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Evaluation of Three Potential Methods for Preventing the Spread of the Salmon Louse, Lepeophtheirus salmonis (Kreyer, 1837)

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EVALUATION OF THREE POTENTIAL METHODS FOR PREVENTING THE SPREAD OF THE SALMON LOUSE *Lepeophtheirus salmonis* (Krøyer, 1837)

By

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An Abstract of the Thesis Presented
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Lepeophtheirus salmonis or salmon lice infections are one of the most prevalent parasitic infections in the salmon aquaculture industry. Salmon lice cause an estimated loss of 3% of the production of Maine's salmon industry annually. Within the State of Maine only a portion of the farm sites experience salmon lice infections on an annual basis, while some sites have never had infections of *Lepeophtheirus salmonis*. Because of the potential impact that salmon lice infections could mean to those areas that to date have been free of *L. salmonis* infections, there has been concern both on the part of the State and the industry to prevent any further spread of the parasite by farming activities. This research evaluated three potential methods for preventing salmon lice from developing to the infective copepodid stage.

In order to evaluate the effects of chlorine, iodine and desiccation on the development of salmon lice eggs a new culture system for *L. salmonis* was developed at the University of Maine. The system allowed for egg strings to be raised within

individual culture chambers and utilized recirculation technology. Before being cultured *L. salmonis* egg strings were exposed to one of eight treatments: 200 ppm of chlorine or iodine for one minute, 500 ppm of chlorine or iodine for one minute or 10 minutes, or desiccation for either four or 24 hours. Egg strings were then raised for six days after hatching in filtered natural seawater at 32 ppt salinity at 12 C. Of the three methods examined desiccation for at least four hours was the only method that prevented *Lepeophtheirus salmonis* from developing to the infective copepodid stage. Transmission electron microscopy (TEM) of the egg membrane and egg sac revealed a complex ultrastructure that would make penetration of chemical agents difficult.

This research would indicate that the allowing all equipment that is transferred between farms to remain completely dry for at least four hours would be an effective method for preventing the spread of salmon lice. The use of either chlorine or iodine to prevent the spread of salmon lice is not effective.

ACKNOWLEDGMENTS

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Chapter 1

INTRODUCTION

Salmon lice have been the cause of one of the most severe disease problems faced by the salmon aquaculture industry in the northern hemisphere. Pike and Wadsworth (1999) report outbreaks occurring in Norway as early as the 1960s. As the industry has expanded so too has the problem. In 1996 losses due to salmon lice and expenses for salmon lice control for three of the worlds top salmon producers was an estimated $74.4 million US dollars (Roth 2000). In Maine the annual cost of salmon lice is estimated at 3% of the total production per year.

It is the ability of the mobile parasitic life stages to roam freely over a fish and even between hosts (Ritchie 1997) that causes the most severe pathological damage. Salmon lice damage their hosts by feeding on the mucus, epithelial cells, and occasionally the blood. When confined to a single location on a host such as in the chalimus stages, damage is minimal. The intensity of the damage increases dramatically once the louse is able to range over the fish (Grimnes and Jakobsen 1996). By feeding mostly on the mucus and epithelium, the fish becomes more susceptible to secondary infection (Wootten et al. 1982; Nolan 1999). Also the ability to maintain homeostasis becomes impaired as noted by the increased activity of the sodium/potasium-ATPase in the gills (Nolan 1999) and elevated plasma chloride levels (Grimnes and Jakobsen, 1996) in the fish. If the parasite load is high enough, mortality can result from these conditions (Grimnes and Jakobsen, 1996). Other dangers to the fish are increased levels of chronic stress (Mustafa et al. 2000), transmission of infectious diseases such as Infectious Salmon

Anemia (Nylund et al. 1993; Nylund et al. 1994) and open wounds, commonly found, on the head of the fish.

Surveys of Maine salmon farms in 1993 indicated that *Lepeophtheirus salmonis* was not a problem (Shaw, 1994). By 1995 though salmon lice were causing problems in the Maine aquaculture industry. Since then the coastal waters of Maine have been divided into three salmon lice zones (Fig 1.1). Salmon lice do not pose a serious threat to aquaculture in zones 2 and 3 at present. To avoid passing pathogens from one farm to another the aquaculture industry follows general biosecurity procedures, which include disinfecting all equipment moving in between farms. The effectiveness of disinfectants such as bleach and iodine against viruses and bacteria is well established. Their effectiveness against salmon lice eggs has never been tested. This thesis examines the effectiveness of bleach, iodine, and desiccation as means to prevent salmon lice from hatching and developing to the infective copepodid stage.

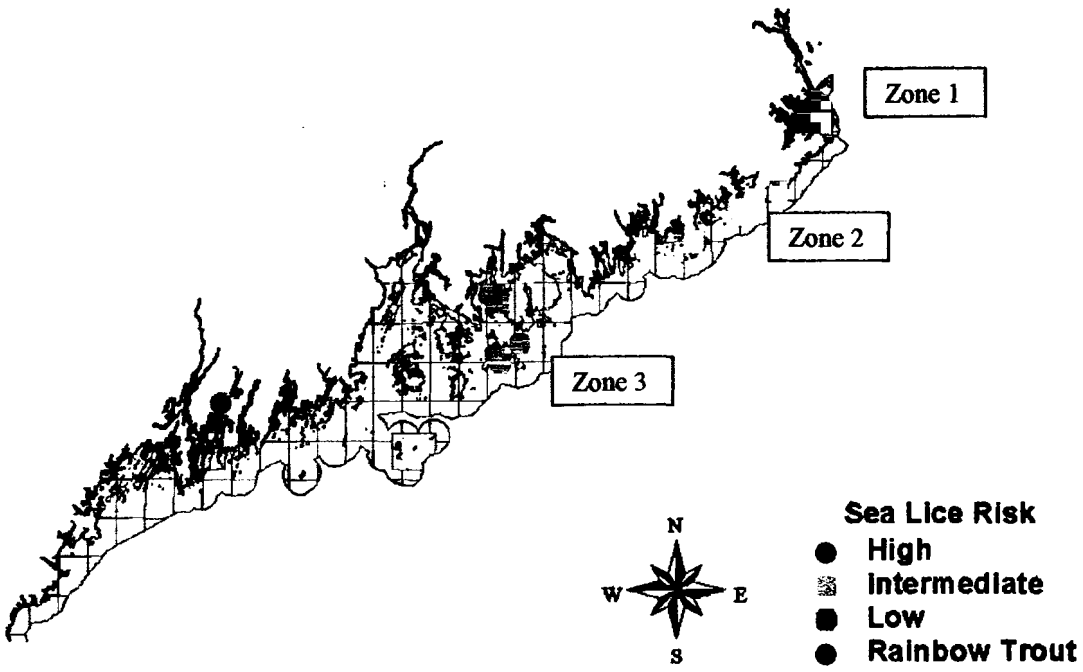

Figure 1.1-Risk for Maine Finfish Sites- A map showing the sea lice risk for all of the finfish sites in the state of Maine.

Chapter 2

LITERATURE REVIEW

2.1. Life History and Morphology

The life history of *Lepeophtheirus salmonis* has three major divisions, the free living or planktonic stages, the attached parasitic stages and the mobile parasitic stages. These three basic stages can be further broken down into eleven life history stages. The free living stages are comprised of the egg, two nauplii stages and the copepodid. The parasitic stages consist of four attached chalimus stages and two mobile pre-adult stages and the mobile adult (Johannessen 1978; Johnson and Albright 1991a). It needs only a single host in order to complete its life cycle, yet it is capable of transferring from host to host (Ritchie 1997; Pike and Wadsworth 1999). Egg strings (Fig. 2.1) are extruded through the oviduct and can vary greatly in length. Besides the variation in the length of each egg string, the number of eggs per string has been shown to vary with temperature (Ritchie et al. 1993) and size of the female. Johannessen (1978) reported a time of 10-12 days at 11.5 C and 30-40 days at 9 C for the eggs to develop from extrusion to hatching. More recent studies report an egg development time to hatching of approximately 8.6 days at 10 C (Johnson and Albright 1991b; Boxaspen and Næss 2000). The stage of egg development can be roughly determined by examining the color of the egg string. As development, progresses and pigments are deposited in the larvae the egg string color will go from white to light brown and finally to a darker brown just prior to hatching (Pike and Wadsworth 1999).

Figure 2.1 –*Lepeophtheirus salmonis* Egg String- A magnified (600 X) section of an egg string from *Lepeophtheirus salmonis*.

After hatching, the organism goes through two nauplii stages (Fig. 2.2). Both are non-feeding, planktonic stages and are extremely similar in appearance. They possess two eyespots about 1/3 of the body width apart (Johannessen 1978). The two nauplii stages lack a mouth, anus (Johannessen 1978) and external segmentation (Johnson and Albright 1991). The second molt can be distinguished from the first by an apical outgrowth on the endopod of the second antenna that is three times larger on the second nauplius (Johnson and Albright 1991a). Johannessen (1978) and Johnson and Albright (1991a) report a slightly longer mean average length for the second nauplius, however in both cases there is some degree of overlap between the size range for the two stages. Johnson and Albright (1991b) report mean duration times of 30.5 hours for the first nauplius and 56.9 hours for the second nauplius at 10 C. It has been observed that under laboratory conditions nauplii that are abnormally round tend not to molt to the second nauplius stage (Johannessen 1978).

The final non-feeding stage is the copepodid (Fig 2.3). Bron et al. (1993a) report that the copepodid stage does possess a complete gut. Once attached to the host it is

capable to begin feeding as a copepodid, but normally molts to the first chalimus stage before feeding. This stage is distinctly different in appearance from the previous stages. The copepodid is larger than both previous stages (length 0.77±0.03 mm, width 0.26 ± 0.04 mm)(Johannessen 1978). The body is divided into a cephalothorax comprised of the fused cepalic segments and the first two thoracic segments. There are ten pairs of setules along the median longitudinal axis and two eyespots on the dorsal shield. The posterior region is made up of the third, fourth, and fifth abdominal segments and a longer segment

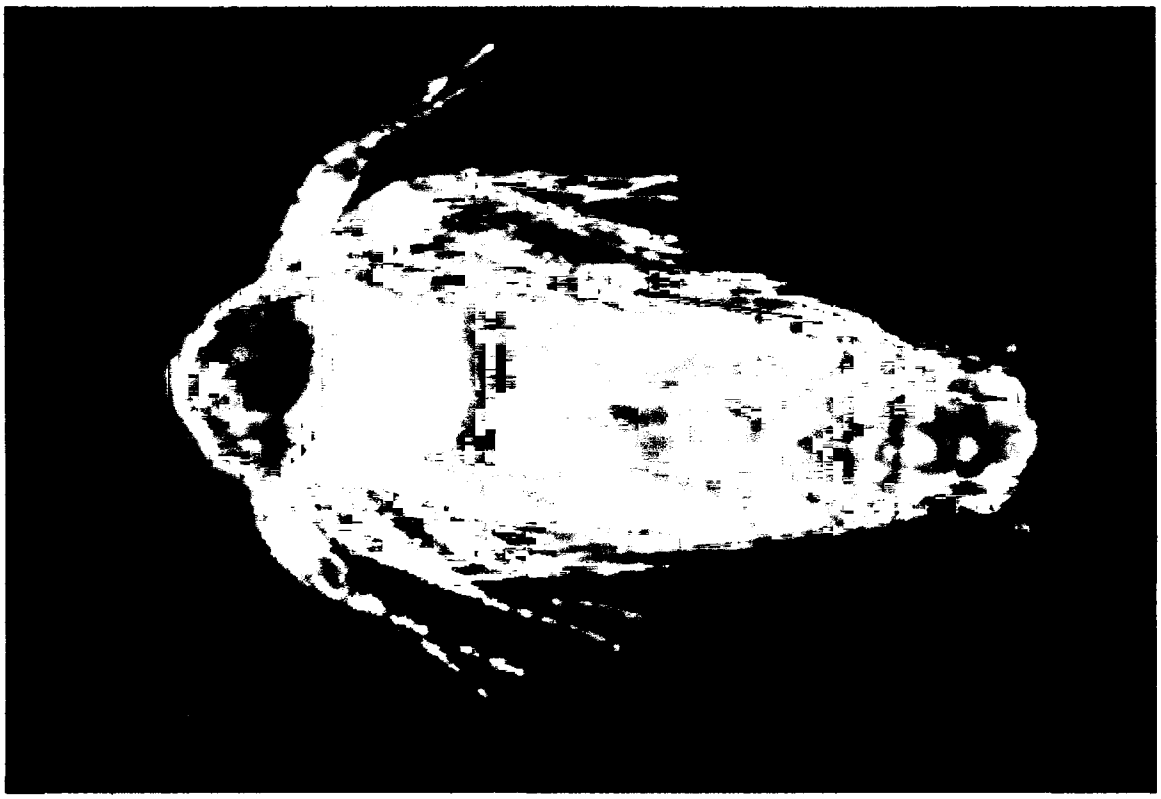

Figure 2.2 - *Lepeophtheirus salmonis* Nauplius- A second stage naplius viewed using dark field microscopy at 250 X.

Figure 2.3 -Copepodid- The infective copepodid stage of *Lepeophtheirus salmonis* seen here at 130X.

that is the sixth abdominal and genital segments fused together (Johnson and Albright 1991a). The second antenna forms a terminal claw that is used for attachment to the host.

Once the copepodid has attached to the host it will develop into four successive stages termed Chalimus I-IV. As the animal molts through all four stages it gets progressively longer and wider (Johnson and Albright 1991a). All four stages are attached to the host via a frontal filament and are capable of feeding. Bron et al. (1991b) describe the attachment of the filament to the epithelial basement membrane via the basal plate of the filament. They also described two other sections of the filament and several organs associated with its secretion.

After the Chalimus IV, stage the parasite molts into the mobile stages of the parasite consisting of two pre-adult stages and the adult. Overall, Johnson and Albright (1991b) report that it takes 39 days at 10 C to reach the second pre-adult stage from hatching. At this point development rates begin to differentiate due to the fact that males will mature before adult females. Adult males appear 31 days post hatch while adult

females did not appear until 41 days post hatch at 10 C (Johnson and Albright 1991b). The reason for the decrease in male development time is the mating behaviors. Ritchie et al. (1996) made detailed observations of the mating behavior. Adult males would pair with pre-adult II females and remain attached to them until they underwent their final molt. At this point the male would attach to the genital segment of the female and transfer two spermatophores. Copulation normally occurs during this final molt of the female; thus, males must mature slightly faster than females. As the lice progress through these final three developmental stages, they move about the host feeding on the epidermis. While they are capable of moving over the entire host, they tend to restrict their distribution to certain areas (Pike and Wadsworth 1999). The preferred regions for feeding are those located around the head of the host. As adult males appear, this preferred region becomes the site of pre-copulatory and copulatory pairs. Other lice of all mobile stages seem to migrate to positions on the posterior dorsal and ventral areas of the fish to avoid adult males.

2.2. Salmon Lice Control

Integrated pest management (IPM) plans are dynamic strategies that combine a number of control methods to keep pest populations under control with minimal use of chemotherapeutants. To accomplish this, regular monitoring of the pest population of interest is essential along with the use of several different control methods. In salmon aquaculture some of the effective methods include site fallowing (Bron et al. 1993c), year class separation, biological control with wrasse (Treasurer 1994, and Treasurer 2000), and co-operative management plans for relevant ecological areas (Smith 1996). A large

amount of research has been focused on various aspects of integrated pest management plans, including vaccine development and salmon lice traps using semiochemicals or lights with the goal of reducing the need for chemical treatments.

Initially the organophosphate dichlorvos (DDVP) sold, as AQUAGARD SLT® (Novartis) was the only effective treatment. Over time resistance has developed to organophosphates (Pike and Wadsworth 1999). There are eleven chemical treatments from five families of drugs used around the world (Roth 2000). Some of these compounds are topically applied in the form of a bath. These types of treatments are being replaced by the avermectins (ivermectin and emamectin benzoate) and benzoylphenyl ureas (diflubenzuron and teflubenzuron), which are applied as feed treatments (Roth 2000). The newest chemotherapeutants developed against salmon lice are emamectin benzoate and the benzoylphenyl ureas (Roth 2000). All of these control measures have various concerns that are associated with their use. The industry is developing IPM practices to address these environmental, regulatory and economic concerns.

2.3. Factors Affecting Infection Success

2.3.1. Host Finding Behaviors and Mechanisms

Successful infection depends on detecting and attaching to the proper host. Bron et al. (1993b) examined sensory organs and behaviors in other parasitic copepods and then looked for these organs and behaviors in *Lepeophtheirus salmonis* copepodids. One of the first behaviors observed by early researchers (Johannessen 1978; Wootten et al. 1982) was the possibility of a phototactic response. Examination of the copepodid eyes

revealed that they were capable of detecting light and even possibly images (Bron et al. 1993b). Their experiments using direct light did show a positive phototactic response. They were cautious to claim a similar reaction occurs in nature as some have suggested positive reactions to a direct light source can be an artifact of the laboratory. Heuch et al. (1995) addressed this question by examining copepodids under natural conditions. They placed plastic enclosures that were 2 m in diameter and 6 m deep or .65 m in diameter and 12 m deep into a bay and added a number of copepodids. The population distribution within each enclosure was monitored for ten days. They found that copepodids displayed a normal diel vertical migration pattern, migrating to the surface during the day and migrating down at night. Heuch et al. (1995) suggest that this behavior might increase the likelihood of an encounter with a host since salmon migrating to the surface at night are forced to pass through the copepodids migrating down and the reverse being true in the morning. Flamarique et al. (2000) confirmed the patterns of diel vertical migration and observed a strong response of copepodids to swim toward the surface when stimulated by light and a passive sinking response to the offset of light. Aarseth and Schram (1999) also confirmed the upward migration of copepodids in response to light, but they noted when UV irradiation was added that the upward response was less pronounced.

Other possible host finding behaviors have been identified. Bron et al. (1993b) examined the possibility that copepodids responded to a change in water pressure. Their experiments revealed that copepodids exhibited a rapid upward swimming response to increased water pressure. The greater the increase, the greater the response. In turn, a decrease in water pressure lead to a return to normal swimming. Further experiments

consisting of injecting water from a pipette near a copepodid showed that it responded by a corkscrew swimming behavior. This behavior was observed when copepodids were placed in a tank with fish and a fish swam close to the copepodid. These observations lead Bron et al. (1993b) to conclude that one major means of finding a host was the sensing of pressure waves from a fish swimming near by.

Heuch (1995) further showed that in laboratory experiments copepodids would congregate at step salinity gradients. Again, this can increase the chances of encounter as olfactory cues will concentrate at these layers and so will fish that are using smell to locate prey.

The final investigations conducted by Bron et al. (1993b) examined the possibility of following some sort of chemical plume present in the water column. This possibility was supported by observations seen in *Caligus minimus* and the presence of numerous chemical sensors in the antennae. Laboratory trials however, revealed no response by copepodids to run off water from hosts. They suggest that these sensors are used to determine the suitability of the host once initial contact is made. If the host is acceptable, then initial attachment occurs using the antennae that are modified into two large hooks.

Environmental factors have also been identified as being important in the successful attachment of the copepodid. Hogans (1995) observed that the infection rates of copepodids on salmon in Canada increased as winter water temperatures got warmer. He also noted that the survival of copepodids seemed to be inhibited below water temperatures of 2 C and that even below 4 C copepodid survival was poor. Boxaspen and Naess (2000) also noted poor copepodid survival below 4 C. Tucker et al. (2000a) found that greater successful copepodid attachment and development occurred at 12 C

compared to 7 C. They also found greater success in higher salinities (34‰) versus lower salinities (24‰). Overall, they believed that the temperature effect could mask any salinity effect.

Tucker et al. (2000b) have indicated that the age of the copepodid is also important to the success of attaching to a host. Through their work on larval salmon lice energetics, they have shown that copepodids aged seven days have a statistically lower chance of attaching to a host than copepodids aged 5 days. They postulate that this lower attachment rate is due to the utilization of fixed energy stores in the copepodid. Once a copepodid has successfully attached, however, there is no difference in survival due to the age of the settled copepodid.

2.3.2. Nauplii and Copepodid Distribution

Lepeophtheirus salmonis is known to infect only salmonid species within the northern hemisphere. Costelloe et al. (1999) observed two main loci of copepodid distribution in a west Ireland bay. The first was in the outer harbor in the vicinity of several salmon farms in the area. This was to be expected as previous sampling by Costelloe et al. (1996) showed that relatively low levels of copepod larvae could be found up to 1 km from farm sites and their model predicted that copepodids could not be found much further than 2 km from salmon farms. In the case of farms, Costelloe et al. (1996) found that they were mainly self-infecting, with greater than 90% of the larval supply coming from inside the net pens. The second major locus was in the shallow waters of the inner bay. The area was sufficiently distant from the salmon farms and it was concluded that it was nearly impossible for salmon farms to be the source of the larvae.

Costelloe et al. (1999) concluded that the most likely source here was from wild salmon, with their own lice or those picked up when passing farms, while waiting for the right conditions to begin migrating upstream.

Tully et al. (1999) were also able to make some inferences on the presence of copepodids along the west coast of Ireland by examining infection rates of sea trout smolts. They divided the west coast of Ireland into five regions. A region was defined as being greater than 100 km in length. Each region was then subdivided into bays that were less than 50 km in size and then further subdivided into estuaries that were less than 10 km in size. Sea trout smolts were sampled for recent salmon lice infections over a period of five years. Statistical analysis of the data revealed that there was a significant variation in salmon lice infections between regions but that variation amongst the bays within each region was consistent with the regional variance over time. The regional variation was also not constant over time. This demonstrates some large-scale process having an effect over infection success. They suggest some type of environmental factor is responsible such as water temperature. This is very reasonable as several authors have demonstrated the effects of temperature on the development of the planktonic stages (Johannessen 1978; Johnson and Albright 1991b; Ritchie et al. 1993). Boxaspen (1997) showed that winter and summer temperature regimes had different effects on the infestation of salmon at various distances from salmon net pens. In the summer there was a negative association between temperature and distance, while in the winter there was a positive association. Furthermore, Boxaspen (1997) and Hogans (1995) both observed that warmer winter water temperatures lead to an increase in the spring infection levels. Within any particular region, the bays displayed a significant amount of variation

between each other. This is consistent with little to no dispersion of larvae between bay systems. Furthermore, the variations between estuaries were also significant indicating migration of planktonic stages between estuaries to be slight. These findings of little dispersal over a large scale (more than 10 km), agree with the findings of Costelloe et al. (1999).

Within a given estuary, Tully et al. (1999) found that the presence of salmon farms caused the infection intensity of sea trout smolts to increase three fold. The dramatic increase in estuaries with salmon farms would agree with earlier work done by Tully and Whelan (1993). They showed that while lice infecting wild salmon had almost twice as many eggs per string as compared to lice on farmed salmon, an estimated 95% of the copepodids found in the bay originated from farmed salmon. This is due to the higher numbers of farmed salmon in the bay versus wild salmon. In estuaries that did not have salmon farms but were adjacent to estuaries with numerous salmon farms, there was a slightly higher infection rate in the non-salmon farm estuary as compared with other non-salmon farm estuaries (Tully et al. 1999). This would suggest some limited transport between estuaries. This transport would be dependant on local hydrography and environmental conditions. When potential hosts are present in an area, a greater availability of planktonic stages leads to increased chances of successful infection.

2.3.3. Reproductive Output

Given the observed limited transport of planktonic larvae, the reproductive output in a specified area can play a significant role in how quickly severe epizootics occur either on new smolts or after treatment. Several factors can play a role in the

reproductive output of an individual female. The female louse will mate a single time, normally when molting between the second pre-adult stage and the adult. In the laboratory, lice have been observed to produce up to eleven pairs of egg strings before dying (Heuch et al. 2000). They also observed that it took between 5 and 10 days, depending on water temperature, for the same female to produce the next pair of egg strings. This is in contrast to Pike and Wadsworth (1999) who reported a period of 24 hours between egg strings. The number of viable eggs per string must be considered along with the number of egg strings produced by a female. Several authors reported an increased number of eggs per egg string with an increase in the body size of the female (Jackson and MacKinon 1992; Tully and Whalen1993). Heuch et al. (2000) reported no correlation between the size of the female and the length or number of eggs per egg string. They also found that the percent of viable eggs was higher with warmer water.

Temperature is one environmental factor reported to affect reproductive output. Both Ritchie et al. (1993) and Heuch et al. (2000) reported longer egg strings at lower temperatures (<9 C). However Ritchie et al (1993) reported that this increase in egg string length is accompanied with an increase of roughly 50-100 eggs per egg string, while Heuch et al (2000) report an increase of only one egg per egg string. Ritchie et al. (1993) also reported that the variation in mean egg length is directly related to the variation in water temperature.

Photoperiod has also been shown to have some effect on hatching. Boxaspen and Næss (2000) demonstrated that hatching egg strings in total darkness resulted in a 10-15% increase in the time to hatch but had no effect on the hatchability. Ritchie et al. (1993) found that egg string length, egg size and number of eggs per string all varied to

some degree with photoperiod. They conclude that the changes they observed were more closely linked to temperature rather than photoperiod but as the two variables are related to each other, it was difficult to determine the effect of one versus the other.

Finally, the number of fish in a given area and the intensity of the lice infection will be a large factor in the reproductive output of an area and hence the number of copepodids available for infection. Heuch and Mo (2001) evaluate a simple model for the reproductive output of salmon lice (measured in eggs produced) in most of Norway from 1971 until 2005. Their model shows that while wild fish and escapees may produce more lice per fish that the sheer volume of fish in farms produces a far greater quantity of eggs. In fact, during the 1990's there were years with well over a hundred billion eggs produced in farms from May 1st until Jan 1st. The model also clearly shows that as the aquaculture industry has continued to expand in Norway, the number of eggs produced on farms will increase again after temporary reductions in the total output due to government limits on the maximum allowable number of gravid females per fish.

Chapter 3

OBJECTIVES

This project had three major objectives.

1) To develop a culture system for salmon lice at the University of Maine.

2) To evaluate the suitability of artificial versus natural seawater for salmon lice studies conducted in recirculation systems.

3) To evaluate the effectiveness of three biosecurity procedures against the transfer of viable salmon lice eggs on farm equipment surfaces.

Chapter 4

MATERIALS AND METHODS

4.1. Salmon Lice Collection

4.1.1. From Farmed Fish

Salmon lice were collected between June of 2000 and February of 2002 from caged salmon in the Cobscook Bay area. All salmon lice were collected from fish that had not been exposed to any type of anesthesia for the purpose of collecting or slaughtering. Gravid females were removed from the host with the use of forceps and placed in sample jars filled with natural seawater. The sample jars were placed in a cooler with crushed ice for the two and a half hour trip back to the University of Maine. At the university lice were separated out and only those females that had a pair of attached egg strings were used.

4.1.2. From Cultured Fish

Salmon lice were raised on fish in the laboratory for use in experimentation. The host salmon were held in artificial seawater at 32-33 ppt and 11-13 C. The salmon were infected via three methods. In the first method, adult and pre-adult lice collected from farmed fish were placed in a tub with salmon and allowed to attach naturally. The lice used in this infection method came from fish that had not been subjected to salmon lice treatments. In the second method, copepodids that had been raised in the larval culture system were placed in an aerated 30 L tub with 5-7 salmon and were allowed to attach naturally for roughly 2 hrs. The time allowed for attachment was dependent on the rise in

water temperature and the level of dissolved oxygen in the tank. The third method of infection involved following the procedures given by Sevatdal (2001) for the alternative infection method.

Lice from experimentally infected fish were allowed to develop into gravid females. Egg strings were collected by anesthetizing the fish using MS-222 and then removing any gravid females. The egg strings were carefully removed from the lice by twisting the base of the egg string up and away from the point of attachment. The lice were then placed back on the host fish and allowed to re-attach. The pairs of egg strings collected were kept separate in 6 well tissue culture plates.

4.2. Culture System

Salmon lice egg strings were hatched and raised in individual culture chambers so that nauplii and copepodids from each egg string were kept separate from those of different eggs strings. The individual culture chamber (Fig. 4.1) was constructed from a 15 mL centrifuge tube which had the sides of the tube cut away leaving only two strips approximately 3-5 mm wide. The two strips that remained ran down the length of the tube and were opposite each other. The cone of the centrifuge tube was filled with sand to provide a counterweight to the tubes buoyancy. The sand was then covered with a layer of silicon to separate it from the developing salmon lice and to hold it in place. The sides of the chamber were then covered with either 105-micrometer mesh in the temperature/salinity trials or with 67-micrometer mesh in all disinfectant trials. The mesh was attached to the top, bottom and two strips left on the side of the tube using hot glue. A second layer of hot glue was applied over all edges to prevent the escape of any

larvae. The mesh size was reduced for the two experiments to prevent the escape of single eggs, which are around 70-75 micrometers in size.

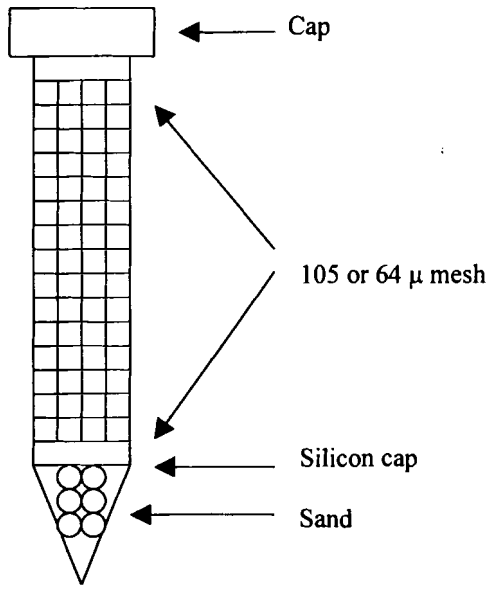

Figure 4.1 –Culture Chamber- Construction of individual culture chamber out of a 15 mL centrifuge tube. The cone of the tube is filled with sand caped with a silicon plug. The sides are cut away except for two strips to allow for the attachment of mesh around the entire circumference of the tube.

Egg strings that had been placed into a culture chamber were then raised in a ten-gallon aquarium (Fig 4.2). Each culture chamber was loosely held in position in the aquarium by being placed in a floating rack made from styrofoam and a pelxiglass plate. Each plate had one-inch holes drilled in a staggered pattern of rows of four holes. This configuration allowed for no tube to be blocking the water flow of another tube. The aquarium had a Whisper II filter system on one of the ends with an activated charcoal bio-bag. Directly below the filter system and against the bottom of the tank a power head was attached to the side. This served to increase flow throughout the tank. An air stone was placed at the opposite the end of the tank from the filtration system. The aquariums were kept in a walk in cooler within 1 C of the experimental temperature. Air

temperature in the cooler was constantly monitored and the daily minimum and maximum were recorded. The water temperature was also recorded daily in each aquarium. Within the cooler the lights were maintained in a 24-hour light pattern to provide consistency. The water within each aquarium consisted of either artificial seawater made from Forty Fathoms Crystal Sea or natural seawater, which was filtered through a one micrometer filter paper (VWR catalog # 410).

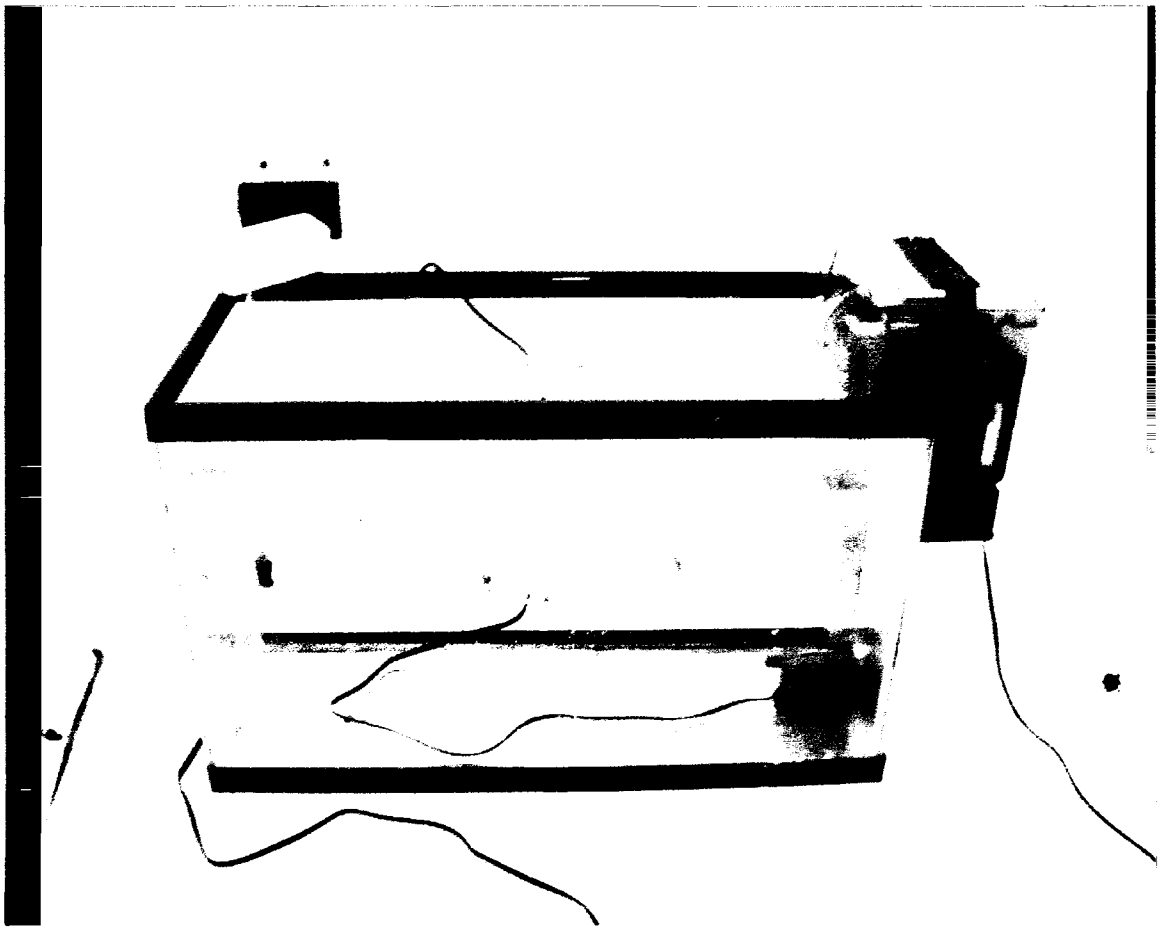

Figure 4.2 –Culture Tank- Photograph of a ten-gallon culture aquarium set up. Notice the powerhead pump to provide circulation, air stone and floating rack to hold multiple culture tubes.

4.3. System Optimization Experiments

4.3.1. Estimation of Eggs per Egg String

Several methods were investigated for estimating the number of eggs per egg string. The first method consisted of counting the number of eggs in a millimeter from over 30 egg strings and keeping a running mean. The overall length of an egg string then multiplied this mean.

The second method involved counting the number of eggs in three separate millimeter segments of each eggs string. The estimated total number of eggs per egg string was calculated by multiplying the average of the egg counts by the total length of that particular egg string (EQ 1).

EQ 1: Estimated number of eggs = (Total length of the egg string) X (Average number of eggs per mm)

The second method was the only method used to estimate the number of eggs per egg string for all replicates in the system optimization studies

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4.3.2. Experimental Design

The initial experiments varied the temperature, salinity and type of water (natural or artificial) to establish the optimal experimental conditions. In the experiments the temperature varied less than ±1 C while the salinity varied less than ±1 ppt. Experiments were conducted at the following temperature and salinity combinations: 10 C & 29 ppt, 10 C & 35 ppt, 12 C & 29 ppt, 12 C & 32 ppt, 14 C & 29 ppt, and 14 C & 35 ppt. Six replicate pairs of egg strings were used at each temperature and salinity combination except 14 C & 29 ppt, which only had 3 replicates.

Experiments were conducted by removing both egg strings from a gravid female collected off of a farmed salmon and estimating the number of eggs in each egg string (EQ. 1). Each egg string was then placed in an individual culture tube and one egg string was raised in artificial seawater while the other was raised under similar conditions in natural seawater. All of the egg strings were checked twice daily until hatching. Once hatched, egg strings were allowed to develop for 5,6, or 7 days at 10, 12, and 14 C respectively. The culture tubes were removed from the aquarium at the end of the development period, the water was drained from the tubes and they were then placed into 70% ethyl alcohol. The contents of each tube was identified to either nauplii (Fig. 2.2) or copepodid (Fig. 2.3) and the total number of each stage present was recorded.

4.3.3. Statistical Analysis

The various variables were evaluated on their ability to allow eggs to hatch and develop to the copepodid stage. From the data collected the percent of lice that developed to the copepodid stage was calculated by dividing the number of copepodids counted by the estimated number of eggs for that egg string. The hatchability was calculated by dividing the number of nauplii and copepodids counted by the estimated number of eggs. A water index was then calculated for each pair of egg strings by subtracting the total percent hatch or the percent of copepodids per egg string in artificial seawater from the same percentage in natural seawater. This index was then used for all further statistical analysis.

A weighted least squares ANOVA analysis was conducted using SYSTAT version 9 software on the treatment (temperature, salinity) and the water index of the

percent survival to the copepodid stage and the total percent hatch. An alpha of .05 was used to determine significance. The weighting factor used was the inverse of the variance. A normal probability plot and correlation was used to determine if the data were normally distributed. Post hoc comparisons were done using Tukey's test to examine potential interactions of temperature and salinity (EQ1).

4.4. Disinfectant Experiments

The effectiveness of two chemical disinfectants and desiccation was evaluated under laboratory conditions. Egg strings were collected from both cultured salmon lice and from farmed fish.

4.4.1. Estimation of Eggs per Egg String

For all replicates in the disinfection study an empirically derived regression (EQ 2)(R^2=0.929) was used to estimate the number of eggs per egg string. The regression, used the total length of the egg string to determine the estimated number of eggs.

EQ 2: Estimated number of eggs=$(7.541+0.534*\text{Length})^2$

The number of eggs in 74 egg strings was estimated by the same method used for the system optimization study. Egg strings in this group ranged from 6 to 25 mm in length. This data was then used to build a linear regression between the length of an egg string and the estimated number of eggs.

Statistical analysis of the regression was conducted using SYSTAT version 9 software. The data were tested for a linear relationship with a two tailed test at 95% confidence interval. Data were examined for outliers in total length (X) using a

histogram. After establishing the regression, the Y variable was analyzed for constant variance, appropriate fit, and outliers by plotting the studentized residuals. A normal probability plot and correlation were used to determine if the data were normally distributed. Various transformations were also examined and a square root transformation of Y gave the best fit, according to R^2, and best satisfied the assumptions.

4.4.2. Experimental Design

4.4.2.1. Chemical Disinfectants. Sodium hypoclorite 5.5% and iodine povidone (Betadine™), two chemical disinfectants used routinely in normal biosecurity procedures in the Maine aquaculture industry, were selected for evaluation. Three treatments were conducted with each disinfectant for a total of six chemical treatments. The three treatments consisted of a 1-minute exposure to a 200 ppm, a 1-minute exposure to a 500 ppm, or a 10-minute exposure to a 500 ppm disinfectant solution. The disinfection solutions were made by diluting either household bleach or Betadine™ with natural seawater to achieve the correct concentration of chlorine or iodine in a total volume of 50 mL. The disinfection solutions were made fresh for each trial.

After calculating EQ 2 the egg string was exposed for the appropriate time and disinfectant concentration. At the end of the exposure period, the egg string was dipped three times into three saltwater baths to remove any chemical residue. The control egg strings were placed into a saltwater bath for the same amount of time before being dipped three times into each of the three saltwater rinses. Treated and control egg strings were placed into culture chambers and incubated for 6 days after hatching. At the end of the development period the culture chambers were placed in 70 % ethyl alcohol (ETOH) and

the lice were counted and identified to stage. A total of 10 replicates were run for each disinfectant's 1-minute exposure at 200 ppm and 9 replicates at 500 ppm. For the ten-minute exposures at 500 ppm, 7 replicates were run for bleach, while 6 were run for iodine.

4.4.2.2. Desiccation Experiments. Desiccation treatments were carried out for 4 hours and 24 hours respectively. Egg strings were collected and the estimated number of eggs (EQ 2) was calculated. After the estimated number of eggs was calculated, both egg strings were damp dried. The control egg string was placed into a culture chamber. The culture chamber was then placed into an aquarium. The treatment egg string was placed on a plastic petri dish and allowed to sit out exposed to the air for the appropriate length of time at ambient temperature between 15-19 C and relative humidity between 57 and 61% over the 24-hour period. At the end of the desiccation period, the egg string was removed from the petri dish and placed into a culture chamber. The eggs were allowed to develop for 6 days after hatching and then were placed in 70 % ETOH and counted. Egg strings that had not hatched 6 days after the last egg string had hatched were terminated and examined for any unseen nauplii or copepodids. A total of 9 replicates were completed for the 4-hour period and 5 replicates for the 24-hour period.

4.4.3. Statistical Analysis

Statistical analysis was done by paired T-tests for each treatment. Using the estimated number of eggs per egg string, the percent of copepodids was calculated. The percent of copepodids in the treatment egg string of each pair was subtracted from the same percent copepodids in the control egg string. This difference was then used in the

T-test with null hypothesis that the difference was equal to zero. The mean of these differences is reported ± the standard deviation. The analysis was repeated using the total percent of eggs hatched. The total number of eggs hatched was derived by adding the number of nauplii and the number of copepodids present in the sample.

4.5. Electron Microscopy

4.5.1. Egg String Fixation and Embedding Method 1

Egg strings collected from farmed fish were fixed for transmission electron microscopy following standard fixture procedures used for other marine invertebrates. Half of the egg strings were fixed at this time and half were placed in fixative and set in a refrigerator for use in the second fixation. Egg strings were fixed in a 3% glutaraldehyde in 0.1 M phosphate buffer solution diluted from a 0.2 M stock solution using microwave enhancement. The stock 0.2M phosphate buffer contained 0.22 g of $NaH_2PO_4 \cdot H20$, 1.19 g anhydrous Na_2HPO_4, 10 g of sucrose and 8 drops of 1% $CaCl_2$ dissolved in 50 mL of distilled water. Acid was added as needed to adjust the pH to 7.4. The fixation process consisted of placing the egg string in fixative and microwaving on high power for 7 seconds, waiting 20 seconds and then microwaving on high for a further 7 seconds. All microwave enhancement was done with room temperature water as ballast in the microwave and the specimen on ice to prevent the specimen from heating. The fixative was removed and the process was repeated with fresh fixative. The fixative was then rinsed away in a 1:1 dilution of the stock buffer solution a total three times using the microwave procedures. After rinsing the egg strings were fixed in a 1% osmium tetraoxide (OsO_4) solution using microwave enhancement and rinsed in distilled water.

Tissue samples were dehydrated through a graded series of acetone using microwave enhancement. Tissues were embedded in Epon-Araldite by allowing samples to sit overnight in a loosely cap vial with a 1:1 mixture of resin/acetone. Samples were transferred to pure resin and placed under a vacuum before being placed in molds and cured for 48 hours at 60 C. Sectioning was attempted on a Sorvall MT2-B ultramicrotome using a glass knife.

4.5.2. Egg String Fixation and Embedding Method 2

A second attempt at fixing the egg strings that had been left in fixative in the refrigerator was made following the failure of the first method to completely penetrate the egg strings. After sitting in fixative for approximately one month the stored egg strings were removed, cut into 1-2 mm lengths and placed into fresh fixative for 10 minutes. After fixing a total of three rinses in diluted phosphate buffer were done, using microwave enhancement. Specimens were fixed in a 1% OsO_4 by completing one cycle in the microwave and leaving in OsO_4 overnight. OsO_4 was rinsed away in three washes using distilled water and allowed to sit for 10 minutes per rinse. Specimens were then left to sit in distilled water for five days. Samples were dehydrated using a graded acetone series where one cycle in the microwave was completed and samples then sat for 30 minutes before the next acetone step. Samples were embedded in Spurr's resign and cured at 60 C.

4.5.3. Sectioning and Staining

Sectioning was done on a Sorvall MT2-B ultramicrotome using a glass knife. Thick sections (.5-1μ) were placed on a glass slide and stained with Toluene Blue. Thick sections were photographed under light microscopy. Thin sections (60 nm) were placed on 200 mesh copper grids and stained with uranyl acetate and lead citrate before observation on a Phillips EM-201 at 60 KV.

Chapter 5

RESULTS

5.1. System Optimization Experiments

The weighted least squares ANOVA showed a highly significant difference between the treatment means ($p<0.000$, $F=73.332$, $df=15$). The correlation test for normality on all treatments showed that the data was slightly non-normally distributed, could not be corrected by transformation. On examining the scatter plot of the studentized residuals (Fig. 5.1) the treatment at 10 C and 29 ppt salinity was where the vast majority of the departure from normality occurred. The correlation was repeated excluding this treatment and the data were found normally distributed. The data were then considered normally distributed for all treatments.

Analysis of the means (Table 5.1) revealed that treatments at 29 ppt for both 10 and 14 C showed no difference in the survival to the copepodid stage in either natural or artificial seawater. However, the treatment of 10 C and 35 ppt showed higher rates of survival to the copepodid stage in artificial seawater, while the remaining three treatments all showed higher rates of survival in natural seawater. Post hoc pair-wise comparisons indicated a number of significant pair-wise comparisons (Fig. 5.2). These significant pair wise comparisons revealed that the treatments at 10 C and 35 ppt and 14 C and 29 ppt produced significantly more copepodids in artificial water than either treatment at 12 C or the treatment at 14 C and 35 ppt while the treatment at 14 C and 35 ppt produced significantly more copepodids in natural sea water than either treatment at 12 C.

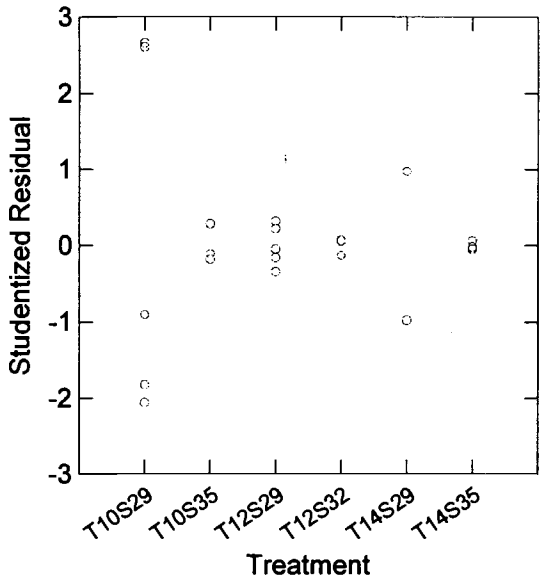

Figure 5.1 –Residual Plot- Scatter plot of studentized residuals used to look for constanct variance, for all treatments by treatment. Treatments are coded so that T-- is the temperature of the treatment and S-- is the salinity of the treatment.

Table 5.1. –Optimization Treatment Means- The least square (LS) means ± standard error (SE) for all treatments and the number of replicates for each treatment. Treatments are coded so that T-- is the temperature of the treatment and S-- is the salinity of the treatment.

Treatment	LS Mean	SE	N
T10S29	7.590	14.858	5
T10S35	-10.127	1.586	3
T12S29	27.735	5.555	5
T12S32	28.296	2.454	3
T14S29	-2.454	4.275	2
T14S35	51.897	2.494	3

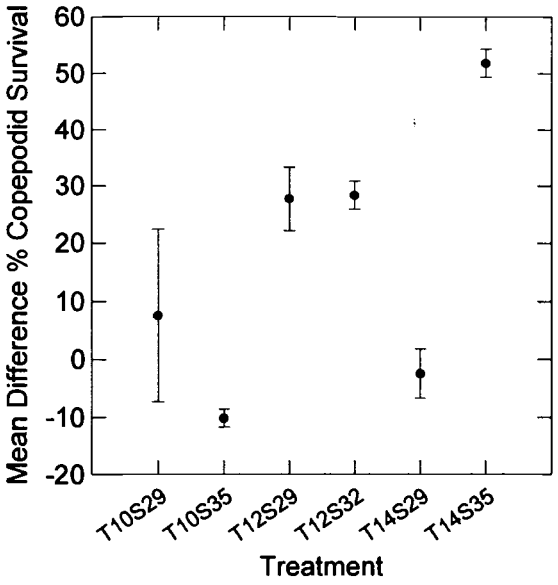

Figure 5.2 –Optimization Mean Difference in Survival- Plot of the least squares means ± the standard error for the difference between Natural and Artificial sea water. Means above zero indicate better copepodid survival in the natural seawater treatment, while means below zero did better in artificial water. Treatments are coded so that T-- is the temperature of the treatment and S-- is the salinity of the treatment.

5.2. Disinfectant Experiments

5.2.1. Chemical Disinfectants

All chemical disinfectant experiments showed that there was no significant difference in the percent of copepodid survival (Fig. 5.3) (Table 5.2) or in the total percent hatch (Fig. 5.4) (Table 5.3) between control egg strings and treated egg strings. All experiments indicated a large degree of variability throughout the studies.

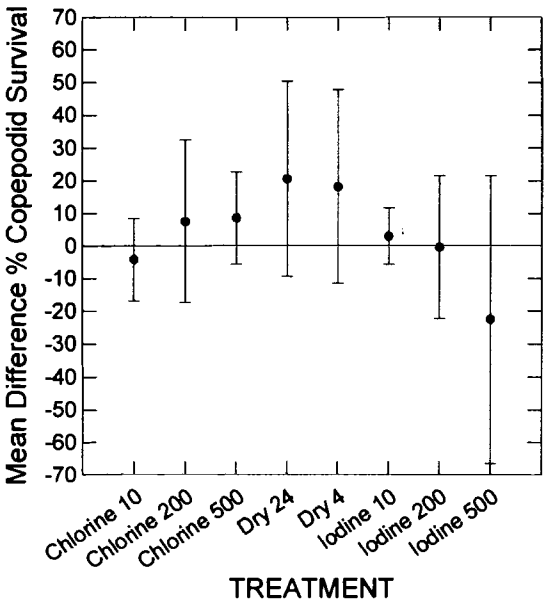

Figure 5.3 –Disinfectant Mean Difference in Copepodid Survival- The mean difference between percent survival to the copepodid stage for treatment egg strings and control egg for all disinfection treatments. Means above zero indicate better copepodid survival in the treatment, while means below zero indicate better copepodid survival in the control. Treatment labels are the treatment applied, chlorine, iodine, or desiccation (Dry) followed by the treatment dose, 200 = 200 ppm for one minute, 500 = 500 ppm for 1 minute, and 10 = 500 ppm for 10 minutes.

Table 5.2. –Least Squared Means for Percent of Copepodid Survival- The least square means ± standard error of the percent survival to the copepodid stage for all of the disinfection treatments. Means were calculated on the difference of the treatment egg string and the control egg string. Means above zero indicate better copepodid survival in the treatment, while means below zero indicate better copepodid survival in the control.

Treatment	Mean	Standard Error	n
1' Chlorine 200 ppm	7.59	25.00	10
1' Chlorine 500 ppm	8.61	14.19	9
10' Chlorine 500 ppm	4.14	12.70	7
1' Iodine 200 ppm	0.37	21.90	10
1' Iodine 500 ppm	22.41	43.97	9
10' Iodine 500 ppm	2.98	8.59	6
4 Hour Desiccation	18.15	29.64	10
24 Hour Desiccation	20.53	29.98	6

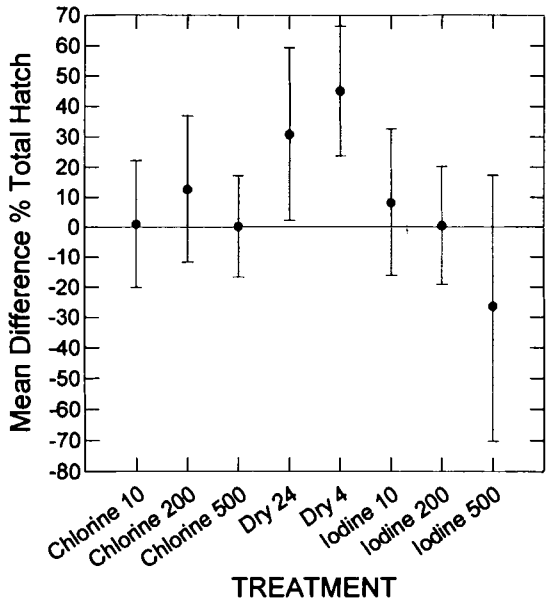

Figure 5.4 –Disinfectant Mean Difference in Hatch- The mean difference for percent of total hatch between treatment egg strings and control egg strings for all disinfection treatments. Means above zero indicate better copepodid survival in the treatment, while means below zero indicate better copepodid survival in the control. Treatment labels are the treatment applied, chlorine, iodine, or desiccation (Dry) followed by the treatment dose, 200 = 200 ppm for one minute, 500 = 500 ppm for 1 minute, and 10 = 500 ppm for 10 minutes.

Table 5.3. –Least Squared Means for Percent of Total Hatch- The least square means ± standard error of the total percent hatch for all disinfection treatments. Means were calculated on the difference of the treatment egg string and the control egg string. Means above zero indicate better copepodid survival in the treatment, while means below zero indicate better copepodid survival in the control.

Treatment	Mean	Standard Error	n
1' Chlorine 200 ppm	12.63	24.31	10
1' Chlorine 500 ppm	0.19	16.97	9
10' Chlorine 500 ppm	1.05	21.12	7
1' Iodine 200 ppm	0.48	19.64	10
1' Iodine 500 ppm	-26.45	43.73	9
10' Iodine 500 ppm	8.14	24.32	6
4 Hour Desiccation	45.04	21.44	10
24 Hour Desiccation	30.77	28.51	6

Initial experiments focused on a short exposure at high and low concentrations. The iodine treatment at 200 ppm clearly showed no difference in the percent of copepodid survival between the treated and untreated egg strings ($p=0.959$, $t=-0.53$, $n=10$) or in the total percent hatch ($p=0.939$, $t=0.079$, $n=10$). In the 200 ppm idoinde treatments the negative mean (mean = -0.370, SD=21.906) for the survival to the copepodid stage indicates a higher survival of copepodids in the treatment eggs strings. The positive mean (mean = 0.489, SD = 19.648) for the total number of eggs hatched indicates that a larger percentage of the eggs hatched in the control egg strings. In the 500 ppm iodine treatments both copepodid survival (mean=–22.417 ± 43.973) and total hatch (mean=–26.460 ± 43.735) were greater in the treatment egg strings vs the control egg strings. Neither mean for the 500 ppm iodine treatment was significantly different when compared to zero (percent copepodid survival: $p=0.165$, $t=-1.529$, $n=9$; total percent hatch: $p=0.107$, $t=-1.815$, $n=9$).

The chlorine treatments also showed no statistical difference between the survival of copepodids exposed to a treatment and those not exposed. The 200 ppm chlorine treatment mean of 7.591 ±25.009 for survival to the copepodids, shows slightly greater survival in the control egg string vs the treatment egg string. There was no statistical difference between the survival of either the treatment or the control ($p=0.362$, $t=0.960$, $n=10$). The mean for the total percent hatched (mean=12.630 ± 24.312) was not significant when compared to zero ($p=0.135$, $t=1.643$, $n=10$), but did show that more eggs tended to hatch in the control egg strings. The 500 ppm treatment did display a

trend to be detrimental to the survival of copepodids with a mean of 8.620 ±14.191, yet was still non significant when compared to zero (p=0.106, t=1.822, n=9). The overall percent hatch for the 500-ppm chlorine treatment was indistinguishable from zero (p=0.973, t=0.035, n=9) with a mean of 0.197 and a standard deviation of 16.974.

5.2.2. Desiccation Experiments

The desiccation experiments did show a statistical difference between the treated and untreated egg strings for the total percent hatch (Fig. 5.4). The 4-hour treatment produced a mean difference between the control string and the treatment string of 45.047 with a standard deviation of 21.446. This was a statistically significant difference (p=0.000, t=6.642, n=10). The 24 hour treatment had a statistically significant difference (p=0.046, t=2.644, n=6) with a mean of 30.780 ± 28.520.

The results for the percent survival to the copepodid stage varied in the experiment. In the process of repeating the experiment the second run produced control egg strings with normal hatching but all egg strings failed to develop to the copepodid stage (Fig. 5.3). This result had significant influence on the evaluation of survival to the copepodid stage. In both desiccation treatments this second trial prevented the finding of any significant difference for the percent survival to the copepodid stage. The 4 hour treatment had a mean of 18.158 ± 29.644 (p=0.084, t=1.937, n=10) while the 24 hour treatment had a mean of 20.536 ± 29.990 (p=0.154, t=1.677, n=6). If the second run is excluded from the calculation for percent survival to the copepodid stage (Fig. 5.5), the 4-hour desiccation period produced a statistically significant difference in survival between the control and the treatment (p=0.01, t=10.097, n=3). The mean of difference

in survival of copepodids from the control egg string and the treated egg string was 60.527 percent with a standard deviation of 10.383. This shows a clear difference in the survival rates of copepodids from the control egg strings over those from the treated egg string. The 24-hour desiccation treatment did not show a statistical difference between treatments ($p=0.151$, $t=2.269$, $n=3$) however there was a much larger variation associated with the mean (mean=41.071, SD=31.358). It should be noted that in all desiccation trials for the time periods, none of the egg strings produced a single nauplii.

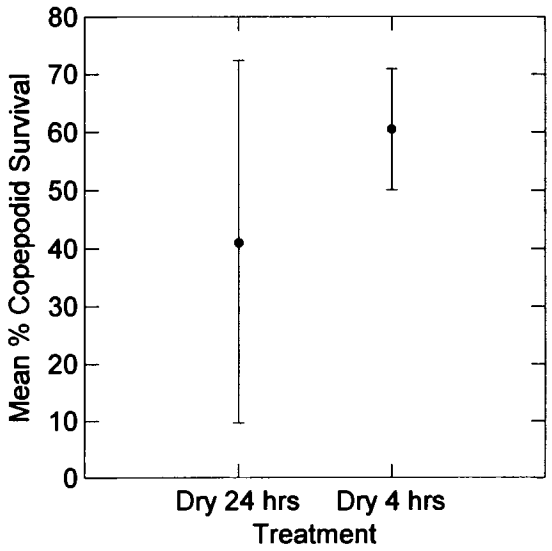

Figure 5.5 –Desiccation Mean Copepodid Survival- The mean percent survival to the copepodid stage ± SD of egg strings exposed to desiccation. This graph is constructed with only the data for the first trial where the control strings were seen to develop to the copepodid stage.

5.3. Ultrastructure of the Egg and Egg Sack

Examination of the membrane ultrastructure of both the egg and egg sack revealed a total of six layers between the outside environment and the egg cytoplasm (Fig

5.6). The egg sack was four layers thick, while the egg possessed a shell and a plasma membrane. The outer most layer was approximately 0.7 μm thick and is termed here the epilayer. The next layer encountered is termed the Bruligund layer and is the largest layer by far (Fig. 5.7). This layer is below the outer layer and makes up a large portion of the egg sack. It consists of fibrous layers that are oriented in different direction to create a spiraled Bruligund pattern. Below the Bruligund layer is the middle layer. It is constructed of fibers layered on the parallel to each other. The final layer of the egg sack is named the inner layer. This layer was comprised of fibers put down in such a manner as to be viewed length wise.

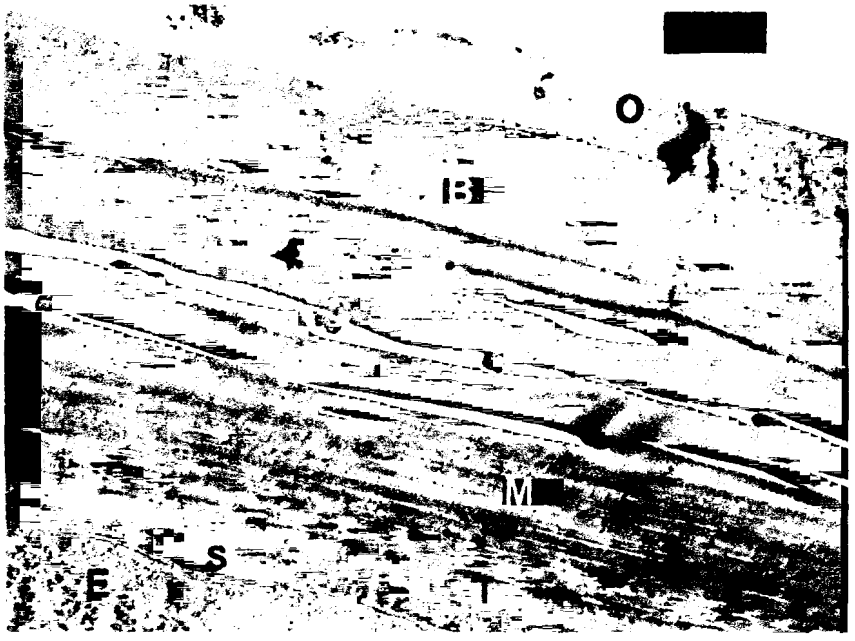

Figure 5.6 –Egg Sac Complex- Egg sac and shell complex (12,000 X). Scale bar equal to 1μm. O=the outer layer, B=Bruligund layer, M=middle layer, I=inner layer, S=egg shell, E=egg cytoplasm

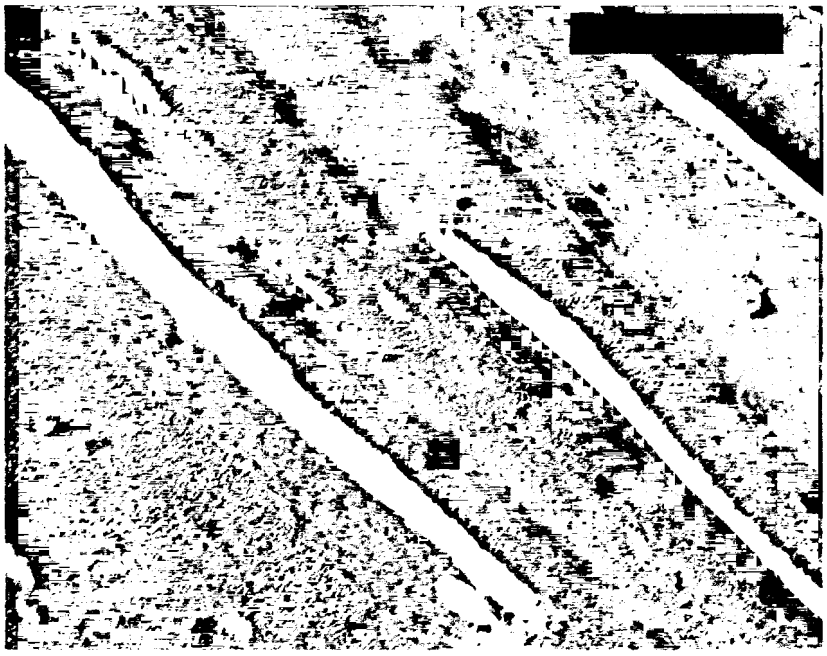

Figure 5.7 –Bruligund Layer- Bruligund layer (53,000 X) comprised of fiber layers in slightly different orientation giving it a spiraled effect. Scale bar equal to 0.5 μm.

The egg itself had two layers (Fig 5.8). The outer most was the egg shell. While the thickness of the shell was rather uniform it was possible to see areas where it had conceivably folded on itself and was two or three times as thick. Also the plasma membrane could be seen in the micrographs. This membrane was the final barrier to the egg cytoplasm.

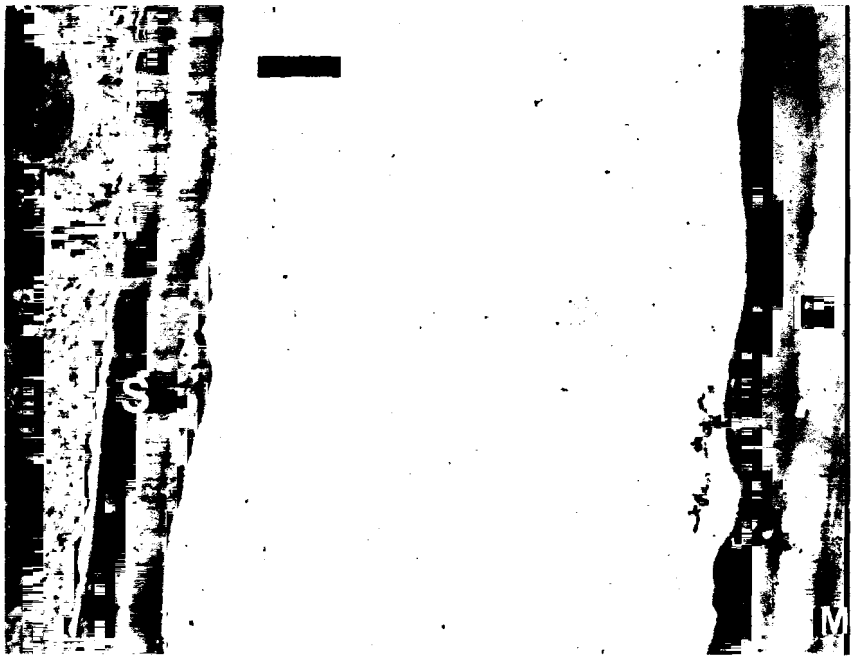

Figure 5.8 –Egg Shell- Two layers of the egg shell (18,000 X). The separation between the egg sac (I & M) and the egg shell is an artifact of the fixing process. Scale bar equal to 0.5 μm. I=inner layer, M=middle layer, S=egg shell, E=egg cytoplasm

Chapter 6

DISCUSSION

6.1. Culture System Design

The first objective of this study was to establish a working culture system for *Lepeophtheirus* salmonis at the University of Maine. The requirements for my experiments included both the ability to raise only a single egg string per culture chamber and the use of recirculation systems. At the beginning of the study all previously published literature described the raising of numerous egg strings and larvae in mesh covered glass beakers in flowing sea water systems (Johannessen 1978; Johnson and Albright 1991b). Systems of this type would not work at the University of Maine due to a lack of flowing seawater on the Orono campus and the lack of individualized culture chambers. An entirely new system that used ten gallon aquariums and individual culture chambers was designed. A year into the study Boxaspen and Næss (2000) reported a method for raising eggs to the copepodid stage in well plates. This new method was suitable for use at the University of Maine however I was unable to achieve any survival in the system even after consulting with Karin Boxaspen.

The use of a recirculation instead of a flow through system posed several questions. First, was there enough oxygen in the water to keep the eggs alive and could enough diffusion of water occur through the mesh of the culture chambers to bring the oxygen to the eggs? An airstone added air to the water while the filter pump and power head provided adequate flow. A dye test showed that the power head provided an increase in the diffusion of water in and out of the culture chambers as compared to diffusion rates with no flow present.

The second problem was the removal of any nitrogenous waste. In a flow through system this waste is flushed out with the used water and does not cause a problem. The amount of nitrogenous waste was assumed to be small because there was no addition of feed and only the metabolism of stored yolk would generate waste. An activated carbon filter system was added to remove any potential nitrogenous waste in the water.

Another question in developing the culture system included how to maintain and manipulate the water temperature. In order to provide consistent temperature regulation, all aquariums were kept in a walk-in cooler that remained within one degree Celsius of the set temperature.

6.2. System Optimization Experiments

The initial objectives were to determine if copepodid survival in artificial water was at least equal to natural seawater and to examine the effects, if any, of various temperature and salinity combinations within the reported optimum temperature and salinity range for salmon lice (Johannessen 1978; Johnson and Albright 1991b). Neither paper examined artificial water or the effects of small changes in salinity or temperature. The proposed plan was to examine all combinations of temperatures 10, 12 and 14 degrees Celsius with 29, 32, and 35 ppt salinity in both natural and artificial seawater. Due to time constraints data was only collected for six of the nine possible combinations.

The best survival was achieved in natural seawater at 12 C and 32 ppt. Later statistical analysis showed that the previous cursory examinations of the best temperature and salinity were statistically true as well. Post hoc pair wise comparisons showed that three out of the six experiments conducted in natural seawater did produce significantly higher percentages of copepodids while in only one case did artificial seawater produce

better results. Previous studies by Johnson and Albright (1991b) looked at survival rates in static natural seawater at 10,15,20,25 and 30 ppt and in flowing natural seawater at 30 ppt. Their results showed no hatching at 10 ppt and 100% hatch at 25 and 30 ppt (static and flowing respectively). Only in 30 ppt seawater did they get active copepodids though. The finer scale work in the present study suggests that natural seawater at 12 C and 32 ppt provides the greatest survivability to the copepodid stage. More replicates of all treatments should be conducted before a stronger suggestion can be made.

The survival rates achievable in my system were equivalent to other culture systems in the literature. The mean percent hatch for all of the egg strings that hatched during these trials was 42.84% with a maximum hatch rate of 71.47% and a minimum of 0.84%. After changing the mesh size from 105 micrometers to 64 micrometers the mean percent hatch increased to 47.04%. Johnson and Albright (1991b) and Boxaspen and Næss (2000) report 100% hatch, but they do not report how they determine this. Johnson and Albright (1991b) report 100% hatch, but only 65.9 % active nauplii and 35.2 percent active copepodids in static seawater at 30 ppt and 10 C. The best survival to the copepodid stage that was achieved in flowing natural seawater was 35.01% in 29 ppt at 12 degrees. Johnson and Albright (1991b) reported 26.8% active copepodids in 30 ppt flowing natural seawater at 10 C. In the present study a 25.19% survival to the copepodid stage in 29 ppt flowing natural seawater at 10 C was achieved.

In the process of calculating the percent of eggs that hatch from a given egg string, several methods of estimating the number of eggs were evaluated. The first was a running average of the number of eggs per mm. In the temperature salinity experiments there could be up to a 9% difference between the two methods used to estimate the

number of eggs per egg string. As the method that involved averaging three counts from each egg string intuitively made more sense that was the method chosen. Before the beginning of the disinfection studies the estimations used in the temperature salinity studies and extra data gathered were used to establish a regression between length of the egg string and the number of eggs in the egg string. There was a very high correlation here with an R^2 value of 0.92. The use of the regression instead of making three counts eliminated any possible negative effect that might have existed from egg strings drying out while being counted under the microscope.

6.3. Disinfection Experiments

The risk of spreading *Lepeophtheirus salmonis* from Cobscook Bay, where they are endemic, to other aquaculture regions is a concern. Since the first epidemic of salmon lice in Maine, the loss caused by lice and cost of treatments has caused a need for additional control measures. This need has become urgent with the outbreak of Infectious Salmon Anemia (ISA) in Cobscook Bay as salmon lice are a potential vector for the ISA virus (Nylund et al. 1993; Nylund et al. 1994). Despite the inability to control the movement of salmon lice attached to fish, farmers have become more aware of their own activities that might inadvertently cause the spread of salmon lice. The best method to prevent the spread of lice between farms is the removal of egg strings and lice from any equipment. The current experiments evaluated the ability of three common disinfecting methods to prevent *Lepeophtheirus salmonis* egg strings from hatching and larvae from surviving to the infective copepodid stage. No previous studies of the effects

of disinfection on the survival of salmon lice to the infective stage have been reported in the literature.

6.3.1. Chemical Disinfectants

Chlorine and iodine disinfectants were selected for investigation because they are used as disinfecting agents in current biosecurity procedures against bacterial and viral pathogens in the aquaculture industry. Exposure of egg strings to either chemical proved ineffective at preventing hatching or molting to the copepodid stage.

Closer scrutiny of the results showed some interesting trends. It was noticed that in the case of iodine used at 500 ppm for a one-minute exposure, the mean would indicate an actual improvement in the survival to the copepodid stage when treated. This observation was unexpected, however, this trend could be similar in mechanism to that in the use of iodine to disinfect fish eggs shortly after fertilization. The failure of the fixative, osmium tetraoxide, and resin to penetrate into the egg in the first attempt at fixation and mounting for electron microscopy demonstrates the resistance of the egg to impregnation of outside chemical agents. The successful examination of the ultrastructure of the egg and egg string revealed at least three layers in the egg sack before penetrating to the egg shell and plasma membrane. These membranes would pose a serious barrier to any chemical attempting to penetrate to the egg.

It is also of interest from a practical point of view that the 10-minute exposures at 500 ppm were also ineffective at reducing the percent of lice that survived to the copepodid stage in treated egg strings. The effectiveness of disinfectants can be affected by the length of exposure, the temperature, the concentration and other factors as well.

Ineffectiveness at increased exposure length and high concentration would also suggest a difficulty of penetrating to the egg tissue.

I observed consistently high variation in all experiments. Johnson and Albright (1991b) also report a large variation in their studies. This variance may be due to, age of the gravid female, age of the egg string since extrusion, the temperature at which the egg strings were extruded, possible exposure to salmon lice treatments, or the number egg strings laid *previously* by that particular female. Karin Boxaspen (personal communication) suspected that the temperature shock from collection, transport on ice to the University and placement into the culture system at 12 C could have played a large role in the variation seen. Attempts were made to culture lice in the lab at the same temperature and salinity used in the experiments to reduce any variation from these unknowns.

In some cases it is possible to estimate the age of an egg string based on its color. This is not true for all egg strings. Pike et al. (1993) found that when hatching eggs of *Caligus elongates* 85% of all the larvae produced in culture hatched within the first three days. Egg strings that were removed too early from the gravid female, i.e.: with more than three days of development left, suffered poor hatching success.

6.3.2. Desiccation Experiments

Desiccation proved to be effective at preventing lice from developing to the copepodid stage. Desiccation was originally chosen as a method for trial due to its impact on the environment and low cost. Desiccation can be a simple technique for some types of gear, like exactas, where egg strings are often observed sticking to the sides.

Other gear such as fish holds in boats can be very difficult to dry out. It is important to note that for desiccation to be effective the surface must be completely dry at the start of the drying time. If the equipment can remain completely dry for at least four hours then any egg strings present should not develop to the copepodid stage.

The desiccation studies completely prevented nauplii from hatching. The statistics do not indicate a difference between the treatment and controls for the survival to the copepodid stage. This was due to the tank effect seen in the second set of desiccation runs. In this set all of the control egg strings produced normal hatches, while all controls failed to develop beyond the nauplii stage. The most likely reason for this was some type of chemical contaminant in the water. From a statistical point of view this tank effect caused the percent survival to the copepodid stage calculation to be zero. In the case of the 4 hour treatment 7 of the 10 replicates contained this experimental error while 3 of 6 replicates at 24 hours were present in the contaminated tank. It is worth noting for the 4 hour treatment if the 7 zeros are excluded then there is a statistically significant difference from zero.

It can be concluded from these experiments that the chemical disinfectants used were not effective at preventing eggs from developing to the infective copepodid stage. When developing farm practices to prevent the spread of salmon lice, all egg strings and lice should be removed from all equipment moving between farms. If it is not possible to remove all of the egg strings then allowing the equipment and egg strings to keep dry for at least 4 hours after completely drying will help prevent egg strings from developing to the copepodid stage.

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Mike Pietrak was born in Albany, Oregon on March 6, 1975. He was rasied in southern Louisiana. Mike attended High School at Aprapahoe HS in Littleton, Colorado, Bakersfield HS in Bakersfield, CA, Lycee Chevier in Angers, France and graduated from the American School in Aberdeen, in Aberdeen, Scotland. He attended Ithaca College where he was a 4yr varsity swimmer and graduated in 1997 with a Bachelor's degree in Biology.

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