampled in British Columbia were transported (in water at the same temperature and salinity as the source site) and maintained off hosts for the same amount of time (24 h) and under the same conditions (10^0C) as those sampled from laboratory salmon.

4.3.3 Incubation conditions

All mucous samples were thawed on ice and diluted 1:1 with sterile seawater (28 ppt, salinity) to a 400 µL volume in 1.5 mL centrifuge tubes. One adult louse was then added to each tube and incubated at 10^0C for 1 h. This was repeated 30 X for each pooled sample of mucus. For individual fish differentiation replicates of three were carried out. For the trials with Pacific coast lice replicates were carried out 10 X for each species using pooled mucous samples. Gender differences between lice were not recorded. Lice were then removed and samples frozen at -80^0C until analysed. Lice were also incubated in seawater (28 ppt salinity) as a negative control or blank. One-hour incubations were chosen similar to studies carried out by Firth *et al* [107].

Prior to incubation, lice were checked for viability. Only lice that were actively swimming, moving or attached to the sides of the aerated buckets were used. This was done to prevent selecting weak or dead lice (which sink to the bottom of the buckets).

4.3.4 Analysis of mucus

Mucous samples were analyzed using the azocasein and alkaline phosphatase assays as well as the protease zymography method, as described in Section 2.3.4 [87]. Determination of positive/negative responses in the protease zymography gels were determined in an identical fashion as was done in Section 3.3.5.

For the individual fish mucus + lice incubations, only five samples (in triplicate) were analysed using gel zymography. All other mucous incubations were analyzed using protease zymography and representative samples chosen from the samples that exhibited the most common mucous protease patterns for each incubation type.

4.3.5 Statistical analysis

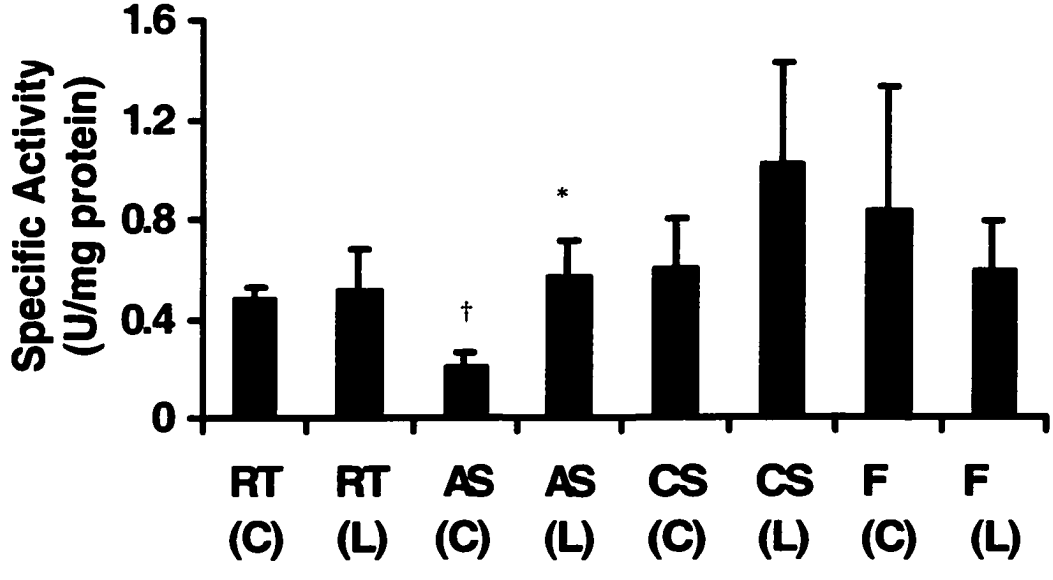
All data was analyzed using 2-way ANOVA to compare control vs. infected fish and to compare species. This was followed by multiple comparison Tukey's tests (Bartlett statistic corrected). The same statistical model as Section 2.3.6 was used. Zymography gels were analyzed for the presence or absence of protease bands, identically to Section 3.3.5 and compared using Chi-square tests.

4.4 Results

Alkaline phosphatase (AP) activity appeared to be higher in coho salmon mucus and lower in flounder mucus following incubation with lice, but neither was significantly different from controls, possibly due to the variation within each sample. Only Atlantic salmon exhibited a significantly higher ($p<0.05$) AP activity following 1 h incubation with sea lice as compared to control mucus (Figure 4.1). Atlantic salmon control mucus also had significantly lower AP activity than all other species ($p<0.05$).

Mucous lysozyme activity was low in the mucus of all pooled samples, in comparison to fish in Section 2.4, prior to and following incubation with lice. There

Figure 4.1: Mean (±SEM) specific activity of alkaline phosphatase in pooled mucous samples of four species of fish incubated for 1 h with live *L. salmonis*. Six fish were pooled for each species. (RT) rainbow trout, (AS) Atlantic salmon, (CS) coho salmon, (F) winter flounder. (C) denotes control mucus and (L) denotes mucus incubated with lice. * Indicates significant differences from control ($p<0.05$),† indicates significantly lower than all other species (n=30).



were no significant differences in mucous lysozyme activity in any pooled mucus samples following lice incubation (Figure 8.2, Appendix B).

Pooled mucous protease activity showed a high degree of variation between different lice incubations. Despite the variation present in the different replicates, rainbow trout mucous protease activity was significantly higher ($p<0.05$) following incubation with sea lice (Figure 4.2).

Protease zymography was used to determine the relative number of lice that would secrete multiple bands of LMW proteases in the presence of pooled fish mucus. There was no significant difference in the number of lice secreting multiple bands of LMW proteases in coho salmon or flounder mucus in comparison to lice incubated with seawater (Figure 4.3). Out of 30 replicates, multiple LMW proteases were observed in only 6 coho salmon, 7 seawater and 11 flounder samples. In comparison, multiple bands of LMW proteases were observed in 26/30 and 22/30 Atlantic salmon and rainbow trout mucus samples incubated with lice, respectively (Figure 4.4). Protease banding in the mid-high molecular weight range appeared to increase in some rainbow trout and Atlantic salmon samples incubated with sea lice as well. These results, however, were observed sporadically throughout different Atlantic salmon and rainbow trout mucous incubations with lice and appeared to have no identifiable pattern.

To observe variation in lice response to individual fish of a particular species, 10 rainbow trout, 10 coho salmon and 10 Atlantic salmon mucous samples were incubated with lice in triplicate. Individual mucous samples exhibited a great deal of variation in AP and total protease activities prior to incubation with lice (Table 4 I).

Figure 4.2: Mean (±SEM) specific activity of proteases in pooled mucous samples of four species of fish incubated for 1 h with live *L. salmonis*. Six fish were pooled for each species. (RT) rainbow trout, (AS) Atlantic salmon, (CS) coho salmon, (F) winter flounder. (C) denotes control mucus and (L) denotes mucus incubated with lice. * Indicates significant differences from control ($p<0.05$) (n=30).

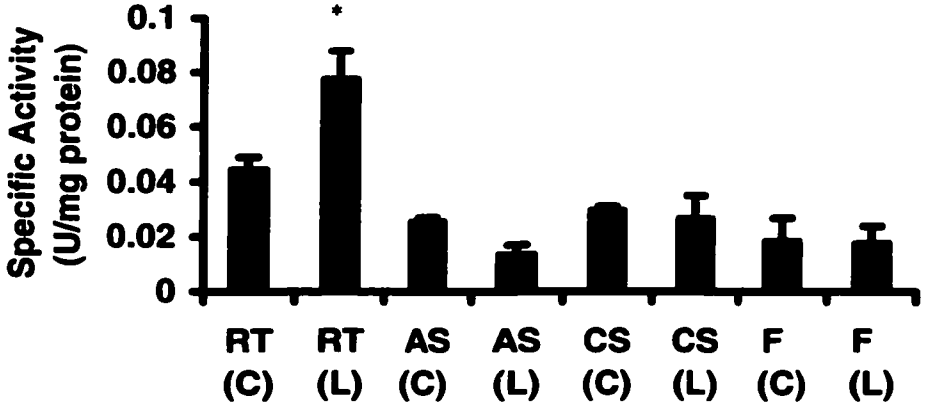


Figure 4.3: Zymogram of protease activity in pooled mucous samples of coho salmon (CS), rainbow trout (RT) Atlantic salmon (AS), winter flounder (F) and seawater (SW) incubated for 1 h without or with (L) live *L.salmonis*. Six fish were pooled for each species. Each well contains 3 µg of mucous protein and samples chosen are representative of each species. Numbers along the side of the gel indicate molecular weights (kDa). (➡) Indicates low molecular weight proteases, (*) indicates single bands of endogenous protease, (+) indicates a positive response, (-) indicates a negative response. All fish samples were analyzed using protease zymography and representative samples chosen exhibited the most common mucous protease patterns for each species.

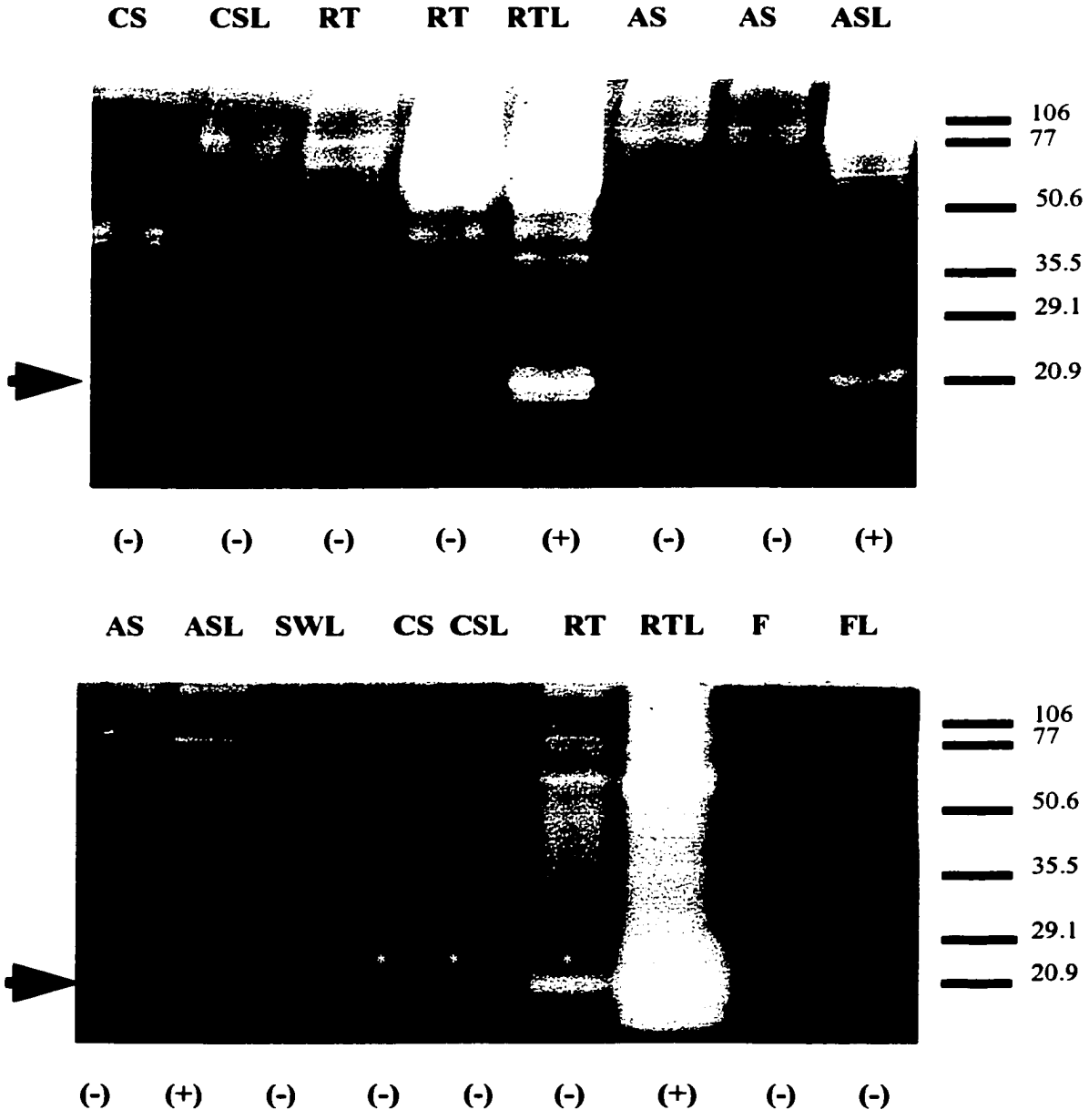


Figure 4.4: Percentage of pooled mucous samples incubated for 1 h with *L. salmonis* showing increases of multiple LMW protease bands. Six fish were pooled for each species. The presence of multiple LMW protease bands is considered a positive response, and results are displayed as a percentage of the total number of replicates in which a positive response occurred (n=30). (SW) indicates lice incubated in seawater, (CS) coho salmon mucus, (F) flounder mucus, (RT) rainbow trout mucus and (AS) Atlantic salmon mucus.

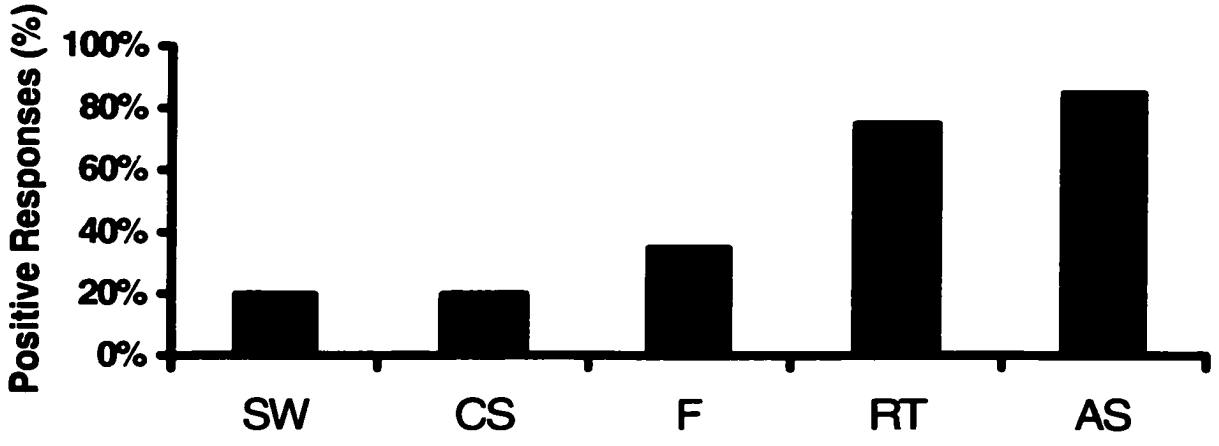


Table 4 I: Mean (± SEM) specific activities (U/mg protein) of alkaline phosphatase (AP) in individual rainbow trout, Atlantic and coho salmon mucous samples incubated for 1 h with and without *L. salmonis*. * Indicates significantly higher AP activity in incubations with lice than without.

Fish #	Atlantic salmon	Atlantic salmon + lice	Rainbow trout	Rainbow trout + lice	Coho salmon	Coho salmon + lice
1	0.41±0.03	0.65±0.03	1.34±0.21	3.06±0.04	0.00	0.00
2	0.15±0.01	0.64±0.04	0.44±0.02	0.49±0.02	0.77±0.04	0.85±0.03
3	0.08±0.02	0.39±0.01	0.55±0.07	0.51±0.03	4.35±0.87	5.13±1.13
4	0.31±0.05	1.00±0.10	0.32±0.03	0.43±0.01	0.94±0.10	1.15±0.05
5	0.18±0.03	0.32±0.02	0.46±0.02	0.53±0.01	0.76±0.04	0.68±0.02
6	0.25±0.04	0.24±0.01	0.74±0.08	1.42±0.04	0.00	0.00
7	0.05±0.01	0.00	2.03±0.31	2.12±0.12	0.30±0.02	0.51±0.03
8	0.13±0.06	0.34±0.04	0.11±0.00	0.17±0.00	0.01±0.00	0.05±0.01
9	0.00	0.00	0.21±0.03	0.17±0.03	0.00	0.00
10	0.23±0.03	1.36±0.07	0.13±0.04	0.12±0.01	0.87±0.05	1.08±0.02
Mean	0.18±0.04	0.49±0.11 *	0.63±0.22	0.90±0.32	0.80±0.38	0.90±0.41

Mean mucous AP and protease levels following lice incubation were similar to levels observed in pooled samples (Tables 4 I, 4 II, Figures 4.1, 4.2). Three trials using five individual fish mucous samples for each species were incubated with one louse and also analyzed using gel zymography. As zymography results were similar in the gels of individual fish mucus and pooled fish mucus (incubations +/- lice), only five individual fish mucous incubations (replicated X 3) were analyzed (Figure 4.5). The total percentage of positive responses to the mucus from individual coho salmon, Atlantic salmon and rainbow trout was also similar to observations with pooled mucous samples (Table 4 III). Again increases in protease banding (mid-high MW range) appeared to occur in particular Atlantic salmon and rainbow trout mucous incubations with lice.

Lice obtained from the Pacific coast of Canada (Vancouver Island, BC) were used to determine whether there were any differences in responses of the Pacific and Atlantic lice populations. Mucous AP activities were not significantly different from pooled Atlantic salmon, rainbow trout and coho salmon samples incubated with Pacific and laboratory grown lice (New Brunswick stock) (Figure 8.3, Appendix B). Zymography gels also did not show any differences between Pacific and laboratory grown lice secretions in the presence of fish mucus from all 4 species (Figure 8.4, Appendix B). However, there were significant differences between laboratory grown lice and Pacific coast lice protease secretions in the presence of fish mucus. Coho salmon mucous protease activity was significantly higher in samples incubated with Pacific coast lice than with Atlantic coast lice or mucous incubated without lice (Figure 4.6). This was not, however, accompanied by the appearance of LMW proteases. Atlantic salmon mucous protease activity also appeared to be higher in samples incubated with Pacific

Table 4 II: Mean (± SEM) specific activities (U/mg of protein) of proteases in individual rainbow trout, Atlantic and coho salmon mucous samples incubated for 1 h with and without *L. salmonis*. * Indicates significantly higher protease activities in incubations with lice than without.

Fish #	Atlantic salmon	Atlantic salmon + lice	Rainbow trout	Rainbow trout + lice	Coho salmon	Coho salmon + lice
1	0.017± 0.002	0.021± 0.001	0.009± 0.000	0.046 ± 0.002	0.041± 0.002	0.042± 0.001
2	0.038± 0.002	0.040± 0.003	0.107± 0.005	0.211 ± 0.018	0.036± 0.001	0.073± 0.006
3	0.022± 0.005	0.024± 0.001	0.060± 0.002	0.055 ± 0.028	0.059± 0.002	0.062± 0.003
4	0.000	0.000	0.046± 0.003	0.050 ± 0.009	0.025± 0.005	0.095± 0.014
5	0.039± 0.001	0.038± 0.003	0.000	0.000	0.054± 0.010	0.061± 0.009
6	0.000	0.000	0.005± 0.003	0.031 ± 0.012	0.000	0.000
7	0.030± 0.004	0.020± 0.001	0.024± 0.007	0.063 ± 0.001	0.130± 0.004	0.120± 0.003
8	0.033± 0.001	0.046± 0.003	0.014± 0.001	0.054 ± 0.002	0.010± 0.001	0.007± 0.001
9	0.000	0.003± 0.000	0.011± 0.003	0.111 ± 0.001	0.000	0.000
10	0.011± 0.002	0.017± 0.002	0.108± 0.001	0.120 ± 0.002	0.000	0.000
Mean	0.019± 0.003	0.021± 0.002	0.038± 0.003	0.074 ± 0.003 *	0.036± 0.008	0.046± 0.006

Figure 4.5: Zymogram of protease activity in individual mucous samples of coho salmon (CS), rainbow trout (RT) and Atlantic salmon (AS) incubated for 1 h without or with (L) *L. salmonis*. Each well contains 3 µg of mucous protein and samples chosen are representative of each species. Numbers along the side of the gel indicate molecular weights (kDa). (◀) Indicates low molecular weight proteases, (+) indicates a positive response, (-) indicates a negative response.

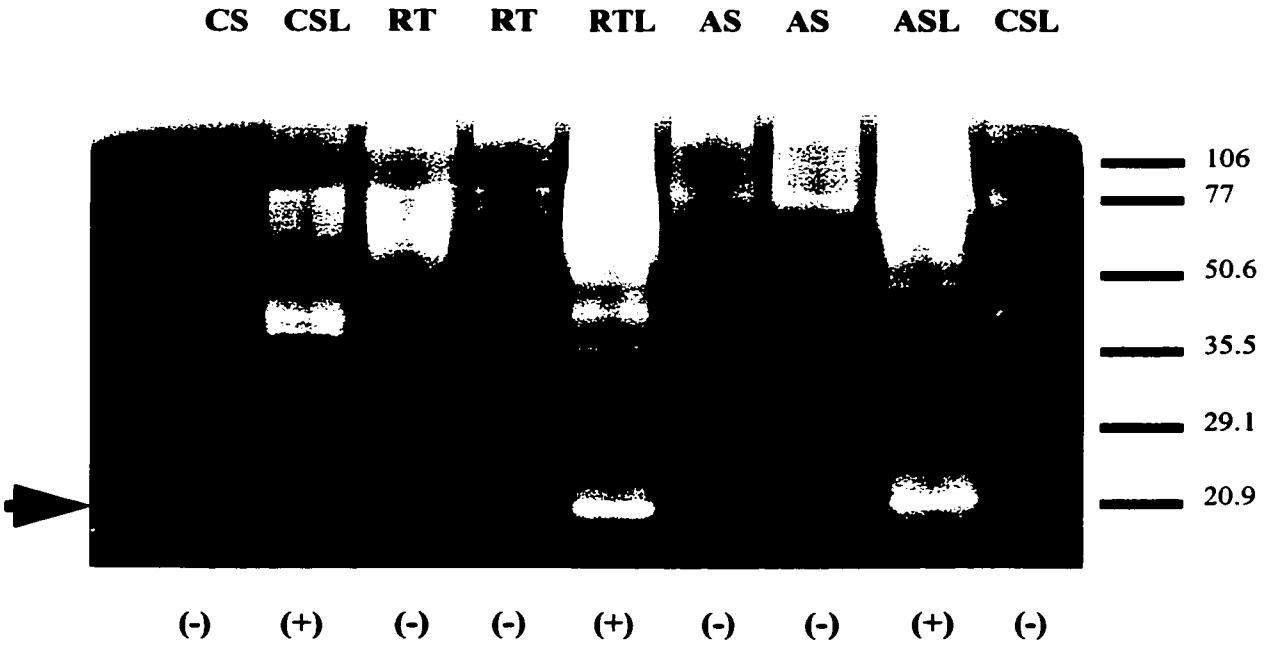
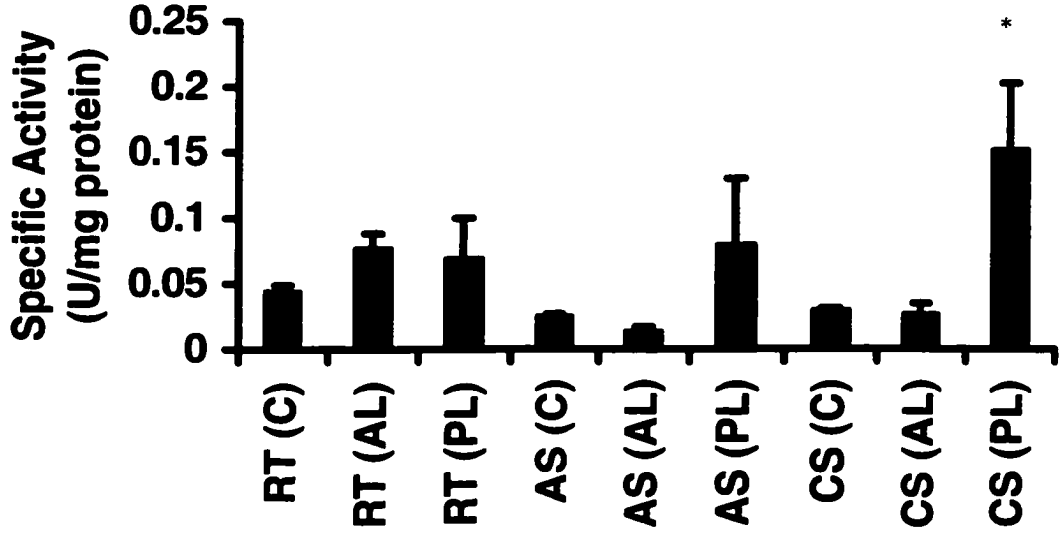


Table 4 III: Mucous samples from individual fish incubated for 1 h with *L. salmonis* showing the presence of multiple LMW protease bands. The presence of multiple LMW protease bands is considered a positive response (+).(-) denotes the lack of multiple LMW protease bands. The left most column indicates individual fish by number. The number of each replicate is along the top of the chart.

	Atlantic salmon			**Rainbow trout**			**Coho salmon**		
Fish #	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1	+	+	+	+	+	-	+	-	-
2	+	+	+	+	+	+	-	-	-
3	+	+	+	+	+	-	-	-	-
4	+	+	+	+	+	+	-	+	-
5	-	+	+	+	-	+	-	-	-
Total number of positive responses		**14**			**12**			**2**	
Percentage of positive responses (individual samples)		**93**			**80**			**13**	

Figure 4.6: Mean (±SEM) specific activity of proteases in mucus of (RT) rainbow trout, (AS) Atlantic salmon and (CS) coho salmon following 1 h incubation with Atlantic coast *L. salmonis* (AL) and Pacific coast *L. salmonis* (PL). (C) denotes mucus incubated without lice. * Indicates significant differences from control and AL ($p<0.05$) (n=20).



coast lice than with the Atlantic coast derived lice; however this difference was not significant (Figure 4.6).

4.5 Discussion

Considerable variation was observed between individual lice responses to pooled fish mucous samples as well as to individual fish mucous samples. Pooled samples were used in order to observe differences among lice to the same conditions. While replicates were also carried out in individual fish mucous incubations with lice, there were much fewer numbers for each (n=3). Lice responses to pooled and individual mucous samples were variable; however, replicates of individual mucous sample incubations with lice were similar to data from pooled mucous sample incubations with lice. The variation observed in these enzymes could be due to differences in the health of the different lice or other factors, such as preference to host substrate. While there is currently no literature on individual variation in salivary components within arthropod parasite populations, particular secretions have been linked to pathogenicity [141,156] and have shown preferences for substrate[197].

The differences could also be due to the condition of the lice. It is possible that in all lice incubations some lice were affected by factors influencing their condition such as the constraints of the incubation tubes. The feeding behaviour of lice is not well understood in terms of time intervals between feeding, length of feeding time, etc. The incubations were carried out for only 1 h, therefore it is possible that some lice may not have needed to feed or had recently released their secretions, resulting in the lice

not secreting enzymes into the sample. It may also be possible that lice do not feed regularly and the 24 h period between removal from the salmon host and incubation was insufficient to allow for products from the host to be completely digested by some lice. Therefore lice nutritional needs may still have been satisfied from the host it had been living on previously. However, other types of arthropod parasites, such as black flies (*Simuliidae* spp.), horn flies (*Haematobia irritans* sp.) and buffalo flies (*Haematobia irritans exigua)*, exhibit intermittent feeding within a 24 h period [146,197,198]. Since there were significant changes in several different parameters, it appears that salmonid mucus did stimulate salivary secretions of sea lice and that the enzymes measured were contributed by secretions from sea lice. The most significant variable was the individual responses of the lice themselves to fish mucus and seawater.

Despite the individual lice variation, differences were observed in lice obtained from different sources. Protease activity was significantly higher in coho salmon mucus when incubated with Pacific coast lice. This may be indicative of an evolutionary difference between Pacific and Atlantic lice populations. Wild coho salmon annually migrate past the farm sites from which the Pacific coast lice were collected (B. Boyce, personal communication). These lice may have evolved to survive on coho salmon resulting in a more active secretion of proteases into coho salmon mucus to assist in infection. Since no corresponding appearance of multiple LMW proteases was observed, the Pacific coast lice may have also adapted altogether different protease secretions from the Atlantic coast. Pacific coast lice have previously been reported on wild and farmed coho salmon, albeit at much lower densities than on rainbow trout and Atlantic salmon [144,199,200]. Genetic differentiation has been observed previously in

populations of lice on farmed salmon in Scotland [201-203]. To determine whether population differences exist in *L. salmonis* from both coasts of Canada much more data would have had to be collected.

The observed increases in mucous proteases in lice incubations with rainbow trout mucus suggest that proteases are present in the secretions of *L. salmonis*. However, it does not appear that these secretions contain sufficient lice protease to have an effect on total protease activity (as measured by azocasein hydrolysis) in the mucus from lice infected rainbow trout (Section 3.4). Several factors such as diffusion into the aquatic environment and greater volume of host mucus may result in *L. salmonis* proteases having little or no effect on total rainbow trout mucous protease activity. The mucus of Atlantic salmon has shown conflicting results regarding the activity of proteases following infection with both increases [87] and no change (Section 3.4) following lice infection. Higher protease activity in rainbow trout mucus incubated with Atlantic coast lice and coho salmon mucus incubated with Pacific coast lice could be a result of lice protease secretion activating latent proteases present in the mucus of these two species. Proteases are salivary constituents of several types of parasites, including nematodes, platyhelminthes and arthropods. The hookworm, *Ancylostoma duodenale*, secretes proteases to interfere with fibrin clot formation and to promote fibrin clot dissolution [147]. A trematode parasite of grazing animals and humans, *Fasciola hepatica*, secretes cysteine proteases into host tissues that may be involved in tissue invasion [162]. Serine proteases have also been discovered in the salivary glands, saliva and mid-guts of insects including the larval blowfly, *Lucilia cuprina* [146,204], the buffalo fly [146], and the mosquito, *Anopheles stephansi* [205]. Sea lice-derived proteases, however, may constitute

very little of the total increase in proteases observed in fish mucus. Endogenous proteases in mucus of these fish may overshadow the presence of lice secreted proteases. It may not always be possible to observe a significant increase in protease activity because of the variation in host mucous protease levels.

Alkaline phosphatase activity was significantly higher in Atlantic salmon mucus following incubation with live Atlantic coast lice. There was no significant differences in AP activities following live lice incubation in the mucus of any of the other species. There was also no significant difference in AP activities in seawater incubated with live lice. Alkaline phosphatase activities of Atlantic salmon mucus were lower than all other species; however, following lice incubation, AP activity was similar to that of the other species. The low AP activity of Atlantic salmon mucus may contribute to Atlantic salmon's greater susceptibility to lice than the other fish. Alkaline phosphatase activities have also been observed to increase in the mucus of lice-infected Atlantic salmon (Section 3.4.3) [87]. The higher AP activity in Atlantic salmon mucus following incubation with live lice suggests that the increase in AP activity observed in Atlantic salmon mucus following infection (Section 3.4.3) is at least partially derived from the sea lice [87]. This suggests that AP may also be a constituent of lice secretions. Alkaline phosphatase has been observed in the salivary extracts of other arthropod parasites such as the cattle tick (*Boophilus microplus*) and the buffalo fly [146]. Conversely, the lack of changes in lysozyme activity following lice incubation of fish mucus, suggest that the observed increases in mucous lysozyme activity in rainbow trout and Atlantic salmon following lice infection (Section 3.4.3) are derived from the host or may take longer than one hour to arise from the louse.

Multiple bands of LMW proteases were observed in the mucus of rainbow trout and Atlantic salmon in 70-80% of the lice incubations in this study. Atlantic salmon mucus incubated with live lice has previously shown multiple LMW protease bands in 100% of lice incubations [107]. The sample size used by Firth *et al.* differed from the present study in that only 6 replicates were carried out [107] in comparison to 30 replicates in this study. Mucous samples were also collected in ammonium bicarbonate by Firth *et al.* [107]. Although the ammonium bicarbonate buffer was removed through lyophilization prior to lice addition, it is possible that residual ammonium bicarbonate may have affected the lice. Finally, lice were maintained in aerated seawater for 4-6 days prior to incubation by Firth *et al.*[107] as compared to 24 h in this study. A span of 4-6 days without access to food may have increased the probability of lice secreting enzymes into mucous incubations. In Section 3.4, the same banding of LMW proteases was observed, late in the lice infection, in the mucus of rainbow trout and Atlantic salmon, but not in coho salmon. Flounder and coho salmon mucus and seawater caused many fewer lice to secrete LMW proteases. Positive responses (ie. presence of LMW proteases) to seawater suggested that handling stress may cause a few lice to secrete LMW proteases. However, the significantly lower number of positive responders (lice) in seawater as compared to Atlantic salmon and rainbow trout suggests that the responses in rainbow trout and Atlantic salmon were not due to lice handling stress. Similarly, the lack of stimulation to secrete proteases by lice exposed to coho salmon and winter flounder mucus suggests that the lice response to rainbow trout and Atlantic salmon was specific to these hosts.

Increased protease banding in the mid-high MW range observed in some Atlantic salmon and rainbow trout mucous incubations with lice, may have been the result of LMW protease secretions activating latent proteases in the fish mucus.

Previous work characterized LMW proteases secreted by lice in the presence of salmon mucus as trypsin[107]. Trypsin is a digestive protease found in the midguts and saliva of several other types of arthropod parasites [146]. Not only could trypsin have a function in digestion of host tissues, but could also participate in evading host defenses and immunosuppression. Huber *et al.* discovered the presence of receptor sites in human monocytes which, when bound to antigen-antibody complexes, induced phagocytosis [206]. Huber *et al.* then eliminated the complement-induced phagocytosis by treating the monocytes with trypsin [206]. Trypsin could possibly be used by the lice to decrease host phagocytic activity and decrease immune response to the ensuing infection. In Section 3.4, a reduced phagocytic and respiratory burst response was observed in lice infected rainbow trout and Atlantic salmon at the same time that the multiple bands of LMW proteases occurred in the mucus. Saliva from other arthropod parasites have shown the ability to decrease macrophage function in their respective hosts. The cattle tick, *Rhipicephalus sanguineus*, has been shown to impair host T cell proliferation and interferon (IFN)-γ-induced macrophage microbicidal activity [152]. Saliva from the sand fly, *Phlebotomus papatasi*, has also shown the ability to inhibit host IFN-γ-induced macrophage activation [207]. The multiple bands of LMW proteases (trypsin) may be linked to virulence of the lice either through immunomodulation or in their capacity as digestive enzymes. Zuo and Woo observed that metalloprotease secretions from the hemoflagellate, *Cryptobia salmositica,* directly lysed fish leukocytes and was also one of

the causes of anemia in salmonid cryptobiosis [208]. Other work by Zuo and Woo has also suggested that the same metalloprotease secretions by *C. salmositica* are related to the pathogenicity of the parasite [141,156]. Several other arthropod parasites have also been shown to take advantage of immunomodulatory effects of salivary gland components[152,207,209,210].

Coho salmon experimentally infected with *L. salmonis* have been reported as mounting an acute inflammatory response as early as 1 dpi and a well-developed hyperplasia at 10 dpi, in the gills [144]. Coho salmon infected with glochidia of the freshwater mussel *Margaritifera margaritifera* have also shown acute inflammation including infiltration of eosinophilic granule cells (EGC) early in the infection and hyperplasia at 14 dpi [211]. Acute inflammation with extensive infiltration of EGCs has been described in other host fish responses to parasitic copepods of the *Lernaea* spp[212,213]. Not only has no inflammatory response been observed in Atlantic salmon infected with *L. salmonis* [144], but in the current study lice did not secrete the same enzymes in response to coho salmon mucus as they did in response to Atlantic salmon mucus. As these findings were made in the absence of active host cells, it suggests that differences exist in the mucous layer of Atlantic salmon and coho salmon apart from cell mediated processes that may account for the differences in resistance to lice.

Overall results suggest that there are factors in rainbow trout and Atlantic salmon that identify these species as more acceptable hosts, whereas lice identify winter flounder and coho salmon as unacceptable hosts and do not secrete the same variety of enzymes into their mucus. However, since lice infect coho salmon at similar densities (Section 3.4.1) to rainbow trout and Atlantic salmon, but for a shorter duration, the

possibility remains that coho salmon resistance is not due to a lack of stimulation of lice secretory products, such as occurs in seawater and winter flounder incubations with live lice. Coho salmon's ability to rapidly reduce lice infection may therefore result from factors in the mucus that block these lice secretions. Further work into fractionation of coho salmon mucus could be useful in isolating a possible lice inhibitory factor. At the same time, fractionation of Atlantic salmon mucus and the mucus from other susceptible hosts may be useful in isolating factors that promote lice infection. The characterization of biochemical components released from sea lice may provide insight into their feeding mechanisms and pathogenesis.

5.0 General Discussion

As host pathogen relationships are continually evolving, fish disease susceptibility and resistance will always be an important area of research and area of concern for farm managers. Rainbow trout, Atlantic salmon and coho salmon contribute most of the farmed salmon production worldwide and variable susceptibility to different disease states has been observed even between these closely related species. The innate immune system plays a major role in host resistance and susceptibility to disease. Comparisons of baseline innate factors in the skin mucus (Section 2.4) showed differences between rainbow trout and coho salmon (protease profiles, mucous and plasma lysozyme activities), rainbow trout and Atlantic salmon (protein profiles, mucous and plasma lysozyme activities) and between Atlantic and coho salmon (protein and protease profiles; plasma and mucous lysozyme activities). This research forms a foundation for further studies into species specific innate immune responses towards particular pathogens. The similarities observed in rainbow trout mucous proteins (ca. 66 kDa protein) and proteases, previously described as having antibacterial properties, with those observed in the mucus of Atlantic and coho salmon, may suggest similar antibacterial properties of these peptides in the latter two species. Future studies on the antibacterial properties of mucous protein and proteases observed in Section 2.4 may elude to the functions of these various peptides in the mucus of rainbow trout, Atlantic and coho salmon.

Differences in enzyme activities between all three species maintained in freshwater as compared to those maintained in seawater (Section 2.4) provide a

basis for further studies into pathogen defense and holding environment. Investigation into other immune factors that may change following smoltification or introduction to seawater may give further insight to evolutionary adaptations to pathogen invasion in different rearing environments.

Following sea lice infection, physiological stress parameters did not change significantly in any of the three species (Section 3.4.2). Changes in enzymatic activities following infection were similar in Atlantic salmon and rainbow trout but differed from coho salmon (Section 3.4.3). Baseline lysozyme activities in the mucus of these species suggest that the increase in mucous lysozyme observed at 1 dpi was a host-mediated response to initial lice infection. Juvenile *L. salmonis* have been shown to cause indirect effects on the skin and gills of rainbow trout following confinement stress [199]. It would be useful in the future to localize the enzymatic activity of the mucus and epithileal layers in order to understand the mechanisms behind the increases of lysozyme, proteases, and alkaline phosphatase. Immunocytochemical localization of lysozyme in intestinal eosinophilic granule cells has been determined in Atlantic salmon using rabbit anti-human lysozome polyclonal antibody that recognized purified rainbow trout kidney lysozyme [214]. Using this technique it may be possible to also localize the production of lysozyme, proteases and alkaline phosphatase in mucus and skin as a response to feeding activities of lice.

The growth of juvenile lice on primary salmon epidermal cell cultures would allow for manipulations of juvenile lice without the need for fish hosts. Addition of salmon mucous fractions to these assays may give insight into the early rejection of lice by coho salmon or the promulgation of lice on rainbow trout and

Atlantic salmon. For instance, frontal filament development of lice could be compared in the presence of coho salmon, Atlantic salmon and rainbow trout mucus. This would permit observations on whether or not coho salmon mucous factors directly attack the frontal filament and prevent lice from remaining attached as easily as they remain on susceptible species.

Chinook and sockeye salmon, sea trout, rainbow trout and other salmonids display susceptibility to *L. salmonis* infection, albeit to a lesser extent than Atlantic salmon [35,144,175], and coho salmon which also acts as a host for up to 10-14 days. It appears that all of these species attract the settlement and attachment of *L. salmonis*. Following attachment the lice undergo several moults until they reach the adult stage of their life cycle and begin to reproduce. However, unlike infection in other salmonids, lice infection quickly decreases on coho salmon (Section 3.4.1) [144]. This suggests that there may be some factor in the susceptible salmonids that encourages the lice infection, but is not present in coho salmon. When looking at incubations of lice in the presence of fish mucus, coho salmon mucus showed similar results to flounder mucus and seawater (Section 4.4). This too suggests that, similar to the mucus from a non-host species and seawater, there is nothing in the coho salmon mucus that stimulates lice secretion of enzymes. However, lice can attach and undergo several moults on coho salmon (Section 3.4.1) [144] whereas this has not been observed on a non-host species such as flounder. Section 3.4.1 also showed no significant differences in the initial numbers of lice per available surface area between coho salmon, rainbow trout and Atlantic salmon. Coho salmon therefore still attract

the settlement and attachment of lice. The lice are able to grow, moult and live on coho salmon, but at much lower numbers than susceptible species.

Previous work has shown that coho salmon injected with cortisol exhibit increased susceptibility to sea lice infection [63]. As cortisol is recognized as a potent immunosuppressant under chronically elevated conditions, this suggests that its injection suppresses the ability of coho salmon to mount an immune response to sea lice, thereby allowing infection similar to other susceptible salmonids. It therefore appears that there is some anti-lice factor produced by coho salmon. This factor works quickly in decreasing the numbers of lice and, since specific immunity is delayed in fish, it is probably already present in the mucus prior to lice infection. The mucus of coho salmon injected with cortisol should be compared to control coho salmon mucus. Protein profiles of test and control fish may show the increased or decreased intensity of certain proteins following cortisol injection that allow lice to remain on the coho salmon host.

Isolation of mucus factor(s) that provide protection to coho salmon may be useful in improving the culture of Atlantic salmon and rainbow trout in sea cages in several ways. A coho salmon inhibitory factor could be used in the production of a treatment for sea lice. Since this factor would be a natural component of coho salmon mucus, concern for tissue residence times in Atlantic salmon and possible environmental risks should be negligible. Investigations into whether Atlantic salmon and/or rainbow trout also produce this factor, but to a degree insufficient enough to afford protection from lice, could lead to manipulation of breeding programs to increase the amount of the protective factor in the mucus of

Atlantic salmon and/or rainbow trout. Although genetically modified fish currently are not well accepted by consumers, Atlantic salmon could be genetically modified to incorporate the genes to synthesize the inhibitory factor of coho salmon mucus, when and if genetic manipulation of farmed animals becomes politically acceptable.

Work needs to be done on *L. salmonis* salivary extracts in determining their contents and capabilities. One important study would be to collect saliva from *L. salmonis* adults and observe its effects on antigen presentation, T cell proliferation and IFN-γ- induced macrophage activity in salmonid cell lines. Further characterization of lice salivary contents may discover the means through which sea lice evade host defenses and lead to a better understanding of host-parasite interactions. Isolation of these salivary constituents may be used as possible vaccine candidates for Atlantic salmon and other susceptible salmonids. All of these possibilities would lead to an eventual decrease in lice treatment costs with lower environmental risks and possible increases in the profit margin for the Atlantic salmon fish farmer.

6.0 CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1) Protein profiles of rainbow trout, Atlantic and coho salmon mucus.
    - Different relative intensities of <14, 18, 31 kDa proteins among species
    - Proteins of similar expression in all three species (66 kDa range)

2) Protease profiles of rainbow trout, Atlantic and coho salmon mucus.
    - Proteases of 42-45 kDa (2) and 65 kDa (possible serine protease activated metalloproteases) in coho salmon mucus
    - 85 kDa metalloprotease in coho salmon mucus
    - 18 kDa serine protease in coho salmon mucus (possible trypsin)
    - Calcium dependent proteases (ca. 60%), serine proteases (65%) and metalloproteases (35%) in coho salmon mucus
    - Rainbow trout mucus proteases mainly consist of serine (75%) and metalloproteases (25%)
    - 45 and 80 kDa serine proteases in rainbow trout mucus
    - 33, 48-73 kDa proteases in rainbow trout mucus (possible serine protease activated metalloproteases)

3) Mucous lysozyme activities are much higher in rainbow trout mucus than in Atlantic and coho salmon mucus.

4) Higher mucous alkaline phosphatase and protease activities are present in all three species when reared in FW as compared to SW.

5) No significant differences in mucous protein profiles, mucous lysozyme activity or plasma lysozyme activity was observed in rainbow trout adapted to SW as compared to being reared in FW.

6) Host susceptibility towards lice follows the order Atlantic salmon > rainbow trout >> coho salmon
    - Lice developed faster on Atlantic salmon as compared to rainbow trout
    - Lice developed faster on rainbow trout as compared to coho salmon
    - Decreases in macrophage function occurred at 14 dpi in Atlantic salmon, but did not occur until 21 dpi in rainbow trout
    - Increases in multiple bands of LMW proteases occurred at 14 dpi in Atlantic salmon but not until 21 dpi in rainbow trout

7) There were no significant changes in physiological parameters during rejection of lice in coho salmon.

8) There was no significant increase in enzymatic activity in the mucus of coho salmon during rejection of lice

9) Lysozyme activity increased significantly in the mucus of coho salmon at 21 dpi, coinciding with the appearance of pre-adult lice on the coho salmon.

10) There was a negligible increase in multiple bands of LMW proteases in the mucus of coho salmon and flounder following incubation with live lice as compared to the mucus of Atlantic salmon and rainbow trout.

11) Alkaline phosphatase increased in the mucus of Atlantic salmon incubated with live lice.

12) Protease activity increased in the mucus of rainbow trout incubated with lice from New Brunswick and in the mucus of coho salmon incubated with lice from British Columbia.

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8.0 APPENDICES

8.1 Appendix A

LIST OF REAGENTS AND RECIPES

Protease Zymography:

Separation gel (12% acrylamide)	Stacking gel (4% acrylamide)
7.05 mL dd H_2O	18.3 mL dd H_2O
7.5 mL 1.5M Tris, pH 8.8	7.5 mL 0.5M Tris, pH 6.8
300 µL 10% SDS [#]	300 µL 10% SDS
12 mL 30% acrylamide/bis	3.9 mL 30% acrylamide/bis
combine ingredients and then degas for 15 min	combine then degas for 15 min
3.0 mL gelatin [#] (50 mg in 5 ml H_2O, boil to dissolve)	150 µL ammonium persulfate
150 µL 10% ammonium persulfate [#]	30 µL TEMED
15 µL TEMED[#]	

In both separating and stacking gels add TEMED last. Slowly swirl to mix and then load solution between two Mini PROTEAN® 3 System glass plates[#]. Allow to polymerize for 1-2 h.

Running Buffer	2X SDS-PAGE Standard Buffer (SB)
add 210 mL 5X SDS-page running buffer (Tris 15 g/L, glycine[#] 72 g/L, SDS 5 g/L) to 840 mL dd H_2O	4.0 mL dd H_2O 1.0 mL 0.5 M Tris-HCl, pH 6.8 0.8 mL glycerol [$] 1.6 mL 10% SDS [#] 0.2 mL 0.05 % bromophenol blue [3] (Combine ingredients)

(Same gel preparation and running buffer for SDS-PAGE except no addition of gelatin) 2X SDS-PAGE SB is also similar for SDS-PAGE analysis with one exception. 50 mM DTT is added to reduce disulphide bonds.

Zymography Washing Buffer
6.075 g Tris base is dissolved in 300 mL dd H_2O, and 6 M HCl added until a pH of 7.5 is reached. Make up to 500 mL by adding dd H_2O.
then add 250 mL 10% Triton X-100 # and 250 mL dd H_2O

1.5 M Tris-HCl, pH 8.8
27.23 g Tris base #
80 mL dd H_2O
Adjust pH to 8.8 with 1M HCl
Make up to 150 mL with dd H_2O

0.5 M Tris-HCl, pH 6.8
6 g Tris base
60 mL dd H_2O
Adjust pH to 6.8 with 1M HCl
Make up to 100 mL with dd H_2O

Acrylamide/bis (30% T, 2.6% C)
add 90 g acrylamide/bis to 300 mL with dd H_2O
Note: Using BioRad's 37:5:1 preweighed mixture

Store both 1.5 M and 0.5 M Tris-HCl and acrylamide/bis (30%T, 2.67% C) solutions at 4^0 C

Macrophage Assays

L-15 (Leibovitz) medium preparation ^

All preparations contain 100 i.u./mL Penicillin-Streptomycin (Pen-Strep) ^

A) 1 L L-15 with 2% Fetal Calf Serum (+100 i.u./mL Pen-strep + 10 i.u./mL Heparin)

L-15	1000 mL
Pen-strep	10 mL (100 i.u./mL; from 10,000 units/mL)
Heparin	10 mL (10 i.u..mL; from 1,000/mL) ^
FCS	20 mL ^

B) 1 L L-15 with 0.1% FCS (+ 100 i.u./mL Pen-strep)

L-15	1000 mL
Pen-strep	10 mL (100 i.u./mL; from 10,000 units/mL)
FCS	1 mL

NBT/PMA preparation

A) Nitroblue tetrazolium (NBT) (1 mg/mL) ^
1 tablet containing 10 mg dissolved in 10 mL L-15

B) Phorbol myristate acetate (PMA stock) (1 mg/mL) ^
1 bottle (5 mg) dissolved in 5 mL ethanol (95%) and aliquotted into 0.5 mL tubes.

C) NBT/PMA solution
10 µL PMA in 10 mL NBT

Bacterial preparation

Bacterial stock (Formalin killed *Yersinia ruckeri* at OD_{350} =1.067 which is ca. $1x10^9$/mL)
(Prepared by Dr. Simon Jones, Immunology Lab, UPEI-AVC)

Preparation: 2 mL of bacterial stock
Wash 2X with phosphate buffered saline (PBS) or L-15
Resuspend cells in 2 mL PBS or L-15
Adjust cells to desired concentration ($1x10^7$ –$1x10^9$)

Phosphate Buffered Saline (PBS) #

NaCl	8 g	#
K_2HPO_4	1.44 g	#
KH_2PO_4	0.24 g	#

Make up to 1L with dd H_2O (pH: 7.2 - 7.4)

BioRad, Richmond, CA

$ BDH, Toronto, ON

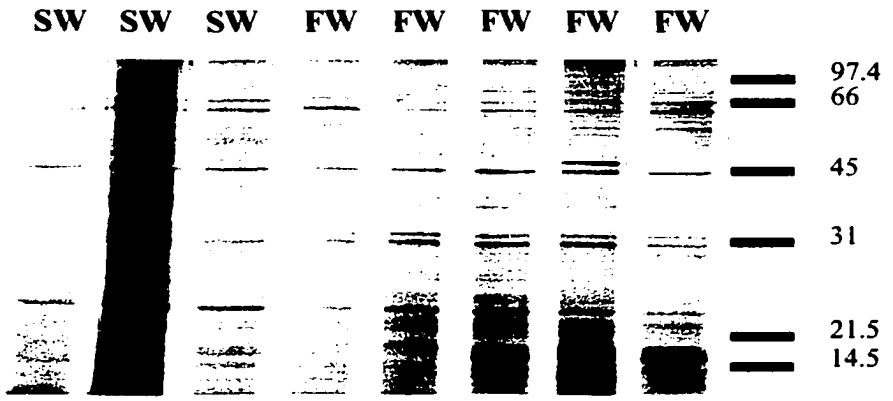
^ SIGMA, St. Louis, MO

8.2 APPENDIX B

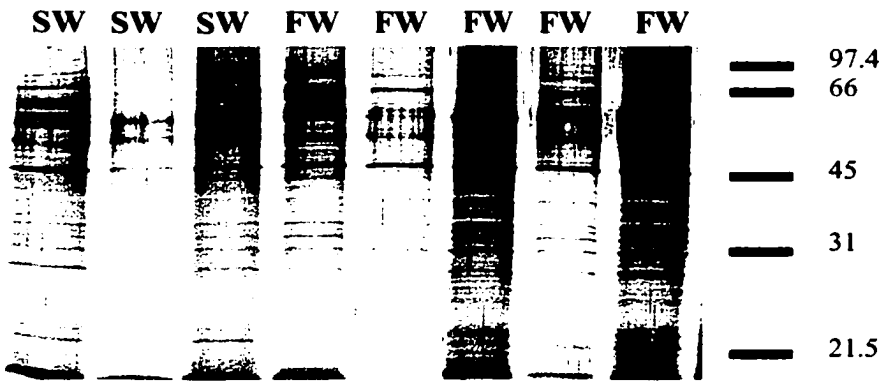
CONTRIBUTING WORK

Figure 8.1: Mucous protein profiles of rainbow trout (RT), coho salmon (CS) and Atlantic salmon (AS) reared in freshwater (FW) and seawater (SW). Molecular weights (kDa) are along the right hand side of each gel. Each gel contains 1 µg of mucous protein. Representative samples of each species expressed here were chosen based on the most common protein banding patterns observed for each species.

RT



CS



AS

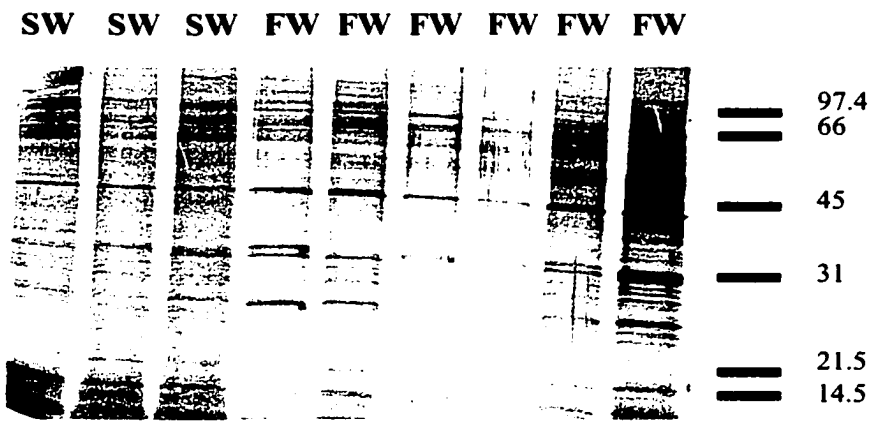


Table 9 I: Effect of specific protease inhibitors on protease activity in the mucus of three seawater-reared salmonid species, using the azocasein assay. Individual tubes containing specific inhibitors were incubated with mucous samples and azocasein substrate for 19 hours. Representative fish (n=5) were chosen for each species and mean ± SEM displayed. Values are expressed as a percent inhibition of the total (100 %) control protease activity. Azocasein assays were analyzed for all the fish sampled and the samples with the most common protease activities determined. Five of these samples were then chosen as representative samples for each species.

Species	***o*-phenanthroline** (10 mM) + **Aprotinin** (0.011 mg/mL)
Rainbow trout	100 ± 0
Coho salmon	100 ± 0
Atlantic salmon	100 ± 0

Table 8 II: Inhibition of trypsin by mucous samples, obtained from salmonids experimentally infected with *L salmonis*, using the azocasein hydrolysis assay. Individual tubes containing mucous samples of each species were incubated with azocasein substrate and with (T) and without (I) trypsin (0.001µg/µL) for 19 hours. Representative fish (n=5) were chosen for each species and mean specific protease activity (U/mg protein) ± SEM displayed. Azocasein assays were analyzed for all the fish sampled and the samples with the most common protease activities determined (closest to the mean). Five of these samples were then chosen as representative samples for each species.

	Rainbow trout		**Coho salmon**		**Atlantic salmon**		**Tryspin**
Fish #	**I**	**T**	**I**	**T**	**I**	**T**	**standard**
1	0.063	0.192	0.043	0.121	0.064	0.148	0.075
2	0.035	0.122	0.053	0.122	0.055	0.187	0.084
3	0.044	0.110	0.032	0.155	0.048	0.168	0.077
4	0.035	0.140	0.034	0.102	0.042	0.126	0.087
5	0.058	0.389	0.038	0.128	0.045	0.123	0.082
Mean	0.047	0.191	0.040	0.126	0.051	0.150	0.081
SEM	0.006	0.057	0.004	0.019	0.005	0.060	0.003

Incubation with trypsin increased the protease activities in all three species similarly, and did not appear to be inhibited by the mucus of the three species of salmon.

Figure 8.2: Mean (±SEM) specific activity of lysozyme in pooled mucous samples of four species of fish incubated for 1 h with *L. salmonis*. Six fish were pooled for each species. (RT) rainbow trout, (AS) Atlantic salmon, (CS) coho salmon, (F) winter flounder. (C) denotes control mucus and (L) denotes mucus incubated with lice (n=30).

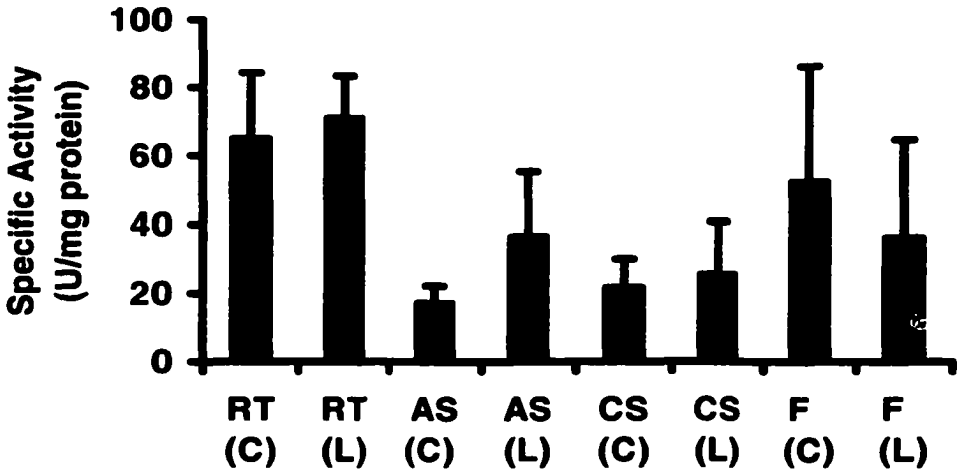


Figure 8.3: Mean (±SEM) specific activity of alkaline phosphatase in pooled mucous samples of four species of fish incubated for 1 h with Pacific coast *L. salmonis*. Six fish were pooled for each species. (RT) rainbow trout, (AS) Atlantic salmon, (CS) coho salmon, (F) winter flounder. (C) denotes control mucus and (L) denotes mucus incubated with lice (n=10).† Denotes 6/10 samples showed increases in activity.

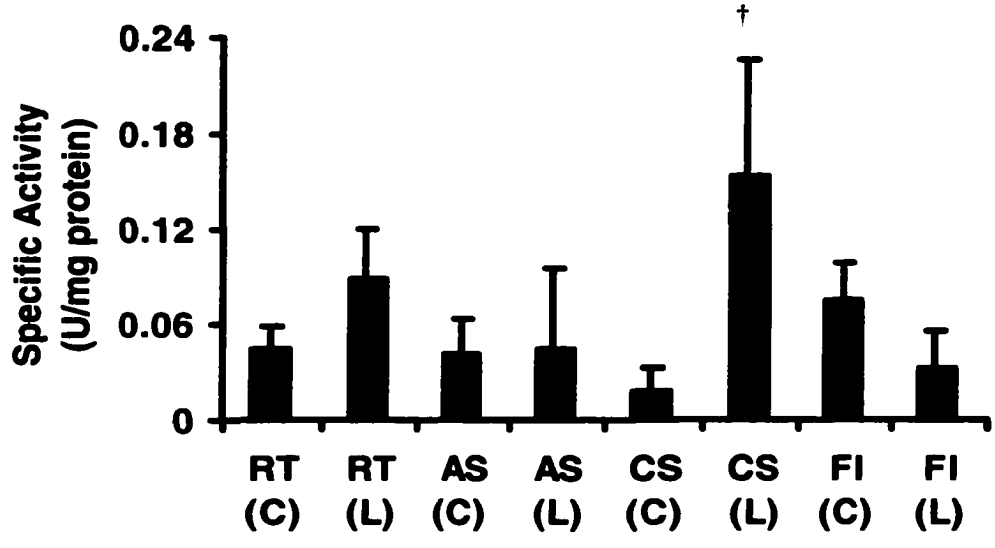


Figure 8.4: Zymogram of protease activity in pooled mucous samples of Atlantic salmon (AS), coho salmon (CS), winter flounder (F) and rainbow trout (RT) incubated for 1 h without and with (L) live Pacific coast *L. salmonis*. Six fish were pooled for each species. Each well contains 3 µg of mucous protein and samples chosen are representative of each species. Numbers along the side of the gel indicate molecular weight (kDa). (➔) Indicates low molecular weight proteases, (+) indicates a positive response, (-) indicates a negative response. All fish samples were analyzed using protease zymography and representative samples chosen from the samples that exhibited the most common mucous protease patterns for each species.