Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science

Screening of blood and mucus parameters towards breeding for resistance to salmon louse (*lepeophtheirus salmonis*) in atlantic salmon

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SCREENING OF BLOOD AND MUCUS PARAMETERS TOWARDS BREEDING FOR RESISTANCE TO SALMON LOUSE (*Lepeophtheirus salmonis*) IN ATLANTIC SALMON

A THESIS SUBMITTED TO THE
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ABSTRACT

Over the last three decades salmon farmers have experienced serious economic hardships due to losses caused by sea lice (*Lepeophtheirus salmonis*) infestations. In the absence of an effective vaccine, inefficiency of biological control methods and growing concerns over the usage of chemical treatments, there is a quest for alternative strategies to combat this problem. This includes selective breeding approach to increase the innate resistance in fish to the parasite. There is hope that easily measured biomarkers that are correlated to sea lice resistance, exist and can be used to ease recording, reduce cost and increase genetic gain for sea lice resistance. Indications of additive genetic difference in lice resistance in Atlantic salmon (*Salmo salar*) have led us to hypothesize that biomarkers of resistance are present in the plasma and/or mucus of fish. High throughput Fourier Transform Infrared (FT-IR) profiling of plasma and mucus together with screening of the selected blood parameters (with the help of i-STAT blood gas analyzer) were coupled with multivariate statistical analysis to investigate the differences between two groups (susceptible (S) and Resistant (R)) of Atlantic salmon that shown different in their susceptibility to sea lice. The assignment was based on the sea lice challenge test results of their full-sibs. 65 rainbow trout (*Oncorhychus mykiss*) were also included in this trial in order to study the species-specific differences for this trait. Fish was challenged twice between late November and early December 2008 and lice were counted for the first time in December 2008 (chalimus sessile stage), followed by second count in January 2009 (pre-adult motile stage) and finally in February 2009 (adult motile stage). A large variation in lice counts both between individual Atlantic salmon and individual rainbow trout was observed. With respect to lice count, S and R groups of Atlantic salmon were different in both sessile and motile stages of lice, indicating genetic background for this trait. Rainbow trout was found to be a more susceptible species than Atlantic salmon in this study, which contradicts most previous studies that suggested Atlantic salmon as slightly more susceptible. Blood parameters are useful measures of physiological disturbance. Most of the measured blood parameters by i-STAT did not show significant differences between the S and R groups of Atlantic salmon, indicating that they were expressed at constitutive levels and not variable due to the low levels of sea lice infection

at the time the sample were recorded. Hematocrit and haemoglobin were found to be very responsive parameters, however, not very specific to the individual lice count. However, the R group of salmon had significantly (P <0.05) higher hematocrit and haemoglobin values than the S group, indicating a genetic background for these parameters. They need to be further examined as the potential inclusion criteria for indirect selection towards increased resistance to sea lice. However, no clustering of S and R groups of Atlantic salmon or Atlantic salmon and rainbow trout with respect to lice count was obtained upon FT-IR spectral analysis of plasma and mucus by Principal Component Analysis (PCA) and Partial Least Squares Regression (PLSR) techniques to differentiate S and R groups of Atlantic salmon or Atlantic salmon and rainbow trout based on number of lice per fish. Moreover, High-throughput FT-IR approach on mucus and blood plasma complemented with classical blood markers at low levels of adult lice present on fish in the final last count did not explain the differences in the number of lice at previous counts. It could be suspected that the variation in FT-IR and i-STAT parameters observed during the last lice count fell within the constitutive (innate) levels of fish. Further studies are still required and metabolomics approach may be more useful at higher level of lice infection to detect the variation in metabolites. However, as the number of lice per grow-out salmon, and thus also breeding candidate, must be kept at very low levels, the metabolomics methods used in this study are not likely to be useful to obtain indirect measures of lice resistance in salmon selective breeding programs.

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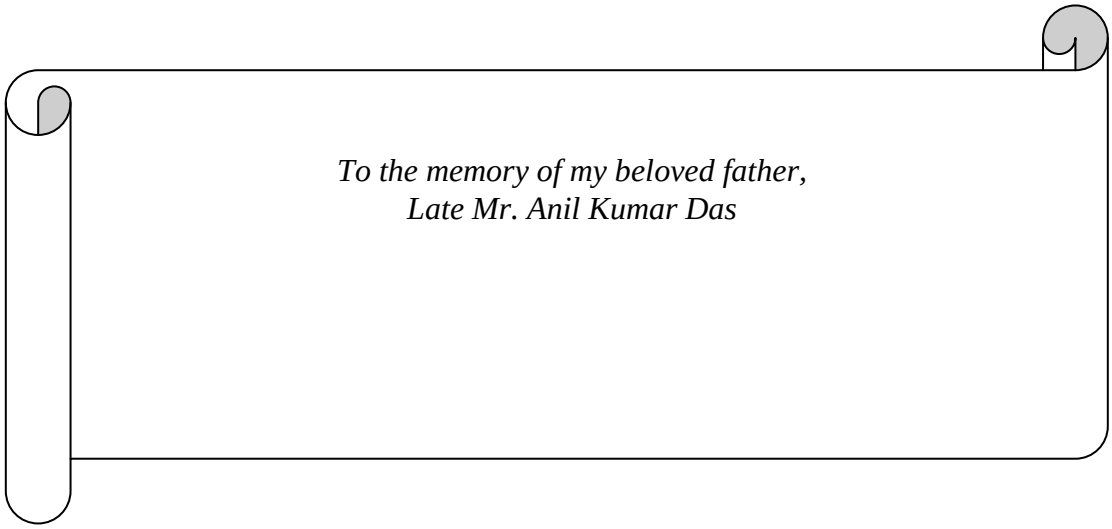
Amit Das

DEDICATION



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ABBREVIATIONS

ANOVA	Analysis of variance
BE	Base excess
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
FT-IR	Fourier transform infrared
Hb	Haemoglobin
Hct	Hematocrit
HSP	Heat shock protein
HTS-XT	High throughput screening eXTension
MLR	Multiple linear regression
MS-222	Tricaine methane sulphonate
NIRS	Near infrared reflectance spectroscopy
NMR	Nuclear magnetic resonance
PO_2	Partial pressure of O_2
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
PCO_2	Partial pressure of CO_2
PCR	Principal component regression
PGE2	Prostaglandin E2
pH	Potentia hydrogenii
PIT	Passive integrated transponder
PLSR	Partial least squares regression
R	Resistant
S	Susceptible
SD	Standard deviations
sO_2	Oxygen saturation
TCO_2	Total CO_2

CHAPTER I

INTRODUCTION

1.1 State of world fishery and salmonid farming

Worldwide, demand for fish continues to increase at a higher rate than wild fish populations can support on their own. When the capture fishery is either stagnated or declining, culture based fishery or aquaculture is growing more rapid than all other animal food-producing sectors, at an average rate of 8.8% per year, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems [FAO 2006]. There are several species of salmonids farmed, the most important being the Atlantic salmon (*Salmo salar*) and significant quantities of Coho salmon (*Oncorhychus kisutch*) and rainbow trout (*Oncorhychus mykiss)* and minor quantities of other species.

Atlantic salmon is one of the most successful species in fin fish aquaculture if measured in terms of value, which has witnessed a growth to over 1.6 million tonnes in 2006 compared to almost nothing in early 1970s. In contrast it can be noted that the wild Atlantic salmon fishery is commercially dead due to extensive habitat damage and overfishing. Thus wild salmon make up only 0.5% of the Atlantic salmon available in world fish markets, predominantly produced from aquaculture in Chile, Canada, Norway, Russia, the UK and Tasmania in Australia, with Norway and Chile together constituting about 77% of the total production.

1.2 What are sea lice and why they are important to study in aquaculture?

Sea lice are common name referring to several species of ectoparasitic caligid copepods, which infect wild and farmed fish in marine environment. They are naturally occurring parasites, which have been reported to co-exist with finfish like salmon, stickleback and herring and they predominantly feed on mucus of the fish, attaching to the skin, fins and to a less extent to the gills [Bron et al. 1991]. Younger and smaller salmonids are more likely to succumb to lice infection. The two most common species of sea lice are *Lepeophtheirus salmonis* (commonly infects salmon) and *Caligus clemensi* (infects a

broad range of finfish including salmon). *Lepeophtheirus salmonis*, which is a common salmon parasite, is more appropriately referred as the salmon louse. To date, no direct 'cause and effect' relationship between sea lice, salmon farms and wild salmon has been established, but in the open marine environment it is not unlikely that copepodids are transported freely between wild and farmed populations [Costello 2006]. Sea lice can lower the fitness of salmon and in many cases lethal if no treatment against the lice is used as the lice may create open lesions on the surface of the fish compromising the fish's ability to maintain its saltwater balance. Sea lice disfigure fish, making them unappetizing and difficult to market, when in abundance they injure and kill fish, reduce growth rates and require expensive therapeutic pharmaceutical and/or biological control treatment.

1.3 Current control methods and the concerns

The two most common ways to mitigate sea lice infection practiced by the salmon farmers currently include the use of bath treatment with synthetic pyrethroids (Cypermethrin and deltamethrin) and oral treatment in which chemicals are used in feed [Grave et al. 2004; Roth 2000; Westcott et al. 2004]. Organophosphates like trichlorfon (Neguvon), dichlorovos (Nuvan) and azamethipos (Salmosan) have been in use extensively for a long time, but in recent years use of bath administered neurotxins (organophosphates and synthetic pyrethroids) have witnessed a decline, where the use of oral preparation of emamectin benzoate (SLICE) has increased considerably. The reason for this is SLICE is effective on all life stages of *L. salmonis*, while synthetic pyrethroids are less effective on chalimus stages [Ramstad et al. 2002; Westcott et al. 2004]. However, a major concern in using chemicals for delousing is development of resistance. Most chemicals used against sea lice are insecticides and resistant populations for these chemicals have been found to occur [Ahmad et al. 2003; Burgess 2004; ffrench-Constant et al. 2004; Waldstein and Reissig 2000]. It is also expected that with time treatment efficiency will reduce from the use of synthetic pyrethroids [Sevatdal and Horsberg 2003]. Moreover, the use of these chemicals have been reasons of concern among farmers and consumers even though they are first being used after going through several approval tests.

1.4 Importance of the study

Over the decades marine salmon farmers have experienced serious economic hardships due to losses caused by sea lice infestations. With the present delousing regime in Norway, when mean number of adult female lice per fish in a cage is more than 0.5 per fish [Holst et al. 2003], or the total number of lice in a cage is more than 5, the farmers need to delouse the fish. The above mentioned concerns over using chemicals for delousing have led to the considerations of different kind of biological control like releasing sterilized male lice and finding a disease organism that would parasitize lice; although it has not yet been realized, the use of wrasse (Labridae) as cleaner fish has been reportedly used by salmon farmers in Scotland [Treasurer 1991], Norway [Costello and Bjordal 1990] and to a lesser extent Ireland [Costello and Donnelly, 1991]. The problem with the biological alternative wrasse that effectively eats the sea lice off fish in the first year of production is that, it is found not to be active during the winter and early spring [Costello 1991], thus farmers have reportedly been reluctant to use wrasse in the second year of salmon production. One potentially important alternative could be development of vaccine against salmon louse, but no studies so far provided tested information on any antigens derived from *L. salmonis* to produce protective antibodies in the salmonid host. Thus in the quest of alternative strategies to mitigate sea lice infection problem in salmon farming industry and fish welfare, increasing innate resistance in fish to parasites through selective breeding can be a future option. However, this is not an easy task, resistance to sea lice is one of the typical hard-to-measure traits which seem to have low heritability or at best low to medium heritability making successful breeding against resistance additionally complicated. Besides, using natural infections of fish as a selection criterion is problematic due to unpredictable timing and magnitude of such infection. Therefore, the measurement of lice number on fish in the controlled environment is still the preferred method for generating data for this trait. However, the use of a standardized protocol for the tank challenges is important for the studies performed under different laboratory conditions to be comparable. Efforts in order to develop a reliable challenge test for the susceptibility of Atlantic salmon to salmon louse aimed to find a test and criterion that could be recorded under controlled conditions and correlated to the sea cage situation have shown positive result. The genetic correlation between the numbers of lice

recorded in the controlled challenge test and during a natural infection was found to be very high (r_g=0.88) suggesting that challenge tests could be used in selective breeding to increase the lice resistance [Kolstad et al. 2005]. It is well known that the delousing measures in the farms makes selection based on field data inefficient. Therefore, controlled infestation tests where the fish is exposed to specific parasites must be carried out. This, as an additional hindrance, makes the selection within families more difficult. In this context it might also be noted that, to reduce costs such challenge test are carried out with a small number of fish and for a short duration of time. Thus, if the infested fish were deloused after the test was completed and reared further to time of selection, only a relatively small fraction of all the breeding candidates would have information on lice resistance, and the data are thus limited between family selection based on sib-testing. Moreover, to include a trait of economic importance, for example, disease resistance in a selective breeding program, the trait must be measured and recorded meaningfully [Fjalestad et al. 1993; Kolstad et al. 2005] which is usually very costly. One way to overcome the difficulties in working with sea lice resistance is to apply the strategy of including correlated traits with no economic value with the purpose to increase genetic gain in the trait of importance [Gjedrem 1967].

However, if we could find specific parameters in blood and mucus that are correlated to lice resistance, then we could use these parameters as traits for indirect selection for lice resistance, making selection within families possible. One inherent problem to this approach is that these parameters are often only induced when there is a challenge, e.g., a pathogen must be present for the organism to exhibit the resistance it may possess. This is to be expected since it would be costly for an organism to continuously produce protective substances that are only needed during a limited period of time. Until recently lice were not a big problem in the salmonid aquaculture industry; however, concurrent with the rise of lice infestations in farmed fish, there have been a number of cases of lice infestation of wild salmonid populations. Such scenario is compatible with the idea that in the evolution of Atlantic salmon there was no need to develop protective mechanisms against lice, as this pathogen did not present a big enough risk for this species. There is hope, however, that some compounds are constitutively expressed in tissues directly

exposed to ectoparasites' attachment such as skin and mucus. In theory blood is also expected to harbour substances of immunity that could show lice resistance. The advantage of using immune parameters as indirect trait is that measurement on each selection candidate allows within family selection of the most resistant fishes [Sahoo et al. 2008]. Supplementing challenge test data with those marker traits would, therefore, make it possible to increase selection intensity and improve selection accuracy [Sahoo et al. 2008]. However, it could be mentioned that so far there has not been found a single immune parameter expressed in non-challenged fish that would correlate well with the susceptibility or resistance towards sea lice, morphological skin parameters were not very helpful either and therefore, classical challenge tests are still very much needed.

The response to an indirect selection will largely depend on the magnitude of the genetic correlation between the measured parameters and the breeding objective trait lice resistance e.g. measured as the number of lice per fish in a controlled infestation (challenge test) or preferably in a field test. Genetic transmissions of innate resistance to several diseases in salmonids and substantial variations in resistance (furunculosis in Atlantic salmon, for example) have been reported [Marsden et al. 1996; Chevassus and Dorson 1990]. In experiments with Atlantic salmon bred for high and low stress responses as measured by blood cortisol levels, the line selected for high cortisol stress response has shown significantly increased mortality when challenged with a pathogen [Fevolden et al. 1992]. A strong genetic basis for innate physiological, and/or biochemical mechanisms conferring resistance to micro-parasites have been reported [Chevassus and Dorson 1990]. Studies also indicate that disease resistance in cold water fish species is correlated with non-specific immune parameters like serum, lysozyme, complement and haemolytic activity, phagocytic respiratory burst and bactericidal activities; all can affect the inherent capacity of fish to resist pathogen prior to showing specific immune response [Sahoo et al. 2008]. A study with *L. rohita* shows significant correlation of family means for survival to aeromoniasis with full-sib family means for four of seven investigated innate, humoral immune parameters in blood [Sahoo et al. 2008]. Several studies investigating immunological and physiological parameters such as lysozyme, haemolytic activity [Roed et al. 2002] and cortisol [Refstie 1986] have shown

variations and thus possible traits for indirect selection when using information of these parameters recorded on the breeding candidates and their full and half-sibs [Fjalestad et al. 1993]. Building on these knowledge, it is of particular interest to determine intrinsic resistance factor(s) that makes some salmonids more susceptible to *L. salmonis* infection than other.

1.5 Objective of the study

Salmonid species show different susceptibility to sea lice infection [Johnson and Albright 1992b; Nagasawa and Takami 1993] and there are indications of genetic variance in resistance to salmon louse in Atlantic salmon [Fjalestad and Gjedrem 1996; Kolstad et al. 2005; Glover et al. 2005]. This has encouraged us to hypothesize that under low infection pressure conditions of sea lice some of the constitutive and/or induced differences are expressed as biomarkers of resistance present in the plasma and/or mucus of fish. Therefore, the quintessential beginning of this study was to search for constitutive and/or induced biomarker(s) of resistance in plasma and mucus of fish that could be correlated to lice resistance and that in the absence of observed lice count on individual fish can be used as an indirect measure of lice resistance in a selective breeding program. A secondary objective of this study was to see if the investigated metabolomics methods were able to discriminate between two groups of Atlantic salmon that were different in their biological susceptibility to salmon louse. In addition, another objective was to see if the same methodology was useful to discriminate between Atlantic salmon and rainbow trout with respect to the same trait.

The idea was that the present trial should be performed under low infection levels, typical of natural sea-cage outbreaks of lice infection. Plasma and mucus could be easily obtained, thus biomarkers present in these tissues hold potential for the indirect selection for lice resistance in the breeding candidates of Atlantic salmon.

Spectroscopic methods can effectively be used for composition analysis of biomolecules and are now an important part of metabolomics approaches. Of all the available spectroscopic methods, Fourier transform infrared (FT-IR) spectroscopy has gained considerable interest within high throughput screening techniques for disease recognition

and biomarker discovery in body fluids [Parveen et al. 2008; Mariey et al. 2001; Naumann 2001; Petrich 2001]. It can be used to analyze very small samples (typically 0.5-20 µl) and gives more detailed chemical information on the sample's composition because it measures the fundamental vibration, while the other available useful spectroscopic methods, like near infrared reflectance spectroscopy (NIRS) measures overtones and combination bands [Dunn and Ellis 2005].

There are no published studies on plasma and mucus analysis by FT-IR spectroscopy in salmonid fishes found during the literature search. Recently a proteomic approach has been applied to better describe the changes in the mucus protein in Atlantic salmon upon infection challenge. However, metabolomics has so far not been used in selective breeding programs for fish in search for constitutively expressed biomarkers that might correlate to variations in sea lice resistance. Taking taken into consideration the advantages of various metabolomics techniques, it was decided that FT-IR technique would be a favourable tool. Therefore, this study also aims to investigate the utility of an FT-IR high throughput profiling coupled with multivariate statistical analysis to determine if it is possible to discriminate fishes showing different lice counts based on metabolic fingerprints of blood plasma and mucus collected from 264 fishes (214 Atlantic salmon and 50 rainbow trout).

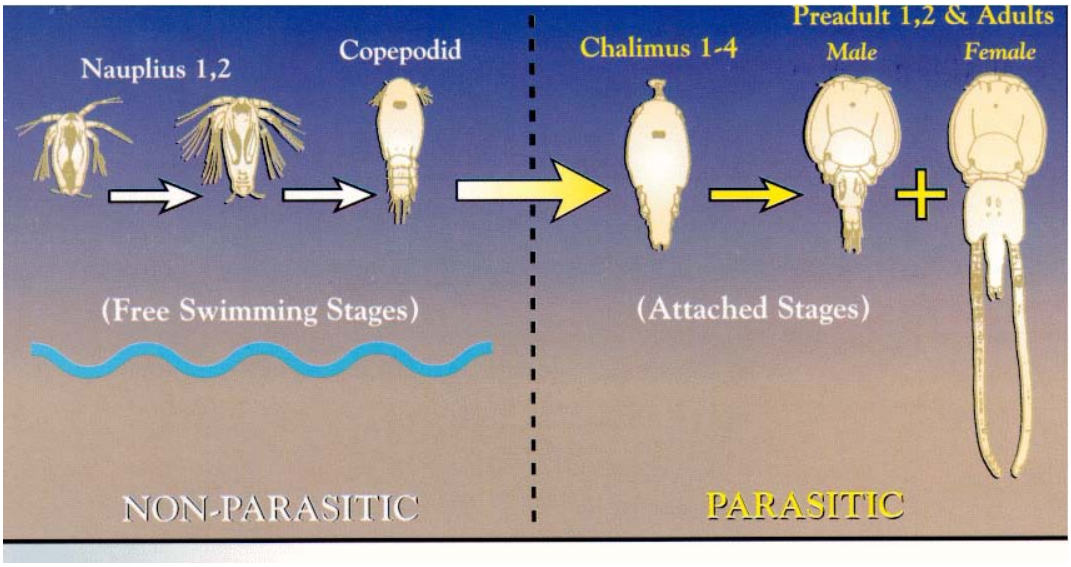
CHAPTER II

LITERATURE REVIEW

2.1 Sea lice biology

2.1.1 Life cycle

Ten different stages (excluding the egg stage) have been described in the life cycle of the salmon louse *L. salmonis*. Each stage is separated by a moult (two non-feeding planktonic naupliar stages, one free swimming infectious copepodid stage, four attached chalimus stages, two pre adult stages and one adult stage [Johannessen 1978; Johnson and Albright 1991]. The entire life cycle for *L. salmonis* is about 7-8 weeks at 10^{o}C.



Figur 1 *The life stages of Lepeophtheirus salmonis*
Image from University of Prince Edward Island.

For a better understanding of host-parasite relationship it is necessary to clearly distinguish several features of different stages of sea lice life cycle. After the sea louse has hatched out of its egg, the first stages are Nauplius I and Naiplius II, which are free swimming and has very noticeable appendages. The next immediate stage is the infectious Copepodid stage, which is also a free-swimming stage but the organism has less noticeable appendages. At this stage the organism locates and establishes itself on

hosts using the second antennae and maxillipeds for maintaining its position on host. It is not yet fully understood the mechanism by which this stage locates its host. When attached to a suitable host this stage starts feeding using a modified mouth structure referred to as ‘oral cone’, which is a characteristic feature of all caligid copepods. Copepodids are believed to be able to change their position on the host until they reach the next developmental stage. Depending on the water temperature, after a period the copepodid stage moults into the parasitic chalimus I-IV stages. Chalimus stages develop a structure called frontal filament, which helps the organism to attach to the fish. It is often seen as hanging off the edge of fins and gills. The frontal filament is replaced at each moult during the chalimus stages I-IV [Gonzalez-Alanis et al. 2001]. But there are earlier findings that state that the original frontal filament is not changed and the lice remain attached throughout the entire chalimus development [Jones et al. 1990]. It is assumed that the ability of sea lice to move during chalimus stages may be advantageous due to the soft nature of tissues to which they attach or possibly to avoid host immune responses [Johnson and Fast 2004]. Chalimus larvae moult into pre adult stages, similar in morphology to the adult stages, which move freely on the surface of fish except for a short time during the moult. This is a sensitive time in the lice developmental cycle when moulting parasites attach to the host by means of a temporary frontal filament in order to keep very close to the fish. Once they moult into the adult stage, the females are inseminated and continue to produce egg strings throughout their life. Adult sea lice can be seen easily with naked eye with the male being smaller, and the female with wider "hips”. Under laboratory condition the life span of female *L. salmonis* is observed to be up to 7 months [Piasecki and Mackinnon 1995].

2.1.2 Attachment and feeding mechanism

Sea louse has modified second antennae, maxillae and to a lesser extent the maxillipeds for attaching to hosts in all of the development stages. They use them to pierce the host tissue and secure firm attachment. Frontal filament plays an important role in the chalimus stages to maintain the position on host. In mobile pre adult and adult stages the post antennary processes, sternal furca and cephalothorax play vital role for the attachment [Jonsdottir et al. 1992; Kabata and Hewitt 1971]. The third leg of louse is modified to form a transverse barrier closing off the posterior margin of cephalothorax

[Kabata and Hewitt 1971]. The mouth tube that is responsible for damaging the host skins described from studies on *C. curtus*, a caligid parasite of gadids [Parker and Margolis 1964] seems to be identical in structure to the feeding apparatus in *L. salmonis.* All the development stages of sea lice found on host surface feed on mucus, skin and blood, which eventually cause the development of lesions and in case of major infections, development of disease. Secretion of enzymes onto the surface of host may also aid in feeding activity [Fast et al. 2003].

Although blood forms a diet component in some species of copepods, its importance as food item is not fully understood in sea lice. Knowledge on blood feeding in different developmental stages of sea lice is necessary for understanding the host-parasite relationship as different host factors is assumed to be present in blood as compared to in mucus or skin. Knowledge of sea lice blood feeding is also important with respect to development of vaccines. Blood feeding as first reported by Brandal et al. [Brandal et al. 1976] shows that 42% of adult females and 10 % of adult males of *L. salmonis* feeding on Atlantic salmon contained blood in their gut which was subsequently identified spectrophotometrically. It is not well understood if blood feeding is important with respect to sea lice fecundity as it is in the case of some arthropod ectoparasites that require blood meal for reproduction [Johnson and Fast 2004].

2.2 Host response to sea lice

2.2.1 Pathological effects of sea lice infection

The disease processes involved with *L. salmonis* infections are dynamic interactions between host and parasite in which parasite stage, and number of parasites present, host age, species, genetic strain, physiological condition and position in the population hierarchy all influence the nature and extent of the effects. Sea lice cause physical and enzymatic damage to their site of attachment and feeding to which the host may or may not respond immunologically. Physical injury caused to the salmonid hosts by *L. salmonis* can be severe and it is seen in both wild and farmed salmon populations. Lesions caused by copepodids and chalimus larval stages are relatively minor with localized damage [Boxshall 1977; Jonsdottir et al. 1992; Mackinnon 1993; Roubal 1994]. But when the chalimus larvae are abundant it is possible that they cause severe damage to

the host, even a complete loss of fins and death [Dawson 1998]. Despite their mobility, the pre-adult and adult stages spend most of their time grazing the epidermis of the host. Younger stages rarely breach the epidermis and therefore cause minor tissue damage only [Johnson et al. 1996; Roubal 1994]. However, in cases of severe damage due to *L. salmonis* infection, it has been found that lice cause extensive areas of skin erosion and haemorrhaging on the head and back and sub-epidermal haemorrhage in the perianal region [Johnson et al. 1996; Pike and Wadsworth 1999]. The development of these large open lesions and extensive areas of gill and fin tissue damage is partly due to secondary infections and the resulting necrosis, in addition to mechanical effects of feeding by the parasite. It has also been suggested that opportunistic infections for example *Vibrio*, could be associated with infection [Wotten et al. 1982].

2.2.2 Species specificities

Microscopic studies of attachment and feeding sites of sea lice demonstrated that tissue responses of naïve chinook, coho and Atlantic salmon to *L. salmonis* are important for its establishment and maintenance on hosts [Johnson and Albright 1992a]. The current theory states that due to their differences in magnitude of inflammatory and hyperplastic responses to presence of parasite, theses species differ in susceptibility to sea lice infection, with Atlantic salmon being the most susceptible one followed by chinook and coho salmon [Johnson and Albright 1992b].

The tissue response of naïve Atlantic salmon to *L. salmonis* was found to be very limited irrespective of stage of development and numbers of copepods present [Johnson and Fast 2004]. Only a mild inflammation in the dermis of some fishes was seen, and no significant fin tissue response was observed in the presence of copepodid or chalimus stages [Johnson and Fast 2004]. In addition, some areas of tissue erosion, minute haemorrhage, and mild inflammation could be observed. On this host the copepods are retained on the gills throughout the chalimus stages, however, the occurrence of lice on gills is probably an artefact of the challenge protocol [Johnson and Albright 1992b]. The epidermis was breached in most cases and underlying dermis and fin rays were exposed [Johnson and Fast 2004]. Inflammatory infiltrate, in cases where inflammatory response was observed, showed abundance of neutrophils and some numbers of lymphocytes

[Johnson and Albright 1992b]. Recently, the involvement of lymphocytes (T cells) was suggested in the response to sea lice in Atlantic salmon by a microarray study [Skugor et al. 2008]. On the other hand the naïve coho salmon was characterized by partial to complete erosion of epithelium, minor haemorrhaging and acute inflammation on the attachment and feeding sites on gills, also mild epidermal hyperplasia at the tips of lamellae was observed on some fishes. In coho salmon the copepods are lost within days from gill tissues [Fast et al. 2002a]. Mild inflammation was found to occur within 1 day post infection in dermis of fins. Inflammatory infiltrate consisted of neutrophils, macrophages and few lymphocytes on both gills and fins. Histopathological studies [Jones et al. 1990] of copepodid and early chalimus stages of *L. salmonis* on naturally infected Atlantic salmon revealed minor host response to the second antennae, maxillipeds or feeding activities of the copepodid stage and no tissue response or mild hyperplasia of the frontal filament in the chalimus stages. However, there was host response to both the filament and the lesions caused by feeding activities when the chalimus larvae was detached from frontal filament in that study. Large variation was observed in the severity of lesions caused by pre-adult and adult stages of *L. salmonis* in their histopathology, which was thought to be due to the mobile nature of the parasites in these stages [Jonsdottir et al. 1992]. However, more heavily damaged tissues under the cephalothorax, and loss of cell surface structure than other region of the copepod's body was observed in general. Besides different thickness of epidermis was reported relative to underlying layers and tissue swelling and in some cases splitting of the epidermal layer above the basal cells was also observed. An inflammatory response to *L. salmonis* was found to be more severe around the periphery of attachment and feeding site than in tissues under cephalothorax [Jonsdottir et al. 1992].

The lack of host tissue response to the attachment and feeding activities of parasites in salmonids except coho salmon is assumed to be due to suppression of immune system caused by stress associated with infection. However, stress is not the only factor limiting the tissue response to sea lice, as there are indications of host responses to frontal filaments and attachment and feeding sites after the copepods have become detached, as well as there are host responses in tissues immediately outside the sites of active

attachment and feeding. This indicates that sea lice can possibly modulate immune response of the host at the sites of attachment and feeding. Lower level of secretions produced by *L. salmonis* in the presence of coho salmon mucus may be the reason that the tissue response is not suppressed in this species [Fast et al. 2003]. There have been reports of imprints of the cephalothoraxes on the surface of the hosts made by pre-adult and adult stages of sea lice [Roubal 1994]. The physical limit of any host immunomodulatory activity may be indicated by the host tissue response around the periphery of cephalothorax. Pre-adult and adult stages have also been reported to cause subtle pathological changes at sites away from attachment and feeding sites viz. increased apoptosis and necrosis of epithelial cells and lower numbers of mucus cells in skin, and swelling of lamellae in gills, detachment of epithelium and apoptosis of chloride cells [Nolan et al. 1999]. These changes are assumed to be resulting from stress response of the host; however it is not unlikely that the enzymes secreted by sea lice on the host surface may cause those changes [Nolan et al. 1999].

2.2.3 Physiological effects of infection

The physiological effects can be different depending on the host species, age and general health, and also the developmental stage of the parasite and the number present, severity of lesions caused by them. Many studies with *L. salmonis* showed that sea lice in general can cause development of lesions (osmoregulatory problems, secondary infections), stress response (reduced growth, reduced swimming performance, increased susceptibility to other diseases) and host death. It is established from earlier studies that the teleost show primary, secondary and tertiary stress responses [Iwama et al. 1999; Bonga 1997]. Primary stress response is neuroenodocrine/endocrine response characterized by increased levels of stress hormones viz. cortisol and adrenaline and cellular response characterized by heat shock protein (HSP) production; secondary stress response is characterized by metabolic changes such as changes in glucose and lactate levels, hydromineral disturbance (imbalance in sodium and chloride contents) and hematological changes, and also possibly cellular response in HSPs production; tertiary stress response is characterized by changes in the whole animal, viz. reduced growth, swimming capacity, disease resistance, reproductive success and reduction in survival [Iwama et al. 1999]. Various challenge studies involving *L. salmonis* included the above

mentioned response criteria for different host species, but there is no standard method reported for carrying out those studies. However, it could be anticipated that stress level on host was influenced by intensity of infection, host size, health condition and possibly host species [Johnson and Fast 2004].

2.2.4 Immune responses to sea lice

Atlantic salmon has very limited cellular and humoral responses to *L. salmonis* infection [Pike and Wadsworth 1999]. Studies conducted so far, in the absence of a good infection model do not provide sufficient information on acquired immunity with respect to sea lice infection that could effectively lead to the production of a vaccine. Most of the studies using single pulses of infection with high infection level per individual used so far may not be appropriate models to study host parasite interaction resulting from a lower magnitude of infection and spread over a significant span of time. Only a few studies suggest the possible roles that the immune system of fish might play in relationship with parasitic copepods, but studies with *L. salmonis* show some evidences that immunity may play role in controlling aspects of reproduction in this species. One study [Grayson et al. 1991] on serum antibody response to *L. salmonis* infection compared immune responses of naturally infected rainbow trout and Atlantic salmon to the responses seen in rainbow trout and rabbits immunized with *L. salmonis* whole body homogenate. No specific antibody responses against the parasite was identified in naturally infected rainbow trout, the naturally infected Atlantic salmon was found to produce antibodies which recognized many antigens in unreduced samples, especially one of molecular weight more than 200kDa. Rainbow trout immunized with whole body homogenate were observed to produce antibodies recognizing larger variety of antigens than seen in naturally infected salmon probably because of 'hidden antigens' in the whole body extract [Johnson and Fast 2004]. No studies so far provided tested information on any antigens derived from *L. salmonis* to produce antibodies in the salmonid host that could be produced as effective vaccine against *L. salmonis*. The only published study on vaccination against *L. salmonis*, using relatively crude preparation of soluble sea lice antigens, did not show significant effect of immunization on sea lice abundance in the laboratory trial. However, the number of eggs produced by the parasite on immunized hosts was 26% less than those that were not immunized, with no difference in hatching rate; but it was not known if the

fish would be naturally exposed to the antigens used in this study [Johnson and Fast 2004].

2.2.5 The host effect on sea lice

Host effect on the biology of *L. salmonis* is not well studied. Observations from other parasite species indicated changes in their distribution on the host, interruption of egg sac production, loss of egg sacs, failure of egg development and lowered infectivity of copepodid stage [Woo and Shariff 1990]. Some studies suggests that *L. salmonis* show different growth rates on different hosts, which correspond well with the studies indicating different susceptibilities of host species, with Atlantic salmon being the most susceptible one [Johnson and Albright 1992b; Woo and Shariff 1990]. Different development rate of *L. salmonis* copepods was reported on different species of naïve hosts and on different regions of the host body [Johnson and Albright 1992b; Fast et al. 2002a] reported slower maturation rate of *L. salmonis* on coho salmon, followed by rainbow trout and then Atlantic salmon. There are also studies indicating variations in the egg number *of L. salmonis* carried by different hosts. A female of *L. salmonis* was found to carry significantly higher number of eggs on Atlantic salmon than those on mature Chinook salmon. Also variation in numbers of eggs was reported on females growing on mature and immature coho salmon (Johnson, 1993). However, it is not well established why there are such differences in growth rates and number of eggs produced by the female lice on different host species. Host nutritional and/or immunological factors in controlling these aspects of sea lice biology should be investigated; in addition it would be valuable to determine if past exposure of the host to sea lice would have any effect on those differences observed [Johnson and Fast 2004].

2.2.6 Host Immunomodulation

There is very limited host tissue response to attachment and feeding of *L. salmonis*, which has led to the view that *L. salmonis* like other arthropod parasites (e.g. ticks), secretes compounds that modulate host tissue responses for ensuring their survival on host. Immunosuppression may be another reason for the absence of host antibody response to sea lice. Many studies suggested that sea lice produce immunomodulatory substances (by gland-like structures associated with oral cone) which are likely to be

responsible for lack of host response in their attachment and feeding sites [Ross et al. 2000]. Mucus collected from infected hosts and mucus samples of susceptible species incubated with live *L. salmonis* showed higher protease and alkaline phosphatase activity indicating secretions being produced by sea lice. Increased protease and alkaline phosphatase activity was reported in the skin mucus of Atlantic salmon infected with *L. salmonis* compared with mucus from non infected fish [Firth et al. 2000]. The reason for this increase is primarily the appearance of a low molecular weight (17-22kDa) protease with trypsin-like activity [Firth et al. 2000].

A well developed inflammatory response help the naïve coho salmon to get rid of their parasites within 10-14 days post infection of *L. salmonis* [Johnson and Albright 1992b], but Atlantic salmon has not been found to show any significant tissue response leading to assumptions that lack of secretory activity of the parasite on coho salmon may reduce parasite feeding, allow tissue responses, or both [Johnson and Fast 2004]. Also differences in mean protease activity and protease responses to coho salmon mucus involving geographically isolated populations suggest that there may be differences between isolated populations of *L. salmonis* on their response to the hosts.

In a previous study using immunocytochemical technique trypsin like activities was observed in *L. salmonis* whole body homogenate and gut epithelial cells [Roper et al. 1995]. Low molecular weight proteases released by *L. salmonis* in the presence of salmon mucus were characterized as trypsin [Firth et al. 2000], which are similar to other crustacean trypsins. These studies suggest that trypsin decreases host phagocytic activity and immune responses following sea lice infection. Reduced phagocytic activity and respiratory burst responses were seen in the absence of elevated cortisol in *L. salmonis* infected rainbow trout and Atlantic salmon in the same time that the multiple bands of low molecular weight proteases found in the mucus [Fast et al. 2003]

Bell et al. [Bell et al. 2000] reported that peroxidases produced from glands associated with oral cone may protect the sea lice from damage caused by reactive oxygen species produced by host immune cells (e.g., neutrophils) as host immune response. It was also

assumed that peroxidases might be involved in the production of prostaglandins [Bell et al. 2000]. A recent study [Johnson, Sea lice workshop, 2004] partially identified and characterized prostaglandin E2 (PGE2) in *L. salmonis* secretions, a substance well known to have major effects on immune function in other arthropod parasites and found to reduce the expression of several genes involved in processes such as inflammation in host. Vast studies on other arthropod parasites provide a foundation to the knowledge on salivary secretions of sea lice as well as gives gateways for strong assumptions that the secretions may contain a wide range of immunomodulatory substances that needed to be identified and characterized using suitable techniques.

2.3 Humoral non-specific defence parameters in blood plasma and mucus

Susceptibility to different diseases among related species such as Atlantic salmon, coho salmon and rainbow trout vary widely [Fast et al. 2002a]. *Oncorhynchus* species are more susceptible to bacterial kidney disease but more resistant to furunculosis in comparison to Salmo species [Kent and Poppe 1998]. There is genetic variation reported between families of Atlantic salmon to different bacterial diseases [Gjedrem and Gjøen 1995)]. The mechanisms for such differences in disease resistance in salmonids are as yet not well understood and for future disease management, it is very important to investigate into the sources of this variability.

The role of humoral innate immune factors in disease resistance has been well reviewed [Ellis 1999]. Differing levels of particular innate factors have been linked to reported differences in susceptibility across and even within species [Fast et al. 2002a]. Blood parameters such as cortisol, glucose, hematocrit, etc., are often used as indicators of stress. Higher plasma levels of α-2 macroglobulin have been attributed to Arctic char's high susceptibility to *Cryptobia salmonisitica* when compared with rainbow trout [Fast et al. 2002a; Zuo and Woo 1997], also α-2 macroglobulin and complement activities appeared to play role in differences of Atlantic salmon families in resistance to furunculosis [Marsden et al. 1996]. Studies reported that different transferrin genotypes of Oregon strains of coho salmon correlated with resistance to *Renibacterium salmoninarum* [Winter et al. 1980]; however, this finding was not confirmed for transferrin genotypes from British Columbia [Withler and Evelyn 1990]. Higher levels of

plasma lysozyme have also been suggested for relative resistance of Kitimat strain of coho salmon to *Vibrio anguillarum* when compared with Quinsam strain [Balfry 1997]

Mucus covering the epidermal surface is the first line defence in fish, which provides a physical and biochemical barrier between the fish and the environment. Mucus, mostly secreted by goblet cells, plays important role in respiration, ionic and osmotic regulation, reproduction, excretion, and protection against micro-organisms, toxins, pollutants and hydrolytic enzymes [Shephard 1994; Macpherson et al. 2005]. The main structural proteins of mucus are high molecular mass (~106 kDa) glycoproteins called mucins [Tabak 1995]. There are numerous studies on innate immune factors in fish blood and mucus, for example the role of proteases, antibacterial agents and other compounds related to the immune system [Bergsson et al. 2005; De Veer et al. 2007; Hjelmeland et al. 1983; Martinez-Anton et al. 2006; Subramanian et al. 2007; Tasumi et al. 2004]. Proteolytic activity is assumed to be due to serine proteases, which can be secreted by pathogens to activate immunological responses that help in invasion processes [Firth et al. 2000]. The peptidoglycan-digesting enzyme lysozyme and other antibacterial proteins have been located within mucus [Cole et al. 1997; Ebran et al. 2000; Patrzykat et al. 2001]. Lysozyme has been reported to occur in rainbow trout tissues rich in leukocytes and the sites with high risk of bacterial infection, such as mucous layer of the epidermis and gills [Lindsay 1986]. Alkaline phosphatase, a lysosomal enzyme is believed to have a wound-healing role (Iger and Abraham, 1994). Increased alkaline phosphatase levels have been observed in mucus under stress in carp [Iger and Abraham 1990] and during parasitic infection in Atlantic salmon [Ross et al. 2000]. Proteases (cathepsins in eel, [Aranishi and Nakane 1997] serine protease trypsin in rainbow trout [Hjelmeland et al. 1983] are found to be important mucus factors contributing to innate immunity. Other potential immune molecules in fish mucus include immunoglobulins, complement, interferon, lectins and vitellogenin [De Veer et al. 2007; Ellis 2001; Tasumi et al. 2004; Tsutsui et al. 2005].

2.4 Variations of salmonids in susceptibility to sea lice

2.4.1 Between species

Coho salmon is reportedly less susceptible to ectoparasitic infection with *L. salmonis* than chinook salmon, Sockeye salmon (*O. nerka*), rainbow trout or Atlantic salmon (Johnson and Albright, 1992a; Johnson et al., 1996). Atlantic salmon is only slightly more susceptible to sea lice infection than rainbow trout [Johnson and Fast 2004]. In several challenge experiments with *L. salmonis,* differences in susceptibility to infection have been observed between Atlantic salmon, brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon and rainbow trout [Dawson et al. 1997; Fast et al. 2002a; Johnson and Albright 1992b].

2.4.2 Within species

Both farmed and wild salmonids are potential hosts for *L. salmonis*. In Norway, farmed Atlantic salmon and rainbow trout in the salt-water phase outnumbers their wild salmonid counterparts by a factor of 100:1 [Heuch and Mo 2001]. Differences in susceptibility to *L. salmonis* infection have been observed within the brown trout populations [Glover et al. 2003], in addition to differences observed in wild and farmed Atlantic salmon populations in a challenge test involving three wild stocks and two farmed strains. A study with two experimental groups comprising mixed Norwegian sea run and freshwater resident population of brown trout infected with *L. salmonis* revealed highly significant differences in lice abundance, and as the non-genetic influences were tightly controlled, it was postulated that the difference might have a genetic origin [Glover et al. 2001]. Studies in Atlantic salmon infected with *Caligus elongatus,* a relative of *L. salmonis* have shown heritability (h^2) of susceptibility to infection to be 0.22 [Mustafa and Mackinnon 1999]. Substantial genetic variation in susceptibility of Atlantic salmon to *L. salmonis* has been reported by Kolstad et al. [Kolstad et al. 2005] which confirms the indication of genetic variation of this trait given by Fjalestad and Gjedrem [Fjalestad and Gjedrem 1996] and result reported by Mustafa and Mackinnon [Mustafa and Mackinnon 1999]. Taken together, these studies suggest that a genetic component exists in susceptibility of salmonids to sea lice infection.

To select fish in a breeding program to be more resistant to sea lice, there should be significant additive genetic variation in the susceptibility to sea lice in e.g., Atlantic salmon [Gjerde and Ødegård in manuscript]. Data obtained from natural infections of Atlantic salmon with *L. salmonis* under commercial rearing conditions show low heritability for the total lice count per fish [0.07±0.02, Glover et al. 2005; 0.14±0.02, Kolstad et al. 2005]. In a controlled challenge test, however, the number of sessile lice recorded have shown higher heritability (0.26±0.07), but obtained from a relatively low number (50) of full sib families [Kolstad et al. 2005]. However, a high genetic correlation (0.88±0.26) has been found between the lice count per fish recorded in a controlled infestation test and during natural infection [Kolstad et al. 2005]. Also a recent study [Gjerde and Ødegård in manuscript] shows that there is substantial additive genetic variation in the susceptibility of *L. salmonis* in Atlantic salmon and it can be potentially utilized in a selective breeding program to reduce susceptibility to *L. salmonis*.

2.5 Scope of metabolomics

Metabolomic measurements report on the actual functional status of the organism (cell, tissue or biofluid) that, in principle, can be mechanistically related to organism phenotype, even though in practicality such relations are not usually straightforward [Bundy et al. 2009]. One additional advantage is by focusing more on questions than hypotheses, metabolomics can discover unexpected relationships and metabolite responses, which in itself can lead to hypothesis generation [Bundy et al. 2009]. Even though many metabolic changes to biological perturbations can be indirect effects, for example as a result of rearrangement of a metabolic network, and observed metabolite changes need not be linked to the original perturbation, conversely, for physiological stress, metabolite changes may be a direct organismal response. A classic example is the accumulation of trehalose to high levels in invertebrates in a dormant state [Clegg 2001]. Thus, metabolomic approaches here have the additional advantage of building on existing knowledge and research [Bundy et al. 2009]. Most of the studies in this field so far have targeted the analysis of specific metabolite classes, sometimes grouped into ‘totals’, such as ‘total carbohydrates’ or ‘total phenolics’ [Davey et al. 2007]. However, non-targeted metabolomics does provide a perceptibly different philosophy, as opposed to analyses of known metabolites [Bundy et al. 2009]. The main advantage with this approach is that a

range of metabolites that are involved with diverse traits and/or stress responses can be detected, and as these metabolites are measured in a non-targeted manner, so unexpected or even novel responses to stressors can be captured [Bundy et al. 2009]. Besides, suitability of this approach for field-sampled organisms (apart from controlled tests) predicted to exhibit considerable inter-individual metabolic variability as a result of their interaction with their relatively uncontrolled environment has been investigated [Hines et al. 2007] in an NMR metabolomics study of a marine mussel. Moreover, when it comes to disease, metabolic effects have been studied in fish and an invertebrate, including bacterial infections in Atlantic salmon [Solanky et al. 2005] and California red abalone [Rosenblum et al. 2005; Viant et al. 2003]. Viant [Viant 2007] described the application of metabolomics approach to study aquatic organisms, in addition to the challenges of measuring metabolites and metabolic variability as well as the importance of genotypic and phenotypic anchoring to facilitate interpretation of multivariate metabolomics data.

Most studies (in environmental metabolomics) so far comprised of the steps like sample collection (mainly laboratory based but with an increasing number of field studies), preparation of samples and measurement of metabolites; application of multivariate statistical techniques (mostly unsupervised) to identify that differences in the metabolic fingerprints of control and stressed organisms, did or did not exist, in addition to few metabolite identification and then attempted rationalization of molecular pathways [Bundy et al. 2009]. This approach, rather harshly called as “fishing”, as described by [Bundy et al. 2009], has been necessary during the emergence of this relatively new field and helped developing a model for many new researchers who have initiated such study. Furthermore, this “collect, grind, measure and analyze” approach [Bundy et al. 2009] could be all that is necessary to answer the question of interest depending on scope of study. In a situation where it was sufficient to detect if differences between metabolic phenotypes occur (or not), relatively rapid, quantitative and non-targeted analysis of the most abundant metabolites for high sample throughput, optical methods of FT-IR was suggested to be appropriate [Bundy et al. 2009]

CHAPTER III

MATERIALS AND METHODS

This study is a follow-up and complementary study of the previous study that was aimed to investigate susceptibility of Atlantic salmon and rainbow trout to the salmon lice *Lepeophtheirus salmonis* [Gjerde and Saltkjelvik 2009] and also to estimate the genetic variation of Atlantic salmon in the susceptibility towards this pathogen [Gjerde and Ødegård in manuscript] in order to design selective breeding programs that would potentially save the farmers from commercial loss and reduce the infestation pressure of salmon lice among wild salmonid population. Detailed methods for the maintenance of fish and parasites exposure were published elsewhere [Kolstad et al. 2005]

3.1 Fish

The initial fish material consisted of 2206 Atlantic salmon individuals from 154 full-sib families (offsprings of 78 sires and 154 dams) of the 2007 year-class from the breeding nucleus of SalmoBreed AS. The fish were start-fed at Sunndalsøra on 5 February to 29 March. In October 2007 (23-25 October), random samples of 15 fish from each family were individually PIT tagged when the average body weight of fish was 54 g. Tagged fish were maintained in a 3mD tank until being vaccinated between 13-19 February 2008. Then the fish were divided in two 3mD tanks with an equal number of fish per family in each tank. On 15 May 2008 the fish were transported to Nofima Averøy where randomly 1100 smolt (7-8 fish/family) from each tank were kept in two separate 3mD onshore tanks with seawater. The fish were fed to satiation with a commercial feed throughout the experimental period.

3.2 Infection trials and counting of lice

On June 20, 2008 a total of 84000 newly hatched copepodids of *L. salmonis* were added to tank 1 (on average 74 copepodids/fish including 58 rainbow trout) and on June 23 42,200 copepodids were added to tank 2 (on average 36 copepodids/fish including 61 rainbow trout). Details of lice production and infection procedure are mentioned elsewhere [Gjerde and Saltkjelvik 2009]. The seawater temperature in the tanks was

12.3°C. The number of lice per fish was counted on anaesthetized fish and individual body weight was recorded during June 30 to July 4, 2008 when the lice were at attached chalimus stage.

Unfortunately all the experimental fish died due to a jellyfish infestation in the tanks that came with seawater inlet pipe. However, another batch of fish from the same family lines were kept in sea cages at Averøy. From these fish in November 2008, 110 fish (10-11 fish per family) from the most lice resistant families and 110 fish from the most lice susceptible families, as identified earlier by lice counts and ranked, were sampled randomly and kept in two 3mD onshore tanks (55 susceptible and 55 resistant fish in each of the two tanks). In addition 65 rainbow trout of the AquaGen breed were added to each tank. In our experiment these two extreme family lines of salmon, which were ranked based on phenotypic observation, are called as susceptible (S) and resistant (R) lines, respectively. On 27 November 2008 these fish were infested with copepodids following the same protocol as mentioned before. 4100 copepodids were added to the Tank 1 (avg.21.6/fish) and 3920 copepodids were added to the Tank 2 (avg. 22.1/fish). Again on 1 December 2008, 5590 (avg. 29.5/fish) copepodids were added to the Tank 1 and 5180 (avg. 29.2/fish) copepodids were added to Tank 2. On 29 December 2008 the lice (sessile lice) count per fish was carried out following the same procedure and individual body weight was recorded, after which the fish were transferred to a sea cage. On 15 January 2009 the number of motile lice per fish was counted on the same fish and individual body weight was recorded. On February 5 and February 6, 2009 the third lice count was carried out for the number of adult lice per fish and individual body weight was recorded and mucus and blood was collected from each of these fish. All the fish were fed with commercial feed in sea cages and maintained at natural seawater temperature during the whole experimental period.

3.3 Sampling of mucus and plasma

3.3.1 Mucus collection

To collect mucus, fish were anesthetized in sea water with 40 mg 1^{-1} tricaine methane sulphonate (MS-222) and placed individually on clean plastic board and mucus was

scrapped off gently along the dorsal side from anterior towards the posterior part of the fish with the help of rubber spatula and collected in sterile 15 ml tubes and immediately placed on ice. This method was followed instead of the one described by Fast et al., [Fast et al. 2002b] where the fish was left for a period of time in the plastic bag containing ammonium bicarbonate buffer in order to collect mucus. The method in our study was preferred to avoid unrinal/faecal contamination. To remove any foreign material and to obtain sufficient mucus, 300 µl of distilled water was added and the samples were centrifuged at 2500 g at 4°C for 15 minutes. Then the mucus was aliquotted and stored at -80°C.

3.3.2 Blood collection and i-STAT analysis

Blood was processed to plasma and stored for the later use with FT-IR. In An i-STAT Portable Clinical Analyzer (Abbott Laboratories. Abbott Park, Illinois, U.S.A) was used at the time of sampling to measure the indicators of stress and disturbed osmoregulation, possibly induced by adult stages of sea lice. i-STAT measurement on whole blood was carried out for 58 randomly sampled Atlantic salmon and 14 randomly sampled rainbow trout preferably with fishes infested with 2 or more number of lice since most of the fishes sampled had few or no lice. However, all the parameters were not obtained for all fishes sampled for i-STAT. Blood samples were collected by caudal venous puncture method inserting the needle attached to a 10 ml EDTA vacutainer system, under the scales of the mid portion of the tail just below the lateral line at a 45° angle to the axis of the fish in a cranial direction. The blood was stored on ice for 20 min prior to i-STAT analysis. pH, base excess (BE), glucose, sodium (Na^+), ionized calcium (iCa^{2+}), potassium (K^+), total CO_2 (TCO_2), partial pressure of CO_2 (PCO_2), partial pressure of O2 (PO2), oxygen saturation (sO_2), bicarbonate (HCO_3), hematocrit (Hct) and haemoglobin (Hb) were measured with this instrument in our experiment as potential indicators of stress and disturbed osmoregulation. Manufacturer recommended procedures and maintenance were followed for the i-STAT analysis. An automatic one-point calibration was done in the indoor laboratory at Averøy just before sample analysis at 20°C. Values for pH and PCO_2 were temperature corrected (following the temperature correction algorithm given in the i-STAT manual for those parameters) to 2°C as the instrument

measures those at 37°C by default. Blood samples obtained from 71 fishes were collected each in 1 ml EDTA syringes and tested immediately on the i-STAT. Single use disposable CG8+ cartridges (containing microfabricated sensors, a calibrant solutions, fluidics system and a waste chamber) were used for all the analysis. Rest of the collected blood was centrifuged for 15 min at 2500 g. Plasma from individual fish was transferred to Eppendorf vials and stored at -80°C.

3.4 Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared spectroscopy has proved to be a powerful tool to study biological molecules. Fourier Transform Infrared (FT-IR) Spectroscopy bases its functionality on the principle that almost all molecules absorb infrared light. Only the monatomic (He, Ne, Ar, etc) and homopolar diatomic (H_2, N_2, O_2, etc) molecules do not absorb infrared light. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared spectroscopy is an excellent tool for quantitative analysis. One of the great advantages of Fourier transform infrared (FT-IR) spectroscopy is that virtually any sample, in virtually any state can be studied. Biological systems such as proteins, peptides, lipids, biomembranes, carbohydrates, pharmaceuticals, foods and both plant and animal tissues have all been successfully characterized by using infrared spectroscopy. FT-IR spectroscopy technique gives important information about the micro-ambient of molecules by measuring vibrations of molecules simultaneously and monitors their different vibrational groups with electromagnetic radiation application. Application of this nondestructive and sensitive technique has been continually rapidly expanding for functional and structural studies.

3.4.1 Principle

In a molecule the atoms are not held rigidly apart, instead they can move, as if they are attached by a string of equilibrium separation. This bond can bend or stretch. When the bond is subjected to a infrared radiation of a specific frequency (between 400-4000^{cm-1}), it absorbs the energy, and the bonds move from the lowest vibrational state, to the next

highest one. Weaker bonds require less energy, as if the bonds are springs of different strengths. If there are more atoms, there will be more bonds, and therefore more modes of vibrations [Stuart 1997].

Most FT-IR spectroscopy uses a Michelson interferometer to spread a sample with the infrared light spectrum and measure the intensity of the light spectrum not absorbed by the sample. This is a multiplexing technique where all optical frequencies from the source are observed over a period called as scan time. The instrument measures the intensity of a specially encoded infrared beam after it has passed through the sample, the resulting signal (which is a time domain digital signal) is called as 'interferogram'. Fourier transform is fixation of modulated light by interferometer and transformation of obtained 'interferogram' to infrared spectrum by Fourier technique. It is briefly a mathematical process, which ensures transformation of complex waves to simple waves by changing one of functions' independent variable. The standard infrared spectrum is calculated from the Fourier transformed interferogram which gives the spectrum in transmittance (%T) vs. light frequency (cm-1).

3.4.2 Sample preparation for FT-IR

Plasma and mucus samples were thawed to the room temperature. 4 µl of plasma and 10 µl of mucus samples were evenly applied to the wells on a pre-blanked silicon plate (Bruker A755-96 plates, Bruker Optics Ltd., Germany) and allowed to dry in the room temperature until moisture content is vaporized completely. Fourier transform spectroscopy was performed in transmission mode by using a Bruker Tensor 27 spectrometer fitted with HTS-XT 96 well plate reader (Bruker Optics Ltd.). Transmission spectra were acquired over the range 4000-370 cm^{-1} using OPUS version 6.5 software (Bruker Optics Ltd.). Spectra were acquired as a mean of 32 scans and at a spectral resolution of 4 cm^{-1}. Both plasma and mucus samples were prepared in triplicates for FT-IR analysis and resulting spectra were averaged to minimize analytical variability. Plate variability was minimized by acquiring background spectra for each well position to be used prior to sample application and this is subtracted from the sample spectra. The absorbance of the spectra were then converted to comma-delimited text files and exported

to software package Unscrambler 9.1 (Camo, Trondheim, Norway) for statistical analysis.

3.4.3 Spectral analysis

FT-IR plasma and mucus spectra (in 4000-400 cm^{-1} field) obtained from 6 randomly sampled Atlantic salmon are shown in Figure 2 and Figure 3 respectively.

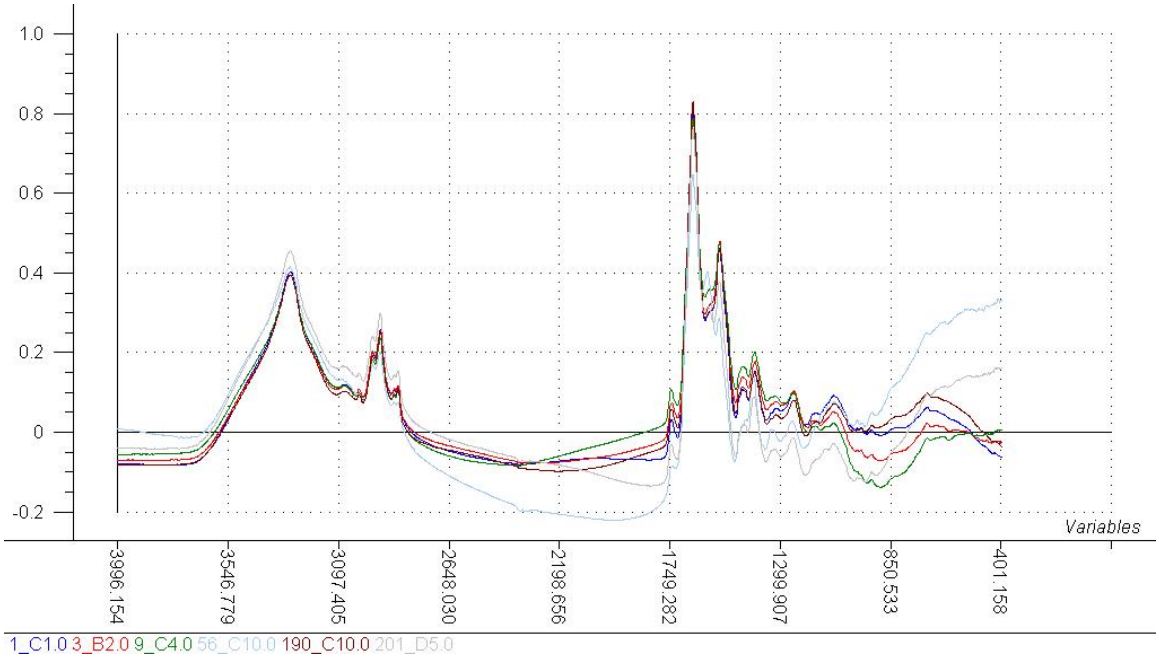


Figure 2 *FT-IR spectra for Atlantic salmon plasma*

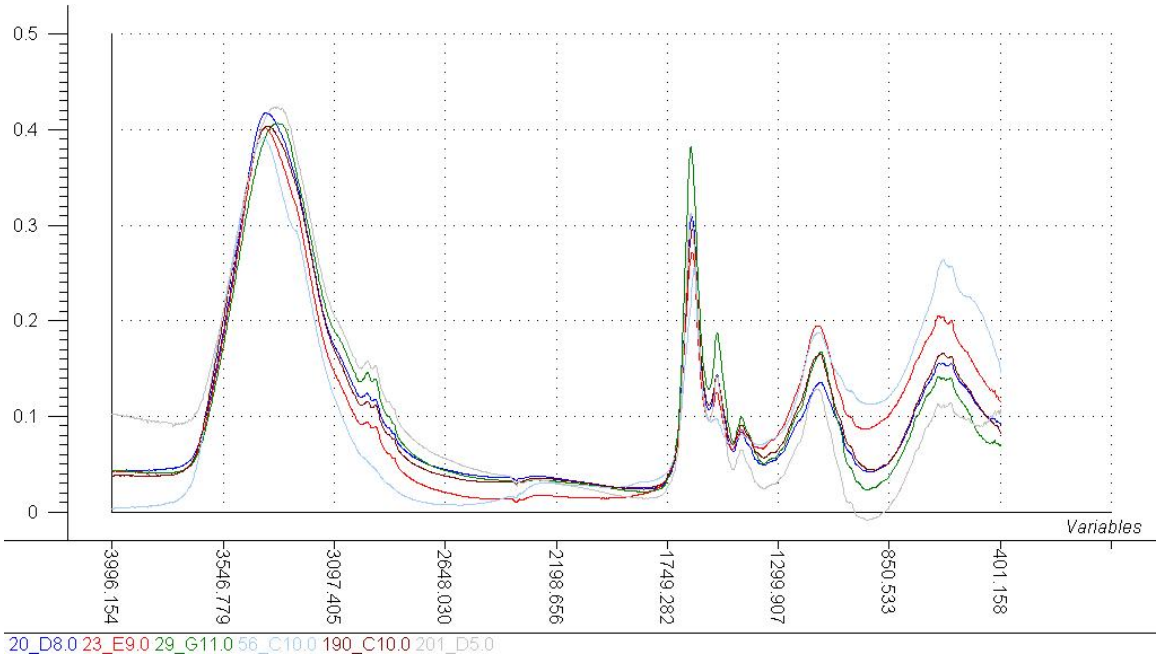


Figure 3 *FT-IR spectra for Atlantic salmon mucus*

We have then selected the spectral range 3580-2794 cm^{-1}, 1755-1000 cm^{-1}, 750-570 cm^{-1} for both plasma and mucus and excluded rest of the spectrum that lies before 3580 cm^{-1}, after 570 cm^{-1} and in between from our analysis where noise was obvious, e.g. it is well known that the excluded regions are not informative due to water and environmental carbon dioxide contamination in the samples. The informative regions in the spectral range kept for plasma is shown in Figure 4. Similarly, informative spectral regions were also selected for mucus (figure not shown) for both species.

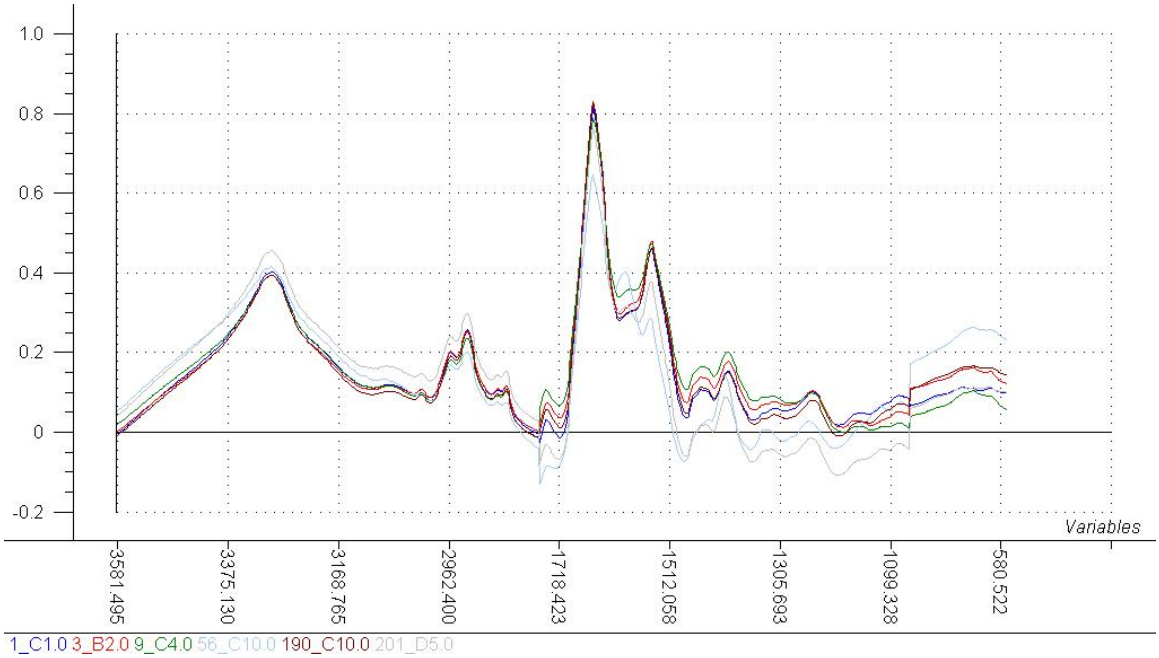


Figure 4 *FT-IR selected spectra for Atlantic salmon plasma*

In the figures, spectrum contains many different bands. Any bond or bond group of a molecule forms characteristic absorption bands in the infrared spectrum. Thus every single band could be assigned to one of the particular bond or bond group. 4000–3000 cm^{-1} region of spectrum contains strong absorption signals originating from stretching vibrations of N–H and O–H. Amines are expected to give rise to N–H stretching and N–H bending absorptions in this region [Stuart 1997]. In 3050-2800 cm^{-1} regions, symmetrical and asymmetrical stretching vibration bands of methylene (CH_2) and methyl (CH_3) groups are found. In the 1800-1000 cm^{-1} region, several bands originated from the vibrations from the amine (N–H) (Amide I and Amide II bands) and polysaccharide groups are located. Increasing the signal intensity signifies increasing the concentration of group equivalent to signal and decreasing the intensity signifies decreasing the concentration of that group [Casal and Mantsch 1984; Fast et al. 2002b]. It is also known that area under the curve is directly proportional to the alteration of matter of concentration. However, since the present study is untargeted FT-IR of plasma and mucus, we do not analyse the band for a spectral vibration given by a known group/groups that would indicate a specific compound, rather it is a broad approach where we look into all possible outcomes from wide spectra obtained from plasma and

mucus samples and we try to explain correlation with lice infection. Thus, band assignments and bond properties generated by FT-IR of plasma and mucus in this study are not discussed.

3.5 Statistical data analysis

Results of this study presented as classical descriptive statistics of differences in lice counts and blood parameters (obtained by i-STAT) for Atlantic salmon and rainbow trout was calculated using Microsoft excel spreadsheet (Microsoft Office 2007 version). Statistical comparisons in lice abundance and blood parameters were carried out by single factor ANOVA and P values <0.05 were considered statistically significant. Means±SE (standard errors) were presented in the figures (charts) whereas SD (standard deviations) was given in the tables in our descriptive statistics.

On the other hand, all the FT-IR data analysis was carried out using a multivariate analysis and experimental design software called Unscrambler (Unscrambler 9.1, Camo, Trondheim, Norway). Traditional statistical methods such ANOVA and MLR are well suited to make regression model from orthogonal data tables. However, the variables in non-designed data matrices are seldom orthogonal, but rather more or less collinear with each other. Methods like MLR will most likely fail in such circumstances, so the use of projection techniques such as Principal Component Regression (PCR) or Partial Least Squares (PLS) of was thought to be appropriate. Thus he Unscrambler multivariate analysis was used for the interpretation of the spectral information from plasma and mucus and i-STAT blood parameters in terms of PCA and PLSR analyses. This approach, known as explorative statistics covers evaluation of the data material under a minimum of model assumptions. In order to differentiate the samples, PCA, one of the most powerful tools in explorative data analysis, has been utilized in this study. In our results the term ‘clustering’ refers to methods applied to search for groups of similar objects or variables. The clustering technique is a part of the explorative data analysis, or unsupervised pattern recognition e.g., PCA is a visual clustering technique. In our experiment we have tried to explain the differences between S and R groups of Atlantic salmon and between Atlantic salmon and rainbow trout based on visual clustering. In this study we considered a data table in Unscrambler with one row for each object (or

individual, or sample), and one column for each descriptor (or measure, or variable). The rows were referred to as samples, and the columns as variables. PCA was used to visualize the main variation and to detect clusters among the samples in the data set based on the FT-IR spectra. PLSR was used to study the correlation between the variation in the FT-IR spectra and the observed lice count. A short description of the multivariate analytic methods used is mentioned in this study, detailed description was given elsewhere [Martens and Stark 1991;Thomas 1994].

3.5.1 Principal Component Analysis (PCA)

Principal component analysis is a multivariate statistical analysis that rotates data to maximize the variability projected onto axes. A set of correlated variables is thereby transformed into a set of uncorrelated variables ranked by variability in the descending order. The resulting uncorrelated variables are linear combinations of the original variables. With PCA, it is possible to reduce dimensionality of a data set while retaining useful information by computing a compact and optimal description of the data set. Following the PCA, the FT-IR spectra is represented in a new co-ordinate system where the first axis represented the direction with greatest variation in data, and the second axis represented the direction with next largest variation and so on. In other words, PCA is a bilinear modeling method that gives an interpretable overview of the main information in a multidimensional data table. The information carried by the original variables is projected onto a smaller number of underlying ("latent") variables called principal components. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on. By plotting the principal components, it is possible to view interrelationships between different variables, and detect and interpret sample patterns, groupings, similarities or differences. The number of principal components possible is equal to the number of variables. A PCA plot, in addition to reducing the number of variables and detecting structural relationship between variables also shows spectral data distribution and thus makes it possible to identify the existence of clusters in the data by looking at score plots of the first principal components from the PCA, making this statistical method very well suited for investigation of clusters in data [Oust et al. 2004].

3.5.2 Partial Least Squares Regression (PLSR)

Partial Least Squares or Projection to Latent Structures (PLS) models both the X- and Y-matrices simultaneously to find the latent variables in X that will best predict the latent variables in Y. These PLS-components are similar to principal components, and also referred to as PCs. □PLS1 deals with only one response variable at a time (like MLR and PCR); PLS2 handles several responses simultaneously. This is a method for relating the variations in one or several response variables (Y-variables) to the variations of several predictors (X-variables), with explanatory or predictive purposes. This method performs particularly well when the various X-variables express common information, i.e. when there is a large amount of correlation, or even collinearity. PLSR is a bilinear modelling method where information in the original X-data is projected onto a small number of underlying ("latent") variables called PLS components. The Y-data are actively used in estimating the "latent" variables to ensure that the first components are those that are most relevant for predicting the Y-variables. Interpretation of the relationship between X-data and Y-data is then simplified as this relationship in concentrated on the smallest possible number of components. PLSR procedure was carried out mainly to remove irrelevant and unstable information in the data matrix X (e.g. FT-IR spectral data) so because only the most relevant part of the variation in X is used in the regression of the reference matrix Y (e.g. lice count) on X. To identify new samples, the PLSR model is used to predict y-values for the samples based on their x-data. The optimal number of PLSR components and the significant variables in the PLSR models were calculated as described by Oust et al. [Oust et al. 2004]

3.5.3 Interpreting results from Unscrambler graphical plots

In the Viewer, data and results are visualized graphically in an interactive manner. Whenever a plot is made, it appeared in a Viewer. A viewer, in manual and Help system of Unscrambler refers to a window where a plot is displayed. The Unscrambler gives a lot of information about the data in the given plot. In our results we have presented two types of plots. In a Score plot, 2D Scatter for any particular compound when the ID line is X-expl: A%, B%; Y-expl: C%, D%, the explained X variance is A% for PC 1 and B% for PC 2. The explained Y variance is C% for PC 1 and D% for PC 2. In the Loading plot, line for a particular compound, if the ID line is PC (X-expl, Y-expl): 1 (A%, B%), it

explains result for that particular compound with the explained X-variance is A% and the explained Y-variance is B% for PC 1. Explained variance is the share of the total variance, which is accounted for by the model. Explained variance is computed as the complement to residual variance, divided by total variance and expressed as a percentage. For instance, an explained variance of 90% means that 90% of the variation in the data is described by the model, while the remaining 10% are noise. Details of PC scores and loadings are not discussed; however, a short description is given below from the Unscrambler user's guide for better understanding of the results.

Scores describe the data structure in terms of sample patterns, and more generally show sample differences or similarities. Each sample has a score on each PC. It reflects the sample location along that PC; it is the coordinate of the sample on the PC. Once the information carried by a PC has been interpreted with the help of the loadings, the score of a sample along that PC can be used to characterize that sample. It describes the major features of the sample, relative to the variables with high loadings on the same PC. Samples with close scores along the same PC are similar (they have close values for the corresponding variables). Conversely, samples for which the scores differ much are quite different from each other with respect to those variables.

CHAPTER IV

RESULTS

4.1 Descriptive statistics

4.1.1 Lice counts for Atlantic salmon

Descriptive statistics for the number of lice per Atlantic salmon are shown in Table 1. Average number of lice per fish was higher in Tank 2 than in Tank 1 in spite of approximately the same number of copepodids per fish added to each tank. Due to the low number of lice per fish in the final lice count this count is not considered in the main statistical analyses.

Pre-adult stages can freely move on the surface of the fish. In our study, lice became mobile during the time of count 2 (pre-adult, January 2009). It was, however, hard to explain that the number of the motile (pre-adult) lice in count 2 was higher than the number of the sessile (chalimus) lice in count 1, both in S and R groups (Figure 5). However, an overall abundance of lice (across tanks, S and R) at lice count 1 is illustrative of the large variation in the lice count per fish (Table 1).

The average body weight of fish recorded at the three different lice counts was 1.22 kg, 1.29 kg and 1.34 kg respectively. The overall correlation between the lice count per fish and body weight was low but positive, 0.05 for lice count 1 and 0.14 for lice count 2. The correlation coefficient was 0.44 between the lice count on the individual fish at count 1 and count 2, 0.22 between count 2 and count 3, and 0.02 between count 1 and count 3.

Table 1: Salmon lice (L. salmonis) counts recorded on Atlantic salmon in different test environment based on their mobility stage
N=number of fish examined, SD=Standard deviation of lice/fish; 1, 2* tank carry over effect from tank 1 and 2, respectively*

Time/stage	Test environment	Tank	S			R		
			N	Mean	SD	N	Mean	SD
December 2008	Tank							
Sessile		1	55	8.80	6.20	53	6.30	3.20
Sessile		2	53	15.40	6.90	53	12.50	6.30
January 2009	Sea cage							
Motile		1*	55	12.04	4.65	53	9.70	5.01
Motile		2*	53	15.60	6.70	53	14.06	6.60
February 2009	Sea cage							
Motile		1*	55	0.58	0.79	53	0.47	0.72
Motile		2*	53	0.55	0.82	53	0.58	0.75

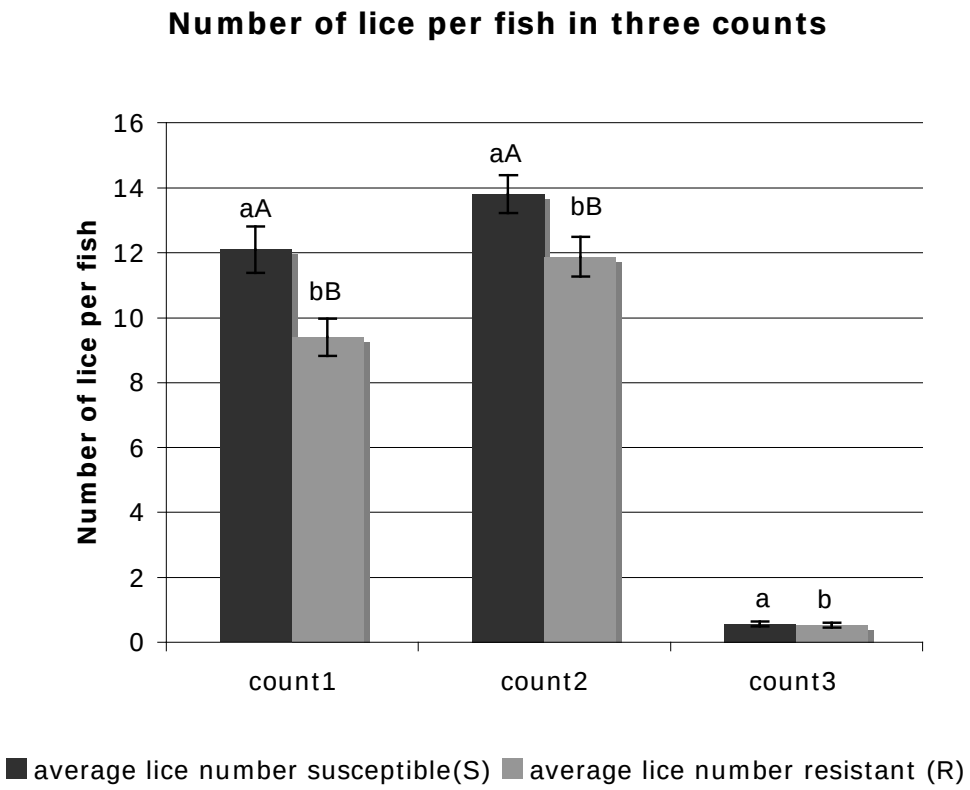


Figure 5 *Average lice count for susceptible (S) and resistant (R) groups of Atlantic salmon*
Error bars represent standard errors of lice/fish; Different capital letters denote significantly different from each other.

The susceptible group shown a significantly ($P< 0.05$) higher number of lice per fish than the resistant group of fish in count 1 and count 2, irrespective of the tank the fish came from and irrespective of the time of counting (Figure 6).

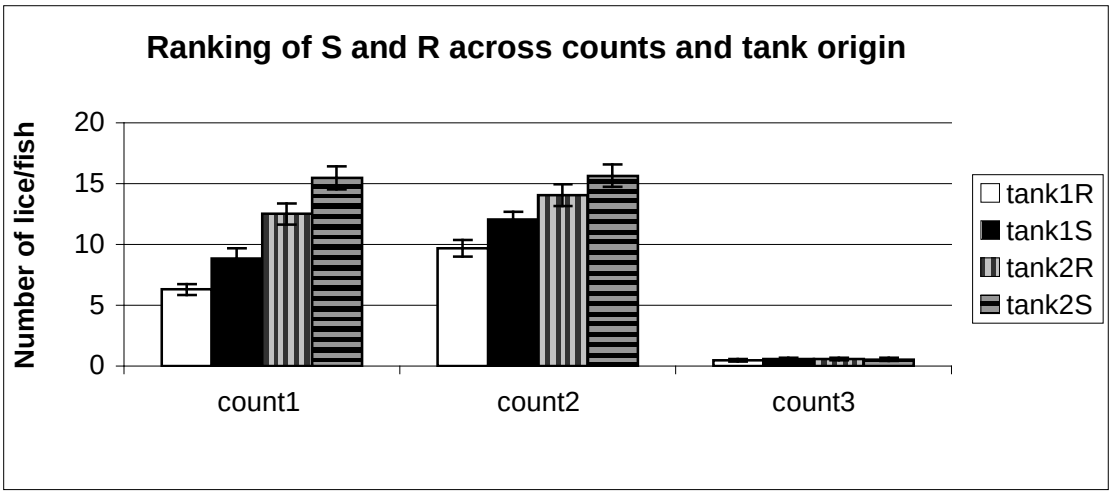


Figure 6 *Ranking of S and R groups of Atlantic salmon based on average number of lice per fish with their tank origin where the fish was infected;*
Error bars represent the standard errors of lice/fish.

4.1.2 Lice counts for rainbow trout

The average number of lice per fish was found to be 30.5 (SD=22.9), 22.8 (SD=10.4) and 1.33 (SD=1.08) in lice count 1, 2 and 3 respectively (Table 2). As for Atlantic salmon, the lice count per fish was higher in Tank 2 than in Tank 1 for the first two lice counts. This average number of lice per rainbow trout for lice count 1 and 2 was significantly higher than that for Atlantic salmon shown in Table 1. Moreover, an overall abundance of lice at lice count 1 and lice count 2 is illustrative of the large variation in the lice count per fish (Table 2).

Table 2: Salmon lice (L. salmonis) count for rainbow trout

N=number of fish examined, SD=Standard deviation of lice/fish; 1, 2* tank carry over effect from tank 1 and 2, respectively*

Time/stage	Test environment	Tank	Lice count on Rainbow trout		
			N	Mean	SD
December 2008	Tank				
Sessile		1	21	21.62	14.78
Sessile		2	18	40.78	26.65
January 2009	Sea cage				
Motile		1*	21	19.24	8.41
Motile		2*	18	26.94	11.16
February 2009	Sea cage				
Motile		1*	21	1.71	1.19
Motile		2*	18	0.89	0.76

The average weight of fish was 2.50 kg, 2.58 kg, and 2.74 kg in count 1, count 2 and count 3 respectively. The overall correlation between the lice count per fish and body weight was negative, -0.02 for lice count 1 and -0.08 for lice count 2. The average number of lice per kg rainbow trout was still higher than that for Atlantic salmon for count 1 (13.8 vs. 9.4), but almost equal for count 2 (10.06 vs. 10.56).

4.1.3 Blood parameters for Atlantic salmon and rainbow trout

The studied blood parameters with their mean and standard deviations are shown in Table 3. Values for iCa^{2+} and K^{+} were also obtained but not presented as they were found to be <0.25 mmol/L and >9 mmol/L respectively which are outside the reportable range in the i-STAT system used. pH, HCO_3 and BE gave acid-base status of the fish while measurement of PO_2, PCO_2, and sO_2 was done to monitor respiratory function.

In Atlantic salmon significant differences (P<0.05) in mean blood parameters was observed only for hematocrit and haemoglobin values between S and R groups (Table 3).

Hematocrit and haemoglobin were observed to be higher in rainbow trout than in Atlantic salmon (S and R together). However, in the absence of a standard baseline reference for blood parameters in Atlantic salmon and rainbow trout, it was only possible to carry out a comparative study within and between species in our study.

Table 3: Blood parameters obtained by i-STAT blood gas analyzer

S=susceptible, R=resistant, N=number of fish examined, SD=Standard deviation. Denotes parameters where S and R statistically different.*

Blood parameters	Atlantic salmon (S)			Atlantic salmon (R)			Rainbow trout		
	N	Mean	SD	N	Mean	SD	N	Mean	SD
PH	23	7.42	0.05	19	7.42	0.05	14	7.45	0.06
pCO_2 (mmHg)	23	0.98	0.14	19	1.00	0.16	14	1.02	0.15
pO_2 (mmHg)	29	2.37	1	23	2.28	0.74	14	1.96	0.63
HCO_3(mmol/L)	23	8.47	1.25	19	8.49	1.27	14	9.19	1.20
BE (mmol/L)	23	-22.78	1.65	19	-22.89	1.56	14	-21.64	1.50
sO_2(%)	22	16.55	11.24	19	14.16	7.60	13	11.54	5.44
Glucose (mmol/L)	29	6.37	0.82	23	6.56	1.03	14	6.48	1.01
Sodium (mmol/L)	29	156.14	2.33	23	155.43	3.29	14	156.79	4.66
TCO_2 (mmol/L)	23	9.57	1.47	19	9.42	1.46	14	10.14	1.35
*Hematocrit (%PCV)	29	25.86	4.02	23	28.35	3.30	14	35.57	4.42
*Haemoglobin (g/L)	29	87.97	13.57	23	96.39	11.26	14	120.93	15.07

4.2a Principal component analysis (PCA)

FT-IR measurements were obtained from mucus and plasma samples collected from 214 Atlantic salmon and 39 rainbow trout with different susceptibilities to *L. salmonis*. The maximum number of principal components (PCs) to be analyzed was set to 20.

Plasma

For Atlantic salmon, for the selected FT-IR spectral area (3580-2794 cm^{-1}, 1755-1000 cm^{-1}, 750-570 cm^{-1}) the most significant components PC1 explained 58% of the variation in the FT-IR variables and PC2 explained 18% variation in the FT-IR variables (for lice count 1) (Figure 7). However, the observed variation in the FT-IR plasma values did not explain a significant proportion of the variation in lice count and consequently did not produce a clustering of S or R groups with respect to lice count 1 (after removing 9 outliers listed by the program Unscrambler). Similarly, the observed variation in the FT-IR plasma values did not explain any significant variation in the lice count 2, thus no clustering was achieved with respect to lice count 2 (figure not shown). Moreover, no clustering of species was found when the Atlantic salmon and rainbow trout plasma data were pooled (figure not shown).

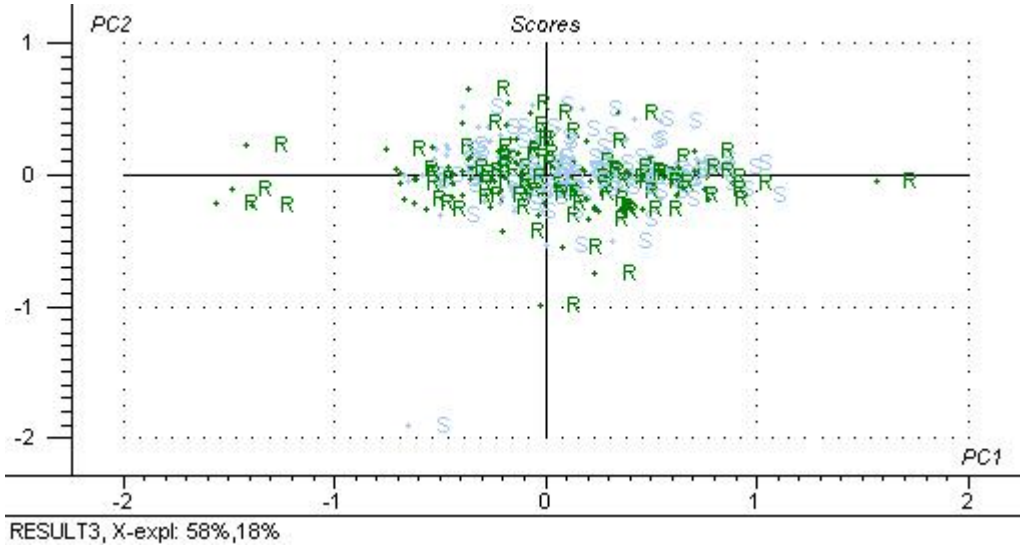


Figure 7 Scores *plot of PCA of FT-IR data (selected spectra) from Atlantic salmon plasma in lice count 1 S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component 2*

Mucus

For Atlantic salmon mucus no significant clustering of the S and R groups with respect to lice count 1 and lice count 2 was found when PCA was carried out for selected spectral area (3580-2794 cm^{-1}, 1755-1000 cm^{-1}, 750-570 cm^{-1})(figures not presented). No clustering of species was found when the Atlantic salmon and rainbow trout mucus data were pooled for lice count 1 and lice count 2 (figure not shown).

Plasma and Mucus

Similarly, no significant clustering of the S and R groups or the two species was obtained when all the Atlantic salmon and rainbow trout FT-IR plasma and mucus data were pooled (figure not shown).

4.2b Partial Least Squares Regression (PLSR)

4.2b.1 PLS 1

PLS 1 gives the effect of one or many explanatory variables (FT-IR plasma and mucus data and i-STAT blood data) on Y response variable (lice count).

Plasma

The selected FT-IR spectral area for plasma did not give any systematic clustering of the scores of individual fish with similar number of attached lice for count 1 or 2. For fish grouped into classes of (0-6.6), (6.6-13.2), (13.2-19.8), (19.8-26.4), (26.4-33) lice per fish(dots) in lice count 1 is shown (Figure 8); whereas for fish assigned to the susceptible (S) and resistant (R) groups non clustering of scores are shown in Figure 9. This is expected considering that the fact as shown in Figure 4.2b.1, PC1 and PC2 explained only 2% and 1% respectively of the variation in the lice count 1 , although PC1 explained 24% and PC2 explained 19 % of the variation in the FT-IR variables respectively (Figure 8).

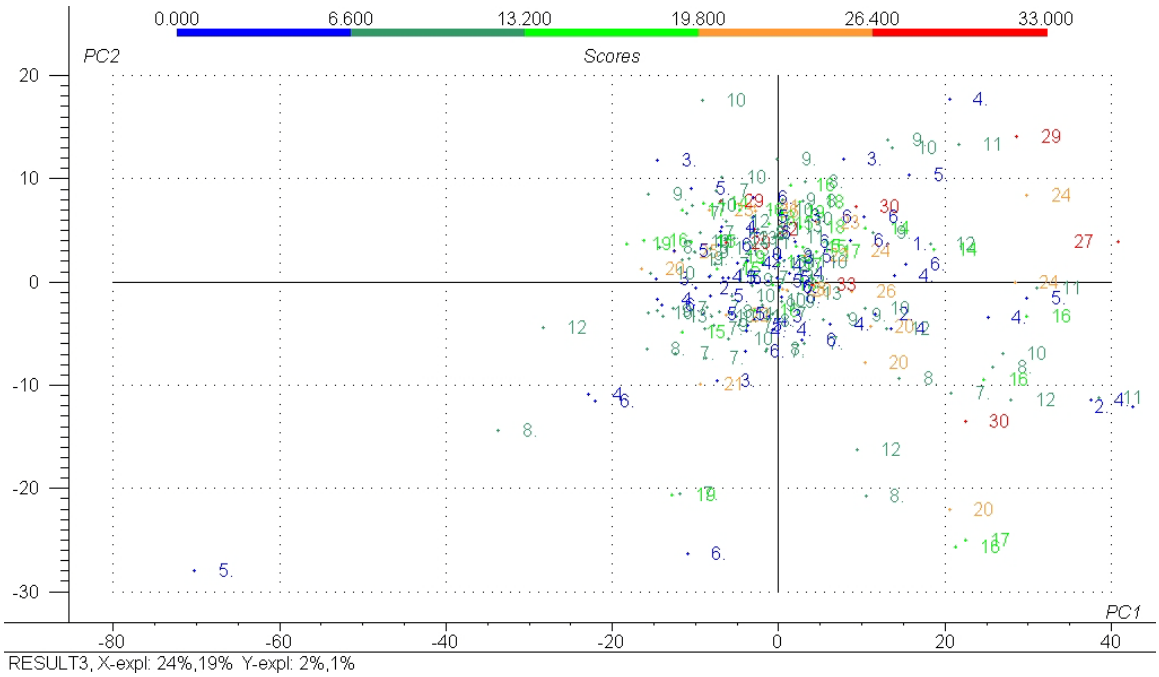


Figure 8 *Scores plot of PLS 1 of FT-IR whole spectra for lice number on individual Atlantic salmon (plasma) in lice count 1 for the different classes of lice count per fish*
Dots represent individual fish; Numbers represent classes of lice number. PC1=principal component 1, PC2=principal component 2

The selected FT-IR spectra (3580-2794 cm^{-1}, 1755-1000 cm^{-1}, 750-570 cm^{-1}) for plasma did not show any significant clustering of S or R groups either with respect to lice count 1(figures not shown) or lice count 2. This was also the case after removing 9 outliers listed by the Unscrambler in lice count 2 (Figure 9). This is obvious as the Figure 9 shows that Y response lice count 2 is explained only 6% for PC 1 and 1% for PC 2 although the explained FT-IR X variance is 11% for PC 1 and 25% for PC 2. However, the observed variance in the FT-IR plasma values did not explain a significant proportion of the variation in lice count and thus no clustering was achieved. No clustering of species was found either when the Atlantic salmon and rainbow trout plasma data were pooled (figure not shown).

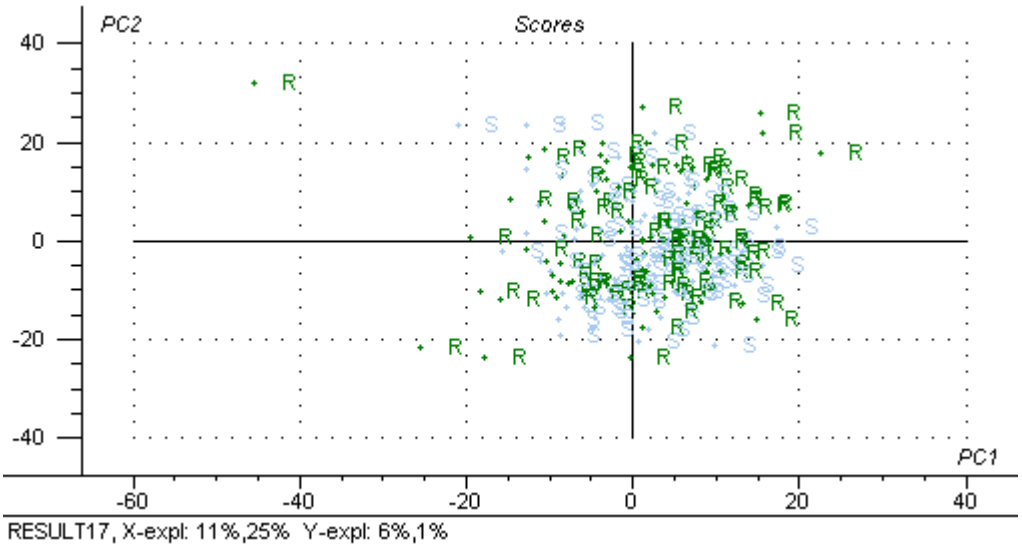


Figure 9 *Scores plot of PLS 1 of FT-IR data (selected spectra) for Atlantic salmon plasma in lice count 2 after removing outliers*
S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component 2

Mucus

The observed variances in FT-IR mucus values did not explain any significant proportion of the variation in lice count and consequently no clustering of S and R groups was seen for lice count 2 (Figure 10). For these data PC1 and PC2 explained only 4% and 2% of the variation in the lice count 2, although PC1 explained 98% and PC2 0% of the variation in the FT-IR variables. No clustering of species was found either when the Atlantic salmon and rainbow trout mucus data were pooled (figure not shown).

Figure 10 *Scores plot of PLS 1 for FT-IR data (selected spectra) for Atlantic salmon mucus in lice count 2 S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component 2*

Plasma and mucus

Similarly, no significant clustering of the S and R groups or the two species with respect to lice count 1 and count 2 were obtained when all the Atlantic salmon and rainbow trout FT-IR plasma and mucus data were pooled (figures not shown). Including body weight as an additional explanatory variable did not improve the clustering (figure not shown).

i-STAT blood parameters

The observed variances in i-STAT values did not explain any significant proportion of the variation in lice count 2 and consequently no clustering of S and R groups was observed (Figure 11). For these data PC1 and PC2 explained only 6% and 3% of the variation in the lice count 2, and PC1 explained 26% and PC2 28% of the variation in these i-STAT variables.

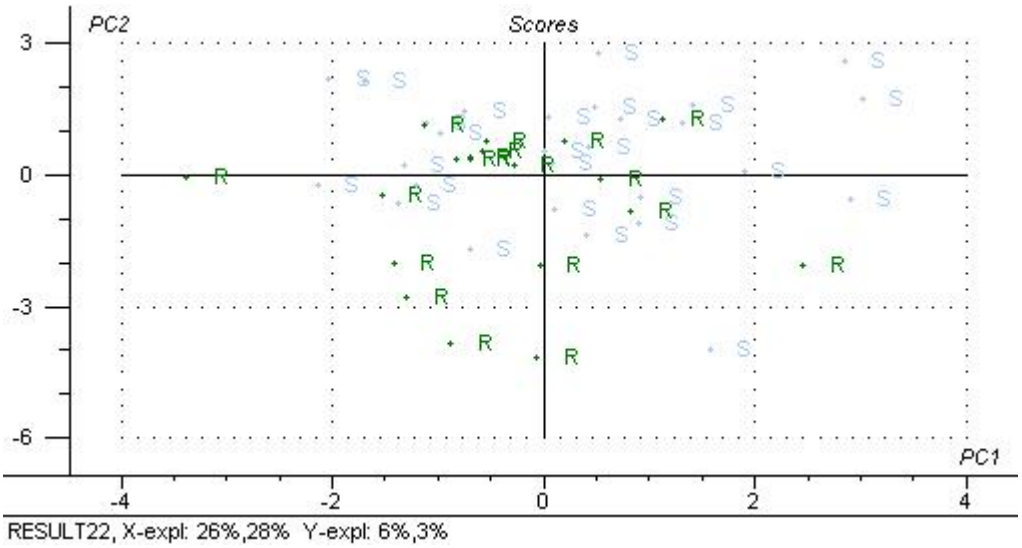


Figure 11 *Scores plot of PLS 1 for Atlantic salmon blood parameters by i-STAT in lice count 2 S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component 2*

However, some clustering of Atlantic salmon and rainbow trout was observed based on i-STAT blood data in lice count 2 (Figure 12) where Y response species is explained 38% by PC 1 and 17% by PC 2 , whereas 40% variation in the i-STAT variables are explained by PC 1 and 12% by PC 2 respectively. However, this did not explain any association with number of lice per fish.

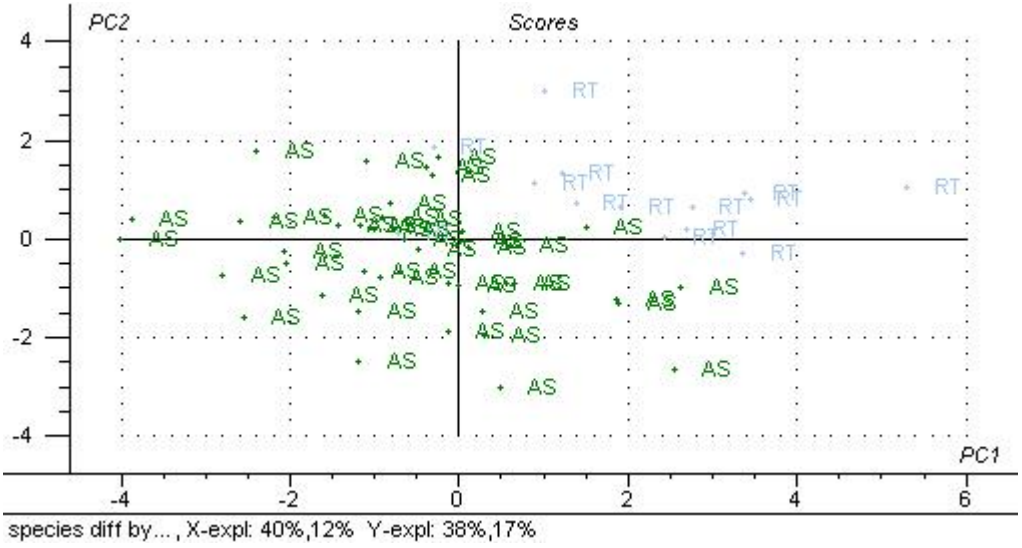


Figure 12 *Scores plot of PLS 1 for species difference by i-STAT blood data AS=Atlantic salmon, RT=Rainbow trout; S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component 2*

FT-IR Plasma and mucus data and i-STAT blood data

Since no clustering of S and R was achieved to explain the variation in the lice count in Atlantic salmon by using individual X variables, we pooled all the FT-IR plasma and mucus data and the i-STAT blood data and tested if the variation in the lice count 2 could be explained by all these X variables combined. However, still no variation in the lice number could be explained by these X variables. In Figure 13, 3% of variation in lice count 2 is explained by PC1 and 2% by PC 2, whereas the variations in the X variables are explained 22% by PC 1 and 13% by PC 2 respectively. However, the observed variances for combined variables did not explain any significant proportion of the variation in lice count and thus no clustering of S and R was observed.

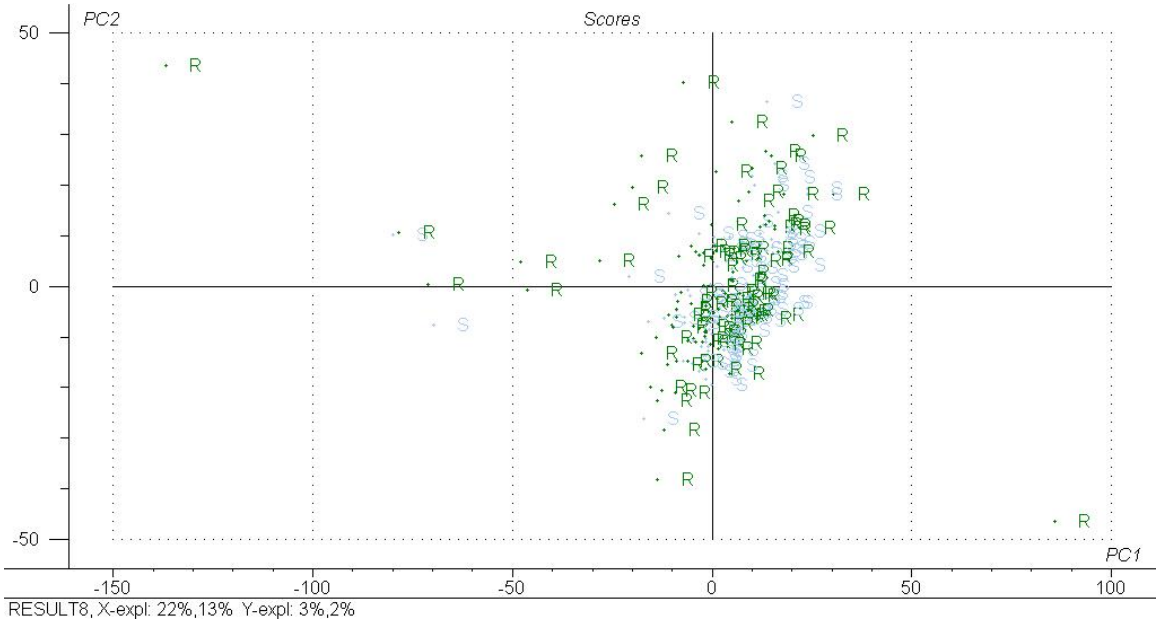


Figure 13 *Scores plot of PLS 1 for FT-IR data of plasma and mucus (selected spectra) and i-STAT data combined for Atlantic salmon in Lice count 2*
S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component2

4.2b.2 PLS 2

PLS 2 gave the opportunity to simultaneously find links between more than one Y response and X variables. In our analysis we tried to explain the variations in all the three lice counts (Y variables) simultaneously by FT-IR data (X variables) first by plasma, then by mucus and finally by plasma and mucus combined for Atlantic salmon. In PLS 2 nine outliers were removed by Hotelling T2 ellipse option from the view menu of

Unscrambler followed by marking of outliers in the scores plot. The recalculated scores plot did not yield significant clustering for differences in lice counts when plasma (Figure 14) or mucus or both plasma and mucus combined (figures not shown) was considered.

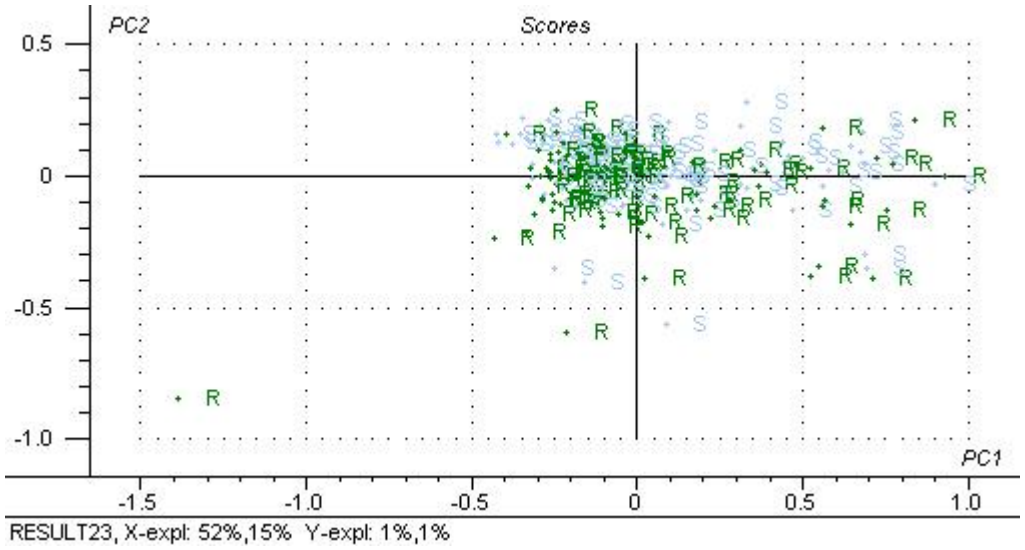


Figure 14 *Scores plot of PLS 2 for FT-IR data from Atlantic salmon plasma (selected spectra) on lice counts*
S=susceptible, R=resistant; PC1=principal component 1, PC2=principal component2

We also tried to explain the differences in lice count 1 and lice count 2 in Atlantic salmon by i-STAT blood parameters, however, no clustering of S and R was achieved. Moreover, the observed differences in the lice count between Atlantic salmon and rainbow trout could not be explained by any of the measured X variables (figures not presented).

CHAPTER V

DISCUSSIONS

Studies suggested that there is significant genetic variation in resistance to salmon louse in Atlantic salmon [Fjalestad and Gjedrem 1996; Glover et al. 2005; Kolstad et al. 2005]. In our experiment we utilized a metabolomics approach in order to improve our knowledge of biological variation in susceptibility to salmon lice among individual Atlantic salmon and rainbow trout possibly reflected in blood and/or mucus with the potential to use it in the future as a high-throughput screening for selective breeding purposes. The Atlantic salmon belonged to two groups, different in their inherent susceptibility to salmon lice.

Given the existing challenges in working with field data in sea lice research and high genetic correlation (r_g=0.88) between the numbers of lice in controlled challenge tests and during natural infections, challenge tests are very useful to obtain a measure of lice resistance in a selective breeding program [Kolstad et al. 2005]. However, challenge tests with a high number of animals need access to a high number of sexually mature lice, it is laborious to count lice on a large number of fish and challenge protocols may also be difficult to standardize. Moreover, as it is not likely to obtain lice count on the breeding candidates, only sib selection can be performed using the challenge test data. Thus, alternative screening methods of parameters or traits that are correlated to lice resistance and that can preferably be applied to non-infected breeding candidates are very much needed.

Use of metabolomics, a systems approach for studying endogenous metabolites in biofluid or whole organism as a screening tool in aquaculture research for detection of changes in the metabolome induced by external or internal stressors has been reviewed by Samuelsson and Larsson [Samuelsson and Larsson 2008]. Metabolomics studies based on the simultaneous measurement of multiple metabolites using techniques like Nuclear Magnetic Resonance (NMR) spectroscopy or mass spectrometry, coupled with

appropriate statistical analysis that typically employs multivariate or else repeated univariate tests, are discussed by Bundy et al. [Bundy et al. 2009]. The authors (2009) suggested that optical methods such as Fourier transform infrared (FT-IR) spectroscopy would be highly appropriate in particular when extremely high sample throughput is required in a situation where it is sufficient to detect if differences between metabolic phenotypes occur (or not). The underlying objective of this study involved the utility of high throughput screening technique FT-IR to evaluate if compounds in mucus and/or plasma of infected and non infected fish could be useful as predictor of lice resistance in Atlantic salmon and rainbow trout. In addition, we have measured a suite of well-established clinical blood parameters to complement the FT-IR data. Blood parameters are useful measures of physiological disturbance.

Our experiment was a time course study. Three lice counts per fish took place over the winter months of December 2008, January 2008 and February 2009 when the lice underwent sessile chalimus (count 1, in tanks on shore), motile pre-adult (count 2, in a sea cage) and motile adult (count 3, in a sea cage) stages of development. It was difficult to explain why the number of lice in count 2 was higher than in count 1. However, since the chalimus stages of *L. salmonis* are very small, it is likely that the numbers of sessile lice were underestimated. Another possible explanation is that motile pre-adult lice came to fish from surrounding sea cages since the fish was already transferred to the sea cage after count 1 that took place in tanks on shore. This illustrates the complexities of working in the real field situation where externalities can influence the data. It was relatively reasonable to deduce that the delousing event in the surrounding cages and fish farms a few days prior to lice count 3 may have caused the extreme low count of lice per fish.

This study evidences the unpredictable nature of the motile stages of lice. Previous studies also warned that motile lice transfer between fish [Ritchie 1997]. A large variation in lice count among individual Atlantic salmon observed in our study indicates that this species differ in their susceptibility to *L. salmonis*. Most important finding in this study was that the average number of lice per Atlantic salmon was always higher in

susceptible (S) group than in resistant (R) group. Clear separation of S and R by number of lice indicated a genetic background for the trait and it is consistent with the previous study [Gjerde and Ødegård in manuscript].

Large variation also observed in lice count among individual rainbow trout indicating their inherent biological differences in sea lice resistance. This study provided contrasting information with most of the previous studies about the response of rainbow trout to salmon louse, which seemed to be significantly more susceptible species than Atlantic salmon. In our study the higher average number of lice per fish in rainbow trout than in Atlantic salmon is not in agreement with the study reporting less or equal susceptibility for rainbow trout in commercial farms in Ireland [Jackson et al. 1997]. This also contradicts the studies suggesting *S. salar* is only slightly more susceptible to infection with *L. salmonis* than *O. mykiss* [Johnson and Albright 1992b]. However this study is in agreement with one previous study [Gjerde and Saltkjelvik 2009] for sessile lice count. This indicates the importance of a long-term study (preferably with equal size group of fish), which should involve all developmental stages of sea lice to see if the mobility of different stages results in differences in lice abundance between the species. However, our study shoes that rainbow trout is always more susceptible. Importantly, lice reached the same developmental stage on rainbow trout as on Atlantic salmon, which was an evidence for similar developmental rates on the two species.

Research in the field of fish parasitology indicated that fish respond to parasite infections by activating different innate and adaptive immune mechanisms [Alvarez-Pellitero 2008]. In spite of the increasing research into the immune response to fish parasites in recent years, there are yet important gaps in the knowledge of the mechanisms involved in protection. Selective breeding of farmed fish for sea lice resistance with the help of molecular markers have not been found so far. An integrated approach like metabolomics holds great promise in this regard as it could provide the much-needed biological markers for indirect selection.

In this study it was hypothesized that changes in some of these measured blood parameters could be induced by adult stages of sea lice. Teleosts exhibit primary, secondary and tertiary stress response. The primary response involves hypothalamic pituitary adrenal (HPA) axis followed by secondary response that is reflected in metabolic changes (for example plasma glucose), hydromineral disturbance (changes in sodium and chloride balance), and hematological changes (hematocrit and haemoglobin) (Johnson and Fast, 2004). The tertiary stress involves even more integrated response at the higher level of biological organization (whole organism) that might affect for example, behaviour or swimming ability. Negative physiological impact of sea lice on swimming performance of Atlantic salmon has been confirmed [Wagner and McKinley 2004].

In the absence of a standard reference with established levels for most of the measured blood parameters for two studied species, we have used i-STAT mainly to infer differences in their mean values and also to include the blood parameters in the regression model to predict the lice count among individuals and between the S and R groups of Atlantic salmon and between Atlantic salmon and rainbow trout. The observed variances in i-STAT values did not explain any significant proportion of the variation in the lice count 1 or 2 in our study by PCA or PLS. This was not surprising since none of the blood parameters shown any direct association to lice count. However, it is also possible that handling and anaesthetic stress masked the stress induced by sea lice, and there is further need to compare the difference with low or non-stressed counterparts. Establishing a reference database for normal levels of blood parameters in fish is very much needed in this regard for future studies.

The typical primary physiological stress response in fish is increased secretion of catecholamines (epinephrine) and corticosteroids (cortisol) by the hypothalamic-pituitary-adrenal (HPA) axis. This response is normally independent of the type of stressor; however, quantitative aspect of the response depends on the intensity and duration of the stressor. The initial endocrine responses cause several metabolic adjustments including changes in plasma osmolyte, glucose, and lactate concentrations

(secondary stress response). No significant difference in osmolyte and glucose levels measured in our study between S and R groups of Atlantic salmon and Atlantic salmon and rainbow trout indicated low stress response possibly due to very low number of lice. However, mean hematocrit and haemoglobin values differed both between S and R, and atlantic salmon and rainbow trout. Higher hematocrit values in rainbow trout than in Atlantic salmon and higher values in R group of Atlantic salmon than in S group could probably not be attributed to a single stressor (sea lice) given that those values can differ depending on environmental changes, handling and transportation, osmotic stress, high stocking density and anesthesia. However, a genetic background for this trait is evident from our data. Since in count 3 there were very few lice on fish, the observed differences in hematocrit are most likely inherited and constitutive. Decreases in hematocrit and hemoglobin have been reported in fishes subjected to an acute cold stress. Decreases in hematocrit and hemoglobin could and also as the result of blood osmoconcentration, as shown by an increase in plasma osmolarity in a study with brook char [Diouf 2000]. In the same study, it was shown that in cold-stressed fish, a decrease in hemoglobin concentration could be the result of hemodilution by body cell water. The lower hematocrit and haemoglobin value in S than R and rainbow trout in our study indicated that these fish could be more prone to anemia. Therefore, it can be said that this is a very responsive and important parameter, however, not very specific. In any case since there was a difference between S and R for hematological parameters irrespective of low level number of lice while measuring those blood parameters, it indicates that these parameters are possibly heritable and should be further examined, preferably at a much higher infection level for better understanding of complex host-parasite relationship. Furthermore, a toxicity study of intraperitoneally injected ivermectin (widely used as a treatment against sea lice infections of farmed salmonids and non-salmonids) in sea bream showed a decrease of the hematocrit value one hour after the injection for most of the doses along with an increase of the hemoglobin concentration [Katharios et al. 2001]. Since our experimental fishes may have been exposed to chemicals owing to delousing events in the adjacent farms, it would be interesting to study if delousing agents at very low concentrations could affect the measured blood parameters in our experiment.

Moreover, this study suggests that, in addition to hematocrit and hemoglobin, there may be other non-specific parameters present in blood but not included in this study that are inherited and can be further examined as the inclusion criteria for indirect selection given a positive and favourable correlation is found with the number of lice. However, the observed mean differences in hematocrit and hemoglobin did not explain the number of lice in the final last count.

Studies where it is sufficient to detect if differences between metabolic phenotypes occur (or not), and when extremely high sample throughput is required as in our study, optical methods such like FT-IR was suggested to be appropriate [Bundy et al. 2009]. We used the explorative techniques of Fourier Transform infrared (FT-IR) spectroscopy for data generation, and multivariate statistics were used to identify significant responses, reduce dimensionality and to uncover structures in the data with the help of the statistical software package Unscrambler. Biological tissue is essentially made up of proteins, nucleic acids, carbohydrates and lipids each of which have their unique IR spectra. If there was a metabolic difference in blood or mucus tissues of fish induced by lice infection, or an observed innate difference without lice, the hypothesis was to observe those differences in terms of phenotypic measurement of the lice count per fish when the blood and mucus tissues were sampled (count 3) or at count 1 and 2. However, no clustering was achieved to segregate the S and R group of Atlantic salmon in our experiment when PCA was carried out for FT-IR spectral data from plasma (Figure 7) or mucus; S and R were sparsely distributed over all the axes. The same was observed in PLS regression. This might be due to the low metabolic variation among individuals for compounds in plasma and mucus. The inability to model the association between FT-IR variables and number of lice based on the obtained data may explain high similarity in Atlantic salmon samples (S and R groups) for all variables studied for plasma and mucus compositions, and indicates, for the given number of lice there might be little or no metabolic changes in plasma or mucus, or there is no innate differences among individuals. It is however, also possible that the non specific parameters in blood and mucus have changed between the time of lice counts (count 1 and count 2) and blood and mucus sample collection (count 3), hence difficult to explain number of lice in those

counts by the samples obtained much later during lice count 3. However, this may be a requirement for the practical application of the method since the samples are needed to be obtained on breeding candidates that may not be infected. Besides, the defence parameters present in blood and mucus are often only induced when there is a challenge for the organism to exhibit the resistance it may possess, extremely low number of lice during lice count 3 may not be sufficient to detect the changes in those parameters by FT-IR. But conversely, higher physiological stress response was expected to be induced by adult lice than chalimus stage. Hence establishing a comparative metabolic profiling involving stress response to sessile and motile lice is necessary for further understanding of fish metabolomics. Inclusion of weight variable in the dataset did not improve the response, indicating there may not be much compositional differences in Atlantic salmon plasma and mucus owing to weight differences, or they are not traceable by FT-IR to separate individual fish and the S and R in consideration with respect to lice resistance.

Moreover, Pooling of the FT-IR information of plasma and mucus and the blood parameters of i-STAT analysis together as X variables to explain Y response number of lice per fish by PLS regression did not cluster S and R groups to discriminate the fishes with respect too their lice susceptibility, further indicating the inability to model based on the values of available variables on the same species. However, interestingly, it was possible to differentiate Atlantic salmon from rainbow trout using PLS 1 with i-STAT blood data as X variables and species as Y response. This was not considered as an objective of our study; rather it indicates the applicability of the method.

CHAPTER VI

CONCLUSION

To conclude, this study provides evidence that resistance towards sea lice is genetically inherited both at sessile (count 1) and motile (count 2) stages of parasitic development and also revealed a substantially higher susceptibility in rainbow trout. However not very specific to the individual lice count, the Blood parameters viz. hematocrit and haemoglobin were found to be very responsive indicating a genetic background and can be further examined as the inclusion criteria for indirect selection in Atlantic salmon. High-throughput metabolomics FT-IR approach on mucus and blood plasma complemented with classical blood markers at low levels of adult lice present on fish (count 3) did not explain the differences in the number of lice at previous counts. Most probably, small number of lice does not cause changes in parameters detectable by the methodology used in this study. It could be suspected that the metabolic variations measured by FT-IR observed during the last count fell within the constitutive levels for these parameters, implying that there are no innately expressed markers of resistance to lice at the given lice infection pressure. Metabolomics methods used in this study would be of limited value in selective breeding for increased resistance towards sea lice under low sea lice infection level.

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