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Lepeophtheirus salmonis, parasitizing three species of pacific salmon (Oncorhynchus spp.): host variations in load, morphology, fecundity, and genetics of the salmon louse

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Lepeophtheirus salmonis, parasitizing three species of pacific salmon (Oncorhynchus spp.): Host variation in load, morphology, fecundity, and genetics of the salmon louse

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Project Summary

The goal of this study was to examine populations of salmon lice *Lepeophtheirus salmonis* in the wild environment as they interact with different host species. Population studies for this organism have become essential to understanding the impacts of salmonid mariculture in various environments worldwide. The following study includes two sections: first an examination of parasite load and morphological aspects of lice on different hosts, then a section relating genetic aspects of salmon lice on different host species. These are two approaches to studying a related concept, thus the study was divided into separate sections. This work was important because most of the current knowledge of this organism has taken place in the North Atlantic Ocean while the parasite is interacting with Atlantic salmonid hosts, or in aquaculture sites that culture Atlantic salmon. The current understanding of population structuring among wild host species for *L. salmonis* has been contradicted and is still somewhat ambiguous.

Chapter 1

Lepeophtheirus salmonis populations on salmonids (*Oncorhynchus spp*.) of the North Pacific, USA: Among host comparison of morphology, fecundity, and parasite load.

Abstract

The purpose of this study is to determine biological parameters of salmon lice (*Lepeophtheirus salmonis*) as they parasitize different species of Pacific Ocean salmonids off the north Pacific coast of Washington State. Parasite load counts of *L. salmonis* were made and individuals were collected from salmonids in their natural environment. Louse morphology and fecundity were examined using microscopy. Parasite loads were equal between different species of salmonids ($p = 0.231$). Lice that infected chinook salmon ($n = 48$) were smaller in total body length ($p < 0.001$), cephalothorax length ($p < 0.001$), and cephalothorax width ($p < 0.001$) when compared to lice that infected coho salmon ($n = 44$) or pink salmon ($n=45$). Lice that infected coho and pink salmon were not statistically different in body length ($p = 0.213$), cephalothorax length ($p = 0.996$), or cephalothorax width ($p = 0.149$). Also, *L. salmonis* produced fewer eggs when infecting chinook salmon ($n = 24$) than when infecting coho salmon ($p < 0.001$) or pink salmon ($p < 0.001$). Whereas, lice that infected coho salmon ($n = 41$) or pink salmon ($n = 22$) produced similar number of eggs ($p = 0.60$). These results indicate that there are factors associated with infecting chinook salmon hosts that reduce the size and fecundity of salmon lice.

Introduction

The salmon louse (*Lepeophtheirus salmonis*) is a marine salmonid ectoparasite that is a major pest to marine aquaculture (Pike 1989; Pike and Wadsworth 1999). Louse populations at aquaculture sites can rapidly increase in size leading to infestation (Tully and Whelan 1993). When parasite load exists at unnatural levels, salmon can experience early mortality (Krkošek et al. 2007). Infections create sores on the host and though there is no definitive evidence of *L. salmonis* serving as a disease vector, these sores can allow for pathogens to enter salmon tissue, increase stress of the host and affect the host's osmoregulation ability (Ritchie *et al*. 1996; Jones et al. 2008, Patterson *et al.* 2009). Furthermore, it has been suggested that *L. salmonis* transfer among wild and farmed hosts as wild salmonids migrate past aquaculture facilities (Castillo 2009; Krkos□ek 2010; Prince *et al*. 2011). The wild Pacific salmonids (*Oncorhynchus spp.*) have varying abilities to resist louse infection (Johnson and Albright 1992) which, makes understanding where the lice originate and how the hosts and parasite interact is important for management of this major seafood industry.

A louse life cycle consists of ten stages (Johnson and Albright 1991a). The life cycle and growth of individual lice can vary greatly based on different environmental factors such as salinity (Genna *et al*. 2005), chemotherapeutics (Tully and Whelan 1993), host infected (Johnson 1993) and water temperature (Nordhagen et al. 2000). After an individual hatches, it is non-feeding and planktonic during the first two instars as a dispersal mechanism. This is followed by an infective copepodid stage that seeks out salmonid hosts. Once attached the louse goes through 3 successive molts as a sessile chalimus usually attached to the dorsal or a pectoral fin of the host. Following the 7th

molt individuals move about their hosts and migrate to the anal fin region where they molt twice more to reach a mature adult terminal instar (Pike and Wadsworth 1999).

Each molt is highly affected by the environment which is dictated by host preferences. Considering that various host species can be ecologically and immunologically distinct from each other, the environmental conditions preferred by the host species can greatly affect the parasite. Pink salmon (*Oncorhynchus gorbuscha*) are a more open-water species preferring to spend the ocean phase off the continental shelf (Takagi 1981). Pink salmon have a protein secretion that acts as a deterrent to infection (innate immunity) and a mild inflammatory response to *L. salmonis* infections (Jones *et al*. 2008). Coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha)* are usually found in the more shallow waters near the shore (Godfrey et al. 1975; Major et al. 1978). Chinook salmon also have an innate immunity to lice infections but do not inflame infected tissues. Coho salmon on the other hand have the combination of innate resistance and an acute inflammatory response to infection that was shown in the laboratory to be very effective in reducing louse longevity on the host (Johnson & Albright 1992).

The objective of this study was to compare parasite load as well as the basic morphology and fecundity of *L. salmonis* among three different salmonids in the North Pacific Ocean, USA. Our null expectations are a) parasite load will be similar between host species, b) morphological parameters are similar between groups of lice found on different species of salmon, and c) fecundity is similar between groups of lice found on different species of salmon.

Materials and methods

The sample site was within the Strait of Juan de Fuca, along the Washington (USA) coast within the 10 kilometer portion defined as Tatoosh Island (western limit) to Neah Bay, Wash. (eastern limit). Samples were collected at 100 - 2000 meters from shore at water depths up to 200 meters however salmon were always located between 0 and 35 meters. All samples were obtained with hook-and-line sampling (Boulding *et al*. 2009, Todd *et al*. 2004). As other methods such as long-line sets or gill netting leave fish vulnerable to increased parasite attack. Three species of Pacific salmon were included in this study: pink salmon (*Oncorhynchus gorbuscha*), coho salmon (*Oncorhynchus kisutch*), and chinook salmon (*Oncorhynchus tshawytscha*). Certain species of fish are more obtainable through hook and line sampling at different distances from shore and water depths. Typically, chinook salmon were sampled before 0700 hours within 200 meters from shore and at depths less than 80 meters whereas pink and coho were almost exclusively sampled after 0700 hours between 1000 and 2000 meters from shore at water depths between 100 and 800 meters. Fish were always within 30 meters of the water surface though the water depth below them varied to a large degree. Over two sampling seasons (2009 and 2010), 89 randomly sampled pacific salmon were obtained.

Of the 379 lice observed from all salmon, 140 were picked at randomly for further analysis. Live salmon were brought to the side of the boat where samplers netted and weighed the fish in the net with a scale. After a weight was obtained a floating fish board was slid underneath the fish while still in the water to avoid over-handling salmon that were to be returned to the wild. This fish board was oversized with large angled sides so samplers could easily observe and collect any lice that were sloughed off the salmon.

While over the gunwale, samplers took fish length and removed lice from fish with forceps and preserved them in 60 ml sample containers filled with 70% ethanol.

We determined louse abundance or parasite load (mean number of parasites on all examined hosts), prevalence (percent of infected hosts sampled) and intensity (number of parasites per infected host) for each host as described by Nagasawa (1987), which are measures of host utilization by the parasite. Parasite load details the average number of lice found in our sample set where the other measures suggest levels of independence between infection events (intensity) and how often individuals are used by *L. salmonis* as a host (prevalence).

In the lab, preserved lice specimens were measured for body size as described by Poulin (1995) and Nordhagen *et al*. (2000). All specimens were measured at a similar interval following preservation to avoid body size variations after exposure to alcohol. An ocular micrometer on a Leica MZ-8 microscope was used to take three separate measurements per individual; total body length (TL), cephalothorax length (CL), and cephalothorax width (CW). Total length was measured from the anterior most portion of the cephalothorax to the posterior most portion of the organism excluding the caudal rami (Poulin 1995). Cephalothorax length was measured from the anterior most portion of the cephalothorax to the posterior most portion of the cephalothorax (Nordhagen *et al*. 2000). Cephalothorax width was measured on the widest part the cephalothorax, not including the hyaline membrane (Nordhagen *et al*. 2000).

The preserved adult gravid females were analyzed for fecundity by estimating an egg count per individual. Total egg numbers were estimated on adults in their final stage of metamorphosis by counting the number of eggs in one millimeter of egg sack and then

extrapolating that to the number contained within the total egg sack length. Due to the variation observed in body size and evidence that female size is correlated with brood size (Poulin 1995); total egg counts were also analyzed after normalizing for body lengths.

Parasite load data were analyzed using a Kruskal-Wallis test with SYSTAT version 12. Sample size for this test was 89 individual hosts separated as follows into 3 groups; 17 randomly sampled chinook salmon hosts, 52 randomly sampled coho salmon hosts and, 20 randomly sampled pink salmon hosts. Morphological and egg production data were both analyzed using a single factor ANOVA with SYSTAT version 12. Costello (2009) and Genna et al. (2005) suggest host body size is a factor in how many lice may be present on an individual fish due to the larger surface area exposed to the environment. To test this in our samples we used a regression analysis of length (independent) to parasite load (dependent) with SYSTAT version 12. Load data was normalized for host body length to give a density of lice measurement and analyzed using a Kruskal-Wallis test with SYSTAT version 12. Then Pairwise Mann-Whitney U-tests of these groups determined similarities and differences independently.

Results

The majority of salmon sampled through the duration of the project were coho salmon (58.4% of sample, n=52) whereas pink salmon (22.5% of sample, n=20) and chinook salmon (19.1% of sample, n=17) were encountered less often (Table 1). Pink salmon had a louse prevalence of 100, an intensity of 5.4, and an equal load (5.4). Chinook salmon had a louse prevalence of 94.1, an intensity of 4.17, and a load of 5.88.

Coho salmon had a much lower prevalence (78.9), intensity (4.1) and load (3.35) then all other examined groups (Table 2).

We failed to reject the null hypothesis that hosts experience similar load values ($p=0.231$; see figure 1). However, a regression analysis suggested that host body size is a determining factor in parasite load ($p < 0.001$, Figure 2) with this we reject the null hypothesis that these populations had the same median values ($n = 89$, $p = 0.023$, Figure 3). A pairwise comparison suggested that chinook salmon hosts have similar loads to coho and pink salmon hosts ($p = 0.384$ and $p = 0.186$, respectively). However, coho salmon hosts and pink salmon hosts were suggested to have dissimilar parasite load ($p < 0.01$).

Ovigerous female lice found on chinook salmon hosts ($n = 48$) were 13.3 % smaller in Total Length, 12.3% smaller in cephalothorax width and, 11.1% smaller in cephalothorax length than lice collected from pink salmon hosts ($n=45$) and coho salmon hosts ($n = 44$). The mean total length of lice collected from chinook salmon was 11.94 millimeters (mm), from pink salmon 13.9 mm, and coho salmon was 13.65 mm. The mean cephalothorax width of lice collected from chinook salmon was 4.12 mm, from pink salmon 4.65 mm, and coho salmon was 4.76 mm. The mean cephalothorax length of lice collected from chinook salmon was 4.69 mm, from pink salmon 5.28 mm, and coho salmon was 5.27 mm. Lice that infected chinook salmon were statistically smaller in total body length ($p < 0.001$), cephalothorax length ($p < 0.001$), and cephalothorax width ($p < 0.001$) when compared to lice that infected coho salmon or pink salmon. Lice that infected coho and Pink salmon were not statistically different in body length ($p = 0.213$), cephalothorax length ($p = 0.996$), or cephalothorax width ($p = 0.149$).

We observed that egg production ranged from 198 - 1208 with an average of 760.3 eggs per brood. The Lice collected from chinook salmon (n = 24) hosts produced 38.6% fewer eggs than lice collected from both pink salmon hosts (n = 22) and coho salmon (n = 41). Egg production was on average 329.2 eggs fewer for lice that were attached to chinook salmon than for lice attached to the other species of salmon sampled with an average for chinook salmon at 521.9 opposed to 851.1 average egg numbers for lice that use coho and pink salmon hosts. Egg production was statistically fewer for lice when infecting chinook salmon than when infecting coho salmon ($p < 0.001$) or pink salmon ($p < 0.001$). Whereas, lice collected among coho salmon and pink salmon hosts produced similar numbers of eggs ($p = 0.60$). A similar trend was also observed in an analysis of eggs per unit body size. Lice collected from chinook salmon hosts produced 31.0% less eggs per unit body size. The mean number of lice collected from chinook salmon was 43.3 eggs per mm of total length whereas lice grand mean of lice from coho salmon and pink salmon hosts was 62.7 eggs / mm total length. This trend was statistically significant ($p<0.001$) while individuals sampled from coho and pink salmon had similar numbers of eggs / unit body size ($p=0.62$).

Discussion

Load, Prevalence and, Intensity

Many studies have considered variability of host susceptibility. Johnson and Albright (1992) Showed that coho salmon produce a cell-based reaction which subsequently killed chalimus larvae. The interaction with coho salmon was compared to chinook salmon and Atlantic salmon (*Salmo salar*). These researchers found that coho

were more resistant to infection than were chinook or Atlantic salmon. In a laboratory experiment by Dawson et al. (1997) showed that Atlantic salmon are more resistant to infection that sea trout (*Salmo trutta*) by comparing sores and longevity of lice attachments. Jones *et al*. (2007) looked at gene expression and cortisol levels in juvenile pink salmon and chum salmon (*Oncorhynchus keta*) and showed that pink are more resistant to infection than are chum salmon. These studies show evidence that suggests the different host species of *L. salmonis* in the pacific have a range of abilities to resist the infection by salmon lice.

Among the Pacific Salmonids coho salmon have the strongest resistance to louse infection, which would likely drive abundance of parasites on this host species down. However, a reduction in parasite load on coho salmon was not observed in the present study. Every Pink salmon sampled in the present study had at least one louse attached, high prevalence. This is consistent with Nagasawa (1987) who sampled salmonids in the high seas of the northern Pacific Ocean. He used long line sampling techniques to assess infection levels and found that pink salmon and chum salmon were very important hosts for *L. salmonis* comprising nearly 90% of the salmon lice observed.

Morphometrics and Fecundity

Lice sampled on chinook salmon were smaller than those observed on other salmon species. The salmon lice observed in this study were of an expected size range. Nordhagen et al. (2000) found from 167 lice on wild fish in a laboratory study, mean total length for lice was 10.4 mm, mean cephalothorax length was 4.6 mm, and mean width was 4.0 mm (n=167). Tulley and Whelan (1993) examined morphometrics of lice on

wild fish compared to farmed fish and found that wild Atlantic salmon lice that are larger than aquacultured lice on the same host. The mean total length of wild lice was 15.2 mm the cephalothorax length was between 5-5.5 mm Ritchie et al. 1993, reported cephalothorax lengths of 4.3 -5.0 mm. In the present study we observed a total length range 9.28 - 16.4mm (grand mean, 13.13mm), a cephalothorax length range of 3.6 - 6.08mm (grand mean, 5.07mm) and a cephalothorax width 3.4 - 5.3 (grand mean, 4.50).

For these measures all egg baring females were measured however, as suggested by Eichner *et al.* (2008) total length measurements may vary depending on the age of the individual in question but the cephalothorax will remain constant once individuals reach adulthood. The variability of total length is related to growth incurred in the genital segment over the course of about 3 days after an individual can start bearing eggs. Fecundity can be highly variable. On farmed fish lice loads are lower, Johnson and Albright (1991b) found an average number of eggs per louse on Atlantic salmon in farms was 344, rarely a female will have as many as 700 (Wooten *et al*. 1982). In the present study of salmon lice from wild host, I would expect to have about 1000 eggs per brood (Tully and Whelan 1993). Egg production was estimated as total number of eggs per individual. Only 87 female lice were usable for egg production as many had severed egg sacks or had just hatched a brood.

Morphological variation and fecundity differences for individual *L. salmonis* have been suggested to be environmental (reviewed by Nordhagen et al. 2000). However, many studies have found genetic differences in lice in the north Pacific but individuals from populations which were different were rarely measured so conclusions about the source of variation in morphology and fecundity are ambiguous (See Chapter 1, Prince et

al. 2011, Boulding et al. 2009). Another possible source of variation in morphology or egg production is phenotypic plasticity (Nordhagen et al. 2000, Lee and Peterson 2002). A future common-garden experiment as per Nordhagen et al. (2000) which considers host origin would be necessary to examine genetics of these traits and to draw conclusions about the source of louse morphological variation in the Pacific Ocean system.

Table 1: Sampling effort over two seasons, 2009 and 2010 in the North Pacific Ocean near the shores of Washington State. Table shows salmon sampled from each season and the percent of each species in the total catch for both seasons

Host species	Number of Fish Examined 2009	Number of Fish Examined 2010	Percent of catch
O. gorbuscha	20	0	22.5
O. kisutch	28	24	58.4
O. tshawytscha	1	16	19.1

Table 2: Occurrence of *Lepeophtheirus salmonis* on three species of salmonids sampled during the summers of 2009-2010.

Host Species	Abundance / Parasite Load	Prevalence	Intensity
O. gorbuscha	5.40	100	5.40
O. kisutch	3.35	78.9	4.17
O. tshawytscha	5.88	94.1	6.25

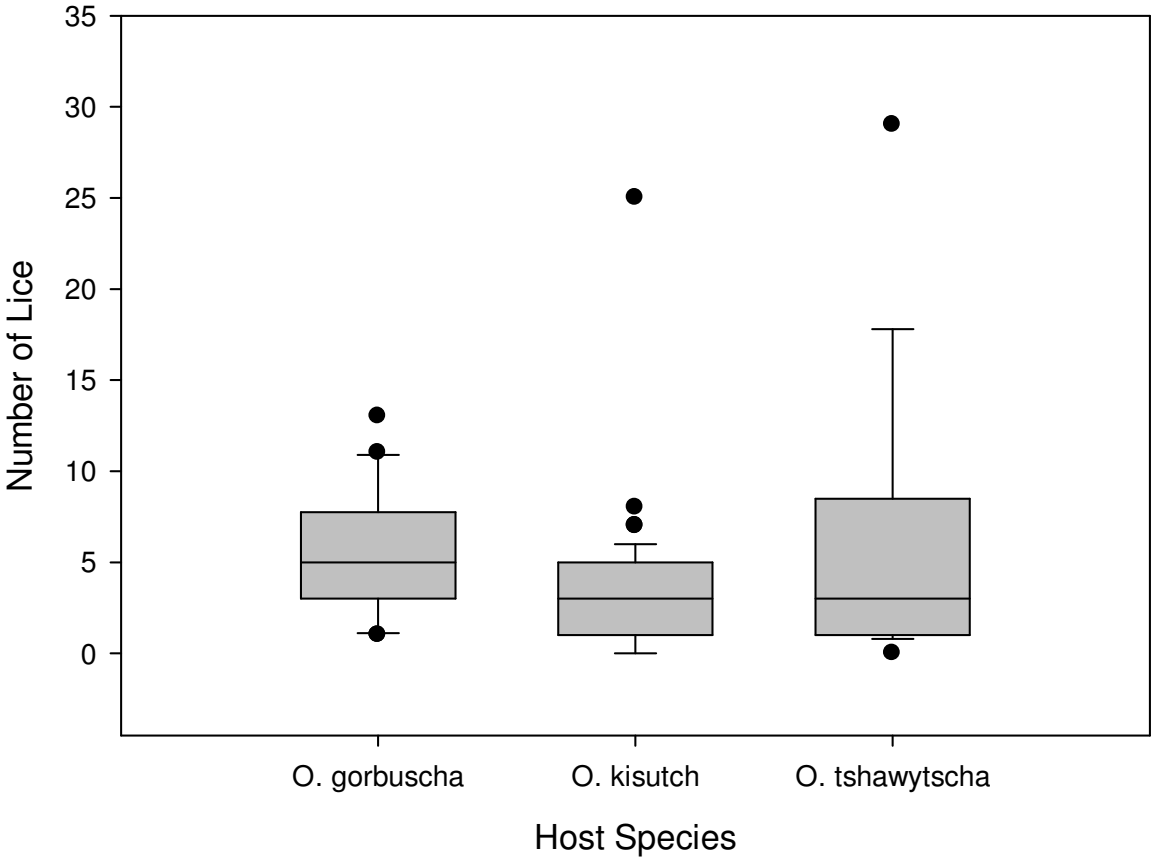

Figure 1: Parasite load (2009 & 2010) from three species of salmonids: *Oncorhynchus gorbuscha* (n=20), *Oncorhynchus kisutch* (n= 52), and *Oncorhynchus tshawytscha* (n=17). Parasite load was not significantly different between species of salmonids (p=0.231).

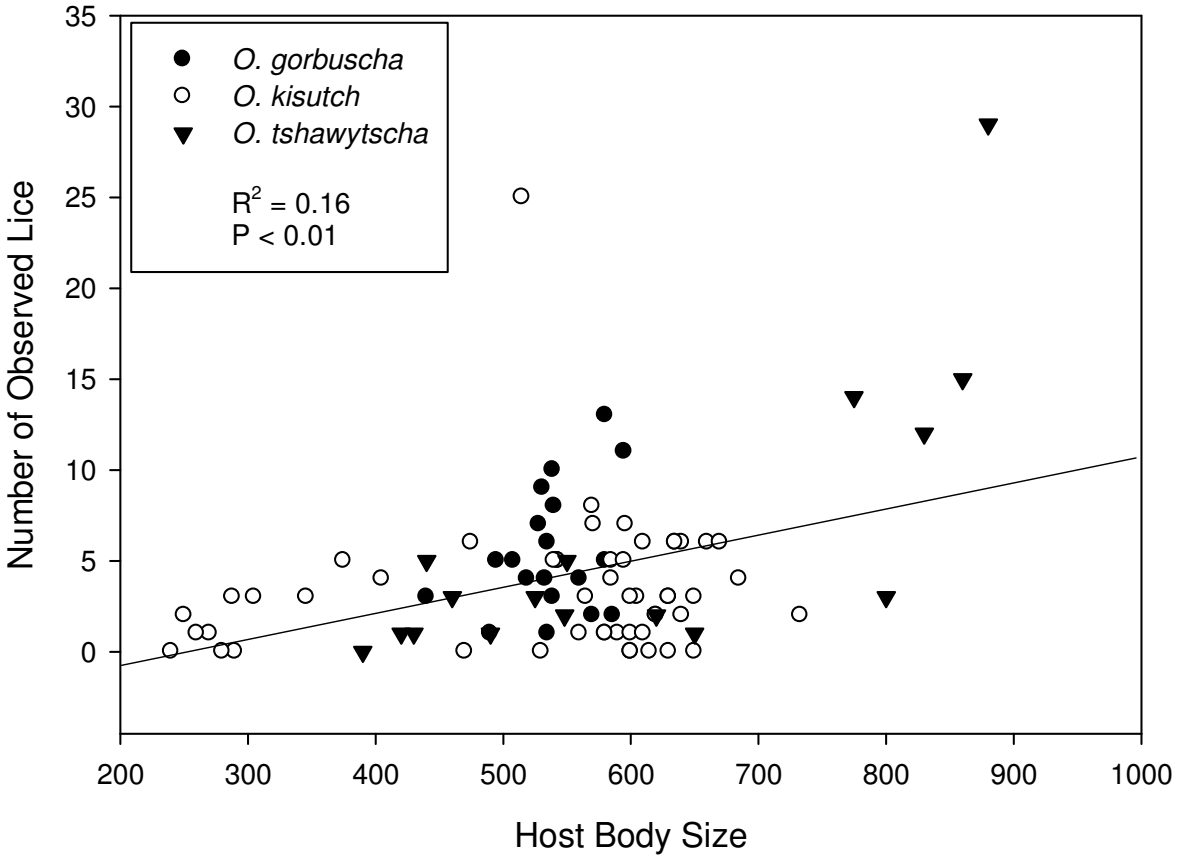

Figure 2: A regression analysis (n=89) of parasite load to body size (2009 & 2010). Dark circles represents *Oncorhynchus gorbuscha* (n=20), Light circles represents *Oncorhynchus kisutch* (n= 52), and Dark triangle represents *Oncorhynchus tshawytscha* (n=17). Body size is significantly regressed with number of observed parasites ($R^2 = 0.16$; $p < 0.001$).

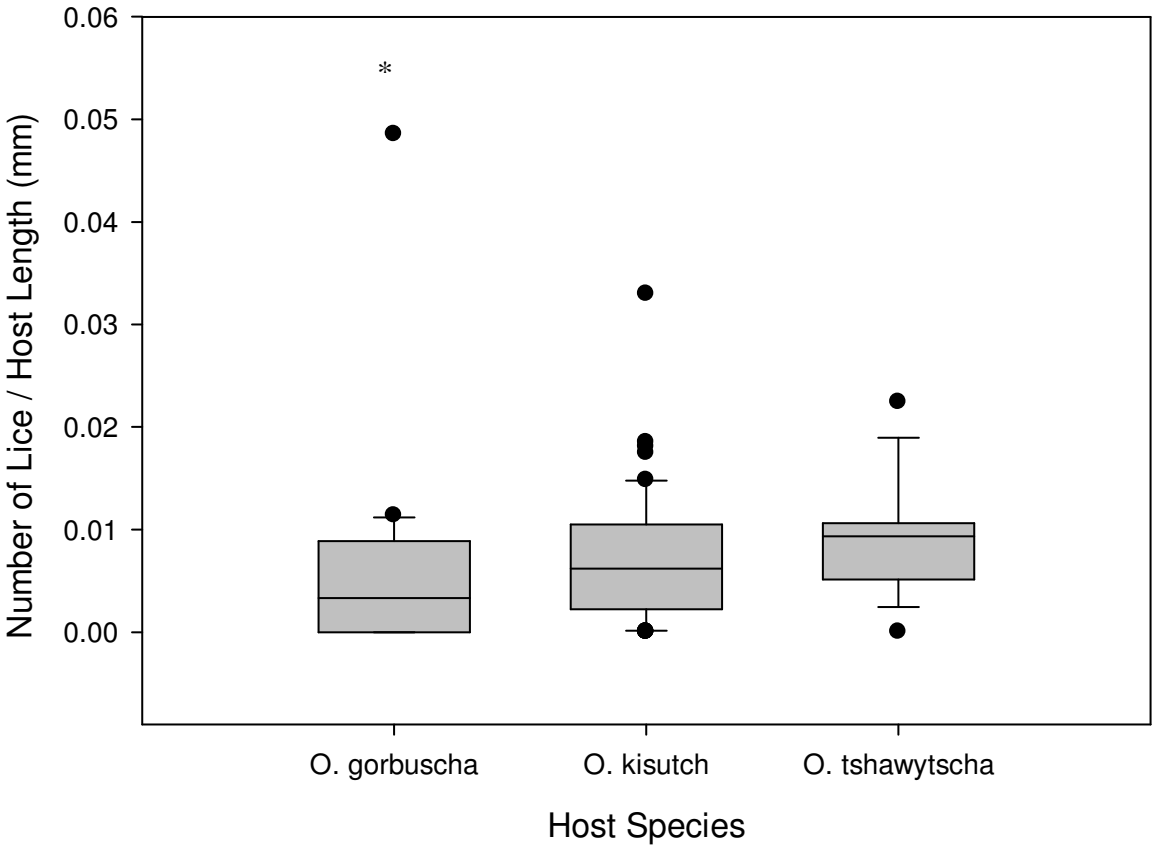

Figure 3: Parasite load (2009 & 2010) from three species of salmonids *Oncorhynchus gorbuscha* (n=20), *Oncorhynchus kisutch* (n= 52), and *Oncorhynchus tshawytscha* (n=17). Parasite load was significantly different between pink salmon hosts and coho salmon hosts ($p < 0.01$) whereas all other combinations were statistically similar..

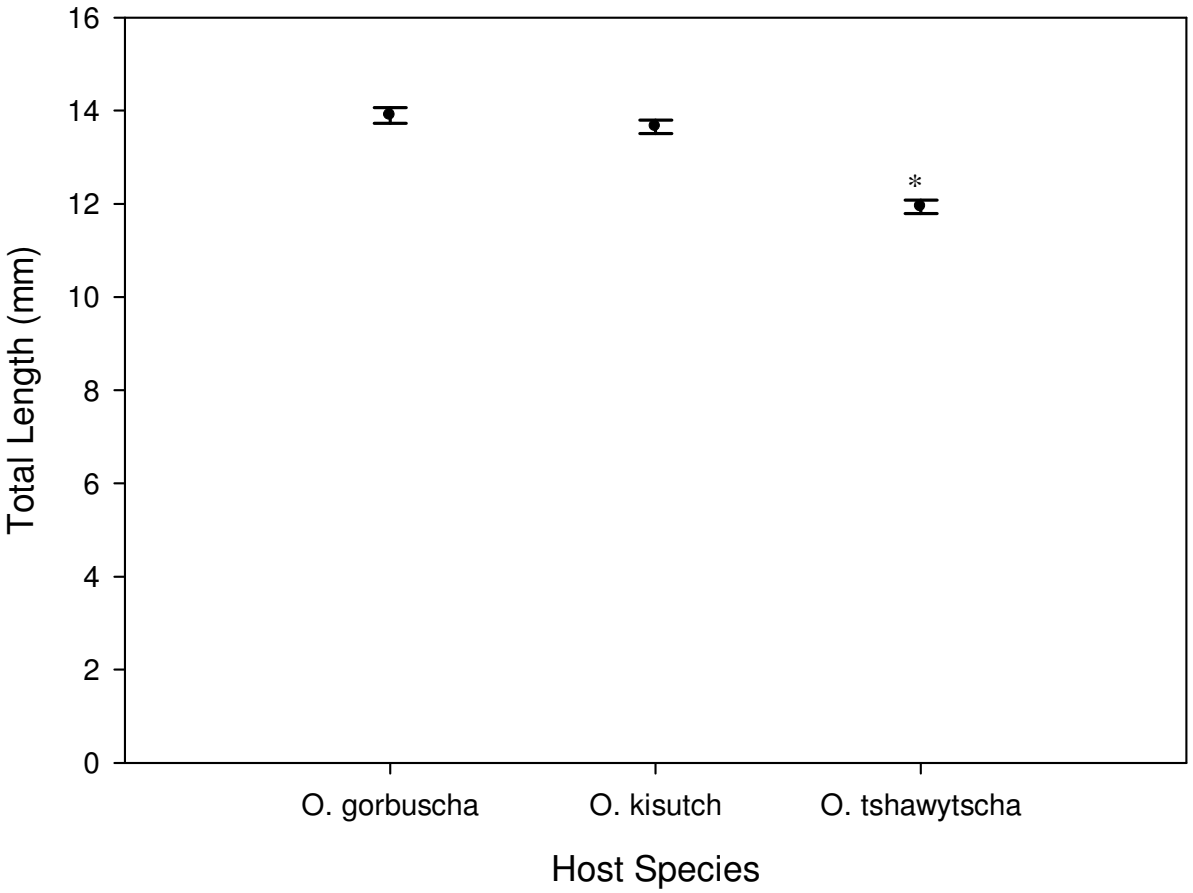

Figure 1: Average total louse length (±SE) of lice found on different host species (2009 & 2010): *Oncorhynchus gorbuscha* (n =22), *Oncorhynchus kisutch* (n = 42), and *Oncorhynchus tshawytscha* (n =24). Louse total length was significantly different among host species ($p < 0.01$).

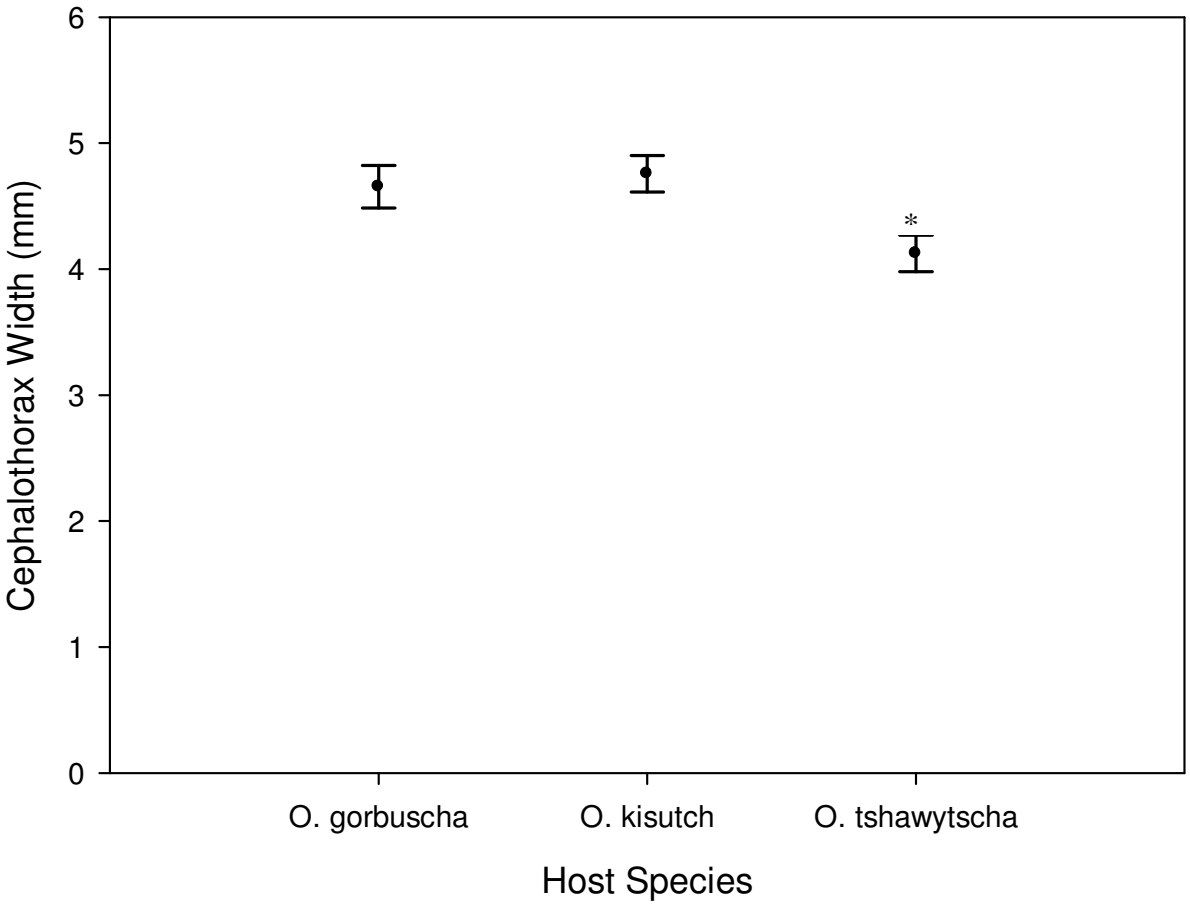

Figure 2: Average Cephalothorax width (±SE) from three host species (2009 & 2010): *Oncorhynchus gorbuscha* (n=22), *Oncorhynchus kisutch* (n= 42), and *Oncorhynchus tshawytscha* (n=24). Lice cephalothorax width was significantly different among host species infected ($p<0.01$).

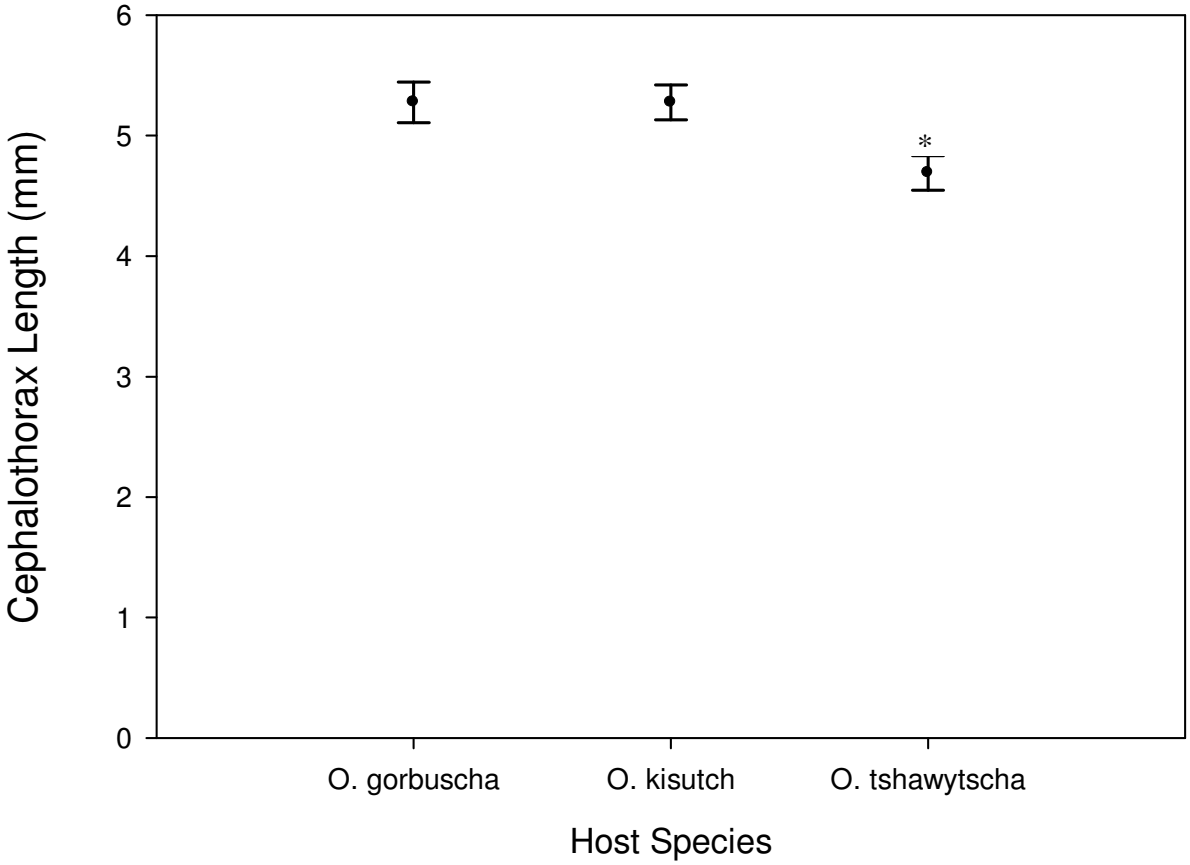

Figure 3: Average Cephalothorax length (±SE) from three host species (2009 & 2010): *Oncorhynchus gorbuscha* (n=22), *Oncorhynchus kisutch* (n= 42), and *Oncorhynchus tshawytscha* (n=24). Lice cephalothorax length was significantly different among host species infected ($p<0.01$).

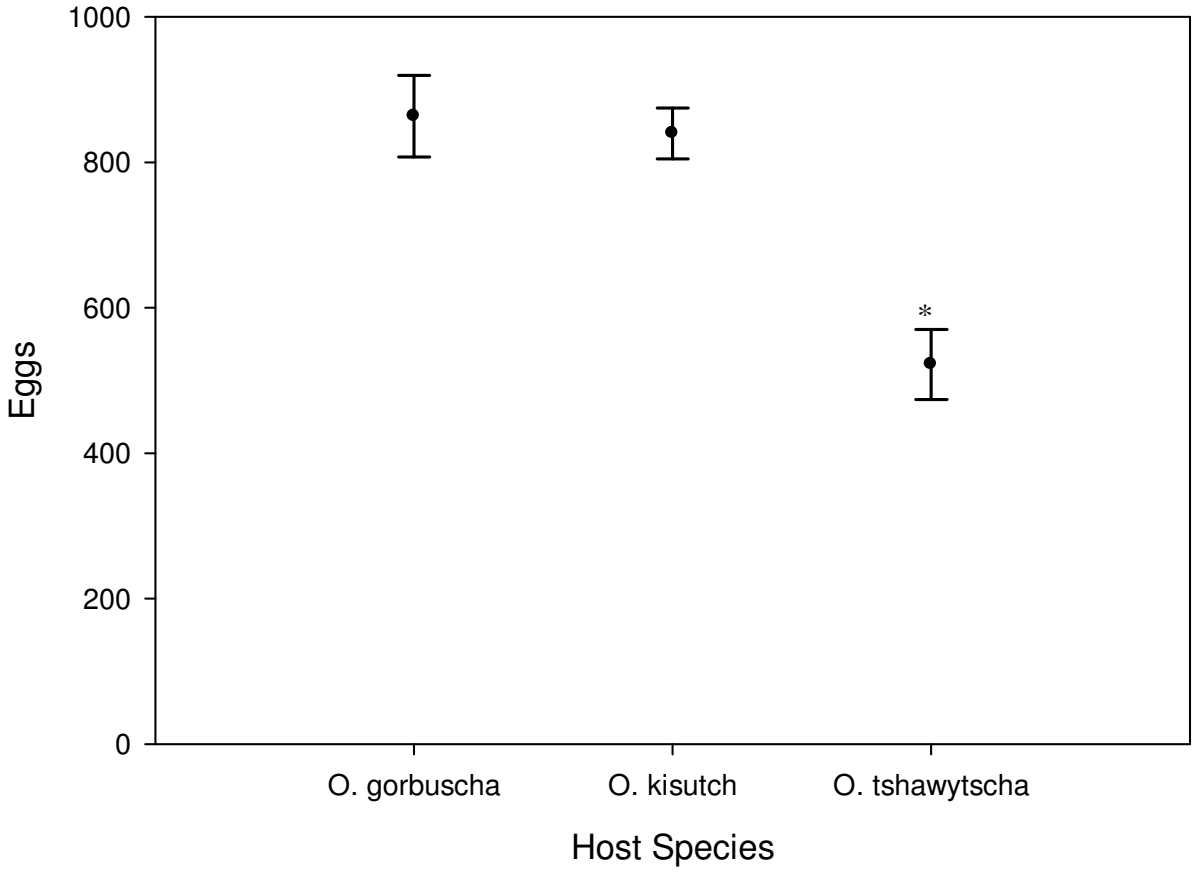

Figure 4: Average Fecundity (±SE) from three host species (2009 & 2010): *Oncorhynchus gorbuscha* (n=22), *Oncorhynchus kisutch* (n= 42), and *Oncorhynchus tshawytscha* (n=24). Lice Fecundity was significantly different among host species infected ($p<0.01$).

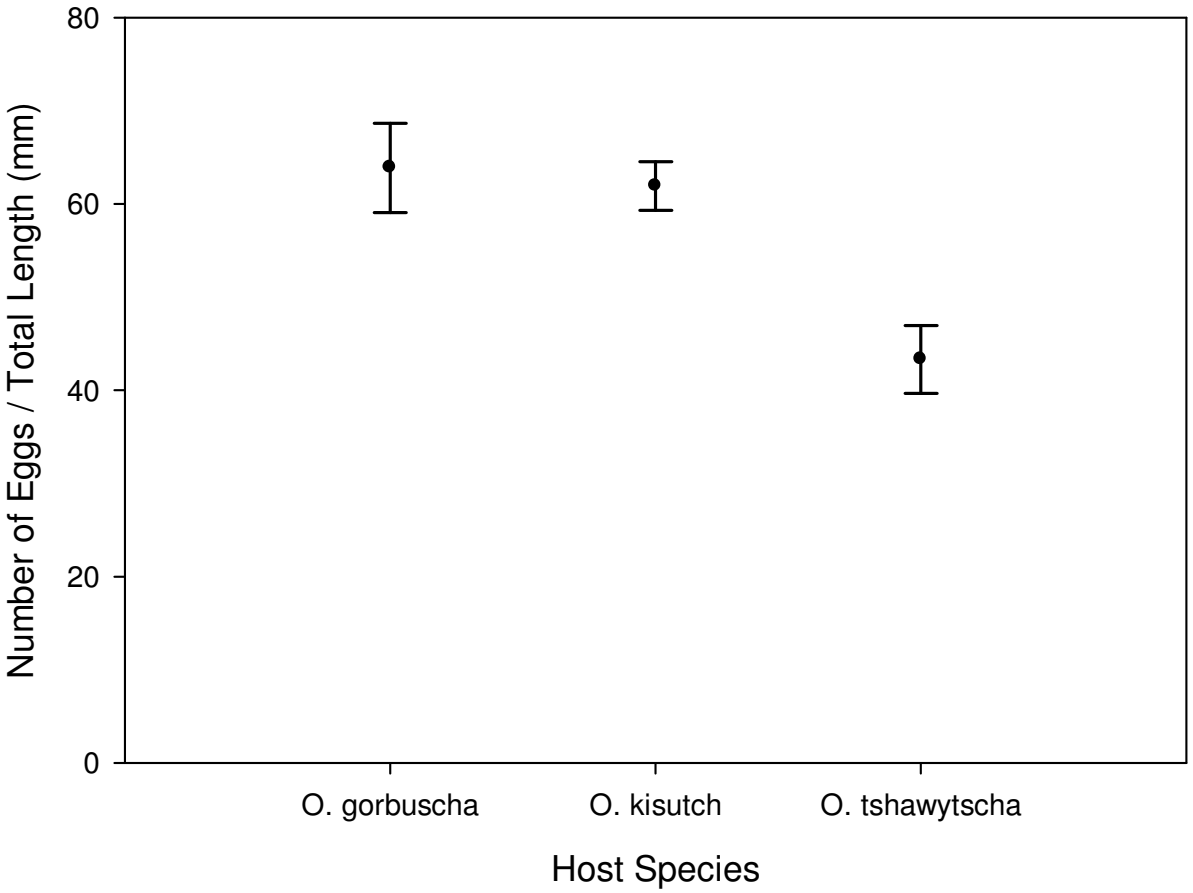

Figure 5: Average lice Fecundity per unit total length (±SE) from three host species (2009 & 2010): *Oncorhynchus gorbuscha* (n=22), *Oncorhynchus kisutch* (n= 42), and *Oncorhynchus tshawytscha* (n=24). Lice Fecundity per unit total length was significantly different among host species infected ($p<0.01$).

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Chapter 2

Examination of population genetics of wild *Lepeophtheirus salmonis* populations on salmonids (*Oncorhynchus spp*.) of the North Pacific, USA

Abstract

This study examined the degree of host-specificity exhibited by the parasitic salmon louse (*Lepeophtheirus salmonis*) when interacting with multiple species of Pacific salmon (*Oncorhynchus spp.*). Lice collections were made in the wild off the Pacific coast of Neah Bay, Washington. We found an overall significant population structuring between lice sampled from different host species. Lice that infected pink salmon were genetically dissimilar from lice that infected chinook ($R_{ST} = 0.2707$) or coho salmon ($R_{ST} = 0.3577$), in contrast the lice collected from coho salmon and chinook salmon were genetically similar ($R_{ST} = -0.0288$). These results might imply that a spatially explicit model for population structuring is in effect in this part of the world. During the migration pink salmon bring parasites from open water to a common area near the shore where genetically different lice are different may be a source of gene flow.

Introduction

This study examined the degree of host-specificity exhibited by the salmon louse (*Lepeophtheirus salmonis*) when parasitizing multiple species of Pacific salmon (*Oncorhynchus spp.*). The salmon louse is a marine ectoparasite that lives, feeds, and breeds on salmonid hosts (Pike and Wadsworth 1999). There are two distinct stages of the *L. salmonis* life cycle: non-feeding planktonic drifters and infective ectoparasites. The first form, comprised of two molts, float in the ocean plankton without feeding. The third molt, the copepodid stage actively pursues its host using chemical cues in the water (Bailey et al. 2006), tactile cues (Bron *et al*. 1993; Heuch and Karlsen 1997) and flashes of light (Genna *et al*. 2005). Once attached *L. salmonis* attempts to stay sessile until maturity when it will migrate to the anal fin in search of mates (Pike and Wadsworth 1999). This organism feeds primarily on salmonid mucus and epithelium until the pre-adult stage is reached where the siphon becomes long enough to reach the blood of the host. This caligid copepod has recently become an important topic of research due to its high reproduction rate at aquaculture sites and its adverse affects on the salmonid hosts (Ritchie *et al*. 1996).

Dense populations of fish at aquaculture sites make mating less challenging for salmon lice resulting in unnatural population increases (Tully and Whelan 1993). Salmon lice can cause sores that allow entry of pathogens into the animal tissue, which can affect the salmon's ability to osmoregulate (Jones et al. 2008; Patterson *et al.* 2009; Ritchie *et al*. 1996). This stress on the host, as indicated by a release of cortisol and increased glucose levels in the blood of the fish (Bowers *et al*. 2000), can reduce the maximum sustainable swimming speed of salmonids (Wagner *et al*. 2003; Wagner *et al*.

2008) which could have implications towards predation for wild fish and reduced ability to reach spawning grounds. Studies of salmon louse populations help determine policies related to aquaculture site locations and the existence of farming industry by giving insight to how salmon lice are transferring from farmed fish to wild fish.

Populations of parasites can become genetically distinct from one another through isolation of specific genotypes due to host-specificity (Todd *et al*. 2004). It is possible for *L. salmonis* populations bred at salmon farms to target specific host species when they reach infective life history stages. Host-specificity is the degree to which parasites specifically target certain species; if these parasites have preferences to specific host species then they will be reproductively isolated which should reflect random gene mutations conserved within populations. The exploitation of specific hosts is usually a reflection of a combination of circumstances between parasite and host. Typically host-specificity will occur when a) parasites have restricted mobility relative to their host, b) the populations are ecologically isolated and, c) parasite populations have differential fitness in specific habitats (Hofsted *et al.* 2004). This has been examined spatially for *L. salmonis* populations but the topic of host-specificity within the family of salmonids, is largely understudied.

There are several distinct habitats for *L. salmonis* throughout the Northern Pacific Ocean in the form of various host species that are ecologically and immunologically distinct from each other. These discrete habitats may provide the previously mentioned criteria for host-specificity. Pink salmon (*Oncorhynchus gorbuscha*) almost immediately leave the continental shelf and head into open waters northwest of the Washington State coast (Takagi 1981). Once at sea, the range of pink salmon will overlap with other

salmonids but not fully coincide leaving much of their range without the other species of salmonids (Quinn 2005). When migrating toward fresh water, pink salmon head directly back to their natal streams without stopping (Takagi 1981). This direct path to the spawning grounds and restricted habitat overlap greatly reduce the chance that lice from pink salmon will interact with coastal salmonids, spatially separated salmon lice. Pink salmon have an innate immunity and a mild inflammatory response to *L. salmonis* infections (Jones *et al*. 2008). Coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha)* stay in the shallower water on the continental shelf for the duration of their time at sea, when migration begins they slowly work their way back to the natal streams (Godfrey et al. 1975; Major et al. 1978; Quinn 2005). Chinook salmon, like most salmonids have an innate immunity to lice infestations but do not have the combination of innate resistance and an acute inflammatory response to infection that coho salmon have shown in the laboratory which is far superior to other resistance mechanisms exhibited by Pacific salmonids (Johnson & Albright 1992). As a result, lice from different groups of salmonids could target the host which provides a more persistent food source, a host-specific model.

The objective of this study is to determine if wild populations of salmon lice express host-specificity by determining population sub-division based on hosts infected. The hypotheses tested are near zero Rho_{ST} and F_{ST} values of parasite populations when tested across salmon species and random union of gametes within populations. If genotypes vary based on host species fixation index values (Rho_{ST} and F_{ST}) between lice from different host species then the values of this indices would approach one

(differentiation), and lice taken from the same host species would approach zero (complete panmixis).

Materials and Methods

Sampling was done off the coast of Washington State in the Strait of Juan de Fuca within a 10 Kilometer radius of Neah Bay, WA. At this location salmon have a limited number of net pens they will pass by on the return from the ocean and are expected to have wild-acquired lice. Fish with wild-acquired salmon lice were obtained by hook and line techniques (Boulding *et al*. 2009; Todd *et al*. 2004). Samples were taken at 100-2000 meters from shore at water depths between 20 – 800 meters. Typically, chinook salmon were sampled before 0700 hours within 200 meters from shore and at depths less than 80 meters whereas pink and coho were almost exclusively sampled after 0700 hours between 1000 and 2000 meters from shore at depths between 100 and 800 meters. This study will include 89 randomly sampled pacific salmon.

From each salmon collections of lice were made and preserved in 70% ethanol. A total of 140 lice were sampled from a pool of 379 lice observed. Researchers identified the hosts and removed lice with forceps while the fish was along the side of the boat in floating fish board. Lice were preserved in 60 ml sample containers filled with 70% ethanol. Each fish within the legal sport-fishing regulations had a dorsal fin clip taken and was preserved in a designated sample container with all lice sampled from that fish. All samples were then taken back to Eastern Washington University in Cheney, Washington and stored at room temperature.

Louse DNA Extraction and purification (Nolan *et al.* 2000)

A quarter of the cephalothorax was excised then placed into sterilized 1.5 mL Eppendorf tubes, frozen with liquid nitrogen and, macerated with individual wooden applicators. To each tube, 95µL of lysis solution (10 mM EDTA pH 8.0, 400mM NaCl, 0.2% SDS, 10mM tris-HCl pH 7.5, 2mM $MgCl_2$) was added to the crushed tissue sample to lyse cells and release its DNA. Proteinase K (0.2 $mg^{-1}mL$) was then added raising the volume to 100µL to digest any contaminating proteins from the solution and incubated at 37^{o}C for 18 hours on a rotary shaker at 190 r.p.m. Samples were treated with 0.04mg mL^{-1} DNAase free RNAase A and incubated for one hour at room temperature to digest RNA. DNA was then extracted using 110µL of chloroform, phenol, isoamyl alcohol (25:24:1). Samples were then mixed and centrifuged for 20 seconds. The supernatant of resulting mixture contained DNA from a single louse. The DNA was precipitated out of this solution by adding equal volume of pure non-denatured ethanol to the sample and was incubated at -80^{o}C overnight. DNA was collected by centrifugation (12,000g) for 30 minutes, removing the liquid from the container and dissolving DNA pellet in 50µl of TE buffer for storage.

PCR, electrophoresis and, experimental controls (Todd *et al*. 2004)

All primers (Nolan *et al*. 2000; Todd *et al*. 2004; Table 1) were purchased through Integrated DNA technologies (IDT) which were received and stored in powder form. When hydrated, concentrations were adjusted according to the Clontech, Terra protocol. Names of microsatellite loci are as follows; Nolan *et al.* (2000): Ls.NUIG.09, Ls.NUIG.14B, Ls.NUIG.20, Ls.NUIG.30; and from Todd *et al*. (2004): LsalSTA1, LsalSTA2, LsalSTA3, LsalSTA4, and LsalSTA5 (Table 1).

The polymerase chain reaction optimal thermal cycling temperatures were provided by the manufacturer of the Taq polymerase, Clontech Terra. The manufacturer provides the protocol for unpurified tissue samples, however there is not enough tissue per specimen to accommodate these guidelines so samples were purified for DNA and 5 µl of purified DNA was used for amplification. PCR work was done using a Techne TC-512 thermal cycler. Included for each reaction along with the louse DNA were 12.5µl of Terra PCR Direct Buffer, 7.5 pmol of each primer (forward and reverse for each loci), Terra PCR Direct Polymerase Mix (1.25 U).

A 10% TBE Polyacrylimide gel was purchased from Invitrogen™ and the DNA was visualized after ethidium bromide staining (1 µg / ml distilled water) using a transilluminator (Todd *et al.*, 2004) and a UV filtered digital camera. Then, F_{ST} and Rho_{ST} values were determined using GENEPOP 4.0.10 (Raymond and Rousset 1995, Rousset 2008). Information on PCR and gel electrophoresis was derived from previous studies (Nolan *et al*. 2000; Todd et al., 2004). Images of all gels were saved in digital format and loci lengths were measured from the photographs using a 100 base pair DNA ladder. For this experiment two controls were used a negative control, salmon tissue was purified in the same fashion and amplified using same primers; for a positive control, PCR products were cleaned with Zymo research and sent to The University of Washington for sequencing to compare DNA sequences to what was expected (Table 1).

Analysis

Pairwise Rho_{ST} and F_{ST} values were computed and tested using GENEPOP version 4.0.10 (Raymond and Rousset 1995; Rousset 2008). When estimates of F_{ST} are

less than 0.05 populations are considered to indicate little genetic differences (Wright 1978). Slatkin's (1995) Rho_{ST} is a newer statistic but is analogous to F_{ST} so it was considered at the same critical number. Expected heterozygosities were calculated using Levene's correction (Levene 1949) and paired with what we observed gives insight into the population genetics. Also tested were Hardy-Weinberg Equilibrium (HWE) exact tests (Haldane 1954) for each locus in each population using fishers method (Fisher 1922). Estimations of p-values are simulated using the Markov chain method for all loci (Guo and Thompson 1992) with 500 batches and 1000 iterations per batch.

Results

In total, 48 alleles were detected across 6 loci in 3 populations of *L. salmonis* in the North Pacific Ocean. Total number of alleles per locus varied from 5 (Ls.alSTA 4) to 12 (Ls.NUIG 14). Our sample sets varied from HWE in all populations and in many within population sets; HWE was recognized for 3 loci (Ls.alSTA 4, Ls.alSTA 5, and Ls.NUIG 14) for *O. gorbuscha* parasite populations. Expected heterozygosities were found or approached in 7 cases (Ls.alSTA 4, *O. gorbuscha*; Ls.alSTA 5, *O. gorbuscha*; Ls.NUIG 14B, *O. gorbuscha*; Ls.alSTA 1, *O. kisutch*; Ls.alSTA 2, *O. kisutch*; Ls.NUIG 14B, *O. kisutch*; and Ls.NUIG 14, *O. tshawytscha*; Table 2). Three of the original 9 primers (Ls.NUIG.09, Ls.NUIG.20, and Ls.NUIG.30) failed to amplify and were excluded in the analysis. Both negative control (attempted amplification of salmon tissue) and positive controls (sequencing a sub-sample of the PCR products; data not shown here) were implemented and assured the researchers of the accuracy of the protocol.

Population differentiation was evident in one population when looking a Rho_{ST} and F_{ST} estimates. Rho_{ST} (Table 3) defines lice populations on *O. gorbuscha* as different from both lice populations found on *O. kisutch* or *O. tshawytscha* (Rho_{ST} = 0.2707, and 0.3577, respectively) and lice found on *O. kisutch* and *O. tshawytscha* as genetically similar (Rho_{ST} = -0.0288). The estimates of F_{ST} (Table 4) suggest a similar relationship with genetic differentiation of lice found on *O. gorbuscha*: lice retrieved from chinook and coho salmon were similar genetically ($F_{ST} < 0.05$) and lice obtained from pink and chinook or pink and coho as genetically distinct (F_{ST}=0.09 and F_{ST}=0.08 respectively).

Discussion

Variation in allele sizes at 5 of the 6 loci that amplified was useful to determine population structuring. The locus amplified at primer Ls.alSTA 4 was not polymorphic across the populations and thus not an informative loci for sub-division (i.e. $Rho_{ST} < 0.05$). At this locus there were only 5 genotypes all within two slippage mutations of one another. This could be an artifact of highly conserved microsatellite locus that is piggy-backing on an important gene or sample size. However, with the same sample size in the same individuals the locus that amplified with Ls.NUIG 14B had 12 genotypes across a large range of base pairs (52) which would indicate highly polymorphic loci. These results indicate the need for more microsatellite development of *L. salmonis* for work in various locations.

In 2000, Nolan *et al*. developed microsatellite primers with the intention of providing scientists with the ability to use *L. salmonis* specific microsatellite loci in ecological experiments. Given lice from Ireland, Scotland and Norway they found that

there was variation within and among groups in allelic frequencies and concluded that these primers would be usable for ecological studies. In 2004, Todd *et al*. used the primers developed by Nolan *et al*. (2000) and found that the variation noted by Nolan et al (2000) was not statistically significant among lice collected from 18 populations around the world examining a total of 1007 lice. In the Northern Atlantic they looked at 7 farms and 8 wild populations around Scotland which included sea-run brown trout (*Salmo trutta*), sea-run rainbow trout (*Oncorhynchus mykiss*), and wild and farmed Atlantic salmon (*Salmo salar*). They also examined wild fish from a single site in northern Norway, one farm site in eastern Canada, and a single sample from a farm in western Canada. To make comparisons they defined populations based on host species and grouped them based on where they were sampled (Scotland, other northern Atlantic sites, and the north Pacific). They compared lice collected from different wild and farmed hosts around Scotland (wild Atlantic salmon to wild sea trout to farmed Atlantic salmon and rainbow trout) finding no significant sub-division among groups (F_{ST} = – 0.0003), among populations within groups (F_{ST}= 0.0006), and within groups (F_{ST} = 0.0003). Then they compared all lice collected in the northern Atlantic (Scotland to Norway to eastern Canada) finding no significant sub-division among groups (F_{ST} = 0.0008), among populations within groups (F_{ST}= 0.0004), and within groups (F_{ST} = – 0.0004). All lice in the North Atlantic Ocean were members of a single population. Then they compared the north Atlantic (pooled) to the north Pacific sample finding very weak sub-divisions among groups (F_{ST}= 0.06) and within populations (F_{ST}= 0.06), while finding similarities among populations within groups (F_{ST}= 0.0006). These finding

suggested that lice infecting Atlantic salmon, sea-run brown trout, or sea-run rainbow trout at any spot in the world are nearly genetically identical.

Conversely, Boulding *et al*. (2009) working with different wild hosts in British Columbia utilized the highly polymorphic mitochondria cytochrome c oxidase subunit 1 (COI) to examine sub-divisions on different host choices of *L. salmonis*. Then they used a neighbor joining tree algorithm to estimate relationships among L. salmonis simulated populations. They estimated that there was subdivision among *L. salmonis* collected from aquaculture sites at different locations and among different species in the wild. Use of this portion of the genome has been criticized as an oversimplification because it has been suggested that DNA barcoding techniques can vary from 2% in vertebrates within a species (Johns and Avise, 1998) to as much as 23% in all animals (Funk and Omland, 2003). These results did indicate that genetic variation might exist in the North Pacific Ocean spatially and based on the host species of salmon lice. In the present study we were able to attribute the variation among wild species to variation in distance through geographic habitat of hosts.

Presented in this paper are both values of F_{ST} and Rho_{ST} to illustrate population genetic differentiation. The original fixation index (F_{ST}) uses the infinite alleles model which suggests that different size alleles are different regardless of length. In the case of microsatellites these mutations would normally be one repeat length larger (or shorter) so size might matter for microsatellite alleles especially in determining differences in closely related organisms (Ellegren, 2004). Rho_{ST} uses the stepwise mutation model (SSM) that assumes each mutation is one evolutionary step further derived. The SSM has the problem of homoplasy but is likely negligible in population studies (Jarne and

Lagoda 1996) and the SSM is conceptually more appropriate for population sub-division studies using microsatellites. When Rho_{ST} values are negative, they suggest that every individual in populations of parasites found on either *O. kisutch* or *O. tshawytscha* were potential partners (panmixia). This was apparent when comparing chinook and coho salmon populations. Whereas, large variation was recognizable between parasites found on either *O. kisutch* or *O. tshawytscha* and parasites found on *O. gorbuscha*.

Salmon lice are very quickly adapting organisms as is evident in the ability of this organism to resist chemotherapeutant pesticides (Denholm *et al*. 2002). As salmon have developed immunity to this pest *L. salmonis* have developed responses to that immunity. Firth *et al*. (2000) and Fast et al. (2003) suggest that proteases secreted while in contact with an Atlantic salmon may be secreted to avoid an immune response from the host. Fast *et al*. (2003) shows that salmon protect themselves from *L. salmonis* infection by secreting a lysozyme and coho salmon may secrete some extra protein in its mucus to help avoid infection. Salmon lice have responded to resistance by mounting a protein arms race in an apparent example of the Red Queen hypothesis (Van Valen 1973). This may be a driving force behind the differences observed in the present. However, coho and chinook salmon hosts were chosen to see if within a location genetic differentiation would occur simply driven by differences in host resistance, a control for the red queen concept. Lice appear to not specify what salmon they are encountering based on the resistance or fitness benefits of certain hosts (See Chapter1) but are probably targeting salmon that are within certain proximities considering that larval dispersal is likely limited to 25 km (Gillibrand and Willis 200). During the migration period, pink salmon bring hosts from open water to a common area near shore these genetically diverse lice may be a source of gene flow.

Heterozygosity excess or deficit can occur after a bottleneck, founder effect or, if heterozygotes have a selective advantage or disadvantage (Cornuet and Luikart 1997). Obviously with microsatellites the latter is of no consequence because they are non-coding regions of the genome. In the present study we recognized a heterozygosity deficit (Table 2) but this is more likely an artifact of sample size or skewed sex ratios of *L. salmonis* (Jacobsen and Gaard 1997) than evidence of a bottleneck event considering the benefit for this organism provided by salmon farms (reviewed by Morton et al. 2005).

Table 1: Loci motif, primer sequence and expected size range taken from Nolan *et al.*, 2000 (Ls.NUIG); and Todd *et al*. 2004 (Ls.alSTA). Ls.NUIG.14B was also used in Todd *et al*. 2004 and adjusted for ease of use.

Primer	Motif	Size (bp)	Primer Sequence
Ls.NUIG.09	$TC_{(10)}$	201	F: CGT CAT TTT GCA TTT GTC R: GAT ATG TGC ACC TTA TCA
Ls.NUIG.14B	$TA_{(10)}$	308	F: GTT CAC GGT CGG GCT ATC TA R: TTT GAG TTA ATT GGT AAG AAA AAT TGA
Ls.NUIG.20	$AT_{(5)}AG_{(7)}$	166	F: AAG ACC AGA AAT CAC TTG R: ATG GTG AAG TGA AAA CGG
Ls.NUIG.30	$AT_{(2)}A_{(1)}$ $AT_{(5)}$	121	F: TGA TAC GCT AAA GAA GAG AG R: TAG CTG AAC ATC CCT AAG G
LsalSTA1	$TC_{(20)}$	202	F: CGT CGA AAT TCT CAT CCA A R: GGG AAA GAT TGG GAG TGA G
LsalSTA2	$TC_{(13)}$	266	F: TCG TGG TGG TTG ACT CTA CT R: AGG AAA TCA GGA GCA AGT G
LsalSTA3	$TC_{(18)}$	232	F: TTA TCC GAA TCC GTC TTA TG R: AGC CTG AAG TAG GTT AGT TGG
LsalSTA4	$GA_{(2)}A_{(1)}$ $GA_{(2)}GT_{(1)}$ $GA_{(8)}$	216	F: AAG GCG TGC GTT GTT AAG T R: CAA TGC GAT CCT GGA GTC T
LsalSTA5	$GA_{(15)}$	240	F: GGG ATA AGT GGC GAG CTA CC R: GTC TCA GCG GCA GAA GTC TC

Table 2: Expected heterozygosities were assessed and visually compared to what was observed during this study.

		Host Species		
		O. gorbuscha	O. kisutch	O. tshawytscha
Ls.alSTA 1	Expected	10.33	12.19	10.52
	Observed	2	8	3
Ls.alSTA 2	Expected	10.37	11.33	11.33
	Observed	2	7	3
Ls.alSTA 3	Expected	11.67	11.41	9.96
	Observed	3	2	5
Ls.alSTA 4	Expected	8.48	10.33	11.26
	Observed	8	4	6
Ls.alSTA 5	Expected	10.74	10.71	11.82
	Observed	7	6	6
Ls.NUIG.14	Expected	12.4074	12.37	11.41
	Observed	12	8	7

Table 3: Pairwise Rho $_{ST}$ estimates of population sub-division.

Estimates for all loci Rho $_{ST}$		
	O. gorbuscha	O. kisutch
O. kisutch	0.2707	
O. tshawytscha	0.3577	-0.0288

Table 4: Pairwise F_{ST} estimates of population sub-division.

Estimates of F_{ST} for all loci		
	O. gorbuscha	O. kisutch
O. kisutch	0.07981	
O. tshawytscha	0.09254	0.00551

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2010 Oral Presentation, Eastern Washington University, Department of Biology, Graduate Student Research Seminar
2007-2010 Lab Instructor, Eastern Washington University, Department of Biology Introduction to Biology
2009 Ocean Port Sampler, Neah Bay, Washington Department of Fish and Wildlife

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Ecological skills; macroinvertebrate sampling, zooplankton sampling, invertebrate keying, boat operation on freshwater and marine environments, stream invertebrate sampling, water quality analysis
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