The Differential Secretion of Proteolytic Salivary Enzymes by the Salmon Louse, *Lepeophtheirus salmonis* (Copepoda: Caligidae) in Response to the Skin Mucus of Various Salmonid and Non-Salmonid Fish Hosts

An undergraduate research project

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ABSTRACT

Lepeophtheirus salmonis is a salmonid-specific marine ectoparasite but has also been documented on white sturgeon, *Acipenser transmontanus*, and three-spine stickleback, *Gasterosteus aculeatus* in coastal British Columbia (BC). Sea lice graze on the mucus and epithelial cells of their host and this initial interaction provides an ideal medium for examining the host-parasite relationship. Previous studies determined that “trypsin-like” enzymes in the mucus of *L. salmonis*-infected fish originated with the parasite and may be differentially secreted in response to various fishes as an indication of host-specificity. Geographic variation between east and west coast lice was also noted. Thus, the main objective of the present study was to establish proteases in the saliva of BC *L. salmonis* and then determine whether secretion was differential in response to pink salmon, *Oncorhynchus gorbuscha*, chum salmon, *Oncorhynchus keta*, Atlantic salmon, *Salmo salar*, white sturgeon, and three-spine stickleback. Mucus was removed from naïve fishes, combined to form pooled species solutions, and then exposed to *L. salmonis*. A “trypsin-sensitive” azocasein assay detected increased enzyme activity in louse-incubated mucus from pink (0.12 ΔOD₄₅₀), chum (0.08 ΔOD₄₅₀), and Atlantic salmon (0.15 ΔOD₄₅₀), white sturgeon (0.13 ΔOD₄₅₀), and three-spine stickleback (0.15 ΔOD₄₅₀) in comparison with non-incubated mucus. Some variation in protease activity was evident between species but the differences were not significant (Kruskal Wallis 1-way ANOVA: $\chi^2 = 10.32$, df = 5, $P = 0.07$). Although not statistically significant, these results inspire new questions. For example, could the indiscriminate release of enzymes confer the trophic status of generalist upon BC *L. salmonis* in contrast with its more specialized east coast relative? Local descriptions of the louse on various marine fish species indicate an expansion of host range with the potential inclusion of paratenic hosts.
INTRODUCTION

Copepods, also known as the “insects of the sea” (Bob Kabata, personal communication) are arguably the most abundant organism on earth, occurring in both marine and fresh water environments. Free-living, filter-feeding copepods form a large part of the zooplankton and serve as an important trophic link between phytoplankton and fish in aquatic ecosystems. Through the process of evolution, many copepods abandoned this common niche to develop the more exploitive lifestyle of parasitism. Various adaptations have evolved among copepods to enhance their parasitic lifestyle creating a diversity of relationships between copepods and their hosts (Kabata, 1988). Despite this immense diversity, the majority are ectoparasitic on the skin and gills of marine fishes (Kabata, 1979). Copepods of the Caligidae (Siphonostomatoida: Copepoda), collectively referred to as sea lice, occur on the skin, fins, and gills, and in the buccal cavity of marine fishes (Jones et al., 2006 a). *Lepeophtheirus salmonis* (Copepoda: Caligidae) is a common ectoparasitic copepod of salmonid species in the northern hemisphere (Kabata, 1979).

Survival in the dynamic environment provided by the external surface of a swiftly swimming salmon has selected for unique morphological adaptations in caligids both for finding and retaining a host. First antennae act as sensory organs to assist in host location. This feature is thought to be enhanced by the functional behaviours of positive phototaxis and vertical migration (Pike and Wadsworth, 1999). Combined, these abilities provide opportunity for the upward moving parasite to contact potential fish hosts (Pike and Wadsworth, 1999). Further parasitic adaptations enhance copepod attachment to the host and include prehensile maxillipeds and second antennae. The cephalothorax and third thoracic segment are dorsoventrally flattened and form a disc-shaped suction cup that assists in adhesion (Roberts and Janovy, 2005). A unique frontal filament provides additional security to the vulnerable parasitic larval stages of the copepod. Adaptations for feeding
combine the saw-like action of serrated mandibles with the suctioning capacity of the mouth tube which allows the parasitic stages of the sea louse to graze on host mucus and epithelial cells (Fast et al., 2003). As pre-adults and adults the parasites become more invasive, gaining the ability to feed on host blood (Firth et al., 2000; Jones et al., 2006a).

Reproductive adaptations complement a direct life cycle and have conferred a high rate of fecundity on caligid copepods. Gravid females extrude egg strings containing hundreds of eggs that are continuously dispersed into the marine environment. Eggs undergo rapid development through two planktonic naupliar stages to the infective copepodid stage in the free-living phase of the 10 stage life cycle (Kabata, 1972; Johnson and Albright, 1991). The parasitic phase of the life cycle begins with successful attachment of the copepodid to a fish host. Attachment is followed by development of the frontal filament and subsequent moulting through four parasitic larval stages; chalimus 1-4 (Kabata, 1972; Johnson and Albright, 1991). While still parasitic, the two pre-adult stages and the adult stage are highly motile and capable of movement on and off hosts.

*Lepeophtheirus salmonis* earned its reputation as an economically important parasite of farmed and wild salmonids throughout the northern hemisphere as their prolific capabilities were expressed on Atlantic salmon farms (Firth et al., 2000; Fast et al., 2002). The crowded conditions typical of aquaculture operations provided caligids, with their direct life cycle, the optimal environment for transmission. Outbreaks of *L. salmonis* infections were observed at fish farms in Norway in the 1960’s; Scotland in the mid 1970’s; and on the Atlantic coast of North America by the late 1980’s (Pike and Wadsworth, 1999). The loss of millions of dollars per year to the salmon farming industry stimulated a large amount of research into sea lice biology in these regions, with a focus on controlling the parasite (Pike and Wadsworth, 1999). Whereas research led to the production of treatments, these met with limited success due to rapid expansion of the industry. Environmental
concerns were raised over the possibility of toxic effects from treatments spreading to other marine life and the ultimate concern over the role farmed fish may play in the epidemiology of the parasite to native salmon species (Pike and Wadsworth, 1999).

Norway, the largest farmed salmon producer in the world, began to record annual epizootics on wild fish as early as 1989 (Bjorn and Finstad, 1998), with salmon farms considered important contributors (Heuch et al., 2005). Subsequent global public awareness, coupled with observations of increased L. salmonis infections on native and farmed salmon in British Columbia (Morton et al., 2004), illuminated the need for data collection on the west coast of Canada. Fisheries and Oceans Canada began monitoring the abundance and distribution of sea lice on juvenile pink and chum salmon in coastal British Columbia’s Broughton Archipelago in 2003 (Jones and Nemec, 2004). A similar survey on adult Pacific salmon began in 2004 (Beamish et al., 2005). Since that time, L. salmonis has been documented on adults of all five Pacific salmon species (Oncorhynchus spp.) (Beamish et al., 2005), and on pink (O. gorbuscha) and chum (O. keta) salmon fry (Morton et al., 2004) in western Canadian coastal waters. Although traditionally considered specific to salmon, L. salmonis has been reported on non-salmonid species (Kabata, 1979) and was recently described on three-spine stickleback (Gasterosteus aculeatus) in coastal British Columbia (Jones et al., 2006 a).

Although carrying a small number of sea lice is not harmful to the fish host, heavy infections have been associated with significant host pathology in both farmed and wild salmonids in the northern Atlantic Ocean (Bjorn and Finstad, 1998). Observed pathology includes osmoregulatory stress and death in extreme cases. The effects of L. salmonis infection vary among host species. Beamish et al. (2005) reported minimal skin damage and scale loss to Pacific salmon in BC coastal waters, with rare exceptions. Jones et al. (2006 b) found no significant correlation between sea lice
intensity and condition factor in sticklebacks; however, it is important to note that stickleback do not typically carry the more invasive adult lice.

Establishing the role of sea lice in the regulation of wild and farmed salmon production has been the principal motivation behind *L. salmonis* research in coastal BC. Elucidation of host-parasite connections necessitates understanding all aspects of the biology of both the parasite and the host. Naturally occurring fluctuations in salmon populations off the coast of BC result in variation from year to year potentially having an effect on sea lice abundance (Beamish *et al.*, 2005). In the Broughton Archipelago the life cycle of anadromous salmon may facilitate parasitic transmission as the return of adults to freshwater spawning grounds overlaps with aggregating coastal juveniles (Richard Beamish, personal communication). Adult Pacific salmon can be infected with *L. salmonis* in the deep ocean and carry their parasitic load back to shallow waters (Bob (Z) Kabata, personal communication). Young pink salmon inhabiting the near shore waters have been found with *L. salmonis* larval nauplii in their stomachs (Richard Beamish, personal communication). Juvenile salmon feeding in close proximity to nauplii, which develop to the infective copepodid stage within 24-48 hours, provides evidence of closely linked host-parasite life cycles. The coincidental proximal location of salmon farms may inadvertently place additional hosts in the middle of a naturally occurring cycle. Researchers at the Pacific Biological Station in Nanaimo BC are currently integrating about 4 years of data on sea lice abundance and prevalence with a similar data set collected by the local salmon farming industry that will provide the foundation for a comparative study (Richard Beamish, personal communication).

Water temperature, salinity, abundance of hosts, and water currents act in combination to influence the prevalence and intensity of *L. salmonis* infections (Jones *et al.*, 2006 b) and all are subject to regional variation. The differential effect of geographic variables on the complex
interplay between parasites and their hosts demonstrates the importance of conducting research locally (Rohde, 1982). Although some important research, including studies on the morphology and biology of *L. salmonis*, originated in BC (Kabata, 1979), to date the vast majority of sea lice research has been conducted in the northern Atlantic region. A number of scientists in Norway and on the east coast of Canada have accumulated data from observations of *L. salmonis* interactions with Atlantic salmon, *Salmo salar* (e.g., Bjorn and Finstad, 1998; Fast *et al.*, 2003). Although this global collection of knowledge serves as a valuable frame of reference enabling the establishment of trends and formulation of hypotheses, it does not negate the need for local observations (Jones *et al.*, 2006 b). British Columbia coastal waters are home to five native salmon species; pink, chum, chinook, coho, and sockeye salmon. *L. salmonis* inhabiting BC coastal waters may be a different strain than those found in the northern Atlantic Ocean (Simon Jones, personal communication). Fast *et al.* (2003) showed that *L. salmonis* from New Brunswick and BC responded differently to the same salmon species. Geographic variation in both host and parasite may result in varied host-parasite interactions.

In recent years researchers on the east coast of Canada have focused on comparative epidemiological studies. For example, Ross *et al.* (2000) demonstrated that resistance to infection with *Lepeophtheirus salmonis* differed between species of salmon. Coho salmon, *O. kisutch*, infected with sea lice, initially showed high levels of infection but rapidly lost their parasitic load suggesting the presence of resistant factors in coho salmon mucus that provide protection from parasitic copepods (Fast *et al.*, 2002). In contrast, Atlantic salmon infected to a similar degree maintained relatively high levels of infection over time and *L. salmonis* has been shown to mature more quickly on this salmonid species (Fast *et al.*, 2002). Such differential resistance was thought to
be due to factors in the mucus (or epithelial layer), or to differences in the immune systems of the host species (Fast et al., 2003).

The mucus layer of fish is the first site of interaction between host and pathogen (Fast et al., 2003) and therefore is an important avenue for research into L. salmonis host specificity. Low molecular weight proteases have been discovered in mucus sampled from L. salmonis-infected Atlantic salmon, but not in mucus from non-infected salmon (Firth et al., 2000; Ross et al., 2000). Fast et al. (2003) demonstrated that L. salmonis secretes or regurgitates proteases onto the surface of its host, possibly as part of the extrabuccal digestion process and/or evasion of host immune responses (Bob Kabata, personal communication). Variation in the release of enzymes by sea lice has been shown in response to different fish hosts (Fast et al., 2003). Discovery of the mechanism and function behind this host-specific response could lead to a clearer understanding of salmon immunology and consequently L. salmonis epidemiology.

Current laboratory work in British Columbia is focusing on salmon immunology and exploring some of the possible mechanisms behind host susceptibility to L. salmonis (Jones et al., 2007). Comparative studies involving pink and chum salmon have demonstrated some degree of variation in immunological competence between the two species (Fast et al., 2006; Jones et al., 2007). The recent establishment of three-spine stickleback as hosts of L. salmonis has initiated the inclusion of that species in comparative assessments (Jones et al., 2006 a; Jones et al., 2006 b). Further studies are required to determine the significance of stickleback susceptibility to L. salmonis and hence their connection to the epidemiology of salmon-sea lice interactions.

This study examines the host-parasite relationship between L. salmonis and various salmonid and non-salmonid hosts including three-spine stickleback. Sea lice secretion of proteolytic salivary enzymes in response to fish mucus is examined for variation relative to host susceptibility to
infection. Local *L. salmonis* were exposed to the skin mucus of several native fish species including, pink and chum salmon, three-spine stickleback, and white sturgeon (*Acipenser transmontanus*). Atlantic salmon mucus was also included for comparative purposes, because of the documented susceptibility and increased enzyme release response of this species (Fast *et al.*, 2003). Thus, the objective of the study was to establish enzyme activity in the skin mucus of various local fish species as it relates to differential and specific secretion by *L. salmonis*.

**MATERIALS AND METHODS**

**Experimental design**

Mucus was collected from juvenile pink, chum, and Atlantic salmon and from two non-salmonid species, the three-spine stickleback and the white sturgeon. Individual mucus samples were collected in a standard volume of 0.5% sodium bicarbonate (NaHCO₃) buffer, pH 8.5 at 37 °C. Pooled mucus samples were then created by combining the individual mucus-buffer samples from 10 fish of the same species. Where necessary, pooled samples were diluted with additional buffer or concentrated by increasing fish sample size, in an attempt to standardize the ratio of biomass: buffer. A negative control was prepared by substituting sterile seawater for mucus in the NaHCO₃ buffer solution.

Eighteen sea lice were then exposed to individual aliquots of pooled pink salmon mucus; 18 were exposed to chum salmon mucus, 18 to Atlantic salmon mucus, 18 to stickleback mucus, 18 to sturgeon mucus and 18 were exposed to the seawater control. The 108 samples containing sea lice were incubated for one hour at 10 °C, together with complementary control samples (without sea lice). Enzyme assays were performed to establish relative enzyme activity among mucus samples.
with trypsin standards run as a positive control. A colorimetric reaction was used to determine protein concentrations. Specific details for each procedure are described below.

**Fish maintenance and collection of mucus**

Naïve fish of each species were maintained in individual tanks at the Pacific Biological Station (PBS) in Nanaimo, British Columbia in accordance with the Canadian Council of Animal Care (CCAC) standards. Pink and chum salmon were maintained in brackish water at 14.5 °C and 15.0 °C respectively. Atlantic salmon, three-spine stickleback and sturgeon were kept in seawater at average temperatures of 16.7 °C, 12.5 °C, and 15 °C, respectively.

Fishes were chosen for this study according to availability, which resulted in fishes of different size and age. Attempts were made to compensate for size differences by standardizing the concentration of mucus samples. A common ratio of mean fish mass: volume of buffer incubate was calculated and applied to each species at the time of mucus collection. Pink salmon (n=10), chum salmon (n=10), and Atlantic salmon (n=10) had average weights (± SD) of 4.3 ± 1.9g, 4.6 ± 2.3g, 127.9 ± 23.5g, respectively. Three-spine stickleback (n=75) were weighed as a group due to the small size of available fish resulting in a total mass of 40.3 grams. The total mass of sticklebacks represents 5 adult fish (~ 1 g each) and 70 juveniles (~ 0.5 g each). White sturgeon needed to be lightly sedated in order to obtain weight data and therefore, in an attempt to keep their stress to a minimum, only a few fish were actually weighed. The average weight for white sturgeon was estimated to be approximately 440 grams based on the actual weights of three individuals and the relative lengths of all 10 fish sampled.

Ten Atlantic salmon were anaesthetized (4 min) separately with a lethal dose of tricaine methanesulfonate, MS 222 (0.2 g/L) and then placed into individual plastic bags containing 10 ml
of 0.5% NaHCO₃ buffer (Ross et al., 2000). Fish were gently massaged and rolled in the buffer for one minute then removed and the bag of mucus placed on ice. Fish wet weight and overall length were recorded. Mucus was transferred by serological pipette from individual bags to sterile labeled 15 ml tubes and centrifuged at 2750 Xg for 15 minutes at 4 °C. Pooled mucus samples were formed by combining the supernatant from all 10 tubes. At this point the pooled sample (1280g/100ml) was diluted to the established standardized concentration of mean fish weight per volume of buffer (5.0 g/ml) by adding an additional 160ml of 0.5% NaHCO₃ buffer. The 260 ml pooled mucus sample was then separated into 250µl aliquots and stored in 1.5 ml labeled microtubes at -20 °C.

The same procedure was repeated for each fish species sampled (pink, chum, sturgeon and stickleback) with slight modifications. For pink and chum salmon, 10 fish of each species were anaesthetized together (4 min) in MS 222 (0.2 g/L) and placed as a group into one bag containing 10 ml of buffer. The resultant pooled mucus sample was then centrifuged (2750 Xg, 15 min., 4 °C), aliquoted and stored at -20 °C. Pink and chum salmon set the standard for mucus concentration in buffer (5 g/ml) with both species having a similar mean weight for 10 fishes sampled (pink salmon, 4.3 ± 1.9 g; chum salmon, 4.6 ± 2.3 g) so further manipulation of mucus was not necessary for these species.

Seventy five sticklebacks, ranging in size from 0.5 – 1.0 gram were sedated as a group (10 min), in Aquacalm (0.0012 g/L) and then placed together in a bag containing 5 ml of buffer. The resultant mucus-buffer solution (40.3g fish/5ml buffer) was diluted with an additional 3 ml of 0.5% NaHCO₃ to form 8 ml of pooled mucus at a ratio of 5 g/ml, fish mass to buffer. This larger sample size of fish (n=75) was used to collect mucus from stickleback to compensate for their small size.

Adult white sturgeon (n=10) were temporarily sedated in an anaesthetic bath of MS 222
(50 mg/L) and then placed into pre-weighed, individual plastic bags containing 25 ml of NaHCO₃ buffer. Each fish was measured for overall length while remaining in the bag of buffer; three randomly selected fish were weighed in the same manner. White sturgeon are a valuable research resource at the Pacific Biological Station. Therefore, the fish were handled as little as possible in efforts to minimize stress. A total of 250 ml of mucus-buffer solution was collected from the ten sturgeon (4400g/250ml) and 50 ml of this solution was diluted with an additional 125 ml of 0.5% NaHCO₃ buffer to form 175 ml of 5 g/ml pooled mucus.

**Preparation of sterile seawater and sterile seawater/ buffer negative control**

Seawater was collected from one of the fish tanks in which fish were being maintained for this study. Seawater was then sterilized to remove any impurities by running it through a 0.2 µm Supor Membrane syringe filter. A 5cc (ml) syringe with a sterile needle attached was used to withdraw seawater from the collection container. The needle was then exchanged for the syringe filter and seawater expelled into sterile labeled 50 ml tubes. Sterile seawater was refrigerated until needed. A negative control was prepared by calculating the average volume of fish mucus present in mucus-buffer solutions produced with fish incubation. The volume of mucus was calculated for each fish species by subtracting the initial volume of buffer (10 ml) from the final volume of buffer (after incubation with fish). The average ratio of mucus:buffer was determined to be 1:5 and a solution prepared replacing the calculated volume of mucus with the same volume of sterile seawater (4 ml sterile seawater: 20 ml NaHCO₃ to form 24 ml of the seawater negative control).
Sea lice collection, maintenance incubation of sea lice in mucus

Sea lice were collected as needed from a fish processing plant in Port McNeill, BC. When available, additional parasites were collected from infected Atlantic salmon held in fish tanks at PBS. Adult and pre-adult *L. salmonis* were removed from live Atlantic salmon with forceps and placed in seawater collected at the site. Lice were transported from Port McNeill to Nanaimo in containers of seawater packed on ice, and provided a constant air supply. The sea lice were maintained at PBS in aerated seawater at 10 °C for 24 hours to allow for the digestion of stomach contents.

Mucus samples (250 µl) were thawed on ice and then diluted 1:1 with sterile seawater to a total volume of 500 µl. Prepared samples were divided into two equal groups, then labeled and numbered (control 1-18 and incubated 1-18). One adult or pre-adult *L. salmonis* was added to each of the tubes labeled for incubation. All 36 tubes were placed in a cold water bath (10 °C) for one hour. Sea lice were removed to labeled tubes containing 95% ethanol for preservation. Both incubated and control mucus samples were centrifuged at 9400 Xg for 2 minutes at 4 °C. The supernatant was carefully removed from each tube using a serological pipette. It was then transferred to sterile, labeled 1.5 ml microtubes and frozen at -20 °C. The procedure was repeated for each fish species and again with the sterile seawater-buffer negative control. Adult and pre-adult lice were used for incubation and specimens were chosen for their vitality. Only lice that were either actively swimming or clinging to the sides of the container were used (Fast *et al*., 2003).

Biochemical Analysis

Mucus samples were thawed on ice and then analyzed for protease activity. Assays were performed using a Bio-Tek microplate reader. Protease activity was analyzed using a colorimetric
test involving azocasein hydrolysis (Charney and Tomarelli, 1947; Firth et al., 2000; Fast et al., 2003). Azocasein is a modified milk protein formed by the joining of a diazotized aryl amine with the original casein molecule. When a proteolytic enzyme is active in a solution of azocasein, peptide bonds are cleaved releasing azo groups into solution. The addition of trichloroacetic acid (TCA) stops the reaction and causes any undigested protein to precipitate out of solution. Azo groups are soluble in TCA and cause the solution to turn yellow allowing enzyme activity to be measured as a function of color intensity. For this assay azocasein was dissolved in 0.5% (w/v) NaHCO₃ by gentle heating and stirring, and the solution adjusted to pH 8.5 with the addition of HCl or NaOH as needed. Equal amounts (125 µl) of 2.5% (w/v) azocasein and mucus were placed in 1.5 ml microtubes and samples were incubated and shaken for 24 hours at 37 °C. The mucus-azocasein solution (200µl) was transferred to sterile eppendorf tubes and 800 µl of 5.0% (v/v) trichloroacetic acid added. Samples were cold centrifuged (4 °C) at 15,400 Xg for 5 minutes and then 50 µl was removed from each tube to a microplate well. Next, 150 µl of 500 mM sodium hydroxide solution (NaOH) was added to each microplate well to neutralize the solution. The solution was mixed by gentle swirling before absorbance was read at 450nm. A set of trypsin standards were prepared through a series of serial dilutions and run alongside the mucus samples. A standard curve was constructed from the trypsin standards of known concentration. One unit of activity was defined as the amount of enzyme required to catalyze an increase in absorbance (at 450 nm) of 0.001 optical density (OD).

**Statistical Analyses**

Statistical analyses were performed using NCSS. Enzyme activity data (OD₄₅₀) were checked for normality through construction of probability plots and histograms. Data that were not normally
distributed were transformed using log (OD$_{450}$ + 1) or arcsin (\sqrt{OD}_{450}). In the absence of normality a non-parametric test, the Kruskal-Wallis one-way ANOVA on ranks, was used to examine the effect of species on the enzyme activity in fish mucus. Results were reported as significant at P ≤ 0.05. Enzyme data were also analyzed to determine the percentage of *L. salmonis* to secrete enzymes into the mucus of each fish species. Conditioned mucus samples exhibiting enzyme activity greater than the mean activity for unconditioned mucus were considered positive responders. Data was thus provided on the number of sea lice responding to a fish species' mucus in addition to the intensity of the response.

**RESULTS**

Proteolytic enzyme activity was determined using a “trypsin-sensitive” azocasein hydrolysis assay and although data were found to be in violation of normality assumptions, some general trends were evident. Protease activity increased in the mucus of pink (0.12 ΔOD$_{450}$), chum (0.08 ΔOD$_{450}$ ), and Atlantic salmon (0.15 ΔOD$_{450}$ ), white sturgeon (0.13 ΔOD$_{450}$ ) and three-spine stickleback (0.15 ΔOD$_{450}$ ) following incubation with live *L. salmonis* (Figures 1A-E, respectively). The level of proteolytic activity remained consistently low in control samples (unconditioned, U) of pink salmon, chum salmon, Atlantic salmon and white sturgeon mucus (0.007, 0.007, 0.004, 0.005, respectively, measured as average OD$_{450}$). After exposure to *L. salmonis*, mucus samples from the same fish species exhibited variable enzyme activity with an increase in overall activity (0.13, 0.09, 0.15, 0.13, respectively, average OD$_{450}$).
Figures 1A-F. Comparative histograms displaying trypsin-like enzyme activity in *Lepeophtheirus salmonis*-incubated mucus (louse) relative to control mucus (no louse). Counts on the ordinate refer to the number of individual lice and enzyme activity data along the abscissa were presented as total color produced in 24 hours and read as optical density at 450 nanometers on a Biotek® plate reader. Figures 1A-E are labeled according to fish species and Figure F presents the control data.
Unconditioned three-spine stickleback mucus displayed greater baseline enzymatic activity than controls from all other fish species sampled (0.17 average OD$_{450}$; see Fig. 1E). However, the range between unconditioned and conditioned samples of stickleback mucus was similar to the other species. Sterile seawater showed the same trend of consistently low activity in controls relative to inconsistent and generally higher levels of protease activity in conditioned seawater (Figure 1F).

In addition to quantifying the relative intensity of enzyme activity, the numbers of sea lice secreting salivary enzymes into each mucus type was evaluated. All *L. salmonis* individuals sampled released enzymes into pink, chum, and Atlantic salmon mucus as well as into seawater, whereas 94% secreted proteases in response to white sturgeon mucus and 89% responded positively to stickleback mucus (Figure 2).

![Figure 2. *Lepeophtheirus salmonis* individuals secreting trypsin-like proteases in response to fish mucus, represented as percentage of responders in total sample (n=18). Lice were considered to be positive responders to fish mucus if enzyme activity in incubated mucus samples was above that of the mean for unconditioned mucus samples.](image-url)
While histograms effectively display both the percentage of sea lice secreting proteases into mucus and the effect of *L. salmonis* secretions on the enzyme activity in fish mucus, a boxplot provides a visual comparison of the variation occurring within and between species (Zar, 1999). Variation in enzyme secretion among *L. salmonis* individuals is evident in the extensive range of protease activity within mucus samples from all fish species and seawater (Figure 3). The placement of medians low in the box region of the plot for pink salmon, chum salmon, Atlantic salmon, stickleback and seawater indicate that one half of the sea lice in those populations were secreting consistently low levels of enzyme activity (Figure 3). The other half of the louse population released higher levels of proteases but did so more erratically, as demonstrated by long whiskers protruding from the top of the respective box plots and accentuated by the presence of obvious outliers (Figure 3). Enzyme activity in white sturgeon mucus was more uniformly distributed throughout the population.

Due to the lack of normality exhibited in the data overall, a reliable analysis of the effect of species required the use of a Kruskal Wallis one way ANOVA on ranks. There was no significant difference in *L. salmonis*-derived enzyme activity between pink, chum, and Atlantic salmon, white sturgeon, three-spine stickleback, and sterile seawater (Kruskal Wallis 1-way ANOVA: $\chi^2 = 10.32$, df = 5, P = 0.07).
Figure 3. The distribution of protease activity in the mucus of Atlantic, chum, and pink salmon, white sturgeon, three-spine stickleback, and sterile seawater. The boxplot layout summarizes the most prominent features of the distribution and allows a comparison of protease activity between species. The length of the box indicates the interquartile range (middle 50% of the data) and the horizontal line running through the box the median. Data points beyond the whiskers (lines protruding from either end of the box) are outliers. Values along the ordinate are measurements of enzyme activity in optical density read at 450 nanometers on a Biotek® plate reader.

DISCUSSION

The presence of enzyme activity was confirmed in the skin mucus of pink, chum, and Atlantic salmon, white sturgeon, and three-spine stickleback. An increase in proteolytic activity following exposure of fish mucus to *L. salmonis*, suggested the enzymes were parasite derived. Proteases have been identified as constituents in the saliva of other arthropods but may also be released into fish mucus by the host salmon as a defence mechanism (Kerlin and Hughes, 1992; Firth *et al.*, 2000). To confidently distinguish sea lice salivary enzyme activity from salmon derived activity, mucus was
removed from naïve tank-reared fishes prior to sea louse exposure. The use of naïve fishes assured that any activity occurring in the mucus was not part of an acquired immune response. Removing mucus from fishes prior to parasitic incubation eliminated active fish cells and subsequently any chance of a cellular immune response. Any factors that may originate as part of a non-specific, innate response would be detectable as protease activity in the control mucus and would not be expected to contribute to a difference in conditioned mucus. Pooling mucus samples to form an homogenous incubate further controlled the effect of fish variation on enzyme activity, allowing confidence that mucosal changes in protease activity detected after *L. salmonis* incubation were parasite derived (Fast *et al*., 2003).

The only exception to the above described protocol was the use of non-naïve stickleback in this study, as lab-reared fish of this species were not available. Recent evidence suggesting three-spine stickleback may be somehow involved in the *L. salmonis* life cycle prompted inclusion of the species despite the fact that only wild specimens were available (Jones *et al*., 2006 b). Use of wild fish can potentially compromise the integrity of experimental results since the health status and immunological competency of the fish is unknown. Three-spine sticklebacks have been documented as susceptible to heavy loads of parasites such as the crustacean *Argulus canadensis*, the plerocercoid of the cestode *Schistocephalus solidus*, and the monogenean *Gyrodactylus canadensis*, in addition to *L. salmonis* (Poulin and FitzGerald, 1989; Marcogliese, 1992; Heins *et al*., 2002; Jones *et al*., 2006 a). Enzyme activity may increase in fish mucus after a parasitic invasion as part of the hosts’ immune response (Firth *et al*., 2000). Previous history of infection may account for the high baseline activity recorded in unconditioned stickleback mucus in this study. The use of naïve fish would control for this effect but unfortunately naïve sticklebacks were unavailable. Since the
difference in activity between control and conditioned mucus was under observation as opposed to baseline enzyme activity, wild fish were included in this study.

Efforts were made wherever possible to anticipate and control for the variation in fishes and parasites under study. A certain amount of variability is inherent in all biological systems and often causes interference that inhibits the “normal” distribution of data. Extreme data points in the enzyme activity of the louse population may be the result of variation in gender, developmental stage, health status or a combination of these factors. Attempts were made to control for as much louse variation as possible by choosing adult and pre-adult *L. salmonis* individuals based on their vitality. This was determined by the presence of active swimming and attachment behaviors as in Fast *et al.* (2003). Following incubation in mucus, individual lice were preserved in 95% ethanol, providing the opportunity for microscopic examination of gender and life history stage. Thus, although time constraints did not allow for it in this study, future analyses could determine whether a correlation exists between individual louse characteristics and specific enzyme activity. However, Fast *et al.* (2003) found no distinct relationship; thus, the assumption was that such factors were adequately controlled for under the present experimental design.

Parasitic arthropods such as mosquitoes (*Anopheles stephensi*) and plant bugs (*Deraeocoris nebulosus*) use “trypsin-like” enzymes to aid in digestion, and some like the cattle tick (*Boophilus microplus*) and the sheep blowfly (*Lucilia cuprina*) contain similar proteases in their saliva (Kerlin and Hughes, 1992; Rosenfeld and Vanderberg, 1998; Boyd *et al.*, 2002). Several authors have reported the release of “trypsin-like” proteases by *L. salmonis* in response to Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), and coho salmon (Firth *et al.*, 2000; Ross *et al.*, 2000; Fast *et al.*, 2002). Low molecular weight proteases (17-22 kDa), similar in weight to trypsin, have been identified using zymography (Firth *et al.*, 2000; Ross *et al.*, 2000). Affinity chromatography and
inhibition studies have determined that these low molecular weight proteases function as chemical
catalysts via a similar serine-activated mechanism as trypsin (Firth et al., 2000). Therefore,
development of a “trypsin-sensitive” azocasein hydrolysis assay in this study, facilitated the
establishment of proteolytic enzyme activity in the salivary secretions of *Lepeophtheirus salmonis*.

Proteases were detected as being released into the skin mucus of pink, chum, and Atlantic
salmon, white sturgeon, and three-spine stickleback by the majority of sea lice sampled. In fact,
100% of *L. salmonis* secreted enzymes into seawater, indicating the behavior may be
indiscriminate. In the only similar study, Fast et al. (2003) found quite the opposite when examining
the response of New Brunswick *L. salmonis* to Atlantic salmon, coho salmon, rainbow trout and
seawater. That study reported differential secretion by the east coast lice with only about 25 and
30% of sampled individuals responding positively to coho salmon mucus and seawater respectively,
while approximately 50% released enzymes into Atlantic salmon mucus and a significantly high
percentage (~70%) responded to rainbow trout. On the other hand, the study recognized the
importance of geographic variation in parasitic interactions, reporting consistently high levels of
protease activity by BC *Lepeophtheirus salmonis* in response to the mucus of all three fish species.

Although the results of the present study were consistent with the general trend for BC “Leps”
recognized by Fast et al. (2003), a greater percentage of positive responders was recorded (100% or
close to it for all types of mucus) and enzyme expression into seawater was noted. Seawater was
carefully filtered through a very fine membrane (0.2 µm) to achieve sterilization, but the presence of
enzyme activity suggests the possibility of contamination. Regardless, this does not negate the
legitimate origin of enzyme activity in fish mucus samples, consistent with the findings of Fast et
al. (2003). That east coast study concluded that the differential enzyme secretions of New
Brunswick *L. salmonis* may be related to host preference or specificity. Following the same logic,
indiscriminate proteolytic activity may confer the trophic status of “generalist” upon British Columbia *L. salmonis*.

Just as the percentage of sea lice secreting enzymes into fish mucus was consistent across species so was the intensity of their protease activity. No significant correlation was found between the intensity of *L. salmonis* salivary secretions and host specificity. Initially it was expected that BC *L. salmonis* would exhibit increased enzyme secretion in response to native co-occurring salmonids with known susceptibility to sea lice infection, similar to the differential secretion demonstrated by New Brunswick *L. salmonis* (Fast *et al.*, 2003). It was hypothesized that enzyme secretion may provide a link between host pathology and parasite virulence suggesting a co-evolutionary “arms race” typical of host-parasite interactions. Proteases were postulated to result from the adaptive development of a chemical mechanism conferring greater virulence upon the parasite; an evolutionary adaptation toward increased success of the species. Indeed, evidence indicates this may be happening in the interaction between east coast sea lice and Atlantic salmon (Fast *et al.*, 2003); however, these data suggest an unexpected and different trend on the British Columbia coast. Whereas some variation in enzymatic activity was evident between fish species, the results were not statistically significant. Still, the results were biologically interesting, and may indicate a trend for west coast sea lice to be less “picky” than their east coast counterparts.

A trend away from specialization may be expected to have implications for both parasite and host. Evolutionary pressures on the host toward the development of parasite resistance could be expected to relax as a dilution effect on *L. salmonis* distribution decreases the cost effectiveness of the energetically expensive adaptive response. Such has not been observed with Pacific salmon to date. The resistance of coho salmon to *L. salmonis* infection is well documented (Johnson and Albright, 1991; Fast *et al.*, 2002), and current research has already shown differential resistance
between pink and chum salmon (Jones et al., 2007). Differential resistance among host species, concurrent with the indiscriminate release of proteolytic enzymes by *L. salmonis*, reflects the ability of some species to block enzyme activity. Selective pressures favoring parasite resistance in fishes are only expected to be a factor in cases of parasite-induced mortality where resistance confers differential survival. Although naturally occurring Pacific salmon appear to be minimally affected by *L. salmonis* infection (Beamish et al., 2005), Atlantic salmon have died following infections (Finstad et al., 2000; Ross et al., 2000). The presence of *L. salmonis* resistance in some salmonids may be the evolutionary product of selective pressures responding to heavy parasitic loads, in combination with extreme pathology.

A parasite evolving toward a lifestyle of decreased specialization may be expected to show an increase in versatility evidenced by an expanding host range. *L. salmonis*, although traditionally considered a salmonid-specific ectoparasite, has been documented locally on Pacific sand lance, *Ammodytes hexapterus*, as well as white sturgeon, and most recently on three-spine stickleback (Kabata, 1973; Jones et al, 2006 a). Sticklebacks are sympatric with Pacific salmon in coastal B.C., harboring extremely high *L. salmonis* intensities but losing the lice prior to the pre-adult developmental stage, while incurring no apparent health cost (Jones et al., 2006 b). Jones et al. (2006 b) suggest that the three-spine stickleback acts as a temporary host for *L. salmonis* during its early developmental stages. Observations of successful settlement and feeding of *L. salmonis* on sea bass (*Dicentrarchus labrax*) prompted Pert et al. (2006) to suggest that *L. salmonis* can use other species as paratenic hosts.

A paratenic host may serve as a temporary environment facilitating the bypass of an ecological barrier to parasitic success. Direct life cycles often negate the need for paratenic hosts but for *L. salmonis* this alternate transmission strategy may act to increase louse survival through the fresh.
water phase of their anadromous host’s life cycle. Stickleback may act as an effective ecological link if detaching pre-adult lice are capable of locating and reattaching to a host more suited to adult louse survival. Although pre-adult and adult sea lice are weak swimmers, easily displaced by the force of ocean currents, movement of motile *L. salmonis* stages between hosts has been documented (Ritchie, 1997). Spawning adult salmon and the aggregation of juveniles emerging from natal streams may provide the crowded conditions required for sea lice to “jump” hosts. An adaptation favoring dispersal of copepodids to an alternate host prior to the imminent death of the salmonid host may have evolved due to selective pressures. Movement of *L. salmonis* between stickleback and salmon is currently being explored to determine whether sea lice may have evolved an alternative transmission strategy as an adaptive response to conditions of host abundance.

In conclusion, this study confirmed the presence of protease activity in the salivary secretions of *L. salmonis* but did not establish differential secretion in response to various salmonid and non-salmonid fish hosts. However, the development of a “trypsin-sensitive” azocasein hydrolysis assay and the establishment of standard protocol for the collection of fish mucus, and maintenance and incubation of sea lice may facilitate future local examination of *L. salmonis* host specificity. Further studies may extend explorations into the potential role of sticklebacks as paratenic hosts in the *L. salmonis* life cycle. Do sticklebacks function as an ecological link in an adaptive transmission strategy evolved to enhance the perpetuation of *L. salmonis* as a species, or are they simply an ecological dead-end? Future investigations may include plankton tows to determine the abundance of free-swimming adult and pre-adult lice in the vicinity of aggregating salmon. The chemosensory specificity of lice to locate hosts may be evaluated through comparative biochemical analyses of fish mucus prior to and following incubation with sea lice. Comparative immunological competence
studies of susceptible versus resistant hosts, as well as infected and non-infected fish, may enhance our understanding of the complex nature of host-parasite interactions occurring in this system.
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