Investigating the molecular basis for resistance to the sea louse, *Lepeophtheirus salmonis*, among salmonids

by

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BSc, Malaspina University-College, 2007
BSc, Vancouver Island University, 2009

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

Supervisory Committee

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Abstract

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Co-evolution between parasites and their hosts result in extremely well-orchestrated and intimate relationships that are characterized by remarkable adaptations in the attack response of the parasite and the defense response of the host. To fully understand host-parasite interactions, these adaptations must be considered in the context of the ecological constraints in which they evolved. As a serious pest to salmon mariculture, *Lepeophtheirus salmonis* has been extensively studied; however, there are still several areas that require further research. Of utmost importance, and the topic of this thesis, is molecular basis for resistance to sea lice. The following chapters investigate this phenomena under the umbrella of ecological immunology using combined modern technologies of transcriptomics, proteomics and functional immunology with a focus on the primary interaction site. In the first chapter, I describe the key players involved in this host-parasite relationship with a focus on the primary interaction site, the louse-salmon interface, where there are responses by the louse (attack) and the salmon host (defense). Previous research indicated that an early aggressive inflammatory response at the louse-skin interface contributes to resistance in coho salmon; however, there are no data characterizing a site-specific response in resistant (pink and coho) and susceptible (Atlantic, chum) species. Accordingly in Chapter 2, I define site-specific cutaneous responses in Atlantic, pink and chum salmon to establish genetic biomarkers of resistance. Chapter 3 focuses on identification of cellular effectors using

histochemical localization of biomarkers to characterize cellular populations activated at the louse-attachment site, while broadening the gene targets. Our notion of pink salmon as a resistant species is challenged by the common observation of migrating pink salmon supporting large populations of *L. salmonis* in the field. Thus the purpose of chapter 4 was to investigate potential mechanisms to explain variations in susceptibility as a function of life history. Host-parasite relationships are a product of both host and parasite responses; therefore, in chapters 5 and 6, I shift focus to the level of the parasite. In chapter 5 I present the first documented large-scale transcriptomic profiling of *L. salmonis* during feeding on both resistant (coho) and susceptible (Atlantic, sockeye) salmon. This was followed (chapter 6) by describing the proteomic profile of *L. salmonis* secretions after feeding on Atlantic salmon. In the seventh and final chapter, I present my conclusions on the molecular mechanisms for resistance to sea lice and discuss potential applications of this information for future louse control strategies.

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Acknowledgments

There have been many people and funding agencies that were involved in specific parts of this dissertation, and who are acknowledged at the end of chapters 2-6.

In addition to these contributions, I have many personal thanks to give.

To my supervisor Dr. Simon Jones…

For his guidance and patience as I learned how to be a scientist, for being an incredible mentor and role model in the fish of aquatic animal science, and for providing me with so many opportunities to explore fascinating problems on my own.

To my co-supervisor Dr. Ben Koop…

For being a strong mentor and source of motivation.

Dr. Terry Pearson…

For his unique perspectives on most things. Thank you.

Dr. Duane Barker…

For his dedication, assistance and support throughout these last 5 years of my life.

Collaborators Dr's Karina Juhl Rasmussen, Ben Sutherland & Stanko Skugor…

Who have helped me by their generous and priceless assistance and advice, and to whom I am eternally grateful.

The members of the Koop lab…

Especially David and Jong. Thank you for all your help and advice on all things bioinformatics.

The many members of the Aquatic Animal Health Division at the Pacific Biological Station…

Particularly Geoff, Laura, Gina and Bill. I was so lucky to have such amazing and knowledgeable people available for my many, many, many questions.

To Holly and Bob…

Thank you for teaching me how to care for a fish and your unrelenting patience with me and my endless projects.

To Brad Boyce, and the crew of the Pacific Joye fleet, for being inconceivably accommodating on my hunt for parasites and without whose help I would not have been able to do any of this work.

Dedication

To Tim & Duane.
For introducing me to spectacular diversity and remarkable adaptation.
To whose supreme character and unrelenting commitment started me down this long road of marvel.
~
To HE, TG, SM, & TK.
With perpetual grace, unmatched beauty and inconceivable devotion.
The strongest women I know.
For showing me the beauty and strength of true friendship.
~
To JT & Nini.
A left and right hand to help me carry the weight of so much.
A constant and unrelenting well of laughter, love, and my stability in most uncertain times.
~
And lastly, but most certainly, not the least.
~
To mom & dad.
The inspiration for this madness.
Whose belief in me through all my days,
And whose tenacity and love ignited the fire of inquiry at all costs.
For carrying my life with deliberate and steadfast devotion.
Pushing and pulling me to excellence in all things.
Giving me the strength to climb higher, stand taller, and go further.
~

LMB

Chapter 1: General Introduction

1.1 Overview and Objectives

The overall aim for this dissertation was to characterize the molecular and cytological mechanisms of salmonid resistance during infection with the salmon louse, *Lepeophtheirus salmonis*. To address this goal, the host-parasite relationship was investigated at its most fundamental level: the louse-skin interface. By using a comparative approach, responses by both resistant (coho and pink salmon) and susceptible (Atlantic, chum and sockeye) salmon were investigated at the louse-attachment site. Furthermore, reciprocal responses by the louse were assessed as a function of attachment to these different host species. There is inadequate understanding of this host-parasite relationship which is the limiting factor for the development of novel and sustainable strategies for parasite control. Development of parasiticide resistance on a global scale necessitates more permanent and sustainable approaches to sea lice treatment, and in the absence of an available vaccine, knowledge pertaining to resistance mechanisms will offer targets for selective breeding or immunostimulation to augment the natural immunity of susceptible species.

The following hypotheses will be tested and discussed in the following six chapters:

1.) Responses at the salmon skin-sea louse interface determine resistance or susceptibility to infection.

2.) Resistance to sea lice is a costly trait and as such will be under the control of bioenergetic trade-offs that occur as a function of life history

3.) Responses by the sea louse differ depending on the host species, and will reflect the susceptibility status of the host.

1.2 Salmon of the Pacific Northwest

1.2.1 Diverse life histories of Pacific salmon

As an economic product and cultural icon, salmon are key species in a network of social–ecological interactions that characterize diverse North Pacific environments. Ecosystem provisioning, cultural and regulating services ultimately may depend on salmon populations that drive nutrient and energy flows in coastal watersheds (Schindler *et al.* 2003; Cederholm *et al.* 2011). Major divergences within *Oncorhynchus* spp. that gave rise to the Pacific salmon occurred between 20 and 6 Ma before the Pleistocene glaciation and coincident with the reorganization of the Pacific rim topography in the late Miocene (Montgomery 2000). Divergent evolutionary adaptations have resulted in diverse life histories observed in the Pacific salmon including variations in freshwater residence, fecundity, growth rate, age of maturation and size at spawning. These different strategies among *Oncorhynchus* spp. coincide with variations in energetic demand throughout each life history phase (Table 1). Consequently, inter- and intraspecific variations in resistance to disease occur that reflect bioenergetic trade-offs, such as that between growth and immunocompetence (Nordling *et al.* 1998). For example, one might expect salmon with higher maturational age and thus longer residence in the ocean to allocate more resources towards feeding and growth and less towards costly immune responses, which in turn results in higher fecundity such as what is observed for chinook (*Oncorhynchus tshawytscha*; Healey 1991) and sockeye (*Oncorhynchus nerka;* Burgner 1991) salmon. In contrast, coho (*Oncorhynchus kisutch*) and pink (*Oncorhynchus gorbuscha*) salmon exhibit the highest growth rate, lowest fecundity and shortest ocean-residence (Heard 1991; Sandercock 1991), and therefore might have more resources to allocate towards immune defenses. Thus, the different ecological constraints associated with each salmon species are concomitant with different strategies of defense against pathogens. This ecological immunology framework

provides context for divergent immune responses among species of salmon that are genetically similar.

Table 1. Comparison of life history traits among the Pacific salmonids.

Species	Common name	Freshwater residence[a]	Age of maturation[b]	Susceptibility[c]	Size at spawning[d]	Fecundity[e]	Growth rate[f]
Oncorhynchus kisutch	Coho	1-2 yrs	3 yrs	+	3	2	2
Oncorhynchus tshawytscha	Chinook*	1-2 yrs	3 - 8 yrs	++	5	5	5
Oncorhynchus keta	Chum	< 1 month	2 - 6 yrs	+++	4	3	4
Oncorhynchus gorbuscha	Pink	< 1 month	2 yrs	+	1	1	1
Oncorhynchus nerka	Sockeye	3 weeks - 3 yrs	4 yrs	++++	2	4	3

[a] Residence in natal streams or lakes prior to ocean phase (Burgner 1991; Healey 1991; Heard 1991; Salo 1991; Sandercock 1991)
[b] Age of reproductive maturation (Burgner 1991; Healey 1991; Heard 1991; Salo 1991; Sandercock 1991)
[c] Susceptibility status towards *L. salmonis* (+ being the least susceptible, ++++ the most susceptible) (Johnson & Albright 1992a; Jones *et al.* 2007; Jakob *et al.* 2013)
[d-f] Size at spawning, fecundity and growth rate all ranked from 1 (least) to 5 (highest) (Burgner 1991; Healey 1991; Heard 1991; Salo 1991; Sandercock 1991)
[*]For brevity, only the ocean-type of chinook is shown

1.2.2 Salmon aquaculture in British Columbia

The global demand for fish will reach 150-160 million tonnes by 2030, yet capture fisheries can only provide 80-100 million tonnes per year on a sustainable basis (FAO 2014). Without aquaculture, a global shortfall of approximately 50-80 million tonnes of fish and seafood is projected. Already over 50% of the world's consumption of fish is sourced from aquaculture (FAO 2014). The intensification of global aquaculture poses potential significant problems in terms of high environmental impacts such as nutrient loading (Verdegem 2013), negative impacts on wild populations (Hansen & Windsor 2006; Torrissen *et al.* 2013) and dependence on chemotherapeutants to treat disease that often accompany high stocking densities (Roth *et al.* 1993; Grant 2002).

In Canada, the Atlantic salmon is the main species of finfish produced by the aquaculture industry, and accounts for ~ 65% of total production. Projections from Fisheries & Oceans Canada place production to reach 197,000 tonnes and $1.2 billion revenue by 2020. In the midst of this growth, it remains imperative for the industry to minimize potential negative ecological and environmental impacts. One of the largest contributors to economic losses in Atlantic salmon farming is disease caused by bacterial, viral, or parasitic pathogens. In addition to industrial losses, the potential for spread of infectious disease from net-pens to nearby populations of wild salmon has been a major impediment for growth of salmon aquaculture in Canada. One pathogen that receives considerable attention is the parasitic sea louse, *Lepeophtheirus salmonis*.

1.3 The parasitic copepod *Lepeophtheirus salmonis*

One of the most common groups of marine ectoparasites of fish are crustaceans belonging to the order Copepoda (Pike & Wadsworth 1999). There are 445 species belonging to the family Caligidae and of these, 107 belong to the species *Lepeophtheirus* (Hayward *et al.* 2011). The sea louse, *Lepeophtheirus salmonis*, parasitizes anadromous members of *Salmo* and *Oncorhynchus* spp. of the Northern hemisphere (Wootten *et al.* 1982; Tully & Nolan 2002; Boxaspen 2006). Genetically distinct varieties of *L. salmonis* exist in the Pacific and Atlantic Oceans and have co-evolved with their respective hosts for the past 2.5-11 million years (Yazawa *et al.* 2008; Koop *et al.* 2008; Skern-Mauritzen *et al.* 2014). Intensive salmon farming is associated with abnormally heavy *L. salmonis* infections which collectively cost the global aquaculture industry an estimated US$740 million per year (Roth 2015). In addition to economic costs, parasites can transfer to and reduce survival of juvenile wild salmon migrating past net-pens which has been observed in the Atlantic localities (Tully *et al.* 1999; Heuch *et al.* 2005; Costello 2006; Todd 2006; Torrissen *et al.* 2013). The biology of the louse as well as the physiology and immunology of this host-

parasite interaction have been the subject of comprehensive reviews (Pike & Wadsworth 1999; Tully & Nolan 2002; Boxaspen 2006; Wagner *et al.* 2008; Fast 2013).

The lifecycle of *L. salmonis* is direct and has the typical developmental stages of a caligid copepod. Larval nauplii hatch from egg strings into the water column and are free-living for two molts before molting into the infective copepodid stage. The copepodid seeks a suitable host fish by a combination of semiochemicals (Devine *et al.* 2000; Bailey *et al.* 2006; Mordue Luntz & Birkett 2009) and positive phototaxis (Bron *et al.* 1993), at which point it penetrates the epithelium with modified antennae (Bron *et al.* 1993). After another molt into the chalimus stage, the louse is anchored in place by the production of a frontal filament for two successive molts (Hamre *et al.* 2013), until finally the mobile preadult emerges and molts twice more before becoming a sexually mature adult (Hayward *et al.* 2011). Much of the damage to the host caused by *L. salmonis* occurs from attachment and feeding behavior, particularly that associated with the larger and mobile pre-adult and adult developmental stages (Jonsdottir *et al.* 1992) feeding on mucus, epidermis, dermis or subcutaneous tissues as well as blood (Grimnes & Jakobsen 1996). The relative amounts and importance of each of these dietary components have not yet been resolved, although the adult female louse is thought to depend more heavily on blood in its diet (Brandal *et al.* 1976). Beyond physical damage, bioactive compounds (e.g. proteases and prostaglandin E_2 (PGE_2)) are secreted into host tissues during feeding (Fast *et al.* 2003, 2004) potentially resulting in immunomodulation, toxic shock (Fast *et al.* 2004), and other unknown effects. Outcomes of infections vary among species of salmon (Wootten *et al.* 1982; MacKinnon 1998; Wagner *et al.* 2008) and negative effects may include epithelium degradation, tissue necrosis, increased mucus production or altered biochemistry (Grimnes & Jakobsen 1996; Wagner *et al.* 2008), reduced appetite and feed conversion, enhanced susceptibility to secondary

infections (Mustafa *et al.* 2000b), anaemia, reduced circulating lymphocytes, elevated cortisol (Mustafa *et al.* 2000a) osmoregulatory failure and ultimately death. Variations in the intensity of louse-associated pathological effects is dependent on host factors (i.e., species, age) and louse factors (i.e., infection intensity, louse development stage), and contributes to differences in host susceptibility to infection (MacKinnon 1998).

Management of *L. salmonis* on farmed salmon is heavily reliant on pesticide treatment although integrated pest management is becoming more frequently applied (Grant 2002; Torrissen *et al.* 2013) given the occurrence of reduced therapeutant efficacy from the development of pesticide resistance (Treasurer *et al.* 2000; Denholm *et al.* 2002). Understanding the basis for this resistance is currently being investigated (Poley *et al.* 2013; Carmichael *et al.* 2013; Sutherland *et al.* 2014b); however, other methods of parasite management focus on augmenting or improving the immunological response to infection such as immune stimulants or selective breeding (Jones *et al.* 2002; Covello *et al.* 2012; Purcell *et al.* 2013). At present there is little evidence that a vaccine will be effective in controlling salmon louse infections (Roper *et al.* 1995; Raynard *et al.* 2002); however, Atlantic salmon immunized with *L. salmonis* homogenate or exposed to natural infections produce serum antibodies and louse fecundity was consequently reduced after immunization (Grayson *et al.* 1991; Reilly & Mulcahy 1993). Potential targets for vaccine development include genes involved in parasite reproduction or feeding activities. For example, immunization of Atlantic salmon with recombinant *my32*, a novel gene isolated from *Caligus rogercresseyii* thought to be involved in transcriptional regulation during all life-stages, resulted in significant abrogation of second generation louse populations (Carpio *et al.* 2011). Novel vaccine targets are being identified using RNA interference (Dalvin *et al.* 2009; Campbell *et al.* 2009; Eichner *et al.* 2014; Trösße *et al.* 2014; Marr *et al.* 2014), and with genomic resources

continually expanding for both Atlantic salmon and *L. salmonis* (Davidson *et al.* 2010; Yasuike *et al.* 2012), promising areas of research are being pursued, both to better understand the defense responses elicited among salmon species and to explore the biochemistry and kinetics of the louse responses during infection. An important development in the context of ectoparasitic infections is the recognition of the extent to which the salmon skin is immunologically active (reviewed in Esteban 2012). A more thorough understanding of the defense mechanisms elicited by the sea louse at the skin attachment site and the extent to which local and systemic responses are integrated will further development of novel management strategies.

1.4 The cutaneous immune system of teleosts

Teleost epithelium is a non-keratinized, metabolically active first line of defense against the external environment and invading pathogens (Jones 2001; Alvarez-Pellitero 2008; Esteban 2012). The skin epidermis consists of an outer and inner epidermis and transdermal scales, whereas the gill epithelium lacks scales. Cells of the skin include rodlet/filament cells, mucus cells/mucocytes, pavement cells, keratocytes and club cells as well as small numbers of lymphocytes, granular cells and resident macrophages (Esteban 2012). Keratocytes are highly motile, rapidly cover wound surfaces within hours of injury (Bullock *et al.* 2009) and in some species have been shown to internalize bacteria (Asbakk 2001). Mucocytes secrete cutaneous mucus composed of water and glycoprotein conjugates known as mucins that functions to trap and slough off pathogens, but which also contains lysozyme, lectins, pentraxins, complement proteins, proteases, antibacterial peptides and immunoglobulins (Jones 2001; Magnadóttir 2006; Whyte 2007; Alvarez-Pellitero 2008). Mucocyte morphology and quantity in fish skin is a useful tool for bio-monitoring (Ledy *et al.* 2003) and for indicating stress (Pickering & Macey 1977; Iger *et al.* 1995; Vatsos *et al.* 2010) due to the rapid turnover of mucus and the large capacity for

responding to external stimuli (Easy & Ross 2010). Furthermore, altered mucus secretion is an important protective factor during parasite infection (Pottinger *et al.* 1984; Jones 2001; Easy & Ross 2009; Marel *et al.* 2010). Epidermal mucus production and composition are known to influence interactions of monogenean, myxosporean and copepod parasites with the host (Buchmann & Bresciani 1998; Urawa 2000; Fast *et al.* 2002b), further highlighting the importance of this interface as a primary determinant of successful colonization and infection.

Cutaneous immunological reactions can be cellular or humoral and during infection by parasites effective activation of the inflammatory cascade is of critical importance (Jones 2001). Early initiation of the innate immune system enables communication between resident surveillance cells (e.g., macrophages or dendritic cells) and cells of the acquired immune system (e.g., lymphocytes). Phagocytic IgM^+ B-cells are present in teleost skin and are suggested to be an integral component of the cutaneous immune system by acting as both an innate and adaptive cellular responder (Li *et al.* 2006; Zhao *et al.* 2008; Salinas *et al.* 2011). It is thought that teleost B-cells represent the common ancestor prior to the divergence of phagocytic macrophages and immunoglobulin-producing B-cells of higher vertebrates (Li *et al.* 2006). In addition to distinctive B-cells, salmonids are equipped with a teleost-specific immunoglobulin isotype, IgT, mainly found in the gut mucosa but found in low concentrations elsewhere (Zhang *et al.* 2010; Salinas *et al.* 2011). The role of IgT during ectoparasite infection is not yet known; however, IgT concentration was found to be higher in naïve Atlantic salmon compared to fish re-infected with *L. salmonis* although this expression does not appear to have protective effects against infection (Tadiso *et al.* 2011).

During infection or wounding, the response of fish skin has been well documented (Iger & Abraham 1990). In contrast to mammals, wound healing in fish results in thinning of the

epithelium as surrounding keratocytes quickly migrate to cover the open area (Karlsen *et al.* 2012). Because of this mechanism wound healing in fish is generally extremely rapid and can occur within hours. Damage to the epithelium causes proteolytic degradation of the extracellular matrix and generates damage associated molecular patterns or alarmins (DAMPs; e.g., *collagen type-1*) that can activate inflammation (Castillo-Briceño *et al.* 2011). Extracellular matrix degradation and remodeling is a critical component of wound healing, and ECM-degrading matrix metalloproteinases (e.g. MMP9) have been described as having a role in initiation and resolution of inflammation in teleosts (Chadzinska *et al.* 2008). Infection with some fish parasites triggers the production of such molecules signifying that anti-parasitic defenses may rely on the activation of phagocytes by damage- or parasite-associated molecular patterns (Alvarez-Pellitero 2008).

Damage by ectoparasites include direct effects due to the attachment and grazing activities of the parasite (Wagner *et al.* 2008), as well as indirect effects from the stress and inflammatory response by the host as a result of infection (Nolan *et al.* 1999). Teleost epithelium responds strongly to mechanical and chemical stressors (Iger *et al.* 1995; Nolan *et al.* 1999; Ledy *et al.* 2003; Fast *et al.* 2006; Caipang *et al.* 2011). Characteristic changes in the skin include increased necrosis, apoptosis, cell migration (mast cells, mucocytes, leukocytes) and cell proliferation, and as these changes are cortisol-dependent, they are not restricted to the site of attachment during parasite infection (Nolan *et al.* 1999). In addition, stress has been shown to induce significant and prolonged disruption of the mucosal barrier leaving the fish host open to secondary infections. Increased susceptibility to secondary infections with bacteria (Bandilla *et al.* 2006), viruses (Jakob *et al.* 2011) and parasites (Mustafa *et al.* 2000b), have been reported.

Despite innate protective mechanisms, the skin and gills of fish are a common food-source for many ectoparasites (Lindenstrøm *et al.* 2004; Sigh *et al.* 2004a; Gonzalez *et al.* 2007c; b; Forlenza *et al.* 2008; Kania *et al.* 2010; Lü *et al.* 2012; Chettri *et al.* 2014). Variations in disease resistance can be linked to genetic differences in the cutaneous mucosal responses (Magnadóttir 2006). For example, differences in the migration of mucocytes, mast cells, and neutrophils have commonly been observed at the attachment site among host species and between individuals of the same species (Nolan *et al.* 1999; Dezfuli *et al.* 2011).

1.5 Host-parasite relationship between salmon and sea lice

1.5.1 Species-specific responses to sea lice – host defenses against attack

Variation in susceptibility to *L. salmonis* involves both host and parasite factors (MacKinnon 1998). Striking inter- and intraspecific differences in susceptibility to infection have been reported for salmon belonging to *Oncorhynchus* spp. and *Salmo* spp. which are likely a product of reciprocal host and parasite responses. Chalimus survival after experimental exposure was higher on juvenile chum salmon (*O. keta*) compared to juvenile pink salmon (*O. gorbuscha*) (Jones *et al.* 2006, 2007), and the parasite is rapidly shed from juvenile coho salmon (*O. kisutch*) in contrast to persistent infections on Atlantic salmon (*S. salar*) or rainbow trout (*O. mykiss*) (Fast *et al.* 2002a). Moreover, development of the parasite is more rapid and its fecundity higher on Atlantic salmon compared to chinook salmon (*O. tshawytscha*) (Johnson & Albright 1992a). This differential susceptibility is also observed among *Salmo* spp. While the mean abundance of lice declined both on sea trout (*Salmo trutta*) and Atlantic salmon, a higher mean abundance was maintained on the sea trout eight weeks following a laboratory exposure, suggesting greater susceptibility (Dawson *et al.* 1997). In addition, although Atlantic salmon are highly susceptible to infection with *L. salmonis* (Johnson & Albright 1992a; Fast *et al.* 2002a), intraspecific heterogeneity in susceptibility occurs among distinct spawning stocks (Glover *et al.* 2005) and

among full-sib families (Glover *et al.* 2005; Kolstad *et al.* 2005; Gjerde *et al.* 2011) which was also observed for the copepod *Caligus elongatus* among full-sib Atlantic salmon families (Mustafa & MacKinnon 1999).

In the Pacific Ocean, juvenile pink salmon acquire and retain a natural resistance to *L. salmonis* before they reach a mean weight of 1 g, despite inadequate nutrition (Jones *et al.* 2008a; b). In a similar study, low louse density of *L. salmonis* was also observed on pink salmon after a simultaneous exposure of co-habited pink, chum and Atlantic salmon (Sutherland *et al.* 2014a). Interestingly, in this study, chum salmon had the highest louse abundance and this was correlated with a decrease in weight gain.

Collectively these comparative studies imply that among *Oncorhynchus* and *Salmo* spp. there are species that are more resistant to *L. salmonis* infections and this enhanced resistance is species- and age-specific (Jones *et al.* 2007). What is not yet clear is how the relationship of the defense response (inflammation) and the physiological factors of a particular host (e.g., skin physiology), in combination to the attack responses of *L. salmonis* on that host (i.e., immunomodulation), contribute to successful attachment. Fast *et al.*, (2002) found that among resistant and susceptible species there was little difference in blood physiology during attachment (and rejection) of *L. salmonis*. Therefore, species-specific differences in the innate defense parameters at the mucosal/epithelial surface may be a critical determinant in successful infection by *L. salmonis.*

The molecular basis for the differential susceptibility to *L. salmonis* among salmon species is related to defense responses of the skin of the host skin; a vigorous and appropriate response will limit louse survival and severity of the infection. Skin erosion at the site of sea lice attachment is a common clinical sign of infection in Atlantic salmon (Wootten *et al.* 1982) and

this is associated with epithelial cell necrosis, increased apoptosis and a decrease in mucus cell density and inflammatory cell recruitment (Nolan *et al.* 1999). Attachment by the *L. salmonis* frontal filament results in weak to absent inflammatory responses in Atlantic salmon (Johnson & Albright 1992a; Jonsdottir *et al.* 1992), while in coho or pink salmon there is a vigorous response characterized by epithelial hyperplasia and acute inflammation (Johnson & Albright 1992a; Jones *et al.* 2007). This evidence supports the role of an inflammatory response at the attachment site as an indicator of reduced susceptibility to *L. salmonis*. This response is species specific and is most exaggerated in coho and pink salmon, followed by chinook salmon and rainbow trout. Chum salmon, Atlantic salmon and sea trout exhibit the weakest response and lack a natural resistance to infection (Jones 2011). Host responses by sockeye salmon (*O. nerka*) have been largely under-researched, but an infection study by Jakob *et al.* (2013) reported severe epithelial erosion with minimal hyperplasia and limited inflammatory infiltrate around or under attached chalimus stages (20 dpi) similar to what was observed for lab-exposed chinook salmon (Johnson & Albright 1992a). As the infection progressed through to pre-adult and adult stages, gross lesions were observed on the head and body of the fish (Jakob *et al.* 2013), comparable to pathology observed in laboratory infected Atlantic salmon (Jonsdottir *et al.* 1992; Grimnes & Jakobsen 1996). Thus from the available data the response of sockeye salmon to *L. salmonis* is characteristic of susceptible species.

Variation in physiological characteristics and innate immune factors among host species likely plays an important role in how each responds to infection with *L. salmonis*. For example, Fast *et al.* (2002) found that Atlantic salmon, a susceptible species, possessed the thinnest epithelial layer with sparsely distributed mucus cells and low mucus enzymatic activity, compared to coho salmon or rainbow trout. Another interesting species-specific difference is

that, unlike Atlantic salmon or rainbow trout, coho salmon skin contains sacciform cells (Fast *et al.* 2002a). Sacciform cells have been reported in the skin of Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta*) infected with *Ichthyobodo* sp. and in that study, were suggested to represent a cutaneous defense system which secretes protective humoral effectors during ectoparasite infection (Pickering & Macey 1977). Coho salmon are resistant to infection with a number of different ectoparasitic copepods (Gonzalez *et al.* 2000); therefore, any unique physiological characteristics may provide mechanisms for this enhanced resistance. Although the capacity of the skin to mount an aggressive inflammatory response appears correlated with protection against ectoparasitic infection, systemic coordination of the inflammatory cell infiltrate and of humoral factors remains poorly understood.

At the site of feeding there is a complex interaction between the host immune response and the sea louse response. The effects of *L. salmonis* infection on gene expression in the skin was studied in Atlantic salmon throughout an entire infection period (Skugor *et al.* 2008). Damaged skin was associated with signs of T_H2-like responses and a decrease in wound healing ability signified by down-regulation of plasma fibronectin and excessive activity of metalloproteases. Delayed wound healing and restricted inflammation is characteristic of Atlantic salmon and is a result of chronic infection whereby the host fish is unable to expel the parasite (Skugor *et al.* 2008). Atlantic salmon up-regulate levels of pro-inflammatory cytokines (*TNF-α, IL-1β*) in fin tissue during copepodid attachment, but this response is insufficient and fails to reduce parasite burden (Fast *et al.* 2006). Conversely, in juvenile pink salmon, heightened cellular repair activation was observed during initial stages of infection (Sutherland *et al.* 2011). Despite these studies, the mechanisms involved in the variable host responses to *L. salmonis* among salmon species are poorly understood.

1.5.2 The counterattack - Louse feeding responses

The likelihood of successful ectoparasitism is increased by reducing the host awareness of the parasite at the cutaneous surface. Thus ectoparasitic arthropods have evolved to secrete molecules to inhibit cutaneous irritation, anti-hemostatic defenses, and suppress cellular immunity (Wikel 1999). Successful modulation of host-immunity needs to ensure survival of both host and parasite, thus this relationship between host response and parasite modulation can be viewed as a dynamic equilibrium at the primary interaction site (Lehmann 1993).

The skin-louse interface is the site of feeding responses by the louse, including the secretion of immunomodulatory molecules during feeding such as PGE_2 (Mustafa *et al.* 2000a; Fast *et al.* 2004, 2007a), trypsin-like proteases (Johnson *et al.* 2002), and cathepsin B (Cunningham *et al.* 2010). These molecules limit the extent of clotting, increase blood flow, and decrease the host inflammatory responses elicited in an effort to expel the parasite. The variable severity of inflammation elicited by *L. salmonis* among host species may indicate that louse-associated immunomodulation is host-specific, affecting some species more than others, and could contribute to the comparatively limited inflammatory response in Atlantic, chum and sockeye salmon (Johnson & Albright 1992a; Jones *et al.* 2007; Jakob *et al.* 2013).

The actions of bioactive molecules in sea lice secretions appear to affect some species more than others (Fast *et al.* 2007a; Wagner *et al.* 2008; Lewis *et al.* 2014). This may explain the limited inflammatory response at the louse attachment site observed in Atlantic salmon, and the suppressed immune competence of Atlantic salmon towards secondary infections (Mustafa *et al.* 2000a; Fast *et al.* 2006, 2007a). It is unclear whether the resistant species are able to resist or counter the biological effects of the secretions, or if the lice are not secreting the same complement of molecules when feeding on these hosts.

The feeding responses of *L. salmonis* may depend on the host species consistent with the host preference shown by copepodids during initial settlement. Planktonic copepodids determine host suitability by examining the skin with sensory antennules (Bron *et al.* 1993). This host-localization strategy is also observed during monogenean infection, and is thought to be mediated by factors in the host mucus (Buchmann & Bresciani 1998), which has also been demonstrated for *L. salmonis* (Fast *et al.* 2003). Interestingly, coho salmon, which mount a severe inflammatory response to *L. salmonis*, produce skin mucus that does not stimulate *L. salmonis* to release the same secretory response compared to the mucus from the susceptible Atlantic salmon (Fast *et al.* 2003). Firth *et al.* (2000) determined the secretions of *L. salmonis* exposed to Atlantic salmon mucus contains trypsin-like proteases. It is possible that resistant host species do not elicit the same secretory components from sea lice as susceptible species. If true, this would likely contribute to the diverse host responses observed among species.

1.6 Defense and attack strategies of host-parasite relationships.

Parasitism is the most common form of life and a major driver of evolutionary and ecological processes (Price 1980; Windsor 1998; Poulin & Morand 2000). Interspecific co-evolutionary interactions between hosts and parasites have resulted in the development of complex immune responses by the host (Grenfell & Dobson 1995) and methods of avoiding recognition by the parasite (Sitjà-Bobadilla 2008). The primary function of immunity is to recognize and clear pathogenic organisms (Medzhitov & Janeway 1997); however, this system is costly both by the resources required (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Bonneaud *et al.* 2003), as well as by the indiscriminate action of inflammation on host cells that commonly results in immunopathology (Lochmiller & Deerenberg 2000). From an ecological immunology perspective the evolution of host defenses are limited by bioenergetic trade-offs that occur

throughout the lifespan of the host (Sheldon & Verhulst 1996). Investment in an effective immune response is a nutritionally demanding process that occurs at the expense of other traits such as growth or reproduction, and thus these trade-offs are necessary when the cost of immunity is outweighed by the benefits of resource allocation to other physiological needs (Zuk & Stoehr 2002; Bonneaud *et al.* 2003; Tschirren & Richner 2006).

Parasite-host interactions have resulted in the development of three main defense strategies by hosts to prevent or minimize the costs of infection: avoidance, resistance, or tolerance (Price 1980; Baucom & de Roode 2011; Medzhitov *et al.* 2012). Avoidance occurs when hosts adapt their behavior to avoid infection, and is a common strategy against bacterial pathogens (Medzhitov *et al.* 2012). Resistance is accomplished by minimizing the number or extent of parasite infection, is a function of the immune system, and has negative impacts on the parasite. Despite the obvious advantage of resistance, susceptibility to parasites is pervasive, and points to investment of resources by hosts to other costly traits such as growth or reproduction (Reznick 1992; Sheldon & Verhulst 1996; Bonneaud *et al.* 2003). In contrast, host tolerance does not affect parasite fitness, but instead acts to limit the host susceptibility to tissue damage and other fitness costs during infection (Best *et al.* 2008; Svensson & Raberg 2010; Baucom & de Roode 2011; Medzhitov *et al.* 2012). Among host-parasite relationships, resistance and tolerance mechanisms are not mutually exclusive, and depending on the system both strategies may be selected for at different life-stages or in different environments. The optimal investment in immunity among hosts to parasites is responsible for shaping life history and is influenced by environmental factors including risk of parasitism (Tschirren & Richner 2006).

Parasites exploit host resources to increase fitness; therefore, maximizing fitness may be achieved by exploiting the most nutritionally rich host, commonly known as “the well-fed

hypothesis" (Christe *et al.* 2003; Tseng 2006; Tschirren *et al.* 2007). However, high host condition is associated with more advanced immune defenses, and thus it may be advantageous for the parasite to exploit hosts with poorer condition (and nutritional value) but with lower immunity, which is known as "the tasty chick hypothesis" (Sheldon & Verhulst 1996). Parasite distribution among host populations or host species is likely related to differences in nutritional value and immunology status. In the same way that hosts have evolved strategies to prevent parasite colonization or pathology, parasites have evolved strategies to circumvent the immunological response of the host.

1.7 Topics of the dissertation

Despite intensive study over the last few decades, there remain many gaps in our understanding of the host-parasite relationship between sea lice and salmon. In particular, the molecular and cytological interactions between *L. salmonis* and the salmon host at the skin-louse interface are largely unknown. Characterizing responses both by the salmon host and the sea louse using an integrated approach is critical, as response by either player do not happen in a mutually exclusive manner. Furthermore, understanding host-parasite relationships should be in the context of the ecological constraints present during evolutionary adaptations within each specific system. With this in mind, the following data chapters focus on elucidating the molecular basis for resistance and susceptibility among salmonids towards infection with the sea louse by asking these general questions:

1.) How do the different species of salmon respond to infection with *L. salmonis* at the louse-fish interface? What are the potential biomarkers for resistance or susceptibility?
2.) Does the louse respond similarly to different species of salmon?

3.) Is the variable host response a product of species-specific louse responses at the skin-louse interface?

Chapter 2: Comparative defense-associated responses in salmon skin elicited by the ectoparasite *Lepeophtheirus salmonis*.

Adapted from: Laura M. Braden[1], Duane E. Barker[2], Ben F. Koop[1], Simon R. M. Jones[3]. *Comparative Biochemistry & Physiology Part D, Genomics* (2012), **7**(2):100-109

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DEB assisted in analysis and edited the manuscript.

BFK conceived of the study, assisted in interpretation and edited the manuscript.

SRMJ conceived of the study, assisted in interpretation and wrote the manuscript.

2.1 Abstract

Susceptibility among salmonids to the ectoparasite *Lepeophtheirus salmonis* is related to inflammatory reactions at the site of parasite attachment. Salmon from two susceptible (*Salmo salar*, *Oncorhynchus keta*) and one resistant (*Oncorhynchus gorbuscha*) species were exposed to adult *L. salmonis*. After 24 and 48 h, skin samples directly below the attachment site and at non-attachment sites were assessed for transcriptomic profiles of select innate defense genes. Abrasion of the skin permitted comparisons between abrasion-associated injury and louse-associated injury. Infection responses were consistently higher than those caused by abrasion. Temporal patterns of expression were evident in all species for the transcription factor *CCAAT/enhancer-binding protein β* (*C/EBP-β*), the cytokine *interleukin-6* (*IL-6*) and the enzyme *prostaglandin D synthase* (*PGDS*) at attachment sites. *O. gorbuscha* was the highest responder in a number of genes while there was an absence of *C-reactive protein* (*CRP*) gene expression in *S. salar* and *O. keta*, indicating an altered acute-phase response. Moreover, *O. keta* displayed distinct *interleukin-8* (*IL-8*) and *serum amyloid P* (*SAP*) responses. Impaired genetic expression or over-expression in these pathways may be evidence for species-specific pathways of susceptibility to the parasite. At *L. salmonis* attachment sites, reduced expression compared to non-attachment sites was observed for *C/EBP-β* (*S. salar*), *CRP* (*S. salar*), *SAP* (*S. salar, O. gorbuscha, O. keta*), *PGDS* (*S. salar, O. gorbuscha, O. keta*), and *major histocompatibility class II* (*MH class II, S. salar*), suggesting local immunodepression.

2.2 Introduction

The salmon louse *Lepeophtheirus salmonis* is one of the most economically important metazoan parasites in salmonid aquaculture (Todd 2006). Control of *L. salmonis* infestations incurs annual costs of $286 million US to salmonid aquaculture operations in the northern hemisphere (Costello 2009). The parasite also occurs on adult Pacific salmon caught in the open ocean (Nagasawa & Takami 1993), as well as in coastal waters of British Columbia (BC) (Beamish *et al.* 2005). Genetically distinct forms of *L. salmonis* occur in the Atlantic and Pacific Oceans (Yazawa *et al.* 2008). Detailed reviews outline the ecology, lifecycle and pathological effects of *L. salmonis* infestations (Pike & Wadsworth 1999; Tully & Nolan 2002; Boxaspen 2006; Costello 2006; Jones & Hargreaves 2007). Susceptibility to infection and its consequences are affected by host size and the post-smolt salmonid is most vulnerable to infection (Finstad *et al.* 2000; Jones *et al.* 2008a). The parasite has been reported on pink and chum (*O. keta*) juveniles shortly after entry into near-shore waters in the vicinity of salmon aquaculture (Morton *et al.* 2004). Although there is a concern that intensively cultured salmonids in net-pens may act as reservoirs of sea lice, more information is needed about the relative risks posed by *L. salmonis* among species of migrating salmon. There is a spectrum of susceptibility to *L. salmonis* among juvenile salmon belonging to different species. The parasites are rapidly rejected from pink and coho (*Oncorhynchus kisutch*) salmon whereas a prolonged retention of infection occurs on Atlantic (*Salmo salar*) and chum salmon (Johnson & Albright 1992b; Jones *et al.* 2007). A component of this variability among host species involves the extent to which an inflammatory response is elicited by *L. salmonis* attachment and feeding activities (Johnson & Albright 1992b; Fast *et al.* 2002a; Jones *et al.* 2007). Epidermal hyperplasia and associated inflammation of underlying tissues assist in the rejection of the parasite and are observed as early as one day post-infection in coho salmon but are reduced or absent in Atlantic salmon (Johnson & Albright

1992b). Recently, transcriptomic analysis revealed a significant size-dependent shift toward immuno-competence among pink salmon following exposure to *L. salmonis* at 0.3, 0.7 and 2.4 g (Sutherland *et al.* 2011). Susceptibility also appears related to the suitability of various host species for development of the louse. For example *L. salmonis* matures at different rates depending on the species of host (Johnson 1993; Fast *et al.* 2002a) and the parasite infects but fails to fully develop on sticklebacks (*Gasterosteus aculeatus*) (Jones *et al.* 2006).

Host sites known to be associated with responses to *L. salmonis* include skin, head kidney, spleen and liver (Fast *et al.* 2007b; Skugor *et al.* 2008). Skugor *et al*. (2008) found transcriptomic evidence for local and systemic mixed inflammatory responses in Atlantic salmon following exposure to copepodids of the Atlantic form of *L. salmonis*. In addition, the expression of pro-inflammatory genes to immature stages of *L. salmonis* differs among host species (Jones *et al.* 2007). To date, skin-associated differences among susceptible and refractory salmonids in response to adult *L. salmonis* have not been investigated. Early innate responses in the skin are expected to determine successful infection or expulsion of *L. salmonis*; therefore, characterizing the interactions at this level is crucial for understanding the molecular basis of resistance.

The aim of this study was to investigate the early transcriptomic responses in the skin of susceptible and resistant salmonids to adults of the Pacific form of *L. salmonis*. We demonstrate the first evidence for temporal expression of key early defense and inflammatory mediators both at the site of fish-louse interaction and at non-interaction sites in the skin of resistant and susceptible hosts. In addition, we differentiate between the responses to infection and mechanical trauma.

2.3 Methods

2.3.1 Fish

Pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon were obtained as swim-up fry from the Quinsam River and Nanaimo River Hatcheries respectively on Vancouver Island, British Columbia. They were reared in equal parts of dechlorinated city water and sand-filtered seawater at the Pacific Biological Station (PBS), Nanaimo. Atlantic salmon (*S. salar*) smolts were obtained from freshwater Marine Harvest Canada Hatcheries on Vancouver Island, British Columbia. The 1800 L tanks were supplied with flow through sand-filtered seawater at 8–10 °C and 33 psu and maintained under a 12 h light:12 h dark photoperiod. All fish were hand fed a commercial diet (EWOS) once daily at approximately 1% mean biomass.

2.3.2 *L. salmonis* collection

Female adult *L. salmonis* were obtained from Atlantic salmon cultured in net-pens located in the Broughton Archipelago, British Columbia. During fish harvest, lice were gently removed with forceps and placed in cold aerated seawater. Lice were transported to PBS and kept overnight in aerated seawater (8–10 °C) until experimental infection the following day. Only lice that were exhibiting active swimming and attachment behavior were used for the infection

2.3.3 Infection trials and mechanical abrasion

Pink, Atlantic and chum salmon were each allowed to acclimate in experimental 300 L tanks for seven days before parasite challenges in three populations (lice-infected, mechanically-abraded, and non- treated fish) of 30 fish per tank, for a total of 9 tanks. Fish were size-matched with a mean fork-length of 21.5 cm. Feed was withheld for 24 h prior to all treatments. For exposure to sea lice, groups of 10 fish were placed in 50 L portable tanks containing 0.5 mg/L Aquacalm (metomidate hydrochloride, Syndel) in aerated seawater. Fifty adult *L. salmonis* were added to the tank and permitted to attach for 20 min as preliminary data concluded a density of 5 lice/fish

results in retention of at least 3 lice/fish after 48h. Each fish was then gently returned to its respective tank and monitored. For mechanical injury, all fish of each species were individually placed onto a wet towel and the lateral flank skin directly anterior to the anus and below the lateral line was subjected to a superficial scratch using sterile forceps. The size and depth of the injury were kept constant to disturb the scales and mucus layer over approximately 15mm^2. Control fish belonging to each species were also subjected to 0.5 mg/L Aquacalm in aerated seawater but were not infected or abraded. The length of exposure was kept consistent among all treatments and species.

2.3.4 Sampling

At 24 h and 48 h post-infection, 15 fish from each group of *L. salmonis*-infected, mechanically-abraded and control fish were sampled. Fish were rapidly euthanized by immersion in 200 mg/L tricaine methane-sulphonate (TMS, Sigma Aldrich) in seawater. Infected fish had a minimum of 3 lice and for each, plugs (5mm^2) consisting of skin and underlying tissues were immediately removed from three sites of louse attachment and from three uniform non-attachment sites using an Acuderm biopsy punch (Dormer Labs). Three tissue plugs from the sites of mechanical injury and three from the same sites on each control fish were also extracted. The three skin plugs from each fish in all exposure or abrasion categories were immediately pooled, snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

2.3.5 Isolation of RNA and cDNA synthesis

Skin from three pooled tissue plugs of 8 fish per group was aseptically separated from underlying muscle. Total RNA was extracted from approximately 10 mg of each skin pool using an RNeasy RNA extraction kit (Qiagen), a sonicator (Qiagen) and on-column DNase digestion (Qiagen) according to manufacturer's instructions. Total RNA was eluted in 50 μL of RNase-free

water and quantified using the NanoDrop-1000 Spectrophotometer (Thermo-Fisher Scientific). A second DNase step was performed on all RNA samples using Turbo DNA-free kit according to manufacturer's instructions (Ambion). All extracted RNA samples had an A260/280 ratio in water of 1.8–2.0. One µg total RNA was reverse-transcribed into cDNA with random hexamers using a High Capacity cDNA synthesis kit (Applied Biosystems) following manufacturer's instructions in a final volume of 40 µL. Following first strand synthesis, samples were stored at -20°C until use in real-time PCR assays.

2.3.6 Quantitative real-time PCR

The choice of gene targets was based on functional gene pathways involved in inflammation and defenses of the host and on previous microarray work (Skugor *et al.* 2008; Sutherland *et al.* 2011). Oligonucleotide primer sequences were based on previously published Atlantic salmon or rainbow trout primers, and on sequences found to be conserved between Atlantic salmon and rainbow trout using Primer 3 software (Rozen & Skaletsky 2000). As there are two isoforms of IL-1β and TNF-α, primers were designed in the variable regions between IL-1β-1 and IL-1β-2 and between TNF-α1 and TNF- α2 (Fast *et al.* 2007b). Forward and reverse primer sequences, accession numbers, and annealing temperatures are listed in Table 15. The target genes were *interleukin-1β* (*IL-1β*), *IL-6*, *IL-8*, *IL-10*, *major histocompatibility* (MH) *class II β chain* (*MH class II*), *C-reactive protein* (*CRP*), *serum amyloid P* (*SAP*), *inducible nitric oxide synthase* (*iNOS*), *nuclear factor κ-B* (*NF-κB*), *CCAAT/enhancer binding protein-β* (*C/ EBP-β*), *matrix metalloproteinase 13* (*MMP13*), *cyclooxygenase-2* (*COX-2*), *prostaglandin D synthase* (*PGDS*), and *tumor necrosis factor-α* (*TNF-α*). The reference genes *elongation factor 1-A* (*EF1-A*), *18S ribosomal subunit* (*18S*), *eukaryotic translation initiation factor 3 subunit 6* (*ETIF3-6*), *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) and *β-actin* (Olsvik *et al.* 2005) were

assessed for stability using geNorm (Vandesompele *et al.* 2002) and used to normalize the results. Quantitative RT-PCR was performed using a Stratagene MX3000P real-time PCR system and Brilliant II SYBR qPCR chemistry according to manufacturer's instructions (Agilent). A reaction volume of 12.5 µL was added to each well of a 96-well plate (Axygen) that contained 6.75 µL Brilliant SYBR qPCR master mix (Agilent), 0.375 µL ROX reference dye (Agilent), 0.75 µL of each forward and reverse primer (0.3 µM) and 1 µL of the first strand cDNA. The final volume was achieved with RNase/DNase free water (Gibco). Wells were performed in triplicate. Non-RT controls were validated for each sample before performing the assays and a no-template control was included on every plate. PCR efficiency for each primer pair was determined from ten-fold serial dilutions of pooled cDNA from control fish using the equation $E=10^{(-1/slope)}$ (Pfaffl 2001). For targets with low quantities, column-purified PCR products were serially diluted as template to achieve a linear regression. Efficiencies from multiple runs for a single primer pair were accepted within 10% of each other. The PCR profile was as follows: initial 10 min denaturation step at 95°C, followed by 40 cycles of denaturation (30 s at 95°C), annealing (xx°C; 60 s) and extension (30 s at 72°C), and a final extension step of 72°C for 5 min. The cycling runs were terminated by a melting curve analysis to ensure single product amplification where the fluorescence was continually measured during a temperature increase from 55°C to 95°C. The fluorescence threshold was set automatically according to MX3000P algorithms.

2.3.7 Data and statistical analysis

The expression data of selected genes were transformed using algorithms outlined in Hellemans *et al.* (2007) that accounts for gene-specific run-to-run variability and multiple reference gene normalization. Gene-specific efficiencies were acceptable at 100±10% (doubling of the product

between every cycle in the log-linear phase). Relative quantities ($RQs=E^{\Delta Ct}$; $\Delta Ct=Ct(ref)-Ct(treatment)$) for every sample were determined using a gene-specific efficiency. Normalized RQs (NRQs) were then calculated with a sample-specific normalization factor using multiple reference genes. Finally, calibrated NRQs (CNRQs) were calculated using a gene-specific calibration factor that minimized variation among technical replicates. Statistical analyses were performed using SigmaStat software (version 11.0). Differences were considered significant at $p < 0.05$. All values shown are means of individuals for each sampling time ± SEM. Two-way analysis of variance was used to determine significance between treatments and species at 24 and 48 h sampling times. If ANOVA was significant, Holm–Sidak post-hoc tests were used to determine the significance of pairwise differences. In all tests, differences were considered statistically significant at $p < 0.05$.

2.4 Results

2.4.1 Reference genes

Three of the five genes screened as internal references were most stably expressed following validation using geNorm (Vandesompele *et al.* 2002). The geometric mean expression of *18S rDNA*, *EF-1A* and *ETIF3* was calculated to produce a sample-specific normalization factor.

2.4.2 Gene expression after mechanical abrasion

Relative to non-abraded controls, local skin abrasion was associated with the significant temporal expression change (i.e., from 24→48 h) of several transcription factors and defense response genes that were host specific. For pink salmon, the expression of *NF-κB* (Figure 1, for statistical significance refer to figure), *TNF-α* (Figure 2) and *IL-10* (Figure 5) was elevated at 48 h. In contrast, at 48 h the expression of *CRP* (Figure 3), *iNOS* (Figure 4) and *MMP13* (Figure 4) was decreased. Among Atlantic salmon only the expression of *COX-2* changed over time (Figure

5). Among chum salmon, the expression of *IL-10* (Figure 5) and *MH II* (Figure 6) was elevated by 48 h whereas that of *SAP* (Figure 3) was depressed.

2.4.3 Gene expression in *L. salmonis* infections

Gene expression associated with *L. salmonis* attachment was analyzed in two ways. For each species, and at each time point, expression was compared between attachment site and non-attachment site skin. Secondly, at both attachment and non-attachment sites, expression between 24 h and 48 h was compared.

2.4.3.1 Pink salmon

Significantly higher expression was observed at louse-attached sites after 24 h and 48 h in *C/EBP-β* (Figure 1), *IL-1β* (Figure 2), *TNF-α* (Figure 2), *IL-8* (Figure 2), *MMP13* (Figure 4) and *COX-2* (Figure 5). Significantly higher expression at attachment sites was also observed for *IL-6* (Figure 2), *CRP* (Figure 3), and *MH class II* (Figure 6) but only at 24 h. Expression was significantly higher at non-attachment sites for *SAP* at 24 h and 48 h (Figure 3), and at 48 h in *PGDS* (Figure 5). Temporal effects (i.e. from 24→48 h) were detected at attachment sites and at non-attached sites. Expression of *NF-κB* (Figure 1) was elevated after 48 h both at attachment sites and at non-attached sites. Conversely, expression of *CRP* (Figure 3) was decreased after 48 h at attached and non-attached sites. *TNF-α* (Figure 2) was only elevated after 48 h at non-attached sites, whereas expression of *COX-2* (Figure 5), *PGDS* (Figure 5) and *MH class II* (Figure 6) were all reduced after 48 h at attachment sites. Finally, expression of *MMP13* (Figure 4) was elevated at attachment sites only after 48 h.

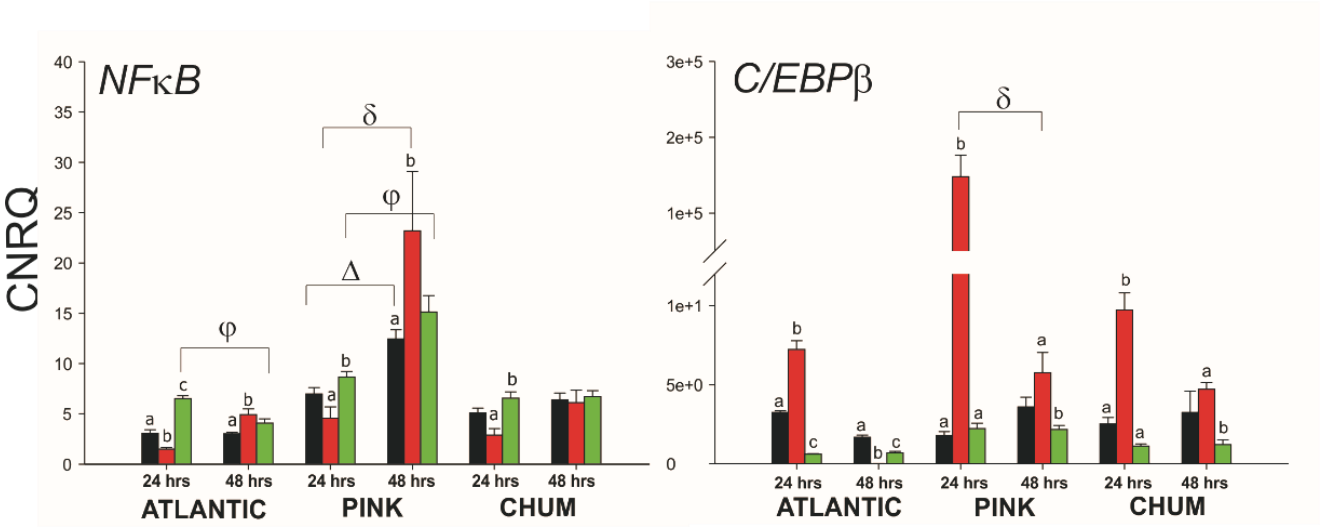

Figure 1. RT-qPCR expression profile of *NF-κB* and *C/EBPβ* in the skin of Atlantic (*S. salar*), pink (*O. gorbuscha*) and chum (*O. keta*) salmon following physical abrasion or infection with *L. salmonis*. Samples were taken at 24 and 48 h following physical abrasion (black bars) and infection with adult *L. salmonis* (red and green bars). Skin from *L. salmonis* attachment sites (red) and non-attachment sites (green) were sampled from the same infected fish. Bars represent the mean ± SEM (n = 8) calibrated normalized relative quantities (CRNQs). Differences between species and time were determined using a two-way ANOVA ($P<0.05$). Post-hoc Holm-Sidak comparisons were performed to determine which groups differed ($P<0.05$). Lowercase letters represent groups significantly different from each other within species and sampling times. Significant temporal differences within species are joined by a bracket and are indicated by Greek letters: physical abrasion (Δ), lice-attachment (δ) and non-attachments sites (φ).

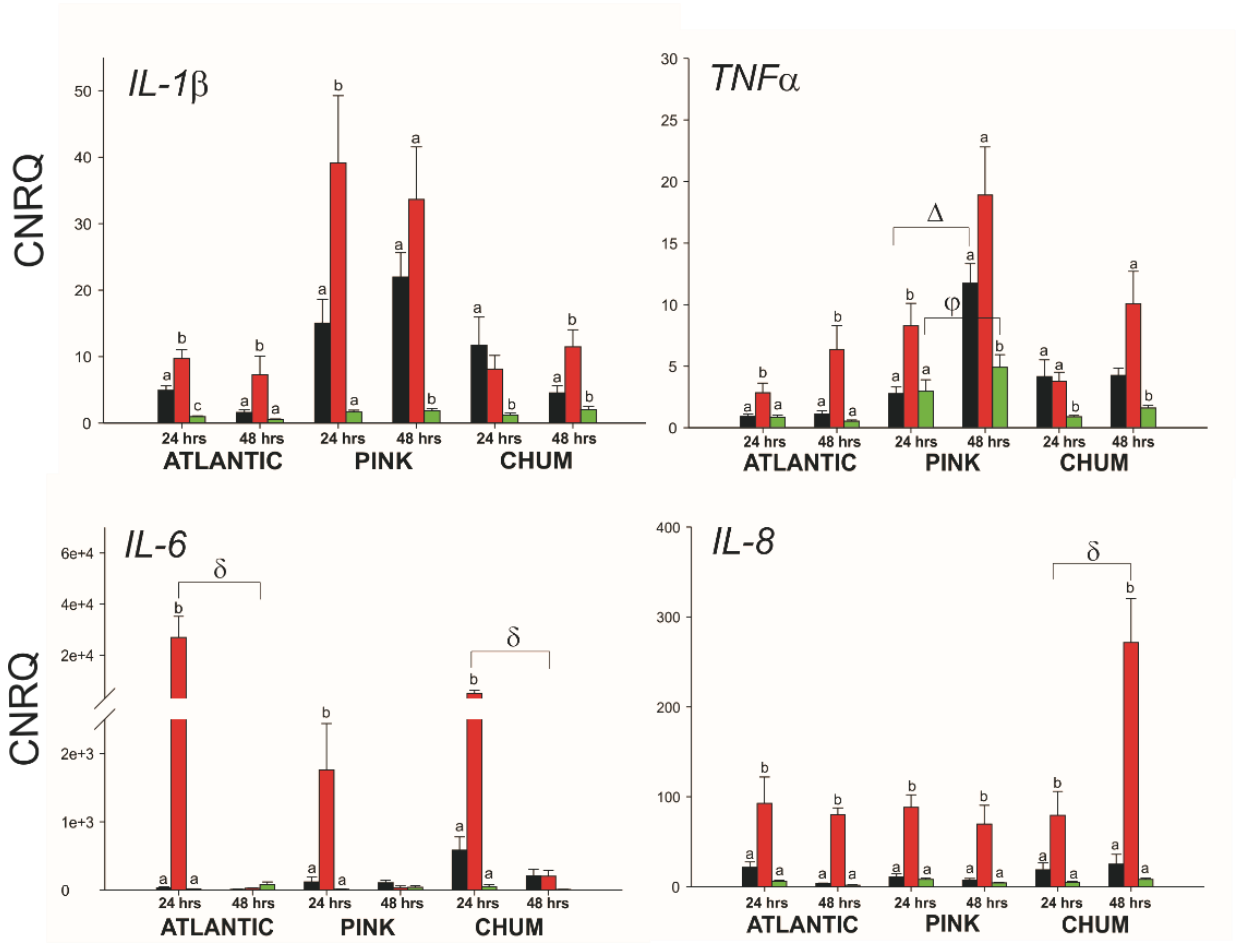

Figure 2. RT-qPCR expression profile of pro-inflammatory cytokines in the skin of Atlantic (*S. salar*), pink (*O. gorbuscha*) and chum (*O. keta*) salmon following physical abrasion or infection with *L. salmonis*.

Samples were taken at 24 and 48 h following physical abrasion (black bars) and infection with adult *L. salmonis* (red and green bars). Skin from *L. salmonis* attachment sites (red) and non-attachment sites (green) were sampled from the same infected fish. Bars represent the mean ± SEM (n = 8) calibrated normalized relative quantities (CRNQs). Differences between species and time were determined using a two-way ANOVA ($P<0.05$). Post-hoc Holm-Sidak comparisons were performed to determine which groups differed ($P<0.05$). Lowercase letters represent groups significantly different from each other within species and sampling times. Significant temporal differences within species are joined by a bracket and are indicated by Greek letters: physical abrasion (Δ), lice-attachment (δ) and non-attachments sites (φ).

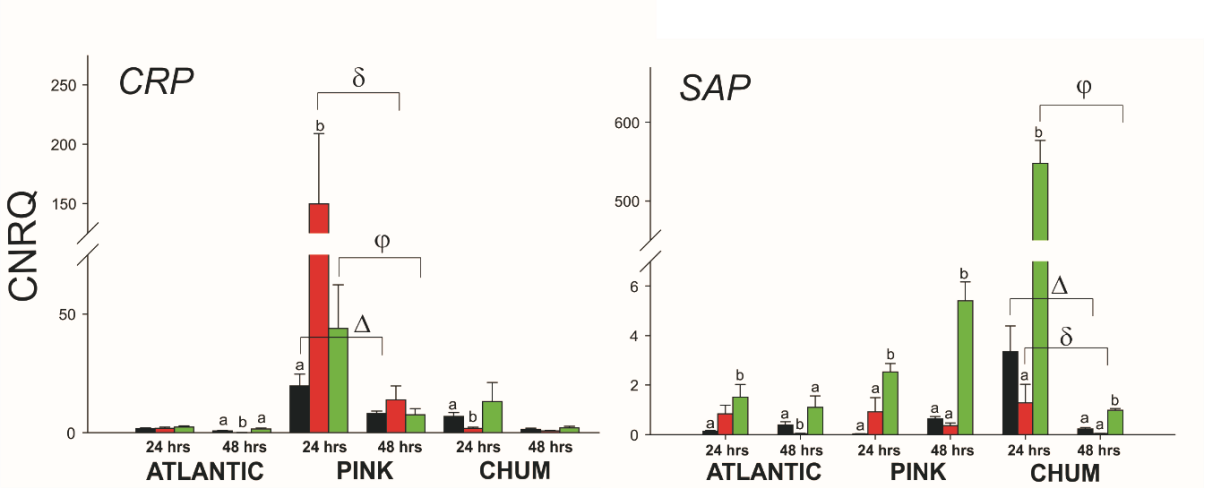

Figure 3. RT-qPCR expression profile of acute-phase proteins in the skin of Atlantic (*S. salar*), pink (*O. gorbuscha*) and chum (*O. keta*) salmon following physical abrasion or infection with *L. salmonis*. Samples were taken at 24 and 48 h following physical abrasion (black bars) and infection with adult *L. salmonis* (red and green bars). Skin from *L. salmonis* attachment sites (red) and non-attachment sites (green) were sampled from the same infected fish. Bars represent the mean ± SEM (n = 8) calibrated normalized relative quantities (CRNQs). Differences between species and time were determined using a two-way ANOVA ($P<0.05$). Post-hoc Holm-Sidak comparisons were performed to determine which groups differed ($P<0.05$). Lowercase letters represent groups significantly different from each other within species and sampling times. Significant temporal differences within species are joined by a bracket and are indicated by Greek letters: physical abrasion (Δ), lice-attachment (δ) and non-attachments sites (φ).

2.4.3.2 Atlantic salmon

Significantly higher expression was observed at attachment sites after 24 and 48 h in *IL-1β* (Figure 2), *TNF-α* (Figure 2), *IL-8* (Figure 2), *MMP13* (Figure 4), and *COX-2* (Figure 5). Higher expression at attachment sites was also observed in *C/EBP-β* (Figure 1) and *IL-6* (Figure 2) but only after 24 h. Significant elevation in non-attached sites was observed after 48 h in *C/EBP-β* (Figure 1), *CRP* (Figure 3), *SAP* (Figure 3), *PGDS* (Figure 5), and *MH class II* (Figure 6). Expression of *IL-10* was significantly higher after 24 h (Figure 5). Temporal effects were detected at attachment and non-attached sites. Decreasing expression after 48 h was observed in *NF-κB* (Figure 1) at non-attached sites and *IL-6* (Figure 2) at attached sites. Expression of *iNOS* (Figure 4) was elevated after 48 h at attachment sites.

2.4.3.3 Chum salmon

Significantly higher expression at attachment sites after 24 and 48 h was detected in *C/EBP-β* (Figure 1), *TNF-α* (Figure 2), *IL-8* (Figure 2) and *MMP13* (Figure 4). *Interleukin-6* expression was higher at louse-attachment sites only after 24 h (Figure 2); whereas *IL-1β* (Figure 2) and *COX-2* (Figure 5) expression was only significantly higher than non-attached sites after 48 h. Significantly higher expression at non-attached sites was observed 24 h in *NF-κB* (Figure 1) and *IL-1β* (Figure 2), and after 48 h in *PGDS* (Figure 5). Expression of *SAP* was significantly higher in non-attached sites at both 24 and 48 h (Figure 3). Temporal effects were observed at attachment and non-attached sites. Decreasing expression was observed at both sites in *SAP* (Figure 3), and at attached sites only in *IL-6* (Figure 2) and *PGDS* (Figure 5). Expression of *IL-8* was elevated after 48 h at attached sites (Figure 2).

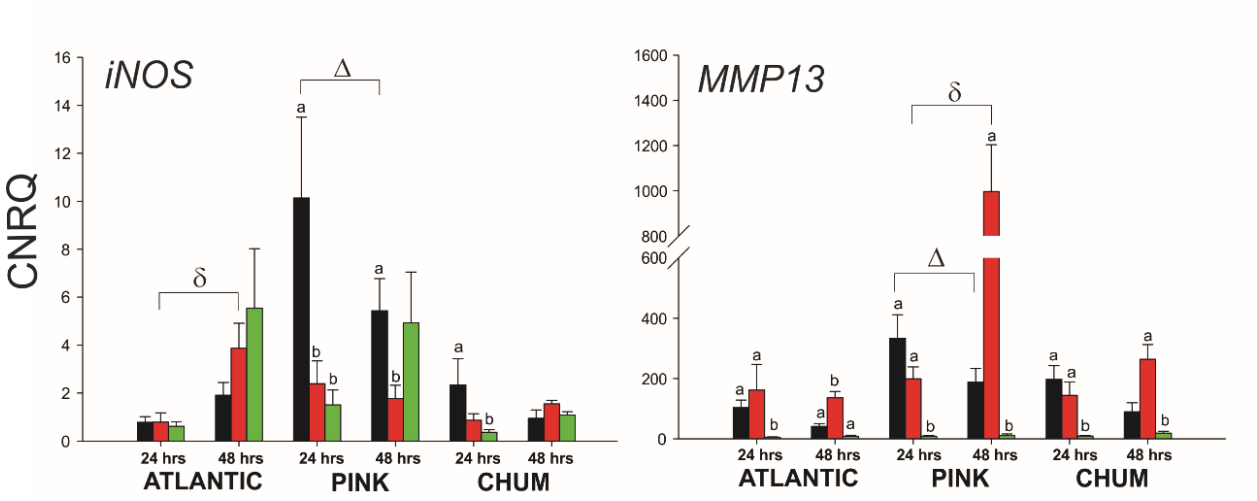

Figure 4. RT-qPCR expression profile of *iNOS* and *MMP13* in the skin of Atlantic (*S. salar*), pink (*O. gorbuscha*) and chum (*O. keta*) salmon following physical abrasion or infection with *L. salmonis*. Samples were taken at 24 and 48 h following physical abrasion (black bars) and infection with adult *L. salmonis* (red and green bars). Skin from *L. salmonis* attachment sites (red) and non-attachment sites (green) were sampled from the same infected fish. Bars represent the mean ± SEM (n = 8) calibrated normalized relative quantities (CRNQs). Differences between species and time were determined using a two-way ANOVA ($P<0.05$). Post-hoc Holm-Sidak comparisons were performed to determine which groups differed ($P<0.05$). Lowercase letters represent groups significantly different from each other within species and sampling times. Significant temporal differences within species are joined by a bracket and are indicated by Greek letters: physical abrasion (Δ), lice-attachment (δ) and non-attachments sites (φ).

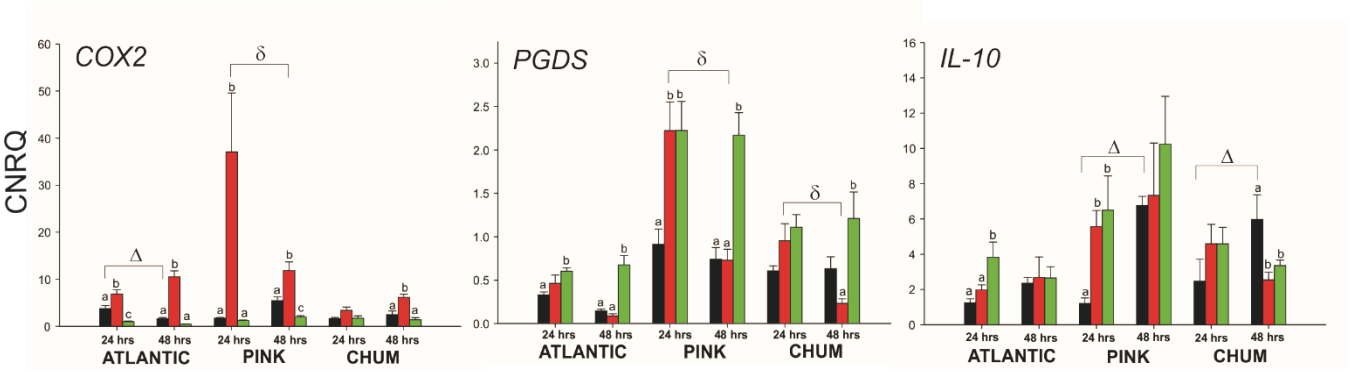

Figure 5. RT-qPCR expression profile of *COX-2, PGDS* and *IL-10* in the skin of Atlantic (*S. salar*), pink (*O. gorbuscha*) and chum (*O. keta*) salmon following physical abrasion or infection with *L. salmonis*.

Samples were taken at 24 and 48 h following physical abrasion (black bars) and infection with adult *L. salmonis* (red and green bars). Skin from *L. salmonis* attachment sites (red) and non-attachment sites (green) were sampled from the same infected fish. Bars represent the mean ± SEM (n = 8) calibrated normalized relative quantities (CRNQs). Differences between species and time were determined using a two-way ANOVA ($P<0.05$). Post-hoc Holm-Sidak comparisons were performed to determine which groups differed ($P<0.05$). Lowercase letters represent groups significantly different from each other within species and sampling times. Significant temporal differences within species are joined by a bracket and are indicated by Greek letters: physical abrasion (Δ), lice-attachment (δ) and non-attachments sites (φ).

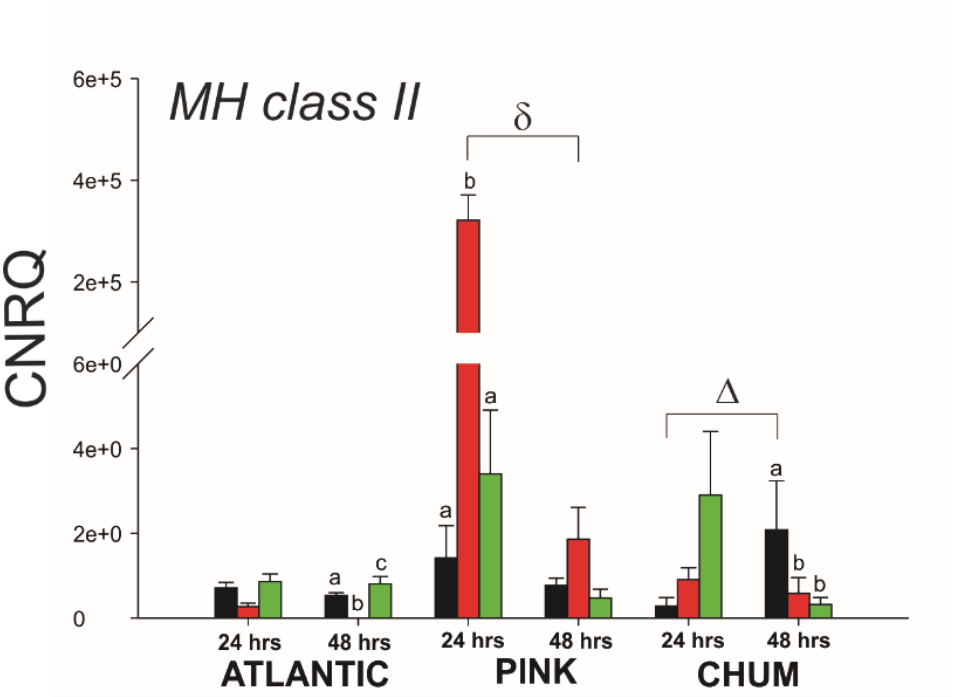

Figure 6. RT-qPCR expression profile of *MH class II* in the skin of Atlantic (*S. salar*), pink (*O. gorbuscha*) and chum (*O. keta*) salmon following physical abrasion or infection with *L. salmonis*. Samples were taken at 24 and 48 h following physical abrasion (black bars) and infection with adult *L. salmonis* (red and green bars). Skin from *L. salmonis* attachment sites (red) and non-attachment sites (green) were sampled from the same infected fish. Bars represent the mean ± SEM (n = 8) calibrated normalized relative quantities (CRNQs). Differences between species and time were determined using a two-way ANOVA ($P<0.05$). Post-hoc Holm-Sidak comparisons were performed to determine which groups differed ($P<0.05$). Lowercase letters represent groups significantly different from each other within species and sampling times. Significant temporal differences within species are joined by a bracket and are indicated by Greek letters: physical abrasion (Δ), lice-attachment (δ) and non-attachments sites (φ).

2.4.4 Relative expression among Atlantic, pink and chum salmon

Species-specific differences in expression were observed during *L. salmonis* infections at local and systemic sites (Figures 1–6). In uninfected controls, basal expression of *MH class II* transcripts in Atlantic salmon was over 1000-times higher than in pink or chum salmon (data not shown). In addition, at louse-attachment sites in pink salmon, the CNRQs of transcripts of *C/EBP-β, IL-1β, MMP13, CRP, COX-2*, and *MH class II* ranged from three (*COX-2*) to over five orders of magnitude (*MH class II*) higher than in chum or Atlantic salmon. Similarly, *IL-6* transcripts in Atlantic and chum salmon were over 10 times higher than in pink salmon at louse-attachment sites, while in chum salmon, *IL-8* transcripts at louse-attachment sites and *SAP* transcripts at non-attachment sites were three times higher than in Atlantic or pink salmon.

2.5 Discussion

This study compared the early transcriptomic responses of immune- and defense-associated transcripts at the site of *L. salmonis* infection on the skin of salmon belonging to three species. The divergent responses of susceptible salmon (Atlantic, *S. salar*; chum, *O. keta*) and those of a refractory salmon (pink, *O. gorbuscha*) provided evidence for early mechanisms associated with resistance and susceptibility to *L. salmonis*. In addition, some genes were preferentially activated at louse-attachment sites at levels higher than were observed following tissue injury, implying louse-associated activation. A consequence of the early sampling times was the difficulty controlling for confounding effects due to possible parasite movement. It was assumed that attachment-site skin samples were inhabited by a louse for precisely the time allotted; however, *L. salmonis* may move on the host and among hosts (Ritchie 1997; Pike & Wadsworth 1999). The possibility of any effect of louse movement was minimized by designing parasite challenges to allow lice to attach for an extended period of time, thus finding the most “suitable” host.

Additionally, three replicate attachment and non-attachment site skin samples were taken from every individual to minimize this source of variability.

2.5.1 Transcription factors NF-κB and C/EBP-β

Up-regulation of *NF-κB* was observed in *L. salmonis*-infected and physically-abraded skin. In louse-infected fish after 24 h, expression was higher at non-attached sites in all species. As both attached and non-attached skin samples were removed from *L. salmonis*-infected fish, systemic responses were expected at both sites, which was not apparent after 24 h. A higher response at louse-attached skin sites suggests louse-associated activation of expression. Conversely, a higher response detected in non-attached sites, suggests either i.) louse-associated reduced expression at attachment sites, or ii.) higher expression limited to distal skin sites only. Higher genetic expression of *NF-κB* at non-attached sites 24 h observed in this study implies a suppressive effect at *L. salmonis*-attachment sites. The expression profile of *C/EBP-β* was distinct from that of *NF-κB* by being associated with early infection in all species. These transcription factors are often synergistic in inflammatory activation; therefore, the differences observed during *L. salmonis* infection implied the presence of a different activational pathway for C/EBP-β. Indeed, nuclear translocation of NF-κB requires release of an inhibitor protein IκB (Didonato *et al.* 1997) while C/EBP-β must be phosphorylated by mitogen-activated protein kinase (MAPK) to become activated (Wynne *et al.* 2008). Despite the temporal similarities in expression, the louse attached site expression of the *C/EBP-β* transcript was over 4 orders of magnitude higher in pink salmon compared with Atlantic or chum salmon. Up-regulation of *C/EBP-β* suggests that all three species are attempting to mount an acute-phase response (Bayne & Gerwick 2001; Agrawal *et al.* 2001); however, the presence of *L. salmonis* may partially block activation of C/EBP-β by MAPK in susceptible species, explaining the observed differential acute-phase responses. NF-κB

and C/EBP-β are phylogenetically conserved transcription factors that are critical for the production of pro-inflammatory genes such as the cytokines IL-1β, IL-6, TNF-α and the enzymes iNOS and COX-2 (Hatada *et al.* 2000; Saeij *et al.* 2003; Wullaert *et al.* 2011). Activation of pro-inflammatory cytokines mediates the synthesis of acute-phase proteins (APPs) and the induction of the acute-phase response (APR) (Agrawal *et al.* 2001). NF-κB and C/EBP-β work synergistically to coordinate early inflammation, as binding sequences are often adjacent or over-lapping (Rebl *et al.* 2011). For example, in rainbow trout (*Oncorhynchus* mykiss), the acute-phase protein serum amyloid A (SAA) promoter has two consensus sites for C/EBP-β and an attachment site for NF-κB (Rebl *et al.* 2011).

2.5.2 Pro-inflammatory mediators and the acute phase response

Expression of *IL-1β*, *IL-6*, *IL-8* and *TNF-α* was clearly associated with louse-attachment in all three species. IL-1β and TNF-α are important inflammatory mediators involved in the teleost immune response to many different pathogens including parasites (Lindenstrøm *et al.* 2003; Sigh *et al.* 2004a). Sigh *et al.* (2004) found that infection with the ciliate *Ichthyophthirius multifiliis* leads to up-regulation of *IL-1β* and *TNF-α* in the skin of trout (*O. mykiss*) Similarly, expression of *IL-1β* and *TNF-α* was shown to be important in anti-gyrodactylid responses in skin of rainbow trout infected with *Gyrodactylus derjavini* (Lindenstrøm *et al.* 2003). A close association between levels of IL-1β1 isoforms and the severity of amoebic gill disease (AGD) also occurs in gills of rainbow trout infected with *Neoparamoeba* sp. (Bridle *et al.* 2006b; a). Our results assign some importance for both IL-1β and TNF-α in site-specific responses to *L. salmonis* infection. However, this response may be limited to adult lice, as expression of *IL-1β* and *TNF-α* did not change in Atlantic salmon skin infected with copepodids (Tadiso *et al.* 2011). Although *IL-1β* gene expression was observed highest in louse-infected skin, expression of *IL-1β* was also up-

regulated in mechanically-abraded skin; similar to what was found after mechanical injury in carp (*Cyprinus carpio*) (Gonzalez *et al.* 2007b) and trout (Ingerslev *et al.* 2010). In mammals, IL-1 is sequestered in the epidermis so that upon injury an immediate induction of inflammation occurs without the lag time incurred with gene activation and protein synthesis (Lee *et al.* 1997). Pro-IL-1β is sequestered in mammals in the inflammasome, which can be rapidly activated through the cleavage of caspase-1 either by PAMPs or DAMPs (Mariathasan & Monack 2007). Considering the importance of IL-1β in teleost epidermal tissue responses together with the immediate and sustained expression at louse-infected sites observed in this study, it seems plausible that there may be a comparable mechanism in fish whereby the immediate release of IL-1β from a reservoir cell induces the inflammatory cascade. The acute-phase response (APR) is activated by tissue injury, infection, trauma and inflammation (Jensen *et al.* 1997; Bayne & Gerwick 2001; Cray *et al.* 2009) and involves an increase in many acute-phase proteins (APPs). In teleosts, APPs include C-reactive protein (CRP), serum amyloid-P (SAP) and serum amyloid-A (SAA), which may increase up to 1000-fold within hours of injury or infection (Jensen *et al.* 1997; Bayne & Gerwick 2001). CRP and SAP are known to be integral components of the innate immune response and evidence for receptors for CRP on macrophages (Tebo & Mortensen 1990) and for SAP on neutrophils (Landsmann *et al.* 1994) suggests a linkage with cellular innate responses. The APR is stimulated by pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) secreted by activated monocytes and macrophages that bind to appropriate receptors and lead to translocation and activation of NF-κB and C/EBP-β (Poli 1998). In the present study, early expression of *IL-6* was clearly associated with the attachment of *L. salmonis* in all salmon species, although the expression in Atlantic and chum salmon was significantly higher than in pink salmon. IL-6 has been shown to be the most potent cytokine in activating synthesis of CRP

(Wegenka *et al.* 1993), therefore high levels of CRP in all three species were expected. On the contrary, very low levels of CRP transcript were observed in Atlantic and chum salmon in both louse-infected and abraded fish, which suggests pathways of CRP activation differ among these species. However in pink salmon, CRP expression was significantly higher in louse-attached sites, indicating louse-associated activation. This pattern was only observed 24 h, similar to IL-6 induction. The absence of IL-6-associated activation of CRP in Atlantic and chum salmon (the most susceptible species) suggests that this is a pathway involved in susceptibility to *L. salmonis* infections in these species. The expression of CRP and SAP during *L. salmonis* infections appeared to be regulated by different mechanisms: CRP expression was highly associated with the site of attachment early in *L. salmonis* infections in pink salmon. In contrast, the expression of SAP was higher only at non-infected sites in all three species. Collectively, this appears to be evidence of a louse-associated local suppression of SAP expression. Furthermore, SAP transcripts were expressed at higher levels in chum salmon than in Atlantic or pink salmon and preceded the expression of *IL-8*. Neutrophil infiltration is known to be an important cellular response in expulsion of *L. salmonis* in resistant species (Johnson & Albright 1992b). Although a relationship between SAP, IL-8 and neutrophils has not been elucidated, extra-hepatic synthesis of APPs in teleosts may occur as in mammals (Cray *et al.* 2009) and initiate a local influx of inflammatory cells that is later reinforced through IL-8 secretions (Whyte 2007; Alvarez-Pellitero 2008). Increased SAA and IL-8 levels have been implicated as an inducer of neutrophil migration in bacteria-infected turtles (Zhou *et al.* 2009).

2.5.3 iNOS

Inducible nitric oxide synthase (iNOS) is produced by stimulated macrophages and functions to synthesize nitric oxide (NO) which acts as a potent antimicrobial molecule (Bogdan 2001).

Expression of iNOS is regulated by cytokines such as IL-1β and TNF-α and is activated by the binding of NF-κB to the gene promoter (Bogdan 2001). This activational pathway suggests that expression of *iNOS* should be similar to levels of *IL-1β* or *TNF-α.* Additionally, high levels of *iNOS* have been reported in the gills and skin of fish infected with other ectoparasites (Lindenstrøm *et al.* 2004; Sigh *et al.* 2004a; Bridle *et al.* 2006a). However, iNOS does not appear to have a definitive role in anti-louse defenses despite pronounced *IL-1β* and *TNF-α* expression in *L. salmonis*-infected skin. Atlantic salmon showed a significant increase in *iNOS* transcript from 24 to 48 h in lice-attached skin but these levels were not different from mechanically-abraded or non- infected sites. In pink and chum salmon, mechanical abrasion elicited the most significant *iNOS* expression. Pronounced activation of *iNOS* in mechanically-abraded pink salmon skin suggests that DAMPs are contributing to early NO production in this species. These data support the view that *iNOS* expression is associated with early pro-inflammatory stages of infection (Bogdan 2001).

2.5.4 MMPs

Matrix metalloproteinases (MMPs) remodel and degrade collagen and remove extracellular matrix (ECM) during pathological processes (Nagase & Woessner 1999). In healthy tissue, MMPs are quiescent and only become activated by pro-inflammatory cytokines after injury or infection. *MMP9* and *MMP13* were up-regulated in skin of Atlantic salmon infected with larval *L. salmonis* suggesting changes in extracellular matrix (Skugor *et al.* 2008; Tadiso *et al.* 2011). Similarly in this study, marked up-regulation of *MMP13* was observed at adult *L. salmonis* attachment sites in all three species and remained constantly induced, with the exception of pink salmon in which there was a massive up-regulation of *MMP13* after 48 h. Interestingly, up-regulation of *MMP13* was also observed in mechanically-abraded sites in all three species,

implying that this gene is activated by both DAMPs and PAMPs. The pronounced expression in attachment site skin in pink salmon suggests a species-specific mechanism in which tissue remodeling capabilities are enhanced despite louse infection.

A distinguishing feature of the susceptible Atlantic salmon during chronic infection with the Atlantic form of *L. salmonis* is the lack of cell proliferation despite increased tissue remodeling (Skugor *et al.* 2008). Very small pink salmon that are still susceptible to *L. salmonis* exhibit high levels of *MMP* expression in the absence of markers for cellular proliferation (Sutherland *et al.* 2011). In pink salmon over 0.7 g, a decrease in the inhibition of proliferation coincides with size-dependent increased resistance to *L. salmonis* (Jones *et al.* 2008a). This data reflects a potential for cellular mobility and proliferation as important factors in conferring resistance to the parasite and genetic expression of associated genes such as MMP13 may be candidates as biomarkers.

2.5.5 Arachidonic acid metabolites

Arachidonic acid metabolism is a critical component of the inflammatory cascade whereby membrane phospholipids are liberated and converted into various prostaglandins (PGs) by the cyclooxygenase enzymes COX-1 and COX-2 (Harris *et al.* 2002), the latter being primarily involved in the regulation of inflammation (Smith *et al.*). *COX-2* was significantly associated with *L. salmonis* attachment sites in all three species and activation of the gene was evidence for increasing PG synthesis. Furthermore, expression of *prostaglandin D synthase* (*PGDS*) implies anti-inflammatory PGs are the dominant form (Harris *et al.* 2002). Moreover, the shift from pro-inflammatory PGE_2 to PGD_2 has been implicated as an important component to the resolution phase of inflammation (Gilroy *et al.* 1999). There was an early and sustained increase in expression of *PGDS* systemically in Atlantic, pink and chum salmon with the highest up-

regulation observed in pink salmon. However after 48 h, the expression of *PGDS* at louse-infected sites significantly decreased in all species. This is in agreement with Skugor *et al.* (2008) where high levels of *PGDS* were detected systemically after 3 days in Atlantic salmon infected with larval *L. salmonis*, but after 33 days down-regulation was observed in louse-attached skin. This evident down-regulation of *PGDS* at louse-infected sites indicates a louse-associated suppressive effect of PGDS and strongly suggests that pro-inflammatory PGs (PGE_2) are dominant specifically at *L. salmonis* attachment sites. Combined expression of *PGDS* and *COX-2 in vitro* results in complete inhibition of NF-κB translocation (Rossi *et al.* 2000). Further, PGD_2 is converted to 15-d-PGJ_2 which in mouse and human macrophages inhibits the translocation of NF-κB (Rossi *et al.* 2000) thus inhibiting the production of IL-1β,TNF-α and iNOS (Castrillo *et al.* 2000). In our study, this implied PGDS is involved in an auto-regulatory feedback loop controlling NF-κB activation of inflammation, thus contributing to resolution of inflammation early during louse infection. PGE_2 enhances the production of type-2 cytokines and antibodies by acting on a variety of innate cell types resulting in an enhanced T helper 2 response (Harris et al., 2002). A strong bias toward Th2-like responses in *L. salmonis*-infected Atlantic salmon was previously suggested (Skugor *et al.* 2008).

2.5.6 IL-10

Interleukin-10 is known to regulate inflammation by down-regulating the intensity of inflammatory mediators activated by IL-1β and TNF-α (Dinarello 2000). Therefore we expected high levels of *IL-1β* to be followed by high levels of *IL-10*. Elevated expression of *IL-1β* at *L. salmonis* attachment sites was not followed by up- regulation of *IL-10*. Rather, comparable levels of *IL-10* were observed in louse-infected fish both at attached and non-attached sites and at sites of abrasion, despite pronounced *IL-1β* expression in attached sites. Although *IL-10* induction was

significantly higher compared to control fish, similar levels in abraded and infected fish may imply that insufficient anti-inflammatory processes are occurring where *L. salmonis* is attached. Inflammatory responses in carp were associated with significant activation of IL-10 in the first 3 h after mechanical injury (Gonzalez *et al.* 2007b). Any up-regulation of *IL-10* due to louse attachment earlier than 24 h would not have been observed in the current study.

2.5.7 MH class II

Major histocompatibility (MH) receptors link the innate immune system to the adaptive response by allowing antigen presenting cells to interact with T-cells to initiate immune responses (Klein & Sato 2000). The significant difference in the basal expression of *MH class II* among Atlantic, pink and chum salmon was of particular interest in the context of *L. salmonis* infections. The basal expression in Atlantic salmon was over 1000-times higher than in pink or chum salmon. However, after *L. salmonis* infection normalized expression in Atlantic and chum salmon did not change significantly whereas that in pink salmon was significantly up-regulated after 24 h. Moreover, this response was only observed at louse-attachment sites and not systemically or in response to mechanical injury. MH receptor variants have recently been implicated in susceptibility to *L. salmonis* in sub-populations of Atlantic salmon (Gharbi *et al.* 2009). MH genetic variation is known to be associated with higher resistance to pathogens in several vertebrate populations (Hedrick 2002; Dionne *et al.* 2009), and in Atlantic salmon certain MH alleles have been linked to higher resistance to *Aeromonas salmonicida* (Langefors *et al.* 2001), infectious salmon anemia virus (Glover *et al.* 2007) and infectious hematopoietic necrosis virus (Miller *et al.* 2004). Decreased *MH class II* gene expression was observed in skin of rainbow trout infected with *G. derjavini* (Lindenstrøm *et al.* 2004) and in Atlantic salmon 15 days after infection with larval *L. salmonis* (Tadiso *et al.* 2011). In the present study, the species-specific

differences in expression of *MH class II* observed in the skin of Atlantic, pink and chum salmon suggest that it is a non-specific biomarker for resistance to *L. salmonis* infection. The rapid up-regulation of *MH class II* at *L. salmonis*-attachment sites among pink salmon is unexpected as this molecule is typically associated with T cells and the adaptive immune response (Dixon & Stet 2001; Whyte 2007). The high levels of *MH class II* in pink salmon skin 24 h following *L. salmonis* attachment suggests that MH class II presenting cells such as macrophages are recruited in large numbers to the site of louse attachment where they likely play a role in inflammatory processes.

2.5.8 Immunomodulatory effects inferred from gene expression in skin

Immunosuppression caused by parasitism contributes to the interaction and coevolution between parasites and their hosts (Yang & Cox-Foster 2005). Reduced transcript levels for several genes of interest at the site of *L. salmonis* attachment were consistent with immunosuppression. This pattern was detected in both susceptible (Atlantic and chum) and refractory (pink) salmon for the transcription factor *NF-κB* at 24 h and for *PGDS* expression, which was significantly lower in all salmon at 48 h. Similarly, *SAP* transcripts were significantly lower at attachment sites in pink and chum salmon. Attachment site suppression of other genes was observed only in susceptible salmon (Atlantic: *IL-10, C/EBP-β, CRP* and *MH class II*; chum: *CRP*). *L. salmonis* secrete trypsin-like proteases and prostaglandin E_2 (PGE_2) (Firth *et al.* 2000; Ross *et al.* 2000; Fast *et al.* 2002a, 2003, 2004) which may modulate immune functions at the site of infection (Harris *et al.* 2002; Fast *et al.* 2003, 2006, 2007a), while enhancing parasite feeding efficiency and survival in a hostile host environment. Our data also showed several examples of a systemic up-regulation of gene expression, in agreement with Skugor *et al.* (2008). The mechanisms of these systemic processes remain unclear and require more research but emphasize that the physiological- and

immunological-related consequences of *L. salmonis* infection are not restricted to the site of attachment.

Expression profiles of 14 defense-relevant genes in the skin of susceptible (Atlantic, chum) and resistant (pink) salmon during early *L. salmonis* infections, provided evidence for distinct pathways of susceptibility between *Salmo* sp. and *Oncorhynchus* sp. and among *Oncorhynchus* spp. The pathways were associated with inflammation and immunomodulation and were often species-specific. Expression of *MH class II, IL-6, CRP, MMP13, IL-1β* and *COX-2* genes was significantly higher in pink compared to Atlantic or chum salmon and therefore appeared to be involved in the early protective response against adult *L. salmonis*.

2.6 Chapter acknowledgements

This work was funded by a Natural Sciences and Engineering Research Council (NSERC) of Canada Strategic Projects Grant (STPGP 372605-08) awarded to DEB and SRMJ in collaboration with Fisheries and Oceans Canada and Marine Harvest Canada (MHC). Brad Boyce from MHC was instrumental in louse collection. The technical assistance of Laura Hawley, Geoff Lowe and Gina Prosperi-Porta, Fisheries and Oceans Canada, is gratefully acknowledged. LMB has an NSERC- CGS-D.

Chapter 3: Signatures of resistance to *Lepeophtheirus salmonis* include a T_H2-type response at the louse-salmon interface.

Adapted from: Laura M. Braden[1], Ben F. Koop[1], Simon R.M. Jones[2]. *Developmental and Comparative Immunology* (2015), **48**:178-19.

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LMB conceived of the study, contributed to experimental design, performed qPCR and IHC work, analyzed and interpreted data, and wrote the manuscript.

BFK assisted in interpretation and edited the manuscript.

SRMJ conceived of the study, assisted in interpretation and wrote the manuscript.

3.1 Abstract

Disease outbreaks with the salmon louse *Lepeophtheirus salmonis* cause significant economic losses in mariculture operations worldwide. Variable innate immune responses at the louse-attachment site contribute to differences in susceptibility among species such that members of *Salmo* spp. are more susceptible to infection than those of some *Oncorhynchus* spp. Relatively little is known about the mechanisms that contribute to disease resistance or susceptibility to *L. salmonis* in salmon. Here, we utilize histochemistry and transcriptomics in a comparative infection model with susceptible (Atlantic, sockeye) and resistant (coho) salmon. At least three cell populations (MHIIβ+, IL1β+, TNFα+) were activated in coho salmon skin during *L. salmonis* infection. Locally elevated expression of several pro-inflammatory mediators (e.g., *IL1β, IL8, TNFα, COX2, C/EBPβ*), and tissue repair enzymes (*MMP9, MMP13*) were detected in susceptible and resistant species. However, responses specific to coho salmon (e.g., *IL4, IL6, TGFβ*) or responses shared among susceptible salmon (e.g., *SAP*, *TRF*, *Cath* in Atlantic and sockeye salmon) provide evidence for species-specific pathways contributing to resistance or susceptibility, respectively. Our results confirm the importance of an early pro-inflammatory T_H1-type pathway as an initial host response during infection with Pacific sea lice and demonstrate subsequent regulatory T_H2-type processes as candidate defense mechanisms in the skin of resistant coho salmon.

3.2 Introduction

Anadromous salmonids of the genera *Salmo, Salvelinus* and *Oncorhynchus* are parasitized by the ectoparasitic copepod *Lepeophtheirus salmonis* (Caligidae) (Pike & Wadsworth 1999; Boxaspen 2006; Torrissen *et al.* 2013). Although present in wild populations, abnormally high infection densities are problematic in salmon net-pens due to high host density (Johnson *et al.* 2004; Costello 2009). The parasite feeds on the mucus and epithelial tissues, and in severe infections grazes on underlying tissues and blood (Brandal *et al.* 1976). The attachment and feeding behavior of *L. salmonis* cause pathology and these effects tend to be more severe on Atlantic salmon (genus *Salmo*) compared to Pacific salmon (genus *Oncorhynchus*) (Johnson & Albright 1992a; Pike & Wadsworth 1999; Johnson *et al.* 2004; Boxaspen 2006). Common pathological impacts during infection include osmoregulatory failure due to compromised epidermis, secondary infections, reduced growth rate, anaemia and chronic stress (Grimnes & Jakobsen 1996; Boxaspen 2006; Wagner *et al.* 2008). In mariculture, global economic costs due to *L. salmonis* infections exceed $500 million USD (Costello 2009). Differences in susceptibility to *L. salmonis* occur among juvenile salmon such that coho (*Oncorhynchus kisutch*) and pink (*O. gorbuscha*) salmon are more resistant compared to Atlantic (*Salmo salar*), chinook (*O. tshawytscha*), or chum (*O. keta*) (Jones 2001; Jones *et al.* 2007; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012). Experimental or natural infections on sockeye salmon (*O. nerka*) cause severe pathological lesions (Johnson *et al.* 1996; Jakob *et al.* 2013); however, the comparative susceptibility of the species is not known.

Among species of fish, physiological variations of the skin determine resistance to other ectoparasites (Esteban 2012); however, the molecular basis for susceptibility to infection with *L. salmonis* is poorly understood. Resistance to *L. salmonis* was shown to be associated with early inflammatory and innate immunological processes (Johnson & Albright 1992a; Fast *et al.* 2006;

Wagner *et al.* 2008; Jones 2011) and this has since been supported in comparative transcriptomic analyses of skin from susceptible and resistant species (Skugor *et al.* 2008; Tadiso *et al.* 2011; Sutherland *et al.* 2011, 2014a; Krasnov *et al.* 2012; Braden *et al.* 2012). A suggested mechanism in susceptible species is a weak pro-inflammatory response characterized by little or no leukocyte infiltration into the site of infection, weak or limited tissue repair, absence of an acute-phase response, and a T_H2-type anti-inflammatory response (Jones *et al.* 2007; Skugor *et al.* 2008; Jones 2011; Tadiso *et al.* 2011; Sutherland *et al.* 2011, 2014a; Krasnov *et al.* 2012; Braden *et al.* 2012). In contrast, resistance to infection has been associated with local inflammation, high levels of infiltrating leukocytes, tissue remodeling, and the acute phase response (Johnson & Albright 1992a; b; Jones 2011; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012). The importance of a functional T_H2-response is well documented during parasite infections in mammals (Allen & Wynn 2011; Medzhitov *et al.* 2012) and a T_H2-like response was evident in the skin of rainbow trout infected with the ectoparasite *Ichthyobodo necator* (Chettri *et al.* 2014), yet this pathway has not been investigated as a mechanism for resistance during *L. salmonis* infection.

The aim of the present study was to build on our previous analysis of the cutaneous genetic response of resistant (pink salmon) and susceptible (Atlantic, chum) salmon during infection with the Pacific subspecies of *L. salmonis* (Yazawa *et al.* 2008; Braden *et al.* 2012; Skern-Mauritzen *et al.* 2014). Here we improve our comparative model by targeting the cutaneous response using both immuno-histochemical and transcriptomic methods in coho, sockeye and Atlantic salmon.

3.3 Methods

3.3.1 Fish and infection trials

All procedures involving the handling and usage of fish in this study were approved by the Canadian Council of Animal Care (CCAC) prior to initiation. Atlantic salmon (*Salmo salar*) parr were obtained from a commercial salmonid hatchery, coho salmon (*Oncorhynchus kisutch*) parr were obtained from the Chase River hatchery, both on Vancouver Island, British Columbia, and sockeye salmon (*Oncorhynchus nerka*) parr were obtained from the Inch Creek hatchery, Chilliwack, British Columbia, Canada. All fish were reared on brackish water (15 ppm) until smolting, after which they were maintained on ultraviolet-treated salt water in single-pass flow-through tank systems on a 12:12 hr light:dark cycle. Fish were fed 1% total biomass daily. Fish were randomly divided among twelve 330 L tanks with 4 tanks for each species (2X infection tanks, 2X control tank), acclimated for approximately 7 days and starved at least 24 hr prior to any manipulation.

Adult female *L. salmonis* were collected during harvest of Atlantic salmon at a commercial aquaculture site on Vancouver Island, British Columbia, Canada. After collection, the lice were rinsed and transported back to the Pacific Biological Station (Nanaimo, British Columbia, Canada) in 8°C aerated sterile sea water. Only lice displaying attachment behavior to the collection vessel were included in the study. The time between collection of the sea lice and initial infection time was < 24 hr.

For infections, the water level of each tank was reduced by half and fish were sedated using 0.2 mg/L metomidate hydrochloride (M-HCl; Aquacalm, Syndel Laboratories). Sedated fish were transferred to a temporary tank containing M-HCl to which 5 adult lice/fish were added and allowed to settle and attach. Fish were gently removed from the infection tank once they

were infected with 5 lice and placed into their original tank. Control fish were sedated and placed into sham infection tanks containing 0.2 mg/L M-HCl, but no sea lice were added.

Samples were obtained at 24, 48 and 72 hr post-infection (hpi). Fish (n = 8) were removed from each tank and immediately euthanized in 200 mg/L tricaine methanosulfonate (TMS, Syndel Laboratories). Blood was sampled by caudal vein puncture into heparinized vacutainers and stored at 4 °C. Once sampling was completed, vacutainers were centrifuged at 3,000 x g for 15 min at 4 °C and the plasma fraction was collected and stored at -80 °C. Three attachment-site skin samples from each fish, obtained using disposable 5 mm AcuDerm® Biopsy punches (Braden *et al.* 2012) were pooled, snap-frozen in liquid nitrogen and stored at -80 °C. Non-attachment-site samples were similarly processed from each infected (n = 3) and non-infected fish (n = 3). Histological samples were similarly collected from infected and non-infected fish, fixed in 10% neutral buffered formalin (NBF) for 24 hr then transferred to 70% isopropanol.

3.3.2 Immunohistochemistry

Six fish were sampled at 24, 48 and 72 hpi for immunohistochemistry. Tissue samples were dehydrated in a graded isopropanol series, cleared in xylene, and embedded in paraffin blocks. Embedded tissues were sectioned using a Leica RM2135 microtome (Leica Microsystems, Germany). Serial sections (5 μm) were placed on SuperFrost UltraPlus (Menzel-Glaser) positively charged glass slides and dried overnight at 40°C. After deparaffinization in xylene and rehydration in graded isopropanol, the sections were either stained using hematoxylin and eosin (H&E) for routine histopathology or with a combined Periodic Acid Schiff/Alcian blue (PAS/AB, pH 2.5) in which sections of control and infected skin were incubated in 1% Alcian blue (pH 2.5) for 20 min, rinsed in distilled water, and subjected to routine PAS reaction.

Sections were examined using a compound microscope (Zeiss Axio Imager) equipped with a digital imaging system (Axiocam MRc5) at 200X magnification (0.9940 mm^2/field of view). Three random fields of view were scanned per fish and the average number of mucus cells was calculated (± standard deviation). The cells were characterized as containing either acidic (blue), neutral (pink) or combined (magenta) pH glycoconjugates as previously described (Bosi *et al.* 2005). Serial sections were also probed with monoclonal antibodies against Atlantic salmon MH II β-chain (α-MHIIβ), surface IgM (α-mIgM) or with polyclonal antibodies to rainbow trout IL1β (α-tIL1β), Pax5 (α-Pax5), or TNFα (α-tTNFα) (Appendix B Table 17). The α-tTNFα polyclonal antibody was generated by Lampire Biological Laboratories. Briefly, rabbits were injected with a peptide covering amino acids 224-239 of rainbow trout TNF-α (TETNRLTDVEPEQGK, Genbank accession #CAB92316.1). The peptide included a C-terminal cysteine for conjugation to KLH for primary immunization and BSA boosting. Rabbits were boosted twice and a final production bleed obtained on day 50. α-tTNFα recognizes both TNF-α1 and TNF-α2 isoforms.

After de-paraffinization and rehydration, sections were heated to 100°C in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0) for 20 min, allowed to cool to room temperature for 10 min in phosphate-buffered saline (PBS) then washed twice in Tris-buffered saline + 0.2% Tween-20 (TBS-T; pH 8.0) for 5 min with gentle agitation. For α-MHIIβ and α-mIgM detection, sections were blocked in protein blocker, and then rinsed gently with TBS-T. The sections were incubated with primary antibody in TBS-T and 1% bovine serum albumin (BSA, Sigma) overnight at 4°C in a humid chamber. After incubation, the sections were washed in TBS-T (2 x 5 min), incubated in a mouse-specifying reagent (EXPOSE Mouse/Rabbit Specific HRP/DAB kit, Abcam) for 10 min followed by a 10 min incubation in hydrogen peroxide blocker. Immuno-labeled cells were detected by 20 min incubation with a goat anti-rabbit HRP conjugate

followed by 5 min with 3,3'-diaminobenzidine (DAB) in PBS with 0.015% H_20_2. For detection of α-tIL1β, α-tTNFα and α-Pax5, a rabbit-specific alkaline phosphatase (AP) detection kit (ABC, Abcam) was used according to manufacturer's instructions. Sections were blocked in protein block for 10 min, washed in TBS-T and incubated with primary antibody in TBS-T + 1% BSA as above. Sections were washed in TBS-T (2 x 5 min), incubated in biotinylated goat anti-rabbit for 15 min, washed in TBS-T (2 x 5 min) then incubated in streptavidin AP for 15 min. After a final wash (2 x 5 min) the sections were developed using the Permanent Red Fast System (Abcam). Sections were counterstained in 1% Alcian blue and diluted Mayer's hematoxylin (1/20), dehydrated in graded isopropanol, cleared in xylene and mounted (Permount). Sections treated with irrelevant antibodies served as negative controls while sections known to contain the target molecules served as positive controls. Species compatibility for each antibody was determined prior to IHC assays.

3.3.3 RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted from pooled skin plugs under liquid nitrogen conditions using the RNEasy Fibrous Tissue kit (Qiagen). Briefly, ~ 10 mg of tissue was homogenized at 30 Hz for 10 min in extraction buffer and then incubated at 55 °C for 15 min with 10 µL of proteinase K (10 mg/ml, Qiagen). Column-purified RNA was treated on-column following manufacturer's instructions with DNase I to digest genomic DNA. Samples were eluted to 30 µL with ultra-pure water and RNA quantity determined using spectrophotometry (NanoDrop-1000) and quality verified using agarose gel electrophoresis (1%). All extracted RNA samples had an $A_{260/280}$ between 1.8 and 2.0.

First-strand synthesis of cDNA was accomplished with 1 µg total RNA with random hexamers using the High Capacity RNA kit (Applied Biosystems) in 40 µL reactions. Controls

for genomic DNA contamination were performed on randomly selected subsamples (n = 48) of RNA by omitting reverse transcriptase (-RT) and all -RT samples failed to amplify products after 35 rounds of PCR. Following synthesis, samples were stored at -20 °C until use in qPCR assays.

Quantitative RT-PCR was performed on gene targets previously found to be differentially regulated in the skin of salmon infected with *L. salmonis*, or known to be important in other ectoparasitic models (Appendix A Table 15). These genes are associated with the early inflammatory response and include cytokines (*IL1β, TNFα, IL4, IL6, IL10, TGFβ, MX1*) and molecules associated with arachidonic acid metabolism (*COX2, PGDS*), acute phase response (*TRF, Hep1, SAA, SAP, CRP, CATH*), cellular remodeling and repair (*MMP9, MMP13*), cellular markers (*MH I, MH II, CD8α, IgM*), and transcription activation in the innate immune response (*C/EBPβ, p38, IRAK1, Pax5*). Reference gene candidates included elongation factor 1-α (*EF1α*), 18S ribosomal subunit (*18S*), eukaryotic translation initiation factor 3 subunit 6 (*ETIF3-6*), glyceraldehyde phosphate dehydrogenase (*GAPDH*) and β-actin. The three most stable reference genes (*ETIF3-6, EF1α* and *β-actin*) were determined using geNorm (Vandesompele *et al.* 2002) with a collective M value of 0.486 and a CV of 0.189; a value within the range typically observed for stably expressed reference genes in heterogeneous samples (Hellemans *et al.* 2007).

To ensure similar efficiency in all species and tissues, a standard curve was generated for each species (n = 5 dilution series) using pooled equimolar amounts from three samples from infected and non-infected salmon. All primers had efficiency values within the range of 85-105% for all three species (Supplementary Table S3). qPCR amplification was performed using Brilliant III Ultra-Fast SYBR® (Agilent) in 12.5 µL reactions in an MX3000P (Agilent) as previously described (Braden *et al.* 2012) with the following thermal profile: 95°C for 3 minutes, followed by a combined annealing and extension step of 60°C for 40 cycles. Amplicons were

checked for single products by melt-curve analysis, and resulting PCR products were sequenced to verify amplicon identity.

3.3.4 Osmolality

Plasma samples (10 µl) were assayed in triplicate utilizing a calibrated (100, 290, and 1000 mmol/kg) vapour pressure osmometer (Vapro 5520, Wescor) following manufacturer's instructions.

3.3.5 Data analysis

Histological analysis. To determine if there was an effect on mucus cells during *L. salmonis* infection, sections from parasitized (n = 6) and non-parasitized (n = 6) fish were examined. Mucus cells were enumerated and evaluated depending on the pH of their contents. The mean number of immuno-reactive cells was estimated from the number observed in three random fields of view at 200X (visual area 0.9940 mm^2; α-tIL1β, α-tTNFα) or 400X (visual area 0.03125 mm^2; α-MHIIβ, α-Pax5) for each sample (n = 6). Therefore, 18 (3 x 6) surface fields were counted for each time point, and kinetics of positive cells were compared to time-matched controls. Counting was standardized to only include immuno-labeled cells within the epidermis and dermis. A two-way ANOVA identified differences in mucus cell density and immuno-labeled cells between species. All statistics were conducted using SigmaPlot (V11.1) with a $p < 0.05$.

Genetic analysis. Data analysis of qPCR results was performed according to the qBASE framework to produce calibrated normalized relative quantities (CNRQs; (Hellemans *et al.* 2007)). Relative quantities ($RQs=E^{\Delta Ct}$; $\Delta Ct=C_{t(ref)}-C_{t(treatment)}$) for every sample were determined using a gene-specific efficiency. Normalized RQs (NRQs) were then calculated with a sample-specific normalization factor using multiple reference genes as described (Hellemans *et al.*

2007). Finally, calibrated NRQs (CNRQs) were calculated using a gene-specific calibration factor that minimized variation among technical replicates. CNRQs were normalized to non-infected samples and calculated for attachment sites and non-attachment sites. To account for differences in magnitude of expression among species we have also expressed gene expression as a ratio of the local response to the systemic response ($\log_2(\text{CNRQ})^{\text{attachment site}}/\log_2(\text{CNRQ})^{\text{internal control}}$). This local activation ratio (LAR) describes the magnitude of gene expression as a function of *L. salmonis* attachment and feeding activities and can be used as a proxy to define either activation or suppression by the louse. Data were $\log_2$-transformed and statistical analyses were performed using SigmaStat software (V11.1). Differences were considered significant at $p < 0.05$. All values shown are means of individuals for each sampling time ± SEM. Three-way ANOVA determined significance between treatments and species at 24, 48, and 72 hpi. If ANOVA was significant, Holm–Sidak post-hoc tests were used to determine the significance of pairwise differences.

3.4 Results

3.4.1 Macroscopic effects of *L. salmonis* infection

Immediately following exposure to the adult *L. salmonis*, all species displayed obvious signs of agitation as evidenced by flashing and jumping behaviors. The number of lice on all salmon sampled during the sampling period was 5 ± 2 and lice density on all species remained sufficient throughout the experiment such that triplicate attachment sites were available during sampling. On sockeye salmon, lice were attached near the dorsal fin and attachment sites were characterized grossly by depigmentation, raised scales, bloody exudate and degraded mucus layers. Signs of epithelial grazing and parasite-induced damage were not observed on coho or Atlantic salmon.

3.4.2 Cutaneous cell populations during infection with *L. salmonis*

In all species, histological assessment revealed the epithelium at louse attachment sites to be degraded with melanin deposits surrounding the scales or in the dermis (Figure 7). In addition, polymorphonuclear cells (PMNs) were observed in the epidermis and dermis of infected salmon from as early as 24 hpi (Figure 7). At 48 hpi, epithelial hyperplasia and cellular infiltration was pronounced in coho salmon, and to a lesser extent in sockeye salmon.

Among species, mucus cell density differed between parasitized and non-parasitized fish. Mucus cells were least dense in naïve Atlantic salmon skin and this number remained the same after infection with *L. salmonis* (Figure 8). Reaction with PAS/AB showed these cells to contain neutral/acidic mucins (Figure 8). The highest number of mucus cells occurred in non-parasitized coho salmon ($p < 0.001$, Figure 8). Mucus cell density in coho and sockeye salmon was significantly reduced following infection and this decreased significantly over time (24 → 72 hpi, $p < 0.001$); the lowest mucus cell density was observed in sockeye salmon at 72 hpi.

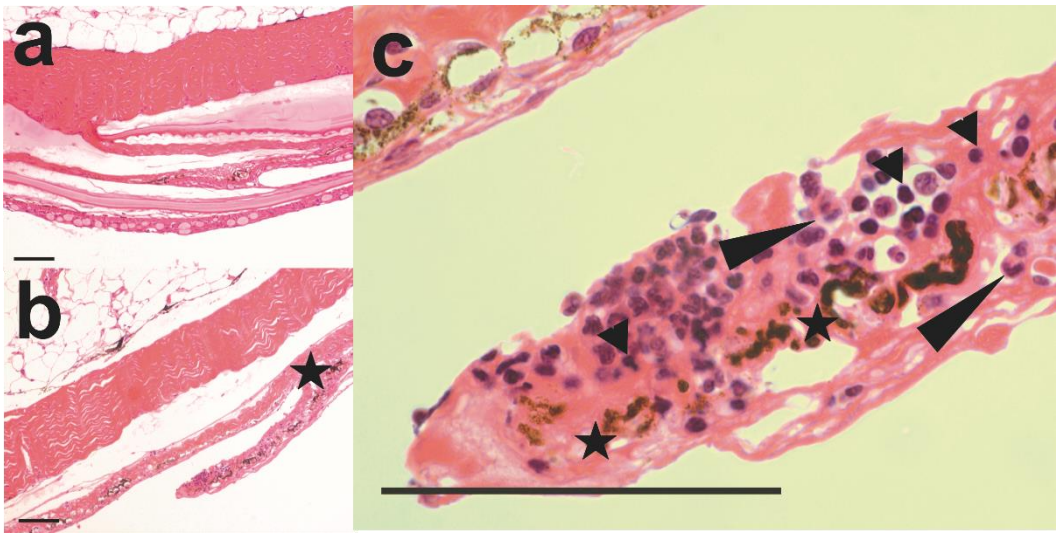

Figure 7. Micrographs of 48 hr non-infected (a) and louse-infected (b) sockeye salmon skin stained with hematoxylin & eosin.

Epithelial accumulation of melanin (star) and degraded epithelium was observed in all species during infection with *L. salmonis*. (c) High magnification (1000×) showing polymorphonuclear cells (long arrowhead) and lymphocyte-like cells (small arrow head) common in louse-infected skin. Bar = 50 μm.

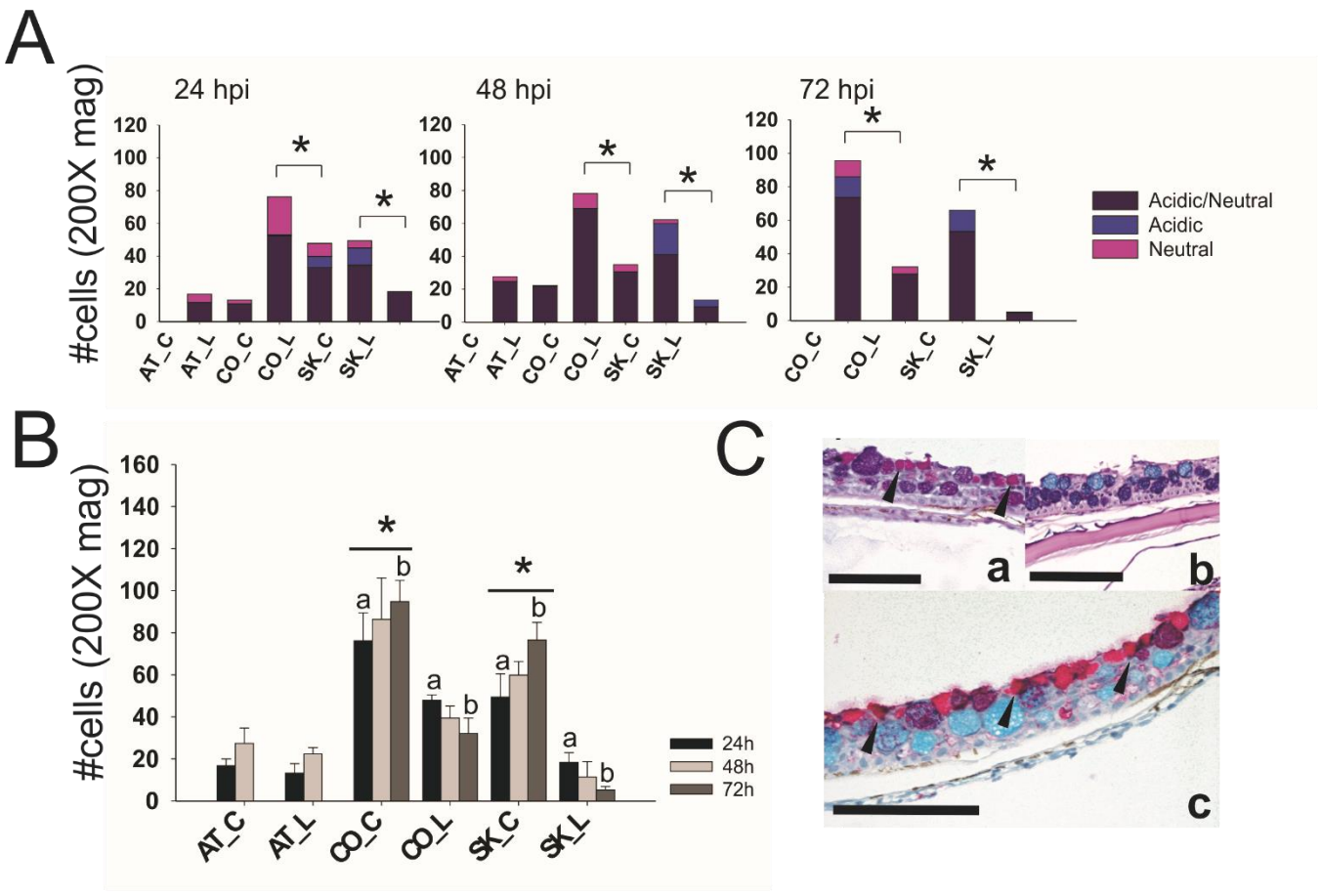

Figure 8. Mucocyte density and acidity in the skin of Atlantic, coho and sockeye salmon during *L. salmonis* infection.

(A) Mucocyte (MC) acidity was determined for Atlantic (AT), coho (CO) and sockeye (SK) non-infected (_C) and infected (_L) fish after 24, 48 and 72 hpi (coho and sockeye only) with magenta cells acidic/neutral (PAS/AB), blue cells acidic and pink cells containing neutral glycoconjugates. (B) MC density in the skin of non-infected and infected salmon over time. Bars represent the mean number of cells (n = 6) ± SEM. Significance determined by a two-way ANOVA, (asterisk, $p < 0.05$). (C) Micrographs of MCs containing (a) neutral (pink) and mixed pH (purple) and (b) acidic (blue) pH glycoconjugates. (c) Serial sections of mucus cells probed with antibodies for IL-1β showing densely packed MC-like cells (pink, arrow) which commonly corresponded to neutral-staining MCs.

3.4.3 Immunohistochemical identification of cell populations in salmon skin

Cross reactivity of α-MHIIβ was demonstrated in rainbow trout and Atlantic salmon (Hetland *et al.* 2010; Olsen *et al.* 2011) whereas α-mIgM (von Gersdorff Jørgensen *et al.* 2011) and α-IL1β (Zwollo *et al.* 2014) have only been shown on rainbow trout. Prior to immunohistochemical assays, antibodies were verified for cross-reactivity in *S. salar, O. nerka,* and *O. kisutch* using hematopoietic tissues that are highly likely to contain the specific targets and which would act as positive control tissues during assays (i.e., head kidney). In all cases, targets were detected in the positive control tissue.

Immunohistochemical reactions were observed at 200X (α-MHIIβ, α-mIgM, α-Pax5) or 400X (α-tIL1β, α-tTNFα) magnification. The average number of cells observed at the respective magnification (± standard error) was determined over time for each species (Figure 9, Supplementary Table 4).

MHIIβ$^+$ cells were detected in the skin of both non-infected and infected Atlantic, coho and sockeye salmon (Figure 9A). There were two common morphologies of MHIIβ$^+$ cells: small (< 10 μm) and round and typical of lymphocytes or monocytes, or diffuse with a dendritic cell-like appearance. There was an increase in MHIIβ$^+$ cells in parasitized coho at 24 hpi and 48 hpi ($p < 0.001$, Figure 9A, 10), while in Atlantic and sockeye salmon there was a decrease at 24 hpi ($p < 0.001$). MHIIβ$^+$ cells observed in putative blood vessels in underlying muscle tissue were not enumerated (Figure 11). We did not detect IgM$^+$ cells in skin of Atlantic, coho or sockeye salmon, either non-infected or infected with *L. salmonis* (Figure 12).

IL1β$^+$ cells were detected in the skin of non-infected Atlantic, coho and sockeye salmon as mucus-like cells in the upper epidermis or keratinocyte-like cells closer to the scales (Figure 9B). After infection with *L. salmonis*, a smaller, lymphocyte-like IL1β$^+$ cell population appeared in the dermis, surrounding the scales and in the epidermis. The number of positive cells

increased during infection in all species. Positive staining was often quite intense, with a diffuse halo surrounding positive cells, often accumulating at the basal membrane or near the scales (Figure 9B, 10).

TNFα^{+} cells were not detected in the skin of naïve salmon, but after infection with *L. salmonis*, small lymphocyte-like TNFα^{+} cells appeared in the skin of all species similar to what we observed for tIL1β^{+} cells (Figure 9C, 10). However, TNFα^{+} cells were only observed in the epidermis and dermis of infected fish and the density of these cells increased over time (24 → 72hpi). There were very few positive-staining mucus-like cells compared to those positive for α-tIL1β.

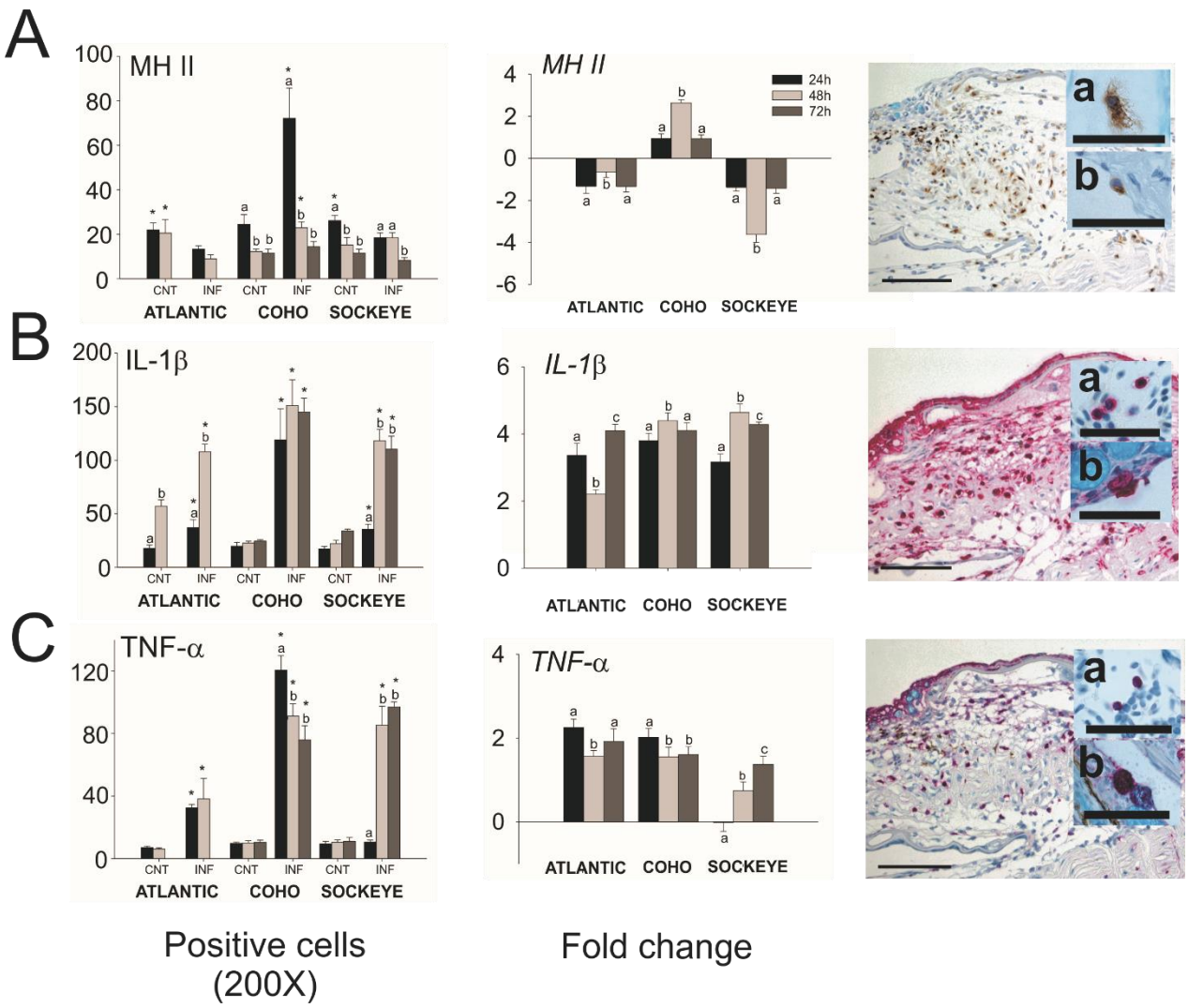

Figure 9. Immuno-reactive cells in control and louse-infected skin in Atlantic, coho and sockeye salmon.

At 24, 48 and 72 hpi expression profiles (RT-qPCR) and immuno-reactive cells were enumerated. (A) MHIIβ$^+$ cells (brown) were commonly observed as (a) larger dendritic-like cells or (b) small lymphocyte-like cells. (B) IL1β$^+$ cells (red) were commonly observed as (a) small lymphocyte-like cells in the epidermis and dermis of infection salmon, while the cells were (b) mucus-like cells in the epidermis of control fish. (C) TNFα$^+$ cells (red) were rarely observed in the epidermis of control salmon but when present they were mucocyte-like (b). After infection with *L. salmonis* small lymphocyte-like cells were abundant in the epidermis and dermis (a). Significance determined by a two-way ANOVA, $p < 0.05$. Lower case letters represent differences within each treatment group, with differences between control and infected fish indicated by an asterisk (*).

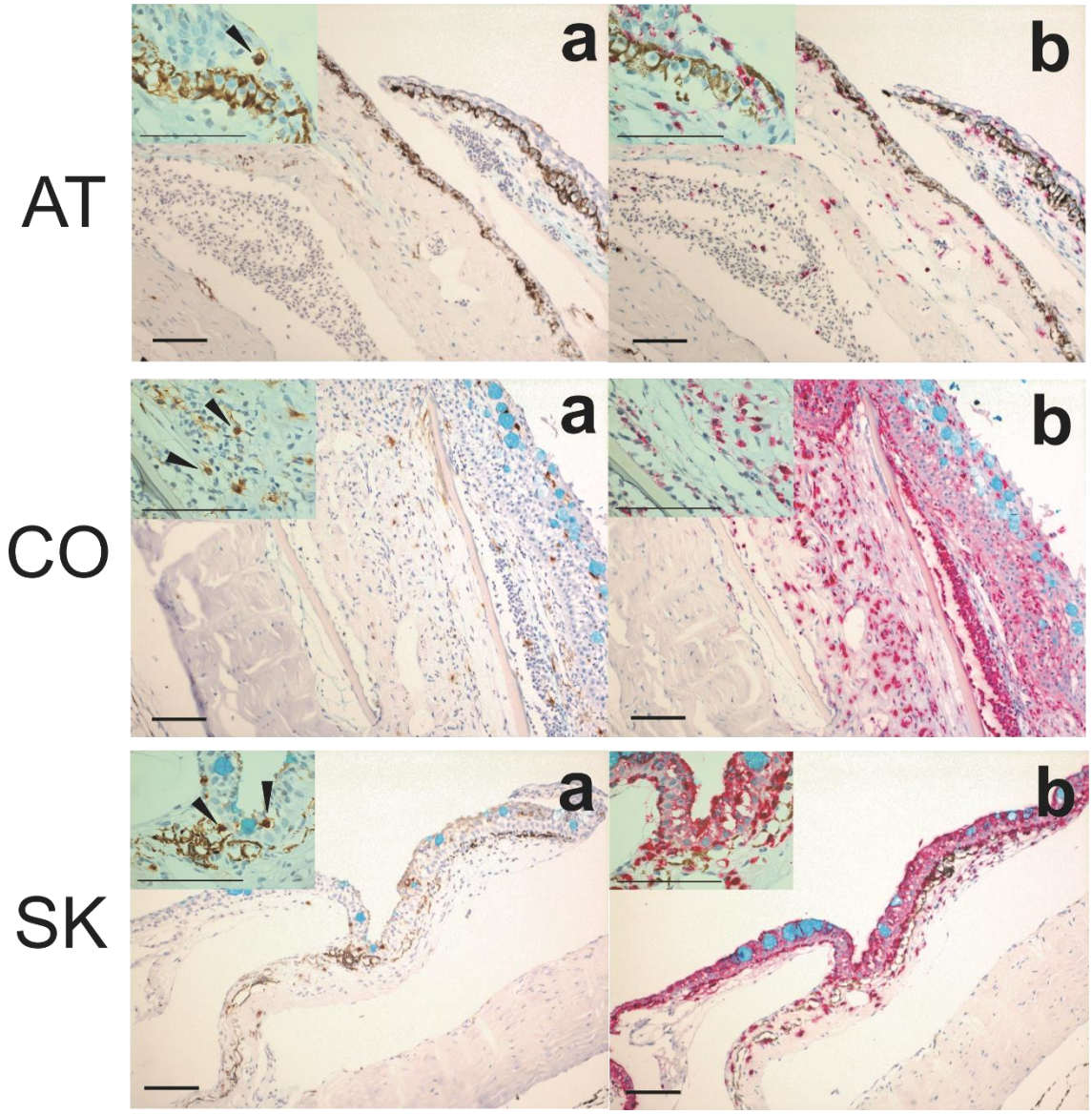

Figure 10. MHII+ and IL1β+ cells in the skin of salmon infected with *L. salmonis*.

Serial sections of Atlantic (AT), coho (CO) and sockeye (SK) salmon at 48 hpi showing brown MHIIβ+ cells (a) or red IL1β+ cells (b). Smaller image is 1000× magnification. Scale bar = 50 μm.

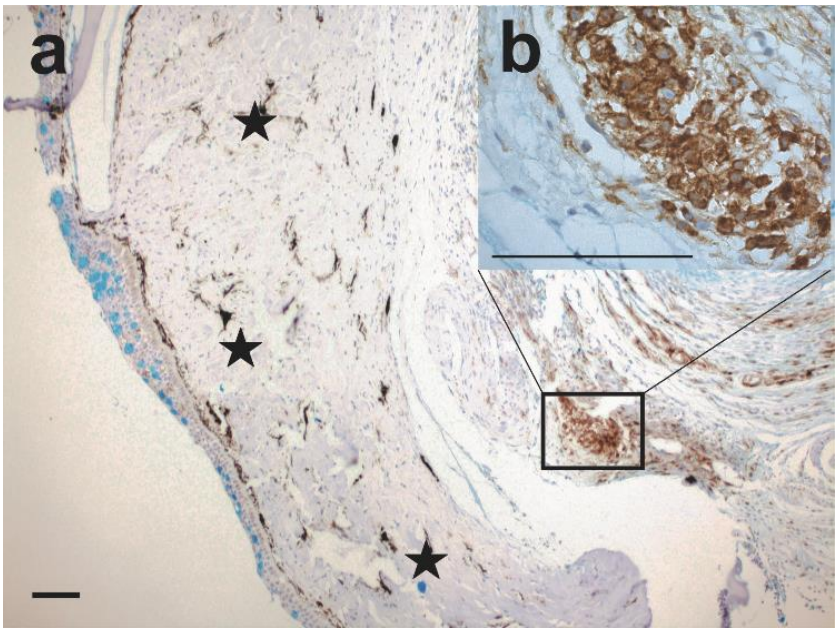

Figure 11. Sub-dermal accumulation of MHIIβ^{+} cells at a louse-attachment site in Atlantic salmon. (a) Low-magnification (100×) showing melanin deposits in the epidermis and dermis (stars), and subdermal accumulation of MHIIβ^{+} cells (brown) at 48 hpi. (b) 1000× magnification of boxed area showing accumulation of MHIIβ^{+} cells. Bar = 50 μm.

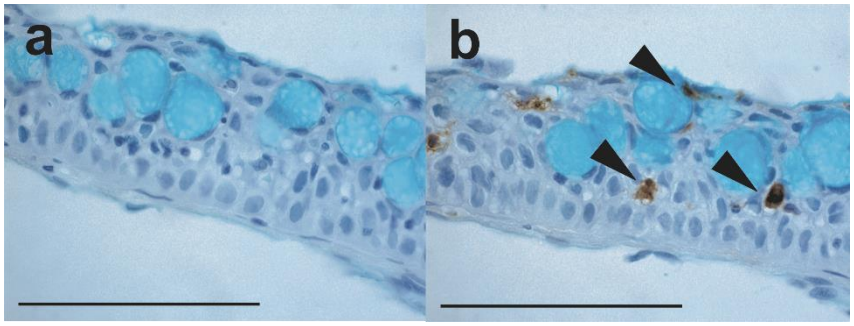

Figure 12. Serial sections of a louse attachment site in coho salmon showing mIgM^{-}/MHIIβ^{+} phenotype.

Immuno-labeled cell populations stained with either (a) α-mIgM or (b) α-MHIIβ (arrows) at 24 hpi. There was no mIgM+ staining detected in all species of control or louse- infected fish. Bar = 50 μm.

3.4.4 Gene expression in lice-infected salmon

Transcriptional changes are described as louse-associated activation or suppression using the ratio of attachment site expression to non-attachment site expression (LAR, see methods), as a function of time (i.e., 24 hpi→72 hpi, Supplementary Table 5), and as differences among species (Atlantic, coho and sockeye salmon).

3.4.4.1 Local differential expression

Activation. *Interleukin 1β* (*IL1β)* expression was significant at attachment sites in Atlantic and coho salmon throughout the experiment, while in sockeye salmon, expression was up-regulated at both attachment and non-attachment sites at 24 hpi (Figure 13, Supplementary Fig. 1A). *Interleukin 6* (*IL6*) expression was significant at attachment sites in coho and sockeye salmon throughout the experiment, whereas in Atlantic salmon, expression was weakly up-regulated. Among the Pacific species, local activation was the highest in coho salmon ($p < 0.001$; Figure 13, Supplementary Fig. 1A). Expression of *interleukin 8 (IL8)* was significant in all species by 24 hpi. Systemic expression of *IL8* was only significant in sockeye salmon throughout the experiment (Figure 13, Supplementary Fig. 1A). Expression of *CCAAT enhancer binding protein β* (*C/EBPβ*) as up-regulated in all species, but only locally activated in Atlantic and coho salmon at 24 hpi ($p < 0.001$; Figure 13, Supplementary Fig. 1A). In sockeye salmon, there was local activation at 72 hpi ($p < 0.001$). Expression of *C/EBPβ* in coho salmon remained consistently high at attachment sites, with local activation increasing over time ($p < 0.001$). *Tumor necrosis factor α* (*TNFα*) expression in Atlantic and coho salmon was specific to attachment sites throughout the experiment, while in sockeye salmon, expression of *TNFα* was not detected until 48 hpi similar to *IL1β* (Figure 13, Supplementary Fig. 1A). Activation of arachidonic acid metabolism, as determined by expression of *cyclooxygenase 2 (COX2)*, was significant in the skin of all species and was specific to louse attachment sites. Similar to *IL1β* and *TNFα*, we did

not detect significant local activation in sockeye salmon skin until 48 hpi. Of the three species, local activation was the highest in coho salmon ($p < 0.001$).

Matrix metalloproteinase 9 (*MMP9*) expression was locally up-regulated in Atlantic and coho salmon ($p < 0.001$). In sockeye salmon, local expression was evident by 48 hpi ($p < 0.001$); however, expression of *MMP9* remained significantly high systemically (Figure 14**,** Supplementary Fig. 1B). A similar pattern was observed in the expression of *MMP13* (Figure 14**,** Supplementary Fig. 1B) with local activation at 24 hpi only significant in Atlantic and coho salmon ($p < 0.001$). At 48 hpi, expression of *MMP13* decreased in Atlantic salmon, but remained activated at attachment sites ($p < 0.001$), whereas local activation in coho and sockeye salmon skin increased ($p < 0.001$). Thus, the pattern of expression for *MMP9* and *MMP13* in sockeye salmon was similar to that of pro-inflammatory mediators in that early local activation was delayed and only significant at 48 hpi (Figures 13 & 14, Supplementary Fig. 1A & B). Expression of *hepcidin 1* (*Hep1*) was locally activated in all species at 24 hpi and remained elevated throughout, with expression in coho and sockeye salmon significantly higher than in Atlantic salmon ($p < 0.001$, Figure 14, Supplementary Fig. 1B).

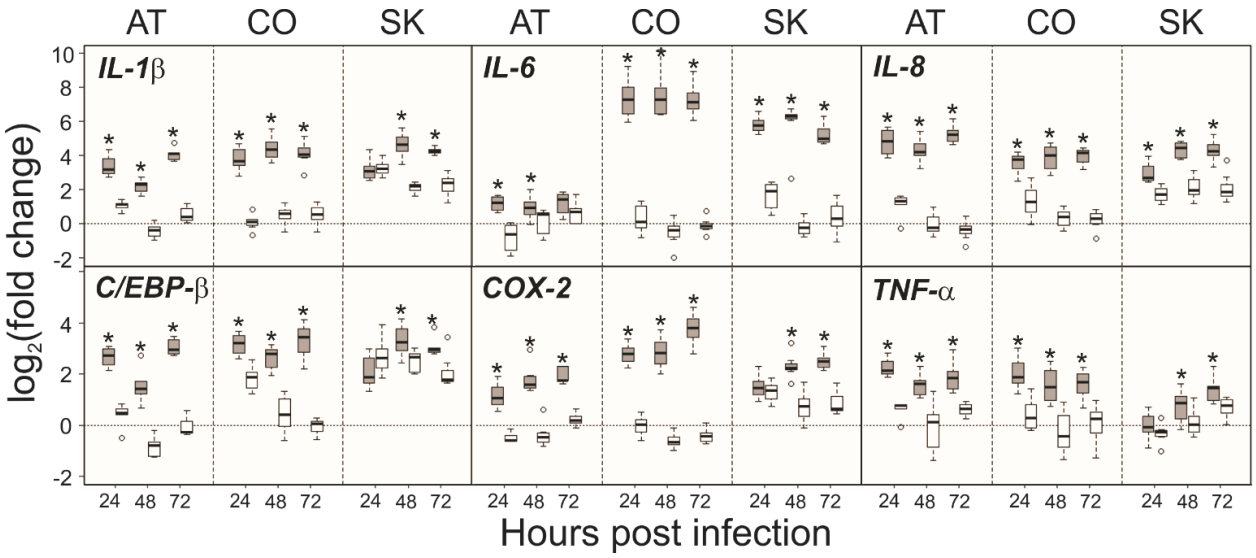

Figure 13. Gene expression of pro-inflammatory mediators in the skin of Atlantic, coho, or sockeye salmon.

Expression from each species is normalized to non-infected individuals from the same species. Boxplots represent the median (n = 8) and interquartile ranges with circles representing outliers in attachment skin (dark gray) and non- attachment skin (white) at 24, 48 and 72 hpi. Significantly elevated expression at attachment sites indicates louse-associated activation whereas if expression is higher at non-attachment sites louse-associated suppression is indicated. A three-way ANOVA determined significance between control and infected groups over time (SigmaStat V11.1; Supplementary Tables S4 & 5).

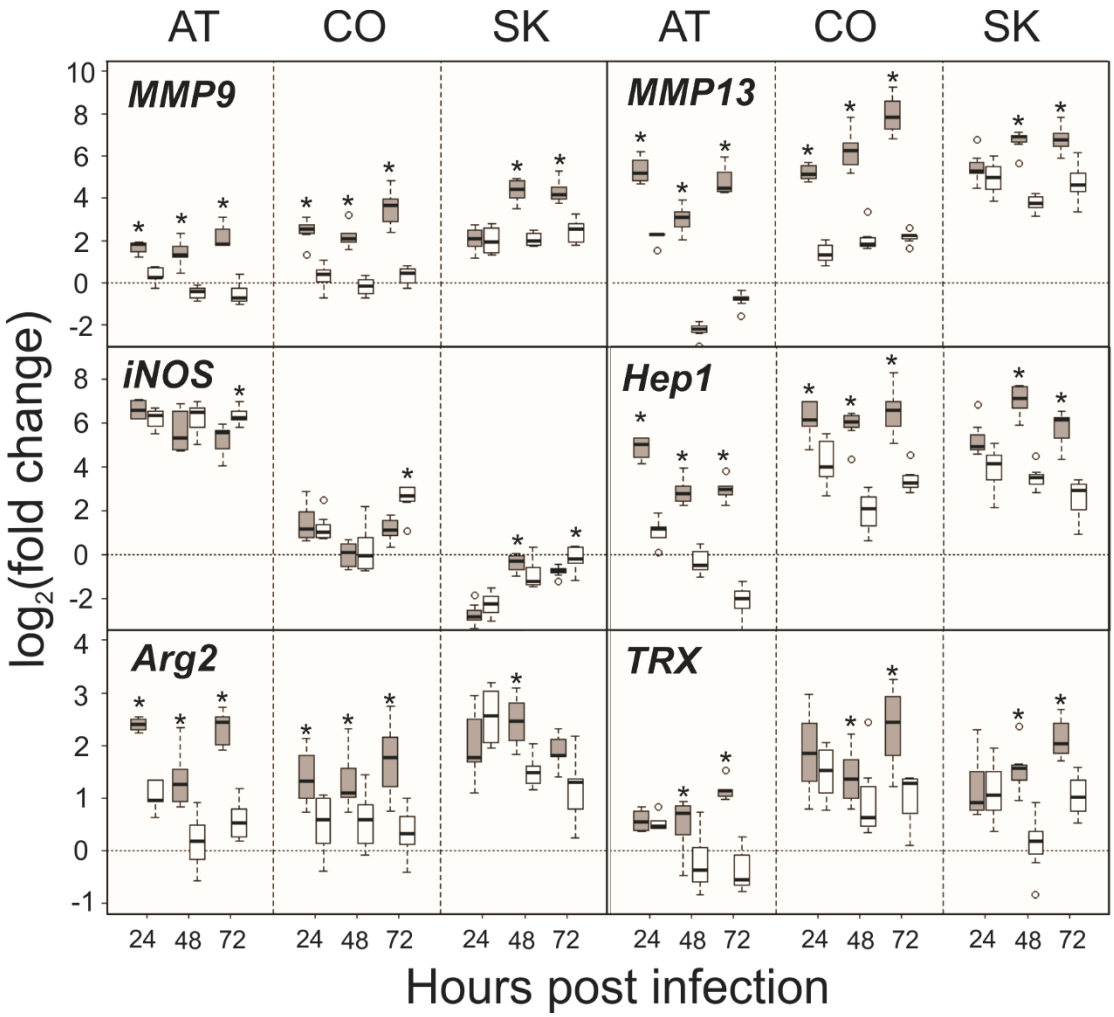

Figure 14. Expression of extracellular killing and tissue repair markers in skin of Atlantic, coho, or sockeye salmon.

Expression from each species is normalized to non-infected individuals from the same species. Boxplots represent the median (n = 8) and interquartile ranges with circles representing outliers in attachment skin (dark gray) and non- attachment skin (white) at 24, 48 and 72 hpi. Significantly elevated expression at attachment sites indicates louse-associated activation whereas if expression is higher at non-attachment sites louse-associated suppression is indicated. A three-way ANOVA determined significance between control and infected groups over time (SigmaStat V11.1; Supplementary Tables S4 & 5).

Expression of *thioredoxin (TRX)* increased in all species; however, significant local activation was only observed at 48 and 72 hpi ($p < 0.001$; Figure 14**,** Supplementary Fig. 1B). Similar to *Hep1*, *TRX* was significantly higher in the skin of coho and sockeye salmon ($p < 0.001$). Activation of *arginase 2 (Arg2)* occurred in all species, with significant local activation observed in Atlantic and coho salmon at all time-points. Local activation of *Arg2* was not observed in sockeye salmon until 48 hpi (Figure 14, Supplementary Fig. 1B). *Transferrin (TRF)* was up-regulated in Atlantic and sockeye salmon throughout the experiment, but local activation was only detected in sockeye salmon at 48 and 72 hpi ($p < 0.001$). *TRF* was not significantly activated in coho salmon skin; however, at 48 hpi, there was systemic down-regulation of *TRF* in coho salmon ($p < 0.001$; Figure 15, Supplementary Fig. 2A). Local up-regulation of *cathelicidin (CATH)* was found in Atlantic and sockeye salmon across time-points, while in coho there was local suppression (24 and 48 hpi) (Figure 15, Supplementary Fig. 2A). There was no local up-regulation of *C-reactive protein (CRP)* in Atlantic, coho or sockeye salmon. Expression of *serum amyloid A (SAA)* was locally up-regulated at 24 hpi in coho salmon, and this expression increased over time ($p < 0.001$). Similar temporal increases were observed in Atlantic and sockeye salmon, but only at 48 hpi ($p < 0.001$; Figure 15, Supplementary Fig. 2A). The expression of *serum amyloid P* (*SAP*) was elevated in Atlantic and sockeye salmon, but local activation was only significant at 48 hpi ($p < 0.001$; Figure 15**,** Supplementary Fig. 2A). Differential expression of *interleukin 4* (*IL4*) was specific to coho salmon and this expression was locally activated across all time-points ($p < 0.001$; Figure 16, Supplementary Fig. 2B). Expression of *transforming growth factor β* (*TGFβ*) was up-regulated in coho and sockeye salmon skin at 48 hpi, with significant local activation ($p < 0.001$; Figure 16, Supplementary Fig. 2B). There was no up-regulation of *TGFβ* expression in Atlantic salmon at any time-point.

Expression of *interleukin 10 (IL10)* was weakly up-regulated in coho salmon at 24 hpi and 72 hpi. In Atlantic and sockeye salmon *IL10* expression was locally suppressed at 72 hpi (Figure 16, Supplementary Fig. 2B).

Suppression. Suppression of inducible *nitric oxide synthase* (*iNOS*) was observed at 72 hpi in all three species, whereas *interleukin-1 receptor-associated kinase 1 (IRAK1)* was locally suppressed in coho (24, 48 hpi) and sockeye (24, 48, 72 hpi) salmon (Figures 14, 15, Supplementary Fig. 1B). Local suppression was observed for *TRF* in Atlantic (72 hpi) and coho salmon (24 hpi), whereas in sockeye salmon *TRF* was activated at 48 and 72 hpi (Figure 15, Supplementary Fig. 2A). Expression of *CATH* was suppressed only in coho salmon at 24 and 48 hpi (Figure 15). *C- reactive protein* was locally suppressed in Atlantic (48 hpi), coho (24 hpi) and sockeye (24, 48, 72 hpi) salmon (Figure 15, Supplementary Fig. 2A).

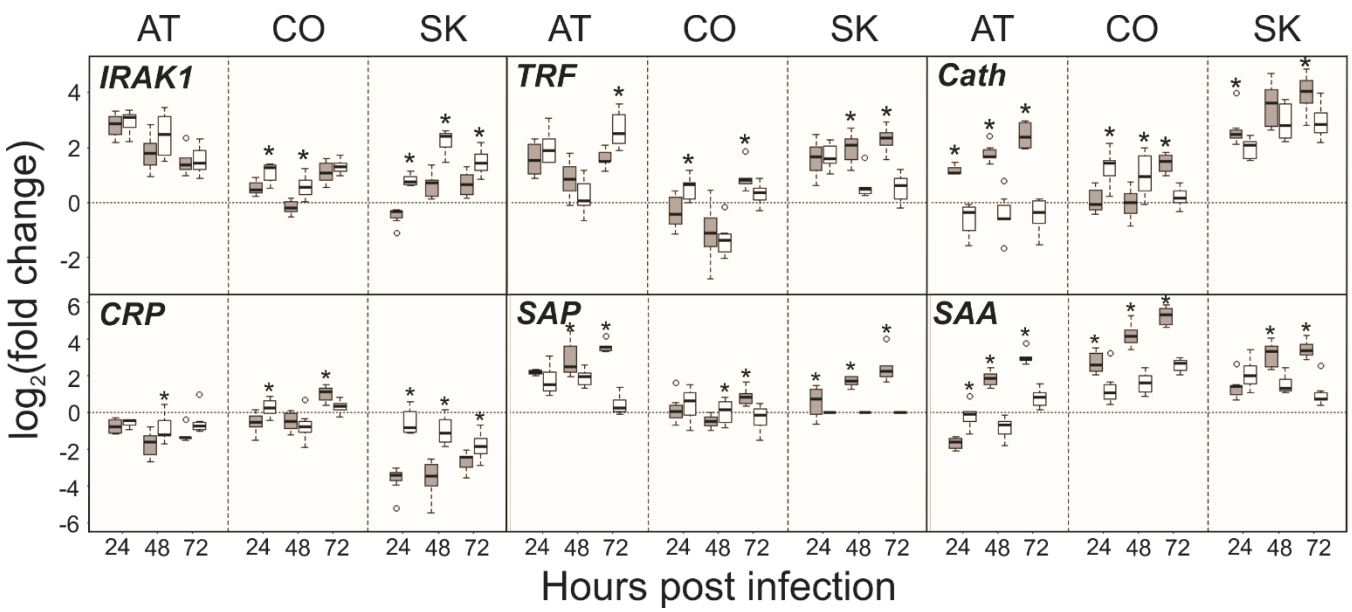

Figure 15. Expression of acute-phase genes in the skin of Atlantic, coho, or sockeye salmon. Expression from each species is normalized to non-infected individuals from the same species. Boxplots represent the median (n = 8) and interquartile ranges with circles representing outliers in attachment skin (dark gray) and non- attachment skin (white) at 24, 48 and 72 hpi. Significantly elevated expression at attachment sites indicates louse-associated activation whereas if expression is higher at non-attachment sites louse-associated suppression is indicated. A three-way ANOVA determined significance between control and infected groups over time (SigmaStat V11.1; Supplementary Tables S4 & 5).

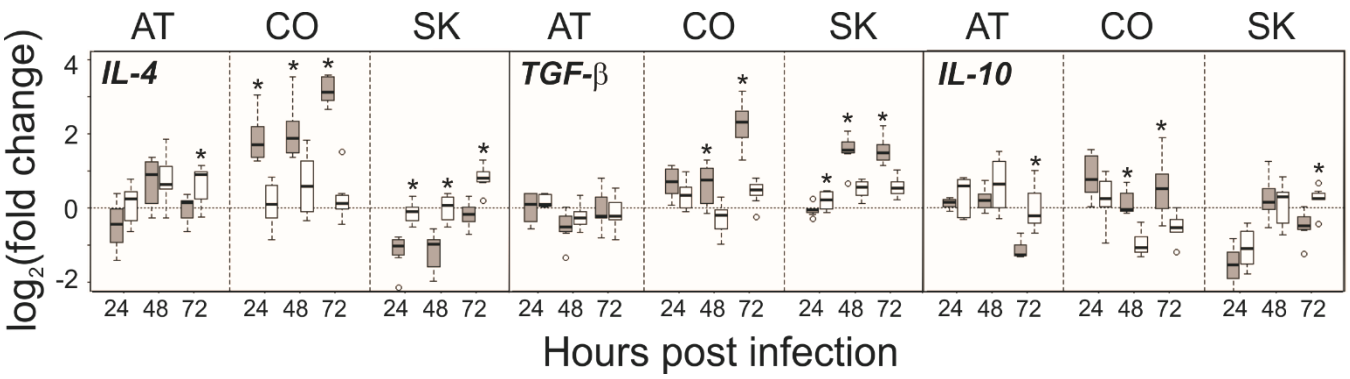

Figure 16. T_H2-type cytokine production in the skin of Atlantic, coho, or sockeye salmon. Expression from each species is normalized to non-infected individuals from the same species. Boxplots represent the median (n = 8) and interquartile ranges with circles representing outliers in attachment skin (dark gray) and non- attachment skin (white) at 24, 48 and 72 hpi. Significantly elevated expression at attachment sites indicates louse-associated activation whereas if expression is higher at non-attachment sites louse-associated suppression is indicated. A three-way ANOVA determined significance between control and infected groups over time (SigmaStat V11.1; Supplementary Tables S4 & 5).

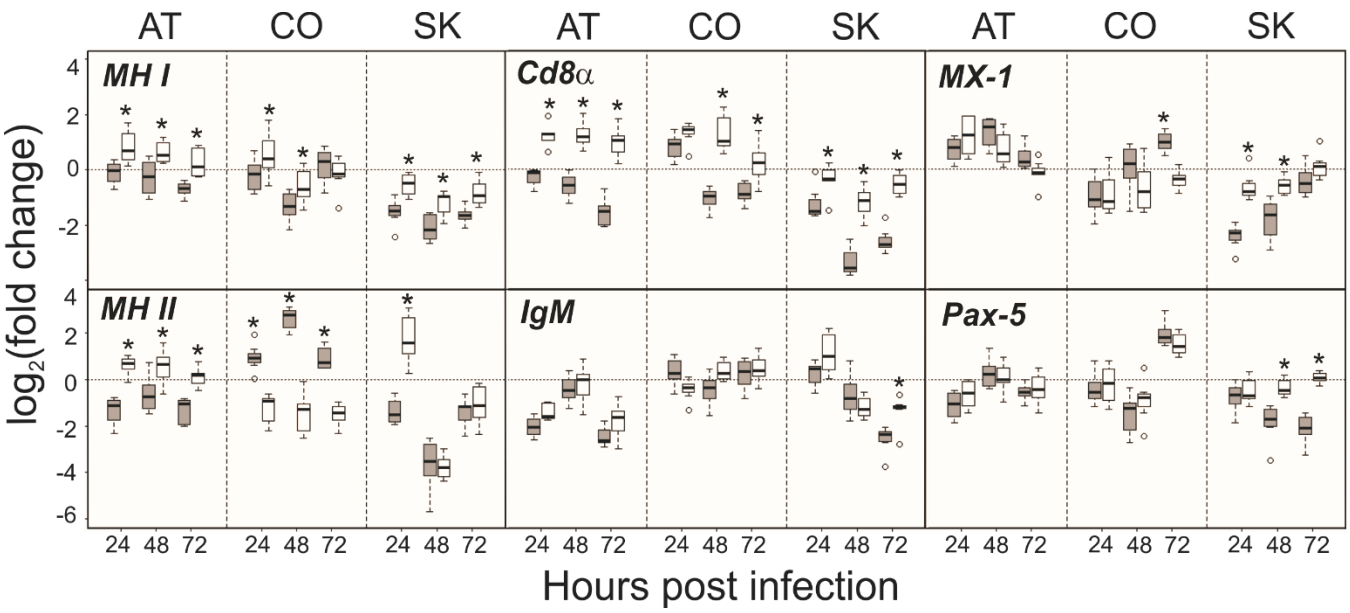

Figure 17. Expression of cellular markers in the skin of Atlantic, coho, or sockeye salmon. Expression from each species is normalized to non-infected individuals from the same species. Boxplots represent the median (n = 8) and interquartile ranges with circles representing outliers in attachment skin (dark gray) and non- attachment skin (white) at 24, 48 and 72 hpi. Significantly elevated expression at attachment sites indicates louse-associated activation whereas if expression is higher at non-attachment sites louse-associated suppression is indicated. A three-way ANOVA determined significance between control and infected groups over time (SigmaStat V11.1; Supplementary Tables S4 & 5).

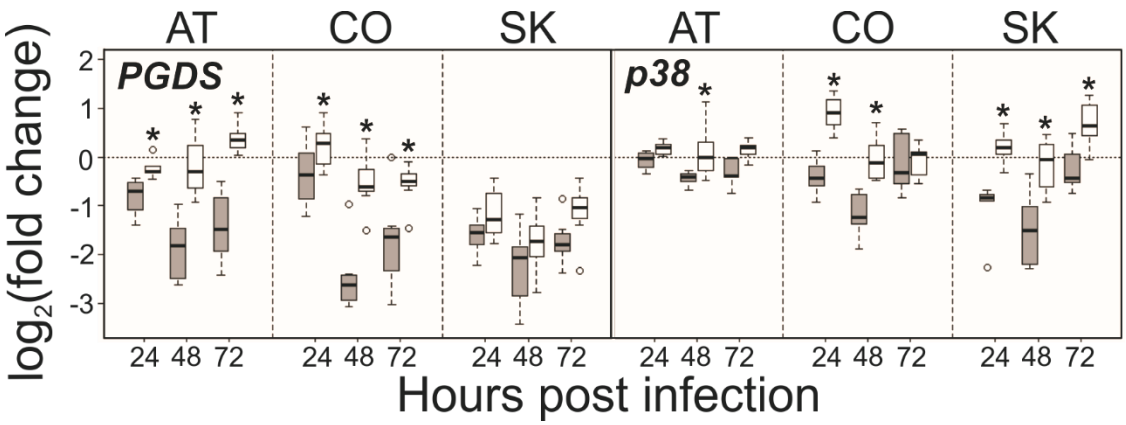

Figure 18. Local suppression of p38 and PGDS in the skin of Atlantic, coho, or sockeye salmon. Expression from each species is normalized to non-infected individuals from the same species. Boxplots represent the median (n = 8) and interquartile ranges with circles representing outliers in attachment skin (dark gray) and non- attachment skin (white) at 24, 48 and 72 hpi. Significantly elevated expression at attachment sites indicates louse-associated activation whereas if expression is higher at non-attachment sites louse-associated suppression is indicated. A three-way ANOVA determined significance between control and infected groups over time (SigmaStat V11.1; Supplementary Tables S4 & 5).

Expression of *IL4* was suppressed in both Atlantic (72 hpi) and sockeye (24, 48, 72 hpi), whereas *IL10* was only suppressed at 72 hpi in Atlantic and sockeye salmon (Figure 16, Supplementary Fig. 2B). Local suppression was observed for all three species in *major histocompatibility class I* (*MH I), CD8α*, and *prostaglandin D synthase* (*PGDS)* (Figures 17-18, Supplementary Fig. 3A & B). Expression of *CD8α* was locally suppressed in all species and this suppression either remained constant (Atlantic salmon) or became more pronounced over time (coho, $p < 0.001$; sockeye, $p < 0.001$; Figure 17, Supplementary Fig. 3A). Expression of *interferon-induced GTP-binding protein (MX1)* was suppressed in coho and sockeye salmon at both 24 and 48 hpi. Significant local suppression of *MX1* was observed in sockeye salmon at 24 and 48 hpi. Expression of *MX1* was locally elevated in coho salmon at 72 hpi ($p < 0.001$; Figure 17, Supplementary Fig. 3A). Similar to *CD8α* expression, the viral response molecule *MH I* was also locally suppressed in all species at across all time points with the exception of coho salmon at 72 hpi, where expression was slightly activated ($p = 0.011$; Figure 17, Supplementary Fig. 3A). Louse-associated suppression of the anti-inflammatory enzyme *PGDS* was observed in Atlantic and coho salmon skin at 48 hpi ($p < 0.001$), and systemic down-regulation of *PGDS* was observed in sockeye salmon skin throughout the experiment (Figure 18, Supplementary Fig. 3B).

Expression of *major histocompatibility class II* (*MH II)* was locally suppressed in Atlantic and sockeye salmon at 72 hpi, but there was significant local activation of this gene in coho salmon ($p < 0.001$; Figure 17, Supplementary Fig. 3A). Suppression was highest in sockeye salmon at 48 hpi ($p < 0.001$; Figure 17). In Atlantic salmon, expression of *IgM* was suppressed at 24 and 72 hpi while in sockeye salmon there was significant local suppression ($p < 0.001$) at 72 hpi. There was no significant differential expression of *paired box protein-5* (*Pax5)* after 24 hpi in any species. After 48 and 72 hpi there was local suppression in sockeye salmon ($p < 0.001$),

and at 72 hpi, there was systemic up-regulation of *Pax5* in coho salmon (Figure 17, Supplementary Fig. 3A).

3.4.4.2 Systemic differential regulation

Up-regulation of expression at both attachment and non-attachment sites (systemic regulation) was observed for several genes, and typically in a species-specific pattern. In Atlantic salmon *IRAK1, TRF* (24 hpi), *iNOS, MX1* (48 hpi), and *serum amyloid P* (*SAP,* 24 hpi) were up-regulated (Figures 14, 15, 17; Supplementary Figs. 1B, 2A, 3A), whereas *IgM* (24 and 72 hpi) and *Pax5* (24 hpi) were down-regulated (Figure 17, Supplementary Fig. 3A). In coho salmon, *TRX* (24 hpi), *IRAK1* (72 hpi), *CD8α* (24 hpi), and *Pax-5* (72 hpi) were up-regulated (Figures 14, 15, 17; Supplementary Figs. 1B, 2A, 3A) and *MX1* (24 hpi) and *Pax-5* (48 hpi) were down-regulated (Figure 17**,** Supplementary Fig. 3A). In sockeye salmon, systemic elevated expression of *IL1β, COX2, MMP9, MMP13, Hep1, TRX, Arg2, TRF, CATH*, and *SAA* was observed at 24 hpi, whereas *iNOS*, *IL10,* and *PGDS* were down-regulated (Figures 13-16, 18; Supplementary Fig. 1-3).

3.4.4.3 Temporal changes in differential expression

In Atlantic salmon, the expression of *IL1β*, *TRX*, *SAA*, *SAP*, and *CATH* increased over time (Supplementary Table 5). Similarly in coho salmon, expression of *IL4, TGFβ, MX1, COX2, CATH, Pax5* and *IRAK1* increased as did *IL4, IL8, IL10, TNFα, TGFβ, MX1, TRX, iNOS, COX2, MMP13, SAA, SAP, CATH, IRAK1, C/EBPβ* and *p38* in sockeye salmon

In Atlantic salmon there was a decrease in the expression over time of *IL10, Hep1, CD8α* and *IRAK1* (Supplementary Table 5). In coho salmon the expression of *PGDS, MMP13, CRP, TRF*, *SAA*, *SAP* and *CD8α* decreased as did *IL1β, CD8α, MH II, IgM* and *Pax5* in sockeye salmon.

3.4.5 Plasma osmolality

There was no significant difference between control and infected Atlantic or coho salmon at 24, 48 or 72 hpi. In contrast, there was a significant increase in osmolality in infected sockeye salmon throughout the study ($p < 0.001$, data not shown). There was a significant increase at 48 hpi among non-infected coho salmon ($p < 0.001$) and non-infected Atlantic salmon had the highest mean osmolality at 24 and 72 hpi ($p < 0.001$).

3.5 Discussion

Using a comparative infection model, we assessed the innate transcriptional and cytological characteristics of the louse-skin interface in Atlantic, coho and sockeye salmon during infection with Pacific *L. salmonis*. By expanding the methodology of our earlier model to include histochemistry we have identified three distinct (MHIIβ+, IL1β+, TNFα+) putative cell populations and demonstrate exaggerated recruitment of these cells in the skin of resistant salmon (coho) during louse attachment. In addition, by expanding our transcriptional analysis to include a more comprehensive suite of gene targets, our data indicate resistance also includes a T_H2-type response (up-regulation of *IL4, IL10* and *TGFβ*) concomitant with wound healing and tissue repair. Infection with *L. salmonis* results in cutaneous lesions and osmotic shock occurs in the absence of or prior to wound repair. Indeed, osmoregulatory failure and distress is a common effect of infection particularly in more susceptible species such as Atlantic salmon (Wootten *et al.* 1982; Grimnes & Jakobsen 1996; Wagner *et al.* 2008). In this study we observed degraded mucus layer and raised scales, and in some cases, bloody exudate as early as 24 hpi in sockeye salmon, similar to those observed by Jakob *et al.* (2013). These lesions were absent in coho salmon or Atlantic salmon. Furthermore, osmolality as a function of *L. salmonis* attachment was significantly increased in sockeye but did not change in either Atlantic or coho salmon, indicating osmoregulatory disturbances in sockeye salmon. A comparison of gross lesions and

physiological, cytological and genetic responses indicates that unlike coho and pink salmon, sockeye salmon belong to a group of susceptible salmon species that also includes chum and Atlantic salmon (Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012).

3.5.1 Histochemistry reveals diverse cell populations activated during sea lice infection.

Mucus cells (MCs) play a role in the protection against ectoparasites (Pottinger *et al.* 1984; Buchmann & Bresciani 1998; Buchmann 2009; Andrews *et al.* 2010), and localized MC responses to parasite attachment include hypertrophy and hyperplasia (Bosi *et al.* 2005) and changes in acidity and composition (Domeneghini *et al.* 1998). Differential acidity among MC-associated glycoconjugates is well-documented and MCs produce antimicrobial peptides, acute phase proteins, immunoglobulins and other defense-related molecules. Site-selection by ectoparasites can be related to the chemical characteristics of MCs (Buchmann & Bresciani 1998). Increased secretion of chemically distinct glycoconjugates by gut mucosa contributes to elimination of internal parasites in brown trout (*Salmo trutta*) (Bosi *et al.* 2005) and MCs are important in defense against *Gyrodactylus derjavini* (Bakke *et al.* 1996; Buchmann & Bresciani 1998; Buchmann 2009). Species-specific patterns of MC density have been observed: density was higher in rainbow trout skin compared with coho and Atlantic salmon (Fast et al., 2002). In the present study, *L. salmonis* induced MC exhaustion in both sockeye and coho salmon which was most pronounced in the former and associated with changes to cellular contents. The observed decrease in MC density may be related to mechanical disruption by the parasite as occurs with other ectoparasites (Buchmann & Bresciani 1998; O'Byrne-Ring *et al.* 2003; Andrews *et al.* 2010; Chettri *et al.* 2014). Stressors such as water temperature, handling or pH also influence proliferation and migration of MCs (Iger *et al.* 1995; O'Byrne-Ring *et al.* 2003; Vatsos *et al.* 2010). Since environmental variables were controlled, the decline in MCs observed

in sockeye and coho salmon is a likely consequence of parasite infection, similar to the observation in Atlantic salmon (Covello *et al.* 2012). Moreover, we have localized IL1β and TNFα to MC-like cells in the epithelium of salmon, suggesting a contribution of MCs to innate immunity.

Class II major histocompatibility (MH II) molecules are expressed on the surface of antigen-presenting cells. Previously we demonstrated up-regulation of MH II genes in the skin of the resistant pink salmon during *L. salmonis* infection (Braden *et al.* 2012). In another study, MH II+ cells were reported in gill lesions caused by *Paramoeba perurans* (agent of amoebic gill disease, AGD) (Morrison *et al.* 2006), supporting the notion that MH II+ cells play a role during cutaneous infections in salmon. Here we report significant infiltration of MH II+ cells in the skin epidermis and dermis of *L. salmonis*-infected coho salmon. Interestingly, we observed infiltration of MH II+ cells in the skin of infected sockeye salmon which was not accompanied by transcriptomic up-regulation. It is possible that in coho salmon *MH II* was up-regulated while in sockeye salmon protein expression of MH II was stable and not induced (Guo *et al.* 2008). These apparently divergent MH II regulatory modes may relate to the observation of MH II+ cells with distinct morphological characteristics: small (< 10 μm) lymphocyte-like cells and larger dendritic-like cells (> 10 μm). Similar observations have been reported in rainbow trout (Olsen *et al.* 2011) and Atlantic salmon (Morrison *et al.* 2006). MH II is induced in a variety of cells including keratinocytes and epithelial cells, but is most commonly associated with APCs such as B-cells (Li *et al.* 2006), monocytes/macrophages (Rieger & Barreda 2011), or dendritic cells (Lovy *et al.* 2006; Bassity & Clark 2012). To identify the MH II+ cells, we performed histochemical localization of α-MHIIβ with α-mIgM, α-Pax5, α-IL1β and α-TNFα. Serial sections of MH II+ tissue failed to detect mIgM+ or Pax5+ cells which indicates this population

is likely not composed of differentiated B-cells (Barr *et al.* 2011). However, significant binding of specific antibodies suggested the presence of IL1β and TNFα, which are expressed by monocytes, granulocytes and dendritic cells. Further work characterizing these two MH II+ cell variants in multiple salmon species is necessary.

Morphologically distinct populations were also observed for cells expressing either IL1β+ or TNFα+ in the skin of Atlantic, coho and sockeye salmon: in both cases lymphocyte-like and MC-like cells were observed. The absence of staining with α-mIgM as well as the lack of significant *mIgM* expression, suggests these cells were likely not activated B cells, plasmablasts or plasma cells (Barr *et al.* 2011), and this was corroborated by the failure to detect evidence of the B-cell marker Pax5. Interestingly we observed a 2-fold increase in *Pax5* mRNA in coho salmon skin 72 hpi. Therefore it is possible that the significant influx of IL1β+/IgM- cells in coho salmon at 72 hpi were activated IgM- B-cells. A population of IL1β+/EBF+/IgM- cells were significantly more abundant in a bacterial cold-water disease (BCWD) resistant strain of rainbow trout (*O. mykiss*) and were suggested to be bi-potential B lymphoid/myeloid progenitor (BMP) cell which can differentiate into neutrophils, monocytes or B lymphoid cells as described in mammals (Nakajima 2011; Zwollo *et al.* 2014). Although we did not assess protein expression of EBF (early B-cell factor), the population of cells we observed in the skin of louse-infected salmon were also IL1β+/IgM-, and may represent a homologous cell population. Interestingly we found the number of putative progenitor B-cells was significantly higher in the skin of two *L. salmonis*-resistant species: coho (data herein) and pink salmon (L. Braden, unpublished). Furthermore, a proportion of these cells was observed to stain positive with α-TNFα, similar to rainbow trout (Zwollo *et al.* 2014). It is unlikely that our IL1β+/IgM- or TNFα+/IgM- are BMPs, as teleost hematopoiesis is primarily found in the anterior kidney. The

predominant labelled cells in the skin of salmon infected with *L. salmonis* are characterized by their small diameter (~ 5-10 μm) and staining characteristics (IL1β+/IgM-, TNFα+/IgM-, MHII+/IgM-). If originating from the peripheral blood, these cells would be easily recruited to the site of attachment and may represent early cellular effectors of the salmonid innate response to infection with ectoparasites.

3.5.2 Genetic responses in the skin during sea lice infection

We assessed transcriptomic responses at the attachment site in both resistant (coho) and susceptible (Atlantic, sockeye) salmon species by focusing on early response targets previously observed during *L. salmonis* infection (Skugor *et al.* 2008; Tadiso *et al.* 2011; Sutherland *et al.* 2011, 2014a; Krasnov *et al.* 2012; Braden *et al.* 2012).

3.5.2.1 Dysregulation of inflammatory regulation in susceptible species

Dysregulated inflammatory processes contribute to overall susceptibility of salmon to *L. salmonis* infection (Fast 2013, and references therein). We measured immediate and exaggerated expression of pro-inflammatory mediators (*IL1β*, *IL8, TNFα, COX2* and *C/EBPβ*) at louse-attachment sites in Atlantic, coho and sockeye salmon; however, in all cases, the ability of sockeye salmon to activate this local response appeared delayed or weak. Activation of inflammation-regulatory pathways (*IL10, TGFβ)* occurred as early as 24 hpi in coho salmon, while in the susceptible Atlantic or sockeye salmon, heightened expression of *IL1β*, *IL8* and *TNFα* was not accompanied by these regulatory pathways. Moreover in Atlantic and sockeye salmon, the lack of *IL10* and *TGFβ* with simultaneous up-regulation of *Arg2*, indicate that the dominant population of cutaneous macrophages at *L. salmonis* attachment sites was M1θ. Moreover, significant expression of *iNOS* in louse-infected Atlantic salmon skin suggests M1θ were involved in extracellular killing. Surprisingly, the high systemic activation of *iNOS* in

Atlantic salmon was not associated with a similar increase in *thioredoxin (TRX)*, which acts to limit oxidative stress during inflammatory events (Ito *et al.* 2011). Tumor necrosis factor α is a T_H1-type cytokine with a dual role, functioning as pro-inflammatory mediator when released by M1θ (Gonzalez *et al.* 2007c; Fast *et al.* 2007b; Jones *et al.* 2007; Forlenza *et al.* 2008; Braden *et al.* 2012) and as an initiator of tissue repair and antioxidant defense when produced by M2θ, (Schwabe & Brenner 2006). Expression of *TNFα* was highly up-regulated in Atlantic and coho salmon at all sampling times, whereas in sockeye salmon *TNFα* expression and infiltration of TNFα+ cells were not detected in attachment-site skin until 48 hpi. Thus the dominant M1θ population in Atlantic and sockeye salmon appear to be exacerbating inflammation (*iNOS, Arg2, IL1β, IL8, TNFα*) in the absence of regulation whereas in coho salmon, the inflammatory response is regulated by *IL10* and *TGFβ*.

3.5.2.2 Acute phase protein expression in salmon skin during sea lice infection

The APR has been implicated in protection against pathogens (Lü *et al.* 2012), as well as during *L. salmonis* infection (Braden *et al.* 2012; Sutherland *et al.* 2014a), and is characterized by hepatic and extra-hepatic production of acute phase proteins (APPs). Up-regulation of the APR inducer *C/EBPβ* was observed in all species suggesting APR activation following exposure to *L. salmonis* (Jensen *et al.* 1997; Poli 1998; Bayne & Gerwick 2001; Agrawal *et al.* 2001). However, local expression of the downstream product, *IL6,* was only detected in coho and sockeye and not in Atlantic salmon. Furthermore, high expression of *IL6* in coho and sockeye salmon was not accompanied by high expression of predicted downstream APPs (eg. *SAP*, *SAA, CRP*). In contrast, we observed species-specific patterns of APP expression similar to earlier comparative studies (Braden *et al.* 2012; Sutherland *et al.* 2014a). Patterns of APP expression were common among sockeye and Atlantic salmon (up-regulation of *SAP* and *CATH*), whereas up-regulation of

SAA was specific to coho salmon skin after 24 hpi and remained the highest compared to Atlantic or sockeye salmon throughout the experiment. SAA is produced by macrophages and attracts leukocytes and stimulates production of cytokines and MMPs (Rebl *et al.* 2011), and has been observed in the skin of rainbow trout during ectoparasitic infection (Chettri *et al.* 2014). The sustained and elevated expression of this pivotal APP in the skin of infected coho salmon suggests a species-specific pathway involving the activation of SAA-associated products that contribute to the resistant phenotype. Specifically, chemotactic recruitment of circulating leukocytes such as monocytes and neutrophils by local *SAA* (and *IL8*) production may provide a mechanism by which circulating IL1β+/IgM- or TNFα+/IgM- cells are recruited into lice-infected skin. Interestingly, in coho salmon we observed the highest number of IL1β+ and TNFα+ cells concomitant with the highest local activation of *SAA* expression.

Induction of iron regulation, determined by expression of *hepcidin 1 (Hep1),* occurred locally in all three species, with the highest level of expression in the Pacific salmon species. Regulation of iron (i.e., nutritional immunity) is an important feature of an efficient APR and hepcidin functions to prevent export of iron from macrophages to the blood (Nemeth & Ganz 2006; Shi & Camus 2006). Expression of another key iron metabolism protein, *transferrin* (*TRF),* was significant at louse attachment sites in susceptible Atlantic and sockeye salmon. These results suggest iron regulation and thus nutritional immunity (i.e., the protection of key nutrients from infectious agents) are important response mechanisms in the skin during sea lice infection, as suggested earlier for resistant pink salmon (Sutherland *et al.* 2014a). Furthermore, results of the present work suggest pathways of iron regulation and acute-phase responses contribute to the resistant phenotype in coho salmon.

3.5.2.3 Tissue remodeling and wound repair in salmon skin during sea lice infection

Wound healing is a critical host response during ectoparasitic infection and compromised wound healing pathways contribute to the susceptible phenotype in salmon. Earlier studies on Atlantic salmon show a delayed healing response after infection with *L. salmonis* despite elevated levels of *IL10* and *TGFβ* (Skugor *et al.* 2008; Krasnov *et al.* 2012). Chronic infections with *L. salmonis* in susceptible Atlantic salmon are characterized by aberrant production of MMPs and reduced cellular proliferation associated with impaired wound healing (Skugor *et al.* 2008; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012). We observed significant activation of MMPs in the skin of Atlantic, coho and sockeye salmon; however, in sockeye the local activation of both *MMP9* and *MMP13* was delayed. Delayed wound healing in a parasite-murine model is associated with ablation of *IL4* expression (Gronert *et al.* 2005). In the present work, expression of *IL4* was only observed in coho salmon, suggesting this molecule may also be important for wound healing during louse infection. Interleukin 4 is known to be important for T_H2 differentiation *in vivo*, but the relevant cellular source is unclear. In rainbow trout, production of *IL4* was localized to unidentified IgM- cells (Takizawa *et al.* 2011). In salmon, the IL4 and IL13 teleost homologue IL4/13A is constitutively expressed in the gills and skin indicating these sites are "T_H2-skewed" environments and thus innately protective against parasites and T_H1-associated damage (Takizawa *et al.* 2011). Sokol *et al.* (2008) showed that murine basophils produced several T_H2-type cytokines after exposure to a cysteine protease, and suggest that the protease activity of certain helminths and ectoparasites stimulate basophils or mast cells to produce T_H2-type cytokines with subsequent wound repair and fibrosis. Proteases are present in secretions by *L. salmonis* (Fast *et al.* 2004, 2007a), and wound repair characterized by a T_H2-type response would be advantageous during sea lice infections (Allen & Wynn 2011). Indeed significant local up-regulation of T_H2-cytokines was observed in the skin of resistant coho salmon during sea lice

infection and it is possible that this response may be mediated by louse-derived proteases, small chitinous molecules or DAMPs such as collagen fragments (Castillo-Briceño *et al.* 2011) that would interact with PRRs on target cells and activate expression of *IL4*, *IL10* and *TGFβ*. Interestingly, *IL4* induces expression of *acidic mammalian chitinase* (*AMcase*) which has a role in feedback attenuation of chitin-induced allergic innate responses (Reese *et al.* 2007) and is a mediator of IL4- and IL13-induced T_H2-type responses (Zhu *et al.* 2004). Previous work has shown expression of *AMCase* is specific to *L. salmonis*-resistant pink salmon (Sutherland *et al.* 2011, 2014a), therefore high levels *IL4* in the skin of resistant coho salmon implies the expression of *AMCase* in this species. The function of *AMCase* in this host-parasite relationship requires further study.

The presence of teleost mucosal dendritic cells (mDCs) has not yet been shown; however, recent functional characterization of dendritic cells in hematopoietic tissues of rainbow trout together with the importance of this cell type in mammalian mucosal immunity suggests the likely presence of a mucosal counterpart in salmonids (Bassity & Clark 2012). Interestingly, in mammalian models of parasite infection, mDCs are key T_H2-type response mediators as they limit secretion of pro-inflammatory cytokines by inducing T_H2 differentiation of CD4+ T-cells into T_H17 T-cells. The latter act specifically at epithelial and mucosal barriers to inhibit pathogen growth and infection (Waite & Skokos 2012), concomitant with suppression of T_H1-type responses (Iwasaki 2007). We observed MH II+ cells with DC-like morphology in the skin of coho salmon which may represent the population responsible for the production of *IL4*.

3.5.2.4 Suppression of immunological pathways in L. salmonis-infected skin

Immunosuppression by internal and ectoparasites may contribute to coevolution between parasite and their hosts (Yang & Cox-Foster 2005), and this phenomenon has been observed for several

fish parasites including *L. salmonis* (Wynne *et al.* 2008; Sitjà-Bobadilla 2008; Fast 2013). Suppression of gene expression at *L. salmonis* attachment sites suggests parasite-associated mechanisms disrupt normal host responses.

For example, suppression of anti-viral pathways have been described during experimental *L. salmonis* infections (Skugor *et al.* 2008; Tadiso *et al.* 2011; Covello *et al.* 2012; Krasnov *et al.* 2012; Sutherland *et al.* 2014a), and in the present study we observed local suppression of *MXI, MH I,* and *CD8α* in Atlantic, coho and sockeye salmon skin throughout this experiment implying that the anti-viral response was compromised at the attachment site of *L. salmonis*. This phenomenon occurs during infection with ticks (*Ixodes* spp.) which suppress host viral response pathways (Ferreira & Silva 1998; Kopecký *et al.* 1999; Oliveira *et al.* 2011). Similar to what is found in tick saliva, immunosuppressive molecules (e.g., PGE_2) have been isolated from *L. salmonis* secretions (Fast *et al.* 2003, 2004, 2007a). The ability of *L. salmonis* to act as a vector for bacterial and viral disease has been previously investigated (Barker *et al.* 2009; Jakob *et al.* 2011) and there is evidence that infection with *L. salmonis* may enhance the risk for viral infection (Petterson *et al.* 2009; Valdes-Donoso *et al.* 2013). As infections with *L. salmonis* in mariculture is often associated with secondary infections, this potential side effect of sea lice infection requires further inquiry.

3.6 Conclusions

Using a comparative infection model together with histochemical and transcriptional analyses, we have advanced the current understanding of the parasite-host relationship between *L. salmonis* and salmon. By utilizing salmonid-specific cellular markers we provide the first cytological evidence for cellular populations (MH II+, IL1β+, TNFα+) activated in the skin during infection, and show that these increase in number in skin of resistant salmon. Our analysis

identified locally elevated expression of several pro-inflammatory mediators (e.g., *IL1β, IL8, TNFα, COX2, C/EBPβ*), and tissue repair enzymes (*MMP9*, *MMP13*) as hallmark responses to infection with *L. salmonis*. However, responses specific to coho salmon (e.g., *IL4, IL6, TGFβ, SAA*, *MH II*) or responses shared among susceptible salmon (e.g., *SAP*, *TRF*, *CATH* in Atlantic and sockeye salmon) provide further characterization of resistance-related pathways. Specifically, differential expression of *IL4* mRNA was suppressed (24, 48 hpi) or non-detectable (72 hpi) in sockeye or Atlantic salmon while in coho salmon *IL4* was significantly activated. This seemingly coho-specific response may represent a resistance pathway, as neither sockeye salmon nor Atlantic salmon, both susceptible species, expressed *IL4*. Our results confirm the importance of an early pro-inflammatory T_H1-type pathway as an initial host response during infection with Pacific sea lice, and demonstrate a concomitant regulatory T_H2-type process as a candidate defense mechanism in the skin of resistant salmon. Thus, resistance to *L. salmonis* may be associated with ability to regulate inflammation, limit pathological effects and switch to a tolerant response as observed in other host-parasite relationships (Medzhitov *et al.* 2012; Fontbonne *et al.* 2013).

3.7 Chapter acknowledgements

This research was funded by Genome BC and Fisheries & Oceans Canada`s Program for Aquaculture Regulatory Research. Monoclonal α-MHCII-β and α-mIgM antibodies were a generous gift by Dr. Karsten Skjoedt at the University of Southern Denmark. Polyclonal α-tIL1β and α-tTNFα antibodies were a generous gift by Dr. Greg Wiens at the National Centre for Cool and Cold Water Aquaculture, and polyclonal α-Pax5 was a generous gift by Dr. Patty Zwollo at the College of William and Mary. We greatly appreciate technical assistance of Karina Rasmussen (University of Southern Denmark) and Bill Bennett (Pacific Biological Station). We gratefully acknowledge industrial cooperation by Brad Boyce (Marine Harvest Canada) in collection of Pacific *L. salmonis*. LMB is funded by an NSERC Alexander Graham Bell Canada Graduate Scholarship. Dr. Ben Sutherland is acknowledged for valuable comments on earlier versions of this manuscript.

3.8 Online material

Supplementary data to this chapter can be found online at

http://dx.doi.org/10.1016/j.dci.2014.09.015

Chapter 4: Reduced abundance of resistant biomarkers in the skin of mature pink salmon, *Oncorhynchus gorbuscha*, infected with the sea louse, *Lepeophtheirus salmonis*.

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LMB conceived of the study, performed immunohistochemical and qPCR work, interpreted the data and wrote the manuscript.

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4.1 Abstract

The purpose of this research is to better understand the apparent contradiction in which juvenile pink salmon greater than 0.7 g rapidly reject the sea louse, *Lepeophtheirus salmonis*, because of rapid and robust innate defense responses; whereas, adult pink salmon captured at sea or shortly before spawning carry large numbers of the parasite. Host species, nutrition and age are important factors in determining the outcome of a host-parasite interaction. Sexual maturation is associated with decreasing immunocompetence in many vertebrates including salmonids; however, a causal mechanism linking increased susceptibility of mature pink salmon to infection with sea lice has not been demonstrated. Here we compare a subset of genetic and cellular biomarkers of resistance to *L. salmonis* in juvenile and mature pink salmon. Down-regulation of *major histocompatibility factor II, C-reactive protein, interleukin-1β, interleukin-8* and *cyclooxygenase-2* was observed in the skin of mature pink salmon compared to juvenile pink salmon. In support of this observation, the louse-attachment site skin of juvenile pink salmon was highly populated with MHIIβ^{+} and IL-1β^{+} cells that were either absent, or at reduced levels in the skin of mature pink salmon. In addition, irrespective of louse infection very low mucocyte densities were observed in the skin of mature pink salmon whereas juvenile pink salmon skin contained high densities of mucocytes that decreased as a function of louse attachment. Divergent genetic and histological responses in juvenile and mature pink salmon helps explain the reduced resistance to infection observed in the maturing pink salmon.

4.2 Introduction

Juvenile pink salmon (*Oncorhynchus gorbuscha*) have one of the shortest freshwater stages of all anadromous Pacific salmonids and migrate to the ocean shortly after emergence from spawning beds (Heard 1991). They are the smallest of all salmon (0.2 g) to enter the marine environment, and are consequently exposed to a variety of marine pathogens including the parasitic copepod, *Lepeophtheirus salmonis,* at a sensitive developmental stage. Juvenile pink salmon have adapted to this precocious pathogen exposure by the early development of functional defense systems. For example, as early as 0.7 g, pink salmon resist infections with *L. salmonis* and this is accompanied by rapid and robust acute phase and inflammatory responses (Jones *et al.* 2008b; Sutherland *et al.* 2014a). Comparative experimental infections show that juvenile pink salmon display a resistant phenotype characterized by parasite rejection and pronounced inflammation at the site of attachment which is absent in the susceptible Atlantic (*Salmo salar*), chum (*O. keta*), or sockeye (*O. nerka*) salmon (Jones *et al.* 2007; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012, 2015). In contrast, pre-adult and adult pink salmon support heavy natural infections of *L. salmonis* (Nagasawa 2001; Beamish *et al.* 2005). A fundamental difference between lab and field observations is that adult fish are preparing to spawn and display physiological changes associated with sexual maturation. Metabolic energy is allocated towards gamete production along with a thickening of the epithelium and absorption of scales (Heard 1991).

The capacity for sex steroids to alter immunological responses to pathogenic agents, including the effects of sex hormones on the function of important cellular effectors, has been described in mammals (Muñoz-Cruz *et al.* 2011) and fish (Chaves-Pozo *et al.* 2012). Elevated levels of steroid hormones was associated with immune-suppression and increased disease susceptibility in salmonid and non-salmonid fishes infected with *Cryptobia salmositica* (Currie & Woo 2007) and *Ichthyobodo necator* (Robertson 1979). Severe ectoparasitic infections

(*Piscicola salmositica*, *Ichthyophthirius* sp., *Scyphidia* sp., *Gyrodactylus* sp.) have been reported in sexually maturing fish (Pickering & Christie 1980; Buchmann 1997; Currie & Woo 2007). In Arctic charr (*Salvelinus alpinus*), immune suppression and increased susceptibility to macroparasites was related to development of secondary sexual characteristics and spermatogenesis (Skarstein *et al.* 2001). Androgenic receptors on salmonid lymphocytes have been suggested to be a mechanism of immune suppression during sexual maturation (Slater *et al.* 1995). There appears to be an additive suppressive effect of androgens and cortisol (Woo *et al.* 1987; Maule & Schreck 1990), and immune suppression seems to operate through a cortisol receptor-mediated system in lymphocytes (Maule & Schreck 1990, 1991; Slater & Schreck 1993). Pickering and Pottinger (1987) reported lymphocytopenia in three strains of sexually mature male and female brown trout. In this latter study, lymphocytopenia was correlated with elevated plasma cortisol levels. As sexual maturation in salmonid fish is associated with an increase in susceptibility to disease (Pickering & Christie 1980; Currie & Woo 2007), it is likely that there is some level of cortisol-induced suppression of lymphocyte activity.

Little is known about the relationship between host maturation and responses during *L. salmonis* infection (reviewed in Nava-Castro *et al*., 2012). The purpose of the present study was to investigate immunological mechanisms in the skin that may help explain the apparent differences in resistance between juvenile and mature pink salmon. We have analyzed the expression profile of a small subset of genetic and cellular markers previously shown to be associated with resistance to *L. salmonis* in the skin of juvenile and mature pink salmon (Braden *et al.* 2012, 2015). Here we present molecular and cytological evidence that may partially explain the switch from a resistant to susceptible phenotype during maturation in pink salmon.

4.3 Methods

4.3.1 Experimental fish and infection trials.

All procedures involving fish were approved by the Canadian Council of Animal Care (CCAC). Pink salmon (*Oncorhynchus gorbuscha*) were obtained as fry from the Quinsam River Hatchery on Vancouver Island, British Columbia. All fish were reared on brackish water (15 ppm) until approximately 25 g, after which they were maintained on ultraviolet-treated salt water (~ 33 ppm) in single-pass flow-through tank systems on a 12:12 hr light:dark cycle. Fish were fed 1% total biomass daily.

The first infection trial was performed on juvenile (ocean phase) salmon in the fall of 2012. Fork length and weights were 18.2 ± 4.2 cm and 119.3 ± 43.9 g, respectively. The remaining fish were maintained in the salt water until the fall of 2013 when indicators of sexually maturation were observed (darkened pigment, absorbed scales, humped-backs, distended abdomens, Figure 19). At this time fork length and weights were 31.7 ± 3.4 cm and 254.1 ± 39.3 g, respectively. For the maturation trial, the fish were sexed and gonad maturity estimated by direct observation. There was a female:male ratio of 65:35, and in all cases necropsy revealed evidence of advanced sexual maturation (well developed eggs, large testes). For both trials, fish were randomly divided in four 330 L tanks (2X infection tanks, 2X control tank), acclimated for approximately 7 days and starved at least 24 hr prior to any manipulation.

Adult female *L. salmonis* were collected during harvest of Atlantic salmon at commercial aquaculture sites on Vancouver Island, British Columbia, Canada. After collection, the lice were rinsed and transported back to the Pacific Biological Station (Nanaimo, British Columbia, Canada) in 8°C aerated filtered sea water. Only lice displaying attachment behavior to the collection vessel were included. The time between collection of the sea lice and initial infection was < 24 hr.

For infections of juvenile or mature salmon, the water level of each tank was reduced by half and fish were sedated using 0.2 mg/L metomidate hydrochloride (M-HCl; Aquacalm, Syndel Laboratories). Sedated fish were transferred to a temporary tank containing M-HCl to which 5 adult lice/fish were added and allowed to settle and attach. Fish were gently removed from the infection tank once they were infected with 5 lice and placed into an infection tank. Control fish were sedated and placed into a sham infection tank containing 0.2 mg/L M-HCl without sea lice.

At 24 and 48 hr post-infection (hpi) six fish were removed from each tank and immediately euthanized in 200 mg/L tricaine methanosulfonate (TMS, Syndel Laboratories). Attachment-site skin samples were dissected from each fish using disposable 5 mm AcuDerm® Biopsy punches (Braden *et al.* 2012) for subsequent RNA and histological analysis. For RNA analysis, three attachment-site samples were pooled, snap-frozen in liquid nitrogen and stored at -80 °C. Non-attachment-site samples were similarly processed from each infected (n = 3) and non-infected fish (n = 3). Histological samples were similarly collected from infected (n = 3) and sham-infected (n = 3) fish, fixed in 10% neutral buffered formalin (NBF) for 24 hr then transferred to 70% isopropanol.

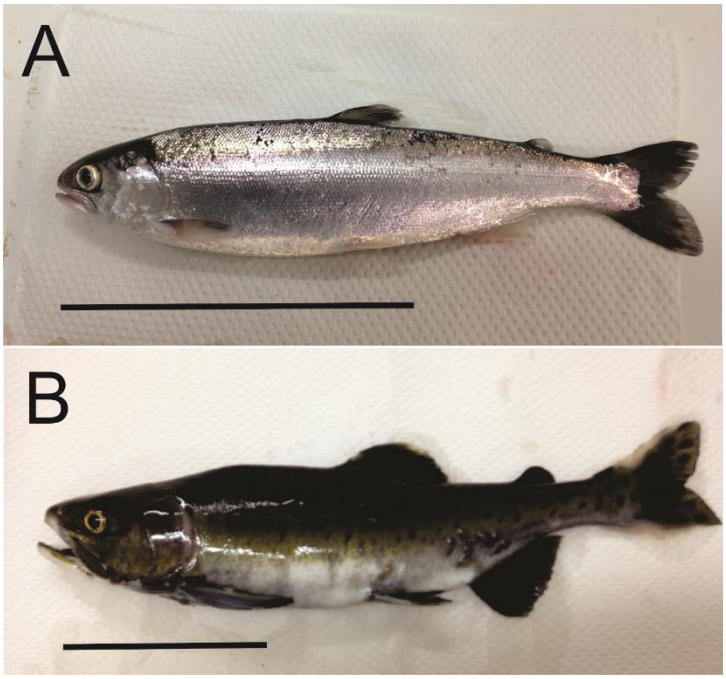

Figure 19. Photograph of juvenile and mature pink salmon showing divergent physiological characteristics.

Juvenile pink salmon (18.2 ± 4.2 cm) (A) were bright silver and lacked developed sexual organs upon necropsy whereas mature pink salmon (31.7 ± 3.4 cm) (B) were much darker, displayed secondary sexual characteristics, absorbed scales, and presented well-developed sex organs following necropsy. Scale bar = 10 cm.

4.3.2 Immunohistochemistry

Tissue samples were dehydrated in a graded isopropanol series, cleared in xylene, and embedded in paraffin blocks. Embedded tissues were sectioned using a Leica RM2135 microtome (Leica Microsystems, Germany). Serial sections (5 μm) were placed on SuperFrost UltraPlus (Menzel-Glaser) positively charged glass slides and dried overnight at 40°C. After deparaffinization in xylene and rehydration in graded isopropanol, the sections were either stained using hematoxylin and eosin (H&E) for routine histopathology or with a combined periodic acid schiff/alcian blue (PAS/AB, pH 2.5) in which sections of control and infected skin were incubated in 1% Alcian blue (pH 2.5) for 20 min, rinsed in distilled water, and subjected to routine PAS reaction. Sections were examined using a compound microscope (Zeiss Axio Imager) equipped with a digital imaging system (Axiocam MRc5) at 200X magnification. Three random fields of view (0.9940 mm^2/field of view) were scanned per fish and the average mucocyte density (cell/mm^2) was calculated (± standard deviation). The cells were characterized as containing either acidic (blue), neutral (pink) or combined (magenta) pH glycoconjugates as previously described (Bosi *et al.* 2005).

Serial sections were also probed with monoclonal antibodies against Atlantic salmon MH II β-chain (α-MHIIβ), surface IgM (α-mIgM) or with polyclonal antibodies to rainbow trout IL1β (α-tIL1β) (Table 17). After de-paraffinization and rehydration, sections were heated to 100°C in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0) for 20 min, allowed to cool to room temperature for 10 min in phosphate-buffered saline (PBS) then washed twice in Tris-buffered saline + 0.2% Tween-20 (TBS-T; pH 8.0) for 5 min with gentle agitation. For α-MHIIβ and α-mIgM detection, sections were blocked in protein blocker, and then rinsed gently with TBS-T. The sections were incubated with primary antibody in TBS-T and 1% bovine serum albumin (BSA, Sigma) overnight at 4°C in a humid chamber. After incubation, the sections were washed

in TBS-T (2 x 5 min), incubated in a mouse-specifying reagent (EXPOSE Mouse/Rabbit Specific HRP/DAB kit, Abcam) for 10 min followed by a 10 min incubation in hydrogen peroxide blocker. Immuno-labeled cells were detected by 20 min incubation with a goat anti-rabbit HRP conjugate followed by 5 min with 3,3'-diaminobenzidine (DAB) in PBS with 0.015% H_20_2. For detection of α-tIL1β, a rabbit-specific alkaline phosphatase (AP) detection kit (ABC, Abcam) was used according to manufacturer's instructions. Sections were blocked in protein block for 10 min, washed in TBS-T and incubated with primary antibody in TBS-T + 1% BSA as above. Sections were washed in TBS-T (2 x 5 min), incubated in biotinylated goat anti-rabbit for 15 min, washed in TBS-T (2 x 5 min) then incubated in streptavidin AP for 15 min. After a final wash (2 x 5 min) the sections were developed using the Permanent Red Fast System (Abcam). Sections were counterstained in 1% Alcian blue and diluted Mayer's hematoxylin (1/20), dehydrated in graded isopropanol, cleared in xylene and mounted (Permount). Sections treated with irrelevant antibodies served as negative controls while sections known to contain the target molecules served as positive controls. Species compatibility for each antibody was determined prior to IHC assays.

4.3.3 RNA extraction, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted from pooled skin plugs under liquid nitrogen conditions using the RNEasy Fibrous Tissue kit (Qiagen) as previously described (Braden *et al.* 2012). Briefly, ~ 10 mg of tissue was homogenized at 30 Hz for 10 min in extraction buffer and then incubated at 55°C for 15 min with 10 µL of proteinase K (10 mg/ml, Qiagen). Column-purified RNA was treated on-column following manufacturer's instructions with DNase I to digest genomic DNA. Samples were eluted to 30 µL with ultra-pure water and RNA quantity determined using

spectrophotometry (NanoDrop-1000) and quality verified using agarose gel electrophoresis (1%). All extracted RNA samples had an $A_{260/280}$ between 1.8 and 2.0.

First-strand synthesis of cDNA was accomplished with 1 μg total RNA with random hexamers using the High Capacity RNA kit (Applied Biosystems) in 40 μL reactions. Control for genomic DNA contamination was performed by omitting reverse transcriptase (-RT) and all -RT samples failed to amplify products after 35 rounds of PCR. Following synthesis, samples were stored at -20 °C until use in qPCR assays.

Quantitative RT-PCR was performed on gene targets previously found to be differentially regulated in the skin of salmon infected with *L. salmonis* (Table 15). These genes are associated with the early inflammatory response and included cytokines *interleukin-1β (IL-1β), tumor necrosis factor-α* (*TNFα*), and *interleukin-8* (*IL8*), the eicosanoid metabolizing enzyme *cyclooxygenase-2* (*COX2*), an acute-phase protein *C-reactive protein* (*CRP*), a cellular remodeling and repair enzyme *matrix metalloproteinase-13* (*MMP13*), and a cellular marker *major histocompatibility factor class II* (*MH II*). Reference gene candidates included *elongation factor 1-α* (*EF1α*), *18S ribosomal subunit* (*18S*), *eukaryotic translation initiation factor 3 subunit 6* (*ETIF3-6*), *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) and *β-actin.* The three most stable reference genes (*ETIF3-6, EF1α* and *β-actin*) were determined using geNorm (Vandesompele *et al.* 2002) with a collective M-value of 0.421 and a CV of 0.132 that falls within the range typically observed for stably expressed reference genes in heterogeneous samples (Hellemans *et al.* 2007).

To ensure similar efficiency among all samples, a standard curve was generated for each gene (n = 5 dilution series) using pooled equimolar amounts from three samples from infected and non-infected salmon. All primers had efficiency values within the range of 85-105% for all

samples. qPCR amplification was performed using Brilliant II SYBR® (Agilent) in 12.5 µL reactions in an MX3000P (Agilent) as previously described (Braden *et al.* 2012) with the following thermal profile: 95°C for 3 minutes, followed by a combined annealing and extension step of 60°C for 40 cycles. Amplicons were checked for single products by melt-curve analysis, and resulting PCR products were sequenced to verify amplicon identity.

4.3.4 Data analysis

Histological analysis. To measure the effect of infection on mucus cell density, sections from parasitized (n = 6) and non-parasitized (n = 6) fish were examined and mucocytes (MCs) were enumerated. The mean number of immuno-reactive cells was estimated from the number observed in three random fields of view at 200X for each sample (n = 6). Thus, 18 (3 x 6) surface fields were counted for each time point, and the number of positive cells was compared to time-matched controls. Counting was standardized to only include immuno-labeled cells within the epidermis and dermis. A two-way ANOVA tested the statistical significance of differences in mean mucus cell density and the mean numbers of immuno-labeled cells between juvenile and mature salmon and over time (SigmaStat, V11.1). Differences were considered significant at $p < 0.05$.

Genetic analysis. RT-qPCR was performed on juvenile and mature pink salmon RNA concurrently to avoid technical bias. Data analysis of qPCR results was performed according to the qBASE framework to produce calibrated normalized relative quantities (CNRQs; Hellemans et al., 2007). Relative quantities ($RQs=E^{\Delta Ct}$; $\Delta Ct=C_{t(ref)}-C_{t(treatment)}$) for every sample were determined using a gene-specific efficiency. Normalized RQs (NRQs) were then calculated with a sample-specific normalization factor using multiple reference genes as described (Hellemans *et al.* 2007). Finally, calibrated NRQs (CNRQs) were calculated using a gene-specific calibration

factor that minimized variation among technical replicates. CNRQs were normalized to non-infected samples and calculated for attachment sites. Data were $\log_2$-transformed and statistical analyses were performed using SigmaStat software (V11.1). Differences were considered significant at $p < 0.05$. All values shown are means of individuals for each sampling time ± SEM. Two-way ANOVA determined significance between treatments at 24 and 48 hpi. If ANOVA was significant, Holm–Sidak post-hoc tests were used to determine the significance of pairwise differences.

4.4 Results

4.4.1 Genetic profile reveals suppression of innate immunity with sexual maturation

Differences in expression over time (24 → 48 hpi) and as a function of maturation (juvenile or mature) were determined (Figure 20). In the skin of juvenile salmon, we observed significant up-regulation ($p < 0.001$) over time of *MH II*, *CRP*, *IL-1β* and *TNF-α* whereas in mature salmon, there was no up-regulation of these genes over time. In the skin of mature salmon, significant down-regulation over time was only observed for *CRP*. At both 24 and 48 hpi, expression in the skin of infected juvenile compared to mature pink salmon was significantly higher ($p < 0.001$) for *MH II*, *CRP*, *IL-8* and *COX-2*. At 48 hpi there was significantly higher expression of *IL-1β* and *TNF-α* in the skin of juvenile pink salmon compared to mature pink salmon ($p < 0.001$).

4.4.2 Cutaneous cell populations during infection with *L. salmonis* in juvenile and mature pink salmon

Histological analysis of attachment site skin revealed polymorphonuclear cells (PMNs) in the epidermis and dermis of juvenile pink salmon at 24 hpi but not in mature pink salmon (Figure 21). There was a significant decrease in the density of mucocytes (MCs) in the skin of juvenile pink salmon as a function of *L. salmonis* infection over time (24 → 48 hpi, $p < 0.001$). MC

density in control mature pink salmon was significantly less than in juveniles ($p < 0.001$), and did not change during infection with sea lice (Figure 22).

There was a significant increase in the density of MHIIβ$^+$ cells in the skin of juvenile pink salmon after infection with *L. salmonis* at both 24 and 48hpi ($p < 0.001$). Two distinct MHIIβ$^+$ cell morphotypes were recognized; a smaller lymphocyte-like cell and a larger dendritic-like cell (Figure 23). We failed to detect MHIIβ$^+$ cells in the skin of non-infected or infected mature fish (Figure 24).

There was a significant infiltration of IL1β$^+$ cells in the epidermis and dermis of juvenile pink salmon at 24 hpi ($p < 0.001$), and these cells increased in abundance over time by 48 hpi ($p < 0.001$). We detected small numbers of IL1β$^+$ cells in the skin of mature pink salmon, but there no significant difference between control and infected fish (Figure 25).

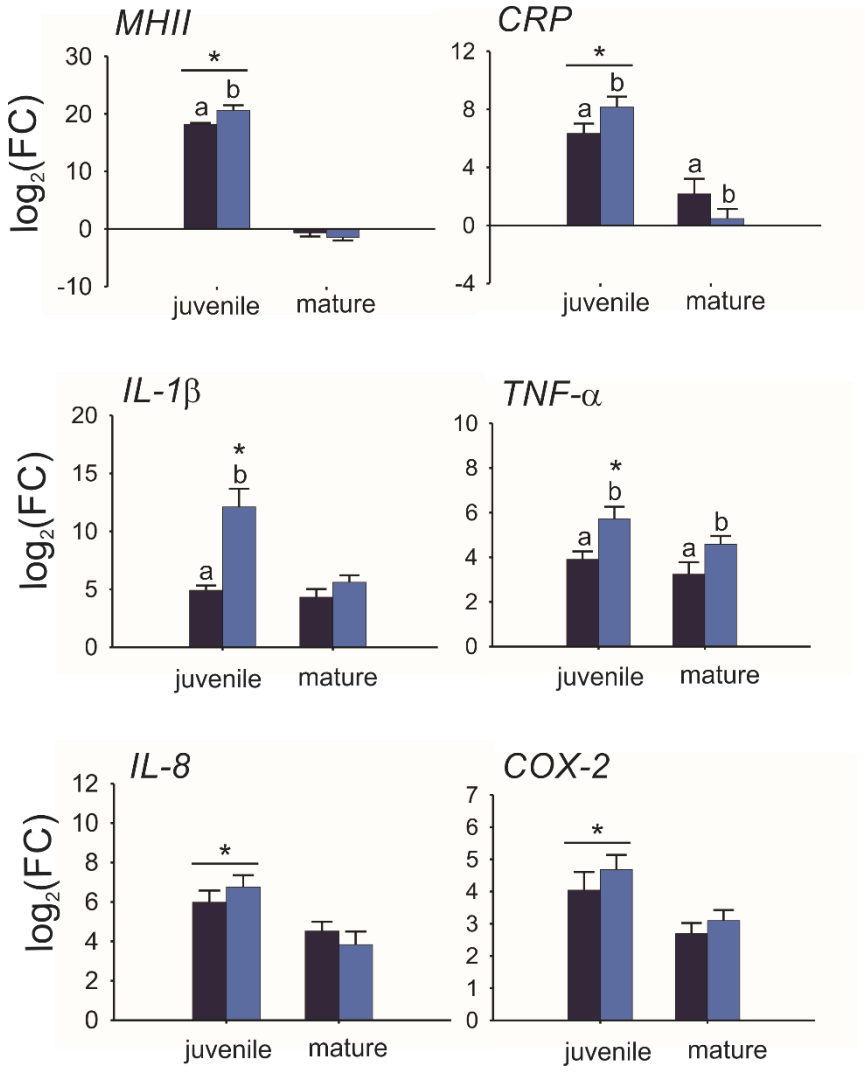

Figure 20. RT-qPCR of innate immune genes in the skin of juvenile and mature pink salmon during infection with *L. salmonis*.

Fold change (FC) is relative to non-infected controls and are $\log_2$-transformed calibrated normalized relative quantities (CNRQs). Each bar represents the mean (n = 6) with error bars as the SEM. Differences between juvenile and mature pink salmon (*), and between 24 hpi (dark blue) and 48 hpi (light blue) (lower case letters) were determined using two-way ANOVA ($p \leq 0.05$). Horizontal bars indicate significant differences between juvenile and mature pink salmon at both 24 and 48 hpi.

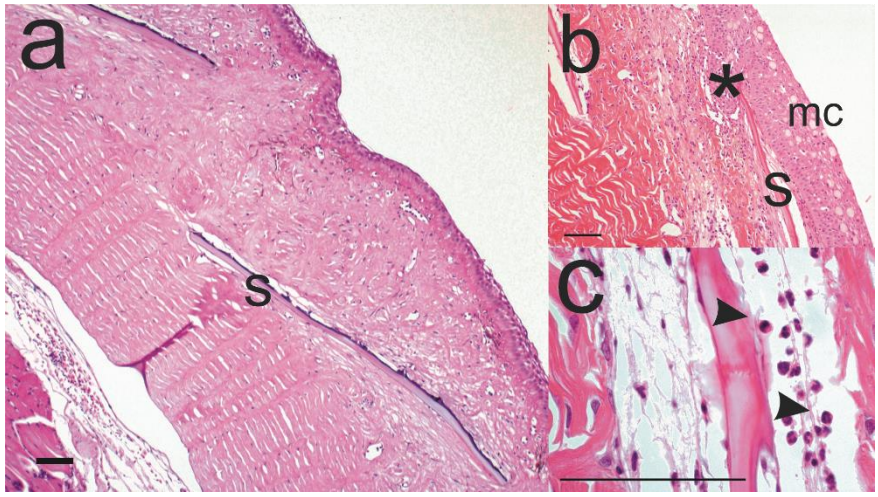

Figure 21. Micrographs of histological preparations of juvenile and mature pink salmon skin during sea lice infection. Hematoxylin and eosin.

At 24 hpi mature pink salmon skin (a) was characterized by sparse mucus cells or infiltrating leukocytes, whereas juvenile pink salmon skin (b) was heavily populated with mucocytes and leukocytes at 24 hpi. (c) High magnification (of area denoted by an asterisk) of juvenile pink salmon skin at 24 hpi showed the presence of polymorphonuclear (PMN) cells (arrows). S = scales, mc = mucocytes. Bar = 50 μm.

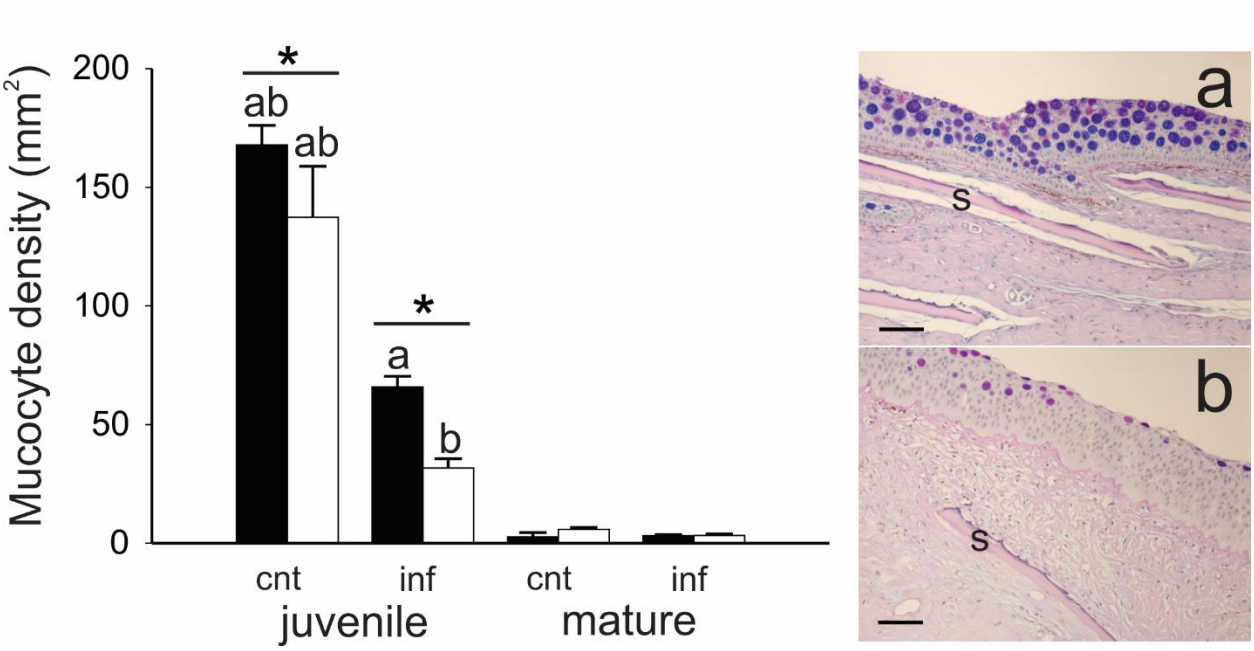

Figure 22. Mucocyte density (per square mm) in the skin of juvenile and mature pink salmon after infection with *L. salmonis*.

Mucocytes (MCs) were enumerated in the skin of control (cnt) and infected (inf) pink salmon at 24 hr (black bars) and 48 hr (white bars). Differences between control and infected individuals over time (lower case letters), or between juvenile and mature pink salmon (*) were determined using a two-way ANOVA ($p \leq 0.05$). (a) Juvenile pink salmon skin densely populated with MCs (dark purple); whereas, MCs were sparse in mature pink salmon skin (b). (s = scale, bar = 50 μm).

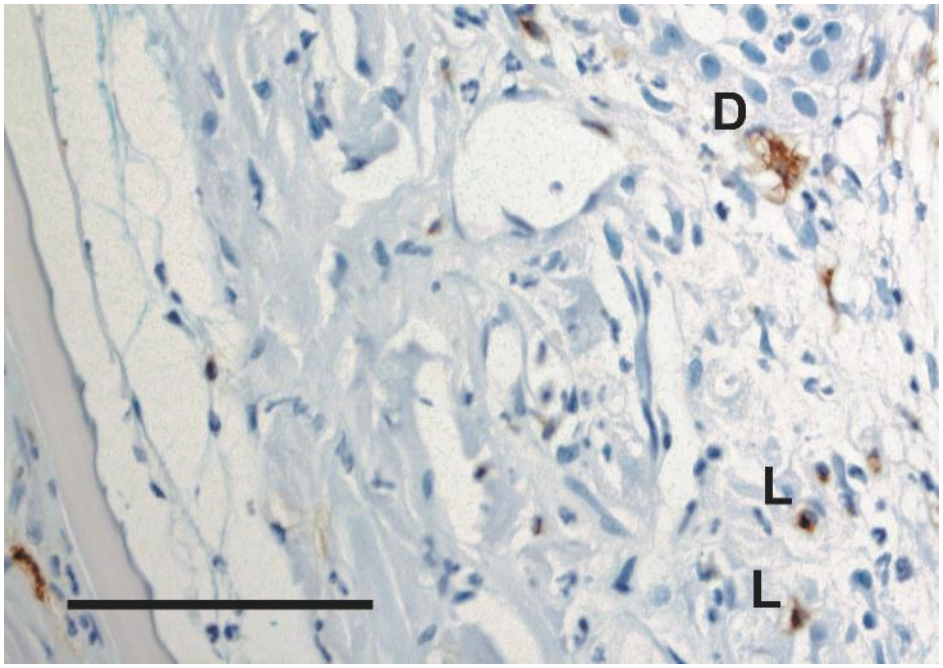

Figure 23. MHIIβ^{+} cell-types in the skin of juvenile pink salmon.

The α-MHIIβ antibody identified two cell-types: a larger, dendritic-like cell (D) and a smaller lymphocyte-like cell (L). Bar = 50 μm.

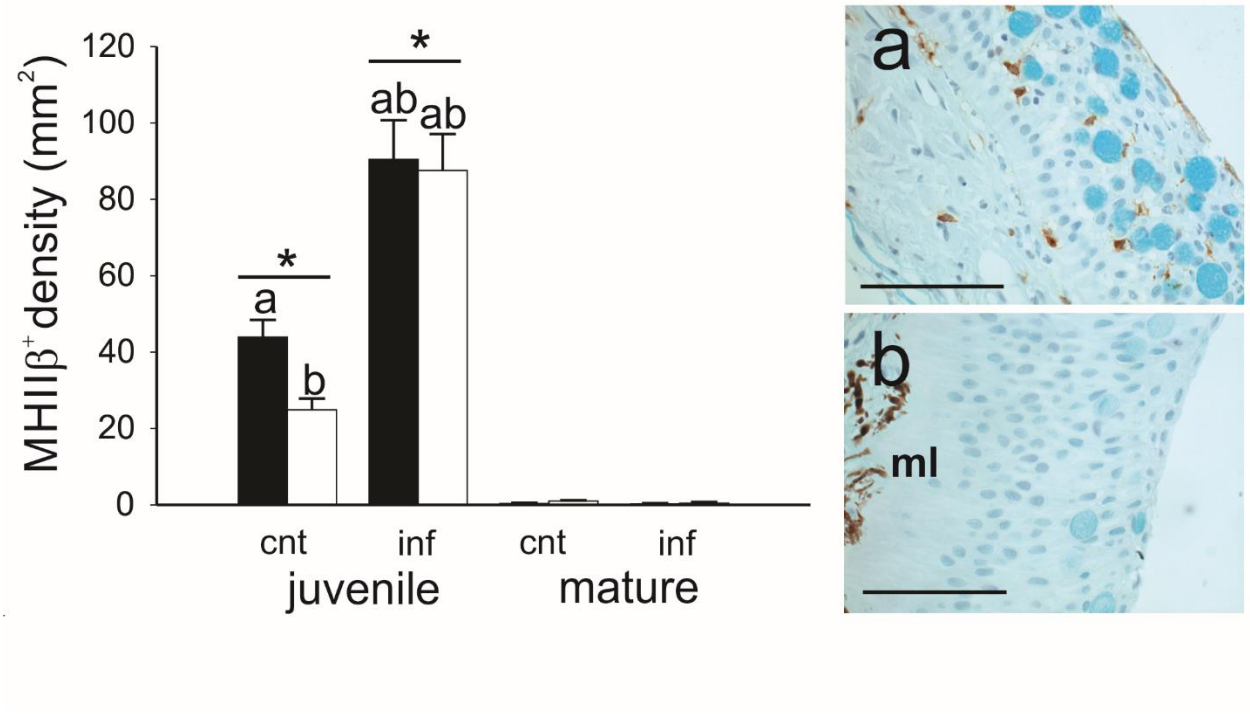

Figure 24. MHIIβ$^+$ cells in the attachment-site skin of juvenile (a) and mature (b) pink salmon during infection with *L. salmonis.*

MHIIβ$^+$ cells (brown) were enumerated in the skin of control (cnt) and infected (inf) pink salmon at 24 hpi (black bars) and 48 hpi (white bars). Differences between control and infected individuals over time (lower case letters), or between juvenile and mature pink salmon (*) was determined using a two-way ANOVA ($p \leq 0.05$). (ml = melanin). Bar = 50 μm.

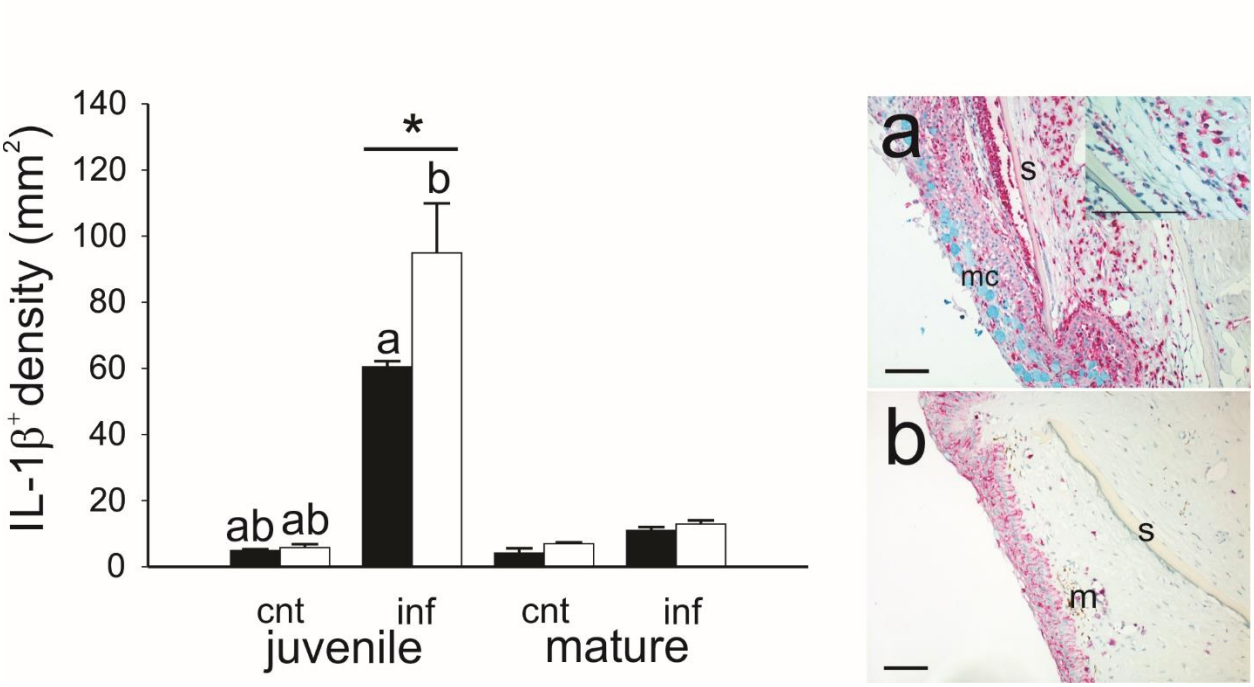

Figure 25. IL1β⁺ cells in the skin of juvenile (a) and mature (b) pink salmon after infection with *L. salmonis*.

IL1β⁺ cells (red) were enumerated in the skin of control (cnt) and infected (inf) pink salmon at 24 hpi (black bars) and 48 hpi (white bars). Differences between control and infected individuals over time (lower case letters), or between juvenile and mature pink salmon (*) were determined using a two-way ANOVA ($p \leq 0.05$). Inset shows higher magnification of (a). (m = melanin, mc = mucocyte, s = scale). Scale bar = 50 μm.

4.5 Discussion

Juvenile pink salmon have heightened resistance towards infection with the salmon louse, *Lepeophtheirus salmonis*. In contrast, adult pink salmon support heavy infestations of *L. salmonis* (Nagasawa 2001; Beamish *et al.* 2005). The aim of the current study was to investigate molecular and cellular mechanisms that might explain these contradictory observations. Resistance to sea lice in juvenile pink salmon is characterized by early inflammation and an acute-phase response coupled with efficient tissue remodeling pathways (Sutherland *et al.* 2011; Braden *et al.* 2012). Up-regulation of *CRP* found in this study is consistent with the activation of an acute-phase immune response that is significantly higher in juveniles than mature pink salmon. Local cutaneous expression of this acute-phase protein has previously been demonstrated in the skin of juvenile pink salmon during L. salmonis infection (Braden *et al.* 2012). Morphological comparison between juvenile and mature pink salmon skin in this study further demonstrates a significant reduction in mucocyte (MC) density in mature fish. Teleost MCs are implicated in the production of immunoglobulins, cytokines, lysozyme C, C-reactive protein as well as other defense-associated molecules (Pickering 1974; Alexander & Ingram 1992; Shephard 1994; Subramanian *et al.* 2007). Thus a potential source of CRP in the skin of salmonids may be MCs, which would explain the reduction in *CRP* expression concomitant with low MC density in the skin of mature pink salmon. MC density and associated immunologically active contents are known to affect parasite localization (Buchmann & Bresciani 1998), and reduced MC density in the epidermis of spawning rainbow trout is suggested as a contributing factor to susceptibility towards *G. derjavini* (Buchmann 1997). In Atlantic salmon, low MC density is thought to play a role in disease susceptibility with *L. salmonis* (Braden *et al.* 2015), whereas the cutaneous layer of resistant pink or coho salmon is characterized by densely populated MCs (Braden *et al.* 2012, 2015). Furthermore, during sea lice infection, the presence

of cytokines (IL-1β, TNF-α) in MC-like cells observed in resistant coho salmon was significantly reduced in more susceptible sockeye or Atlantic salmon (Braden *et al.* 2015). In this study we observed comparable density of MCs in the skin of juvenile pink salmon to that of coho salmon. However, the skin of mature pink salmon was sparsely populated with MCs, and in some cases MCs were not observed. Given the apparent importance of MCs and the associated immunologically active products, we propose the significant reductions of MCs in mature pink salmon skin is an important contributor to enhanced susceptibility to sea lice infections.

Braden *et al*. (2012) found that the *major histocompatibility factor class II* (*MH II*) gene was significantly upregulated in the skin of infected juvenile pink salmon. Subsequent analysis of coho salmon (*Oncorhynchus kisutch*) skin corroborated this cellular marker as an important determinant of resistance both in transcript abundance as well as histochemical identification with monoclonal antibodies (Braden *et al.* 2015). In the present study, we confirmed the presence of MHIIβ^{+} cells in juvenile salmon skin but failed to detect these cells in mature pink salmon skin. The absence of these cells was concordant with suppression of *MH II* expression in mature pinks after infection with *L. salmonis*; whereas, in juvenile pinks, expression of this molecule was 8-fold higher than controls. Host response and expression of *MH II* is well-documented for many parasite-host systems (Sigh *et al.* 2004b; Bridle *et al.* 2006b; Hetland *et al.* 2010), and allelic variants of *MH II* have been associated with heightened resistance to *L. salmonis* in some families of Atlantic salmon (Glover *et al.* 2007; Gharbi *et al.* 2009). We have documented modulated expression of *MH II* in coho (Braden *et al.* 2015) and pink salmon, both transcriptionally (Braden *et al.* 2012) and now at the protein level, and propose that MHIIβ^{+} cells are key cellular effectors at the primary infection site that provide protective effects during infection with *L. salmonis*. Therefore, observations of reduced expression of MH II at the

transcriptional and protein level in mature pink salmon suggests a mechanisms for reduced resistance in adult migrating pink salmon.

As in coho salmon (Braden *et al.* 2015), we detected significant infiltration of IL1β^{+} cells in *L. salmonis* infection of juvenile pink salmon that increased over time whereas in the mature fish, presence of these cells did not increase after infection. These cells may represent a bi-potential B lymphoid/myeloid progenitor cell that can differentiate into neutrophils, monocytes or B lymphoid cells, as suggested earlier (Nakajima 2011; Zwollo *et al.* 2014) and they have been associated with resistance towards bacterial cold-water disease in rainbow trout (Zwollo *et al.* 2014). We suggest that the IL1β^{+} population in the skin of coho and pink salmon represents another important determinant of resistance to sea lice.

Although there have been several studies describing the suppressive effects of sex hormones on immunocompetence in vertebrates (Pickering & Pottinger 1987; Hou *et al.* 1999; Saha *et al.* 2004; Currie & Woo 2007; Muñoz-Cruz *et al.* 2011; Nava-Castro *et al.* 2012), the molecular pathway responsible for this suppression in teleosts is largely unknown. Sex hormones play an important role as modulators of immunity, including the regulation of differentiation, function, and survival of important cellular effectors (Chaves-Pozo *et al.* 2012; Nava-Castro *et al.* 2012). Significant reductions of MHIIβ^{+} and IL1β^{+} cells observed in the skin of mature pink salmon may be a result of the regulatory effects of sex hormones. As we did not measure plasma levels of testosterone or estrogen we cannot make conclusions as to the relationship between sex hormones and these cell populations. However, the presence of well-developed sex organs together with physiological indicators of sexual maturation (e.g., darkened pigment, absorbed scales) indicated sexual maturation in these fish was advanced. Thus we postulate these physiological indicators are accompanied by expression of sex hormones, and further work is

required to determine the regulatory effect of these hormones on cellular effectors of pink salmon during infection with *L. salmonis*.

Sex-specific differences in susceptibility to parasites has been reported in salmonids and is related to differences in the regulatory effects of testosterone and estrogen (Klein 2004; Nava-Castro *et al.* 2012). In rainbow trout, susceptibility to infection with *C. salmositica* is heightened in spawning females compared to males (Currie & Woo 2007). Sexually mature male Atlantic salmon are more susceptible to *Gyrodactylus* sp. (Buchmann 1997) and in Arctic charr, parasite prevalence was significantly higher in spawning males compared to that of females (Skarstein *et al.* 2001). Our study was not designed to detect differences in host responses to *L. salmonis* between sexes in pink salmon; however, a recent study found no difference in susceptibility to *L. salmonis* among post-smolt Atlantic salmon fed diets supplemented with either 17β-estradiol or testosterone (Krasnov *et al.* 2015). Interestingly, the same study found a significant protective effect after the administration of either androgen in Atlantic salmon. Variation in responses among these studies may be explained by differences in host development, nutritive status, and pathogen exposure. Furthermore, regulation of the immune response by sex hormones is context-dependent and can be either inhibitory or stimulatory (Klein 2004; Muñoz-Cruz *et al.* 2011; Krasnov *et al.* 2015).

During the early seawater-phase, juvenile pink salmon invest resources to growth and immune-competence; however, as they sexually mature, resources must be shifted away from immunity towards the development of sex organs and secondary sexual characteristics. Immune responses can be energetically costly due to the metabolic demands of cellular effectors and to the effects of tissue damage during inflammation (Zuk & Stoehr 2002; Bonneaud *et al.* 2003). Therefore, immune responses can divert finite resources from other important physiological

functions such as reproduction (Sheldon & Verhulst 1996; Norris & Evans 2000). Increased reproductive effort is associated with increased susceptibility to infectious diseases, including parasites (Moller 1993; Nordling *et al.* 1998). With respect to infection with *L. salmonis*, juvenile pink salmon are shown to mount an aggressive, and thus energetically expensive, inflammatory response during initial infection that is followed by pathways of tissue remodeling and wound repair (Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012). Juvenile pink salmon enter the marine environment as the smallest among Pacific salmon species and exhibit the fastest growth rate coincident with the shortest seawater residence (Heard 1991). Thus allocating resources to contribute to heightened resistance against pathogens during this critical time would be advantageous and increase overall fitness. Later in mature migrating pink salmon, this costly resistance would eventually need to be traded for investment in other physiological functions, such as development of gonads and secondary sexual characteristics.

The high energetic cost of mounting an immune response (Sheldon & Verhulst 1996; Bonneaud *et al.* 2003) combined with strong selection pressures exerted by parasites on the host has resulted in a wide range of defense strategies and variations in susceptibility among hosts (Price 1980; Poulin & Morand 2000; Zuk & Stoehr 2002). This has contributed significantly to shaping host life histories (Zuk & Stoehr 2002). Hosts activities are often energy-limited and trade-offs must be made among physiological demands including host defense to optimize the cost-to-benefit ratio while minimizing impairment of host-fitness (Tschirren & Richner 2006).

4.6 Conclusions

This study presents, for the first time, a potential molecular mechanism that may explain observed heightened susceptibility towards a parasite during maturation of a pink salmon, a resistant species.

4.7 Chapter acknowledgements

This research was funded by Genome BC and Fisheries & Oceans Canada`s Program for Aquaculture Regulatory Research. Monoclonal α-MHIIβ and α-mIgM antibodies were a generous gift by Dr. Karsten Skjoedt at the University of Southern Denmark. Polyclonal α-tIL1β antibodies were a generous gift by Dr. Greg Wiens at the National Centre for Cool and Cold Water Aquaculture. We greatly appreciate technical assistance of Karina Rasmussen (University of Southern Denmark) and Bill Bennett (Pacific Biological Station). We gratefully acknowledge industrial cooperation by Brad Boyce (Marine Harvest Canada) in collection of Pacific *L. salmonis*. LMB is funded by an NSERC Alexander Graham Bell Canada Graduate Scholarship.

Chapter 5: The tasty fish – transcriptomics reveal an exaggerated feeding response of the salmon louse Lepeophtheirus salmonis on its host, the Atlantic salmon

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BFK conceived of the study, assisted with data interpretation and edited the manuscript.

SRMJ conceived of the study, interpreted the data, and wrote the manuscript.

5.1 Abstract

Host-parasite systems are the sum of co-evolutionary adaptations that result in an optimum balance between maximal exploitation by the parasite with minimal harm to the host. To maximize benefits to the parasite, a host with the highest nutritional value and lowest immunological response is preferred. Differential host-preference and host-susceptibility suggest that the salmon louse, *Lepeophtheirus salmonis* displays preferences among its salmon host species. Thus the aim for this work was to test the whether host-susceptibility is reflected in the transcriptomic responses of the parasite. We applied a 38k oligonucleotide array to measure the feeding response of Pacific *L. salmonis* after parasitizing susceptible (Atlantic salmon, *Salmo salar*; sockeye salmon; *Oncorhynchus nerka*) and resistant (coho salmon, *Oncorhynchus kisutch*) hosts. Analysis of gene expression profiles reveal enhanced responses to Atlantic salmon that are characterized by significant enrichment of virulence factors, energy metabolism enzymes and reproductive genes. In contrast, responses to coho or sockeye salmon were benign and more similar to the starvation response. Thus, despite the high susceptibility of sockeye salmon, our results indicate that the sea louse is more successful parasitizing Atlantic salmon compared to Pacific salmon, which is not explained by parasite acclimation. Rather, we suggest there are Atlantic salmon-specific factors presumably related to high nutrition, low immunity, or a combination of both, that permit an exaggerated feeding response by the sea louse that is otherwise absent in coho or sockeye salmon. Molecular data from the present work supports earlier behavioral host preference studies that suggest the Atlantic salmon is a more desirable host for *L. salmonis*, which likely involves differences in nutritive status and immune defenses among *Salmo* and *Oncorhynchus* spp.

5.2 Introduction

Parasite fitness is negatively correlated with host immune-competence while positively correlated with host nutritive value (Roulin *et al.* 2003; Bize *et al.* 2008). In some hematophagous parasites this relationship can be observed by the smaller blood-meals extracted from nutritionally-deprived or immune-stimulated hosts compared to non-manipulated controls (Bize *et al.* 2008). On the contrary, parasites of hosts that are experimentally supplemented with food display higher reproductive output (Christe *et al.* 2003; Tseng 2006; Tschirren *et al.* 2007). One strategy by which the host limits parasite infestation is through the combined defense mechanisms of innate and adaptive immunity. Earlier work determined that younger and less immune-competent house martins (*Delichon urbica*) were more likely to be infected with ectoparasites than older more immune-competent nest-mates, later coined "the tasty chick hypothesis" (Christe *et al.* 1998). However, this immuno-ecological view may provide only a partial explanation for host resistance, as other factors such as facultative anorexia, may also confer resistance to parasites (Exton 1997). In contrast, parasites may choose instead to favor hosts that offer high-quality energetic and nutritive resources at the expense of exposure to more developed defense strategies, known as "the well-fed hypothesis" (Christe *et al.* 2003; Roulin *et al.* 2003; Valera *et al.* 2004). Thus there is mixed empirical evidence for host-choice strategies by parasites. For example, the mite *Spinturnix myoti* prefers bats with higher nutritive status (Christe *et al.* 2003), whereas Ixodid ticks parasitize hosts with a combination of high host nutritive status and low immunocompetence. Therefore, to maximize reproductive success and survival, parasites are expected to select hosts based on a balance of immune-competence and the nutritional benefits derived from feeding (Tschirren *et al.* 2007; Bize *et al.* 2008). The extent to which parasites choose to exploit more vulnerable hosts (i.e., the tasty chick hypothesis) or

hosts with higher condition factor and thus higher quality of resources (i.e., the well-fed hypothesis) appears to be case-specific.

The salmon louse (*Lepeophtheirus salmonis*) is a common pest of salmonid mariculture (Boxaspen 2006; Costello 2006; Torrissen *et al.* 2013), with different sub-species occurring in the Pacific and Atlantic oceans (Skern-Mauritzen *et al.* 2014). The louse feeds on the epidermis, mucus and blood of the fish host, and in heavy infestations degrade the cutaneous barrier causing osmoregulatory distress, anaemia, lethargy and a generalized stress response (Grimnes & Jakobsen 1996; Fast *et al.* 2006; Wagner *et al.* 2008). Divergent host responses to *L. salmonis* occur among juvenile Pacific salmon (*Oncorhynchus* spp.) such that coho (*O. kisutch*) (Johnson & Albright 1992a; Braden *et al.* 2015) and pink salmon (*O. gorbuscha*) (Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012) display a resistant phenotype characterized by a well-developed inflammatory response at the attachment site followed by rejection of the parasite and limited pathology. In contrast, Atlantic (*Salmo salar*) (Skugor *et al.* 2008; Tadiso *et al.* 2011; Sutherland *et al.* 2011, 2014a; Krasnov *et al.* 2012; Braden *et al.* 2015), chum (*O. keta*) (Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012), and sockeye (*O. nerka*) (Jakob *et al.* 2013; Braden *et al.* 2015) display a more susceptible phenotype characterized by a weakened inflammatory response, higher parasite burden and associated pathology. Based on an ecological immunity view, we expect that sockeye, chum and Atlantic salmon are preferred host species and this may be tested using measures of behavioral or physiological responses by the parasite. Indeed, host-preference experiments using Y-tubes have shown *L. salmonis* larvae to swim more rapidly towards the mucus of Atlantic salmon compared to that of turbot (*Scophthalmus maximus*) (Devine *et al.* 2000; Ingvarsdóttir *et al.* 2002). Similarly, Atlantic *L. salmonis* release more low molecular weight proteases when exposed to mucus from Atlantic salmon or rainbow trout compared to

coho salmon or a non-salmonid species (Fast *et al.* 2002a), suggesting that the parasite may be more attracted to Atlantic salmon or rainbow trout compared with coho salmon. Secretions from *L. salmonis* elicited by dopamine or Atlantic salmon mucus also contain prostaglandin E_2 (PGE_2) and trypsin proteases, potent immune-modulators (Fast *et al.* 2004). However, it is not known whether the secretion of these virulence factors is influenced by host species. The impaired immune response observed in Atlantic, chum and sockeye salmon during infection may result from immunosuppressive molecules secreted into the attachment sites in these species that is absent in the attachment site on coho salmon. Initial responses by the host at the attachment site likely influence whether infections are successful; and we are exploring whether louse feeding behavior and associated secretions are a determinant of the host response. Thus susceptibility to infection by *L. salmonis* may result from reciprocal responses by the host and the louse.

In this study, we apply a 38k oligonucleotide array (Sutherland *et al.* 2012, 2014b) and quantitative reverse transcriptase PCR to test the hypothesis that the feeding response of *L. salmonis* is related to the susceptibility status of the host species. We predict that highly susceptible salmon will elicit a more aggressive response reflected both in the degree of host damage and in the magnitude and diversity of parasite feeding-associated pathways, compared with those observed on less susceptible host species. Further, we expect that on susceptible species the feeding responses will include up-regulation of virulence factors such as proteases, as well as pathways associated with reproduction and energy metabolism.

5.3 Methods

5.3.1 Experimental fish and infection procedures

5.3.1.1 Host-effect hypothesis experiment

All procedures involving the handling and usage of fish in this study were approved by the Canadian Council of Animal Care (CCAC) prior to initiation. Atlantic salmon parr were obtained

from a commercial salmonid hatchery, coho salmon parr were obtained from the Chase River hatchery, on Vancouver Island, British Columbia, and sockeye salmon parr were obtained from the Inch Creek hatchery, Chilliwack, British Columbia, Canada. All fish were reared on brackish water (~15 ppm) until smolting, after which they were maintained on ultraviolet-treated salt water (~33 ppm) in single-pass flow-through tank systems on a 12:12 hr light:dark cycle. Fish were fed 1% total biomass daily. Fish were randomly divided among twelve 330 L tanks with 2 tanks for each species (2X infection tanks), acclimated for approximately 7 days and starved at least 24 hr prior to any manipulation.

Adult female *L. salmonis* were collected during harvest of Atlantic salmon at a commercial aquaculture site on Vancouver Island, British Columbia, Canada. After collection, the lice were rinsed in fresh sea water and transported back to the Pacific Biological Station (Nanaimo, British Columbia, Canada) in 8°C aerated previously sterilised sea water. Only lice firmly attached to the collection vessel were included in the study. The time between collection of the sea lice and initial infection time was < 24 hr.

For infections, the water level of each tank was reduced by half and fish were sedated in seawater containing 0.2 mg/L metomidate hydrochloride (M-HCl; Aquacalm, Syndel Laboratories). Sedated fish were transferred to a temporary tank containing M-HCl to which 5 adult lice/fish were added and allowed to settle and attach. Once infected with 5 lice, fish were gently removed from the infection tank and returned to their original tank. Another population of lice were maintained at 8°C in aerated sea water and thus served as the non-attached population.

After 24 and 48 hr, one louse was removed from each of ten Atlantic, coho and sockeye salmon and from the non-attached population (n = 10) and snap frozen in liquid nitrogen. At the time of sampling, the Atlantic salmon were 218 ± 29 grams, the sockeye salmon were 167 ± 17

grams, and the coho salmon were 192 ± 35 grams. Lice recovered from an experimental host were considered to have been feeding whereas non-attached lice were considered to have been starved (Figure 26).

5.3.1.2 Acclimation hypothesis experiment

This experiment was design to determine whether the exaggerated response of *L. salmonis* to Atlantic salmon was related to parasite acclimation or to an Atlantic salmon-specific factor. Atlantic and sockeye salmon were divided between two tanks (n = 15 per species per tank) and allowed to acclimate for 7 days. Adult female *L. salmonis* collected from either Atlantic salmon during harvest at a commercial aquaculture site (Atlantic-acclimated. *L. salmonis*-Atl) or from sockeye salmon during a test fishery (sockeye-acclimated, *L. salmonis*-Sox) were transported back to the Pacific Biological Station in 8°C sterile sea water. Infection proceeded as described above with salmon in one tank for each species infected with *L. salmonis*-Atl (n = 5 per fish) and those in the second tank for each species infected with *L. salmonis*-Sox (n = 5 per fish). After 24 and 48 hpi, lice (n = 8) were removed from fish in each tank and frozen in liquid nitrogen (Figure 26**).**

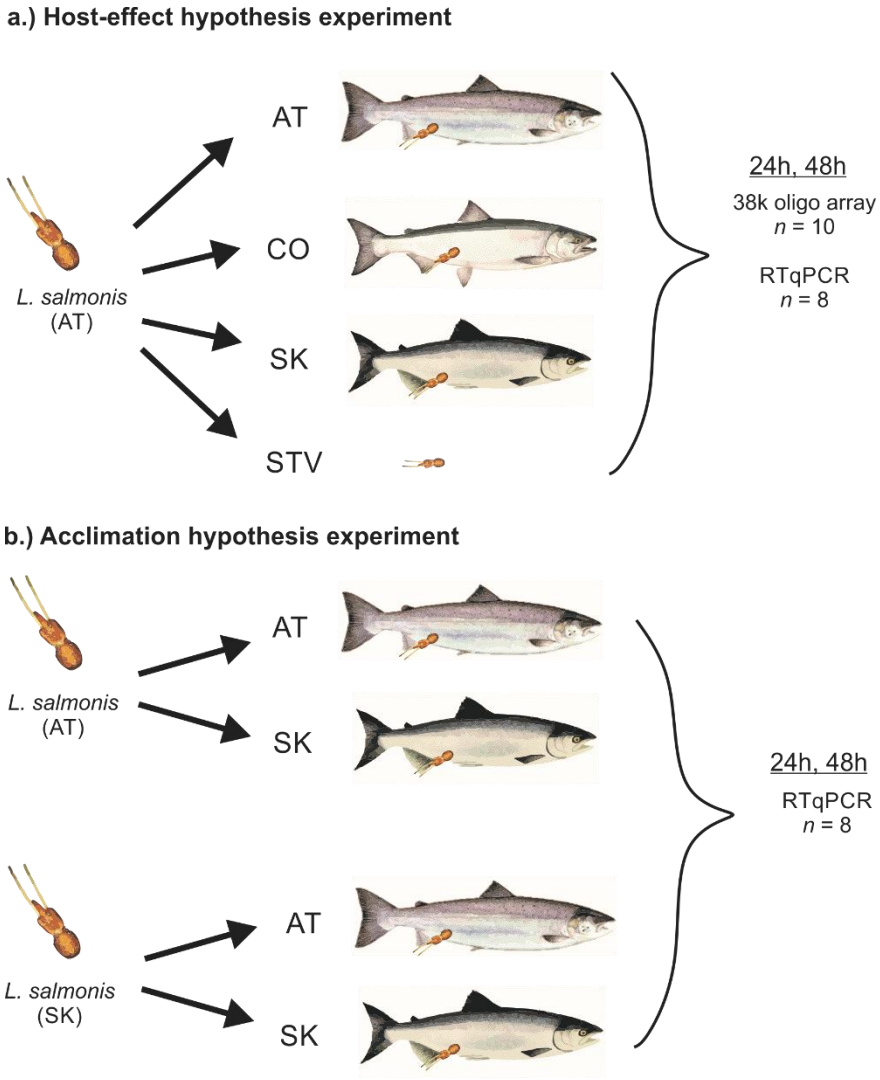


Figure 26. Experimental design of the host-effect hypothesis and acclimation hypothesis experiment.

In the host-effect hypothesis experiment (a) Atlantic (AT), coho (CO), and sockeye (SK) salmon were infected with *L. salmonis* sourced during commercial Atlantic salmon harvest. A sub-set of unattached lice served as the starved (STV) population. At 24 and 48 hr lice (n = 10) were removed from every population and processed for down-stream microarray and RT-qPCR analysis. In the acclimation hypothesis experiment (b) *L. salmonis* were sourced from Atlantic salmon during harvest as well as from sockeye salmon from a test fishery. Lice from each population (AT, SK) were used to infect both Atlantic and sockeye salmon. At 24 and 48 hrs, lice (n = 8) were removed and processed for down-stream RT-qPCR analysis.

5.3.2 RNA extraction

Frozen lice were homogenized using 5 mm stainless-steel beads and a Tissue-lyser (Qiagen). RNA was extracted using TRIzol (Invitrogen) following manufacturer's instructions with modifications. Following the organic phase extraction the supernatant was removed and RNA purified using RNeasy spin columns (Qiagen) with an on-column DNase I digestion to remove genomic DNA. Total RNA was eluted in 30 μL ultra-pure water and quantified by spectrophotometry (Nanodrop-1000, Thermo Fisher). RNA quality was determined using a BioRad Experion and RQI values were < 9 which is an indicator of high quality RNA.

5.3.3 cRNA synthesis and reference pool generation

Purified total RNA (200 ng) was reverse-transcribed to cDNA and then transcribed to labelled cRNA using Low Input Quick Amp Labeling kits (Agilent), as per manufacturer's instructions for hybridization to a 4-pack oligo gene expression microarray. Labelled cRNA was purified through RNeasy columns as per manufacturer's instructions (QIAGEN) and quantified using spectrophotometry (NanoDrop-1000), ensuring specific activity of all samples > 6 pmol dye per microgram cRNA (Agilent). Samples were kept at -80°C until hybridization. A reference pool of Cy3-cRNA was synthesized by amplifying experimental samples as described previously, but with Cy3-CTP-labelled nucleotides (Perkin Elmer). For each experiment, a reference pool was generated using equimolar cRNA from each experimental condition.

5.3.4 Microarray hybridization, quantification, normalization and filtering

A 38K oligo microarray was designed using previously annotated ESTs from both Pacific and Atlantic *L. salmonis* (Yasuike *et al.* 2012) using eArray (Agilent) with selection of probes preferentially at 3′ untranslated regions. Sample and reference combinations (825 ng cRNA each) were fragmented and then hybridized at 65°C for 17 h at 10 rpm as per manufactures' instructions (Agilent) using SureHyb chambers (Agilent). Washing was performed as per

manufacturers' instructions, using the optional protocol to prevent ozone degradation. All slides were transferred to a dark box and kept at low ozone until scanned on a Perkin Elmer ScanArray® Express at 5 μm resolution using PMT settings optimized to have the median signal of ~1–2% of array spots saturated (Cy5: 65; Cy3: 68). Images were quantified in Imagene 8.1 (Biodiscovery) using an eArray GAL file (Design ID: 024389; Agilent). Poor spots and control spots were flagged by the software for downstream filtering. A block-specific background correction was performed by subtracting the average median signal for negative control spots from each signal median. Sample files were loaded into GeneSpring 11.5.1 (Build 138755; Agilent). Each experiment was normalized and filtered separately as follows: raw value threshold of 1.0; intensity-dependent *Lowess* normalization; and baseline transformation to the median of all samples. Control spots and any probes not passing the following filter were removed from the analysis: raw values ≤ 500 in at least 65% of samples in any one condition and no flags in at least 65% of samples in any one condition.

5.3.5 Differential expression and functional analysis of microarray data

Array probes were tested for significance using a two-way ANOVA without equal variance assumption, followed by a post hoc Tukey's HSD ($p < 0.01$). Probes were filtered for fold change difference ≤ 1.5 from control. All probes passing significance and fold change filtering were used as an input for K-means clustering (Euclidean distance metric; 5 clusters; 50 iterations; GeneSpring 11.5.1 Agilent). Gene ontology (GO) and pathway enrichment were performed on annotated probes using the DAVID online bioinformatics tool (modified Fisher's exact test; (Huang *et al.* 2009)), with Uniprot accession numbers of clustered probes compared to a background list of all probes passing quality control filters (15,718 entities). Overlap between differential lists was evaluated using *VENNY* (Oliveros 2007). GO Trimming was performed on

the significantly enriched GO lists to reduce redundancy during analysis. This algorithm reduces redundancy by removing overlapping terms from the enriched GO category list through the removal of parent terms if they contain less than 40% unique entity content when compared to the child term. This procedure is fully described elsewhere (Jantzen *et al.* 2011), and does not change enrichment values of terms, but rather just systematically selects a subset of terms to be discussed.

5.3.6 Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

The same RNA samples analyzed with microarrays in the feeding and starvation experiment were used for RT–qPCR. Synthesis of cDNA was performed with 2 μg of total RNA in 20 μl reactions using oligo (dT) primers and AffinityScript cDNA Synthesis kits (Agilent), as per manufacturer's instructions. Each cDNA sample was diluted 10-fold. To generate a standard curve, one sample from each of the four conditions (starved, Atlantic-fed, coho-fed, sockeye-fed) was randomly selected and synthesized as described previously. These samples were then pooled and diluted 10-fold. This pool was then used for a serial dilution (5-point, 10-fold each point) for efficiency tests. qPCR amplification was performed using Brilliant UltraFast SYBR III® (Agilent) in 20 μl reactions with 0.1 μM of each primer using the following thermal regime: 95°C for 3 minutes, followed by a combined annealing and extension step of 60°C for 40 cycles. Genes of interest were selected from the microarray results based on biological relevance, high significance level or presence in significantly enriched GO categories. Reference gene candidates were selected from microarray results and indicate stable expression across conditions, consistency across replicate spots and moderate levels of expression as well as from previous literature (Sutherland *et al.* 2012). Primers were designed in Primer3 (Rozen & Skaletsky 2000) selecting amplicon sizes of 80-150 base pairs (Table 16). Amplicons were checked for single

products by melt curve analysis and were sequenced to confirm identity. RT–qPCR data analysis was performed using qbase-PLUS (Biogazelle). Stability of reference genes was tested using geNorm (Vandesompele *et al.* 2002). Selected reference genes included the previously identified gene *structural ribosomal protein S20* and *tubulin beta chain*, with a collective M value of 0.382 and CV of 0.146, which is within the range typically observed for stably expressed reference genes in heterogeneous sample (Vandesompele *et al.* 2002). Other tested reference genes that were not used to normalize due to higher variability included the following: *elongation factor 1-α* and *HPGRT* (data not shown). NTC and RT controls showed no amplification. Statistical significance was identified by two-way ANOVA ($p < 0.05$) with pairwise significance determined by post-hoc Tukey test (SigmaPlot V11.1). Correlation between methods (RT–qPCR and array) were checked using a linear best fit lines of $\log_2$ expression values for RT–qPCR samples vs. microarray $\log_2$ expression ratios (Cy5/Cy3) for the probe corresponding to the contig used for primer design.

5.4 Results

5.4.1 Starvation and feeding responses

We first analyzed the overall feeding response of *L. salmonis* without differentiating among host species. Comparison between feeding and starved lice at 24 and 48 hrs resulted in 553 and 546 up-regulated differentially expressed genes (DEGs) during feeding, whereas starvation resulted in 182 and 487 up-regulated DEGs, respectively. Enrichment analysis of DEGs significantly associated with feeding revealed significant over-representation in 12 categories after 24 hr feeding (data not shown), and 17 after 48 hr feeding (Table 2). The three most significantly enriched GO terms after 24 hr feeding were proteolysis, female pregnancy, and digestion while after 48 hr they were proteolysis, response to nutrients and collagen catabolic process. In contrast, GO analysis of DEGs associated with starvation revealed significant over-

representation of 5 categories after 24 hr starvation (data not shown), and 27 after 48 hr starvation (Table 2). The three most significantly enriched categories after 24 hrs were membrane invagination, endocytosis and negative regulation of transcription and after 48 hr they were glucose metabolic process, alcohol catabolic process and cellular carbohydrate catabolic process.

K-means clustering of significantly expressed transcripts in feeding and starved lice resolved 5 clusters. The two main clusters contained 433 and 46 features respectively, and showed fold changes > 1.5 across all conditions. Expression profiles of starved lice revealed down-regulation of proteolytic enzymes (e.g., *prss1, cpb1, mmp1*) oxidative reduction enzymes (e.g. *cox2*) and reproductive associated genes (e.g., *pp11*), while up-regulation in starved lice was observed for stress-related genes (e.g., *Tcp, hsp*) and genes involved in transcriptional regulation (*uap1, rps2, ef1-α*) (data not shown).

Temporal regulation of gene expression revealed that genes up-regulated between 24 and 48 hr were involved in muscle metabolism (*myh, tpm, tnn*), transport (*pmr, slc6a7*), and collagen-association processes (*col4a1, mmp1*). Of the total number of genes up-regulated over time, muscle-associated expression accounted for ~22.8% (13/57). Genes that were down-regulated over time involved energy metabolism (*cox2*), transcription (*znf16, rpl29*), and proteolysis (*ctra, nas-7*) (data not shown).

Table 2. Gene Ontology enrichment of transcripts up-regulated in feeding or starved *L. salmonis* at 48 hr.

Biological function categories that were over-represented in feeding or starved *L. salmonis* at 48 hr are shown along with the number of genes in each category (FC ≥ 1.5) and fold enrichment for each category.

48hrs upregulation - Feeding *L. salmonis*

GO Term	Biological Function	# genes	p-value	FE[a]
GO:0006508	Proteolysis	31	6.47E-07	2.59
GO:0007584	Response To Nutrient	6	0.000	10.45
GO:0030574	Collagen Catabolic Process	5	0.000	15.48
GO:0055114	Oxidation Reduction	21	0.001	2.26
GO:0007586	Digestion	4	0.006	10.13
GO:0035188	Hatching	3	0.007	20.90
GO:0050679	Positive Regulation Of Epithelial Cell Proliferation	3	0.007	20.90
GO:0006979	Response To Oxidative Stress	6	0.007	4.78
GO:0040007	Growth	7	0.011	3.61
GO:0007596	Blood Coagulation	4	0.012	7.96
GO:0030198	Extracellular Matrix Organization	4	0.012	7.96
GO:0001501	Skeletal System Development	5	0.031	4.10
GO:0019748	Secondary Metabolic Process	5	0.031	4.10
GO:0006766	Vitamin Metabolic Process	4	0.032	5.57
GO:0006814	Sodium Ion Transport	4	0.032	5.57
GO:0007565	Female Pregnancy	3	0.047	8.36
GO:0045471	Response To Ethanol	3	0.047	8.36

48hrs upregulation - Starved *L. salmonis*

GO Term	Biological Function	# genes	p-value	FE[a]
GO:0006006	Glucose metabolic process	10	7.3E-05	5.31
GO:0046164	Alcohol catabolic process	7	6.3E-04	6.34
GO:0044275	Cellular carbohydrate catabolic process	7	0.001	6.05
GO:0016052	Carbohydrate catabolic process	7	0.001	5.78
GO:0034637	Cellular carbohydrate biosynthetic process	6	0.002	6.37
GO:0043508	Negative regulation of JUN kinase activity	3	0.002	37.15
GO:0006096	Glycolysis	5	0.005	6.88
GO:0006072	Glycerol-3-phosphate metabolic process	3	0.007	22.29
GO:0042692	Muscle cell differentiation	5	0.008	5.99
GO:0006091	Generation of precursor metabolites and energy	10	0.013	2.58
GO:0006007	Glucose catabolic process	5	0.014	5.16
GO:0006796	Phosphate metabolic process	12	0.015	2.24
GO:0031399	Regulation of protein modification process	5	0.017	4.89
GO:0005978	Glycogen biosynthetic process	3	0.018	13.93
GO:0016481	Negative regulation of transcription	7	0.018	3.25
GO:0001503	Ossification	4	0.022	6.46
GO:0046034	ATP metabolic process	5	0.022	4.53
GO:0060348	Bone development	4	0.025	6.19
GO:0045934	Negative regulation of nucleic acid metabolic process	7	0.027	2.99
GO:0031327	Negative regulation of cellular biosynthetic process	7	0.034	2.83
GO:0009165	Nucleotide biosynthetic process	6	0.037	3.18
GO:0009890	Negative regulation of biosynthetic process	7	0.037	2.77
GO:0010558	Negative regulation of macromolecule biosynthetic process	7	0.037	2.77
GO:0034404	Nucleobase, nucleoside and nucleotide biosynthetic process	6	0.041	3.10
GO:0034654	Nucleic acid biosynthetic process	6	0.041	3.10
GO:0022604	Regulation of cell morphogenesis	4	0.044	4.95
GO:0006071	Glycerol metabolic process	3	0.045	8.57

[a]Fold enrichment

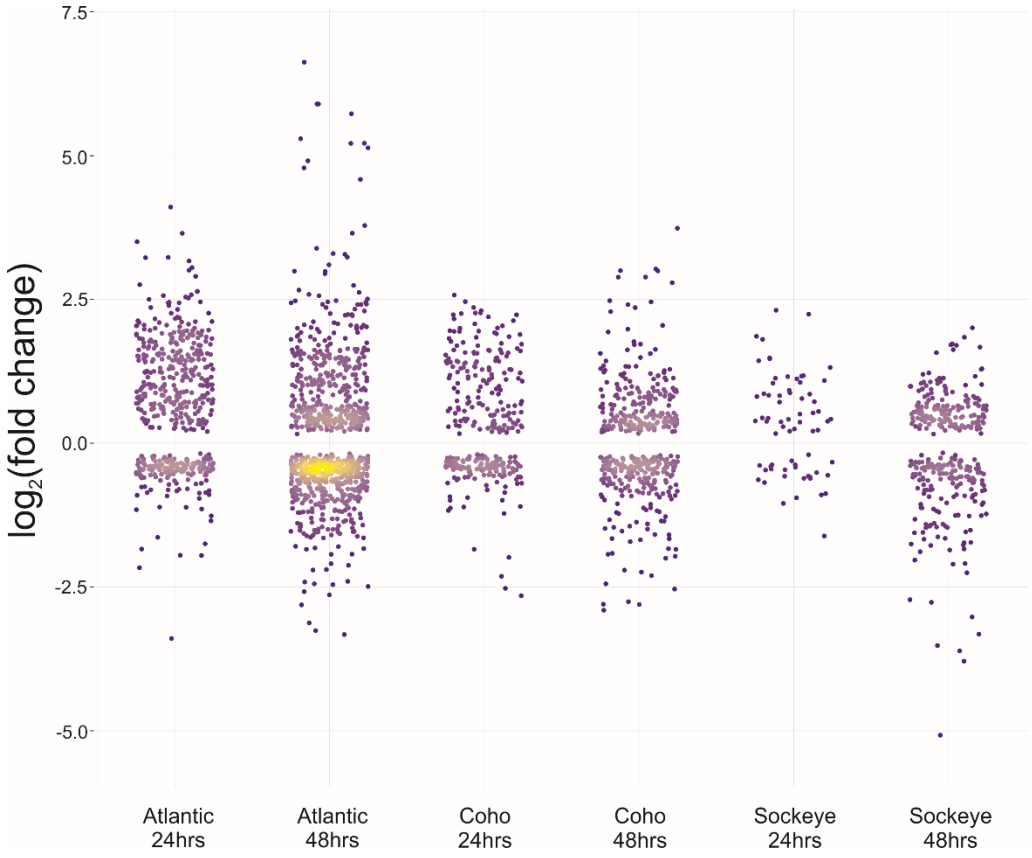


Figure 27. Heat-plot showing differentially expressed genes in *L. salmonis* feeding on different species of salmon compared to starved controls.

Each point represents the highest expression of a given transcript such that each transcript is only represented once in all 6 categories. Atlantic-fed *L. salmonis* had the highest number of genes with the highest fold change (no FC filter) at 24 hr (495) and 48 hr (867). Coho-fed *L. salmonis* had the second highest at 24 hr (304) and 48 hr (440). Sockeye-fed *L. salmonis* expressed the least number of genes at 24 hr (69) and 48 hr (334). Color of the plot changes with density of genes from purple to yellow.

5.4.2 The response of *L. salmonis* on different species of salmon

Responses of *L. salmonis* while feeding on different species was quantified. There were species-specific responses in the number of DEGs expressed by *L. salmonis* after 24 and 48 hr on Atlantic, coho or sockeye salmon (Figure 27).

After 24 hr feeding, 85 up-regulated transcripts were common to all three species, 28 were specific to Atlantic-fed lice, 12 to coho-fed lice and 3 to sockeye-fed lice. There were 26 transcripts specific to Atlantic- and coho-fed lice, 3 specific to coho- and sockeye-fed lice, and 6 specific to Atlantic- and sockeye-fed lice (Figure 28A). Compared with starved lice, 20 transcripts were down-regulated in lice feeding on all three species, 19 were specific to Atlantic-fed lice, 16 to coho-fed lice and 6 to sockeye-fed lice. Ten transcripts specific to Atlantic- and coho-fed lice were down-regulated, 5 specific to coho- and sockeye-fed lice, and 8 specific to sockeye- and Atlantic-fed lice (Figure 28B). After 48 hpi, 93 up-regulated transcripts were common to lice feeding on all three species, 33 were specific to Atlantic-fed lice, 9 to coho-fed lice, and 14 to sockeye-fed lice**.** There were 23 transcripts shared by Atlantic- and coho-fed lice, 14 shared by coho- and sockeye-fed lice, and 5 shared by Atlantic- and sockeye-fed lice (Figure 28A). After 48 hr, 64 down-regulated transcripts were common to all three species, 32 were specific to Atlantic-fed lice, 5 specific to coho-fed lice, and 9 specific to sockeye-fed lice. There were 18 transcripts down-regulated both in Atlantic- and coho-fed lice, 12 down-regulated in coho- and sockeye-fed lice, and 4 down-regulated in both sockeye- and Atlantic-fed lice (Figure 28B).

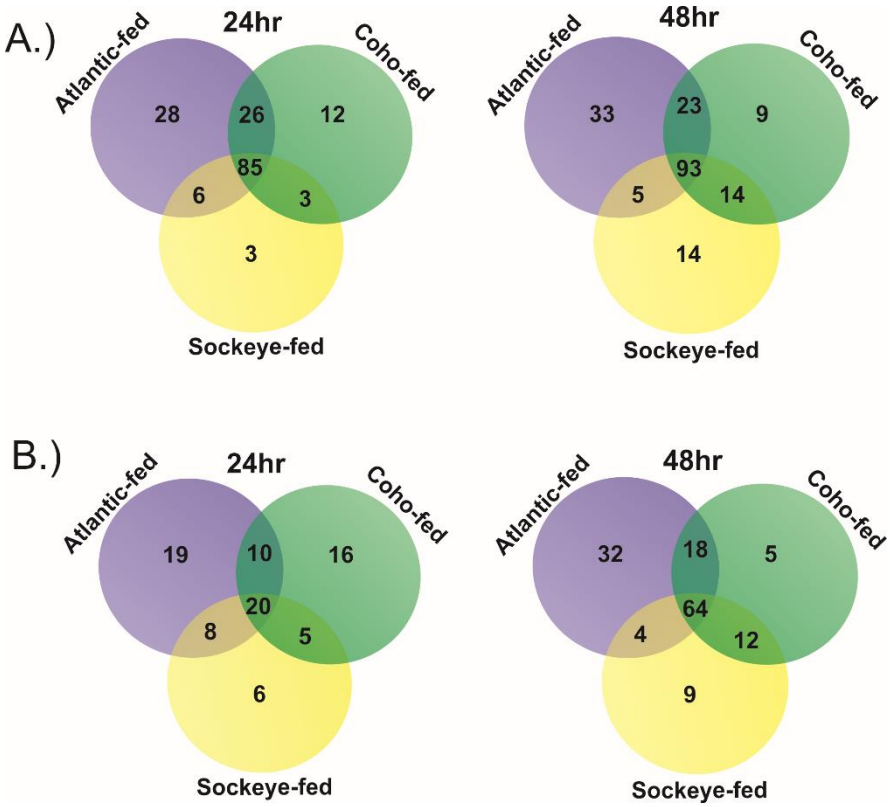


Figure 28. Differentially expressed genes unique or common to *L. salmonis* feeding on different species at 24 and 48 hr.

Venn diagrams displaying the numbers of up-regulated (A) and down-regulated (B) differentially expressed transcripts (FC ≥ 1.5, $p \leq 0.05$) in *L. salmonis* feeding on Atlantic, coho or sockeye salmon compared to starved controls at 24 or 48 hr.

Enrichment analysis was performed on significantly expressed transcripts from lice feeding on Atlantic, coho and sockeye salmon (Table 3). The biological process categories of proteolysis, oxidative reduction, and reproduction were consistently over-represented and contained the most significantly enriched and highest populated categories. Expression profiles of genes in these categories were compared. Although there were similarities in the genes up-regulated in lice feeding on all three species, the fold-change in Atlantic-fed lice was commonly the highest compared to coho or sockeye, and this trend was observed in proteolytic- (Table 4), oxidative reduction- (Table 5), and reproductive-associated genes (data not shown) at 24 and 48 hr.

Table 3. Gene Ontology enrichment of up-regulated transcripts in *L. salmonis* feeding on Atlantic, coho or sockeye salmon compared to starved controls at 48 hr.

Up in Atlantic compared to starved - 48hr				
Term	**Biological Function**	**# genes**	**p-value**	**FE[a]**
GO:0006508	Proteolysis	34	1.04E-07	2.64
GO:0055114	Oxidation Reduction	22	8.22E-04	2.15
GO:0070085	Glycosylation	5	0.02	4.52
GO:0009100	Glycoprotein Metabolic Process	5	0.05	3.55
GO:0007565	Female Pregnancy	4	0.01	10.50
GO:0050817	Coagulation	4	0.01	7.50
GO:0007599	Hemostasis	4	0.01	7.50
GO:0006766	Vitamin Metabolic Process	4	0.04	5.25
GO:0042060	Wound Healing	4	0.04	5.00
GO:0006026	Aminoglycan Catabolic Process	3	0.01	19.68
GO:0044243	Multicellular Organismal Catabolic Process	3	0.04	8.75
GO:0050678	Regulation Of Epithelial Cell Proliferation	3	0.04	8.75
GO:0006022	Aminoglycan Metabolic Process	3	0.04	8.75
GO:0044259	Multicellular Organismal Macromolecule Metabolic Process	3	0.04	8.75
Up in coho compared to starved - 48hr				
Term	**Biological Function**	**# genes**	**p-value**	**FE[a]**
GO:0006508	Proteolysis	30	1.28E-06	2.57
GO:0055114	Oxidation Reduction	22	1.99E-04	2.38
GO:0043413	Biopolymer Glycosylation	6	0.00	5.99
GO:0006486	Protein Amino Acid Glycosylation	6	0.00	5.99
GO:0007599	Hemostasis	4	0.01	8.27
GO:0050817	Coagulation	4	0.01	8.27
GO:0050878	Regulation Of Body Fluid Levels	4	0.02	7.24
GO:0031667	Response To Nutrient Levels	4	0.04	5.04
GO:0009991	Response To Extracellular Stimulus	4	0.05	4.83
GO:0035188	Hatching	3	0.01	21.72
GO:0009312	Oligosaccharide Biosynthetic Process	3	0.02	12.41
GO:0044259	Multicellular Organismal Macromolecule Metabolic Process	3	0.04	9.65
GO:0030574	Collagen Catabolic Process	3	0.04	9.65
GO:0050678	Regulation Of Epithelial Cell Proliferation	3	0.04	9.65
Up in sockeye compared to starved - 48hr				
Term	**Biological Function**	**# genes**	**p-value**	**FE[a]**
GO:0006508	Proteolysis	26	2.69E-05	2.41
GO:0055114	Oxidation Reduction	19	0.00	2.22
GO:0006812	Cation Transport	8	0.03	2.67
GO:0009611	Response To Wounding	5	0.03	4.35
GO:0050817	Coagulation	4	0.01	8.95
GO:0007584	Response To Nutrient	4	0.02	7.37
GO:0042060	Wound Healing	4	0.03	5.97
GO:0009991	Response To Extracellular Stimulus	4	0.04	5.22
GO:0035188	Hatching	3	0.01	23.49
GO:0009312	Oligosaccharide Biosynthetic Process	3	0.02	13.43
GO:0007565	Female Pregnancy	3	0.04	9.40

[a]Fold enrichment

Table 4. Expression profiles of genes enriched in the category proteolysis in Atlantic-, coho-, or sockeye-fed *L. salmonis*.

The highest fold change among Atlantic, coho or sockeye is in bold. Genes that are considered virulence factors in other parasite systems are indicated by an asterisk. FC ≤ 1.5 indicated by a hyphen.

	Atlantic-fed		**Coho-fed**		**Sockeye-fed**	
Gene	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
*nas7**	-	**5.35**	1.92	3.26	-	2.42
cpa1	**3.6**	2.1	1.93	-	1.69	-
k02a2	1.87	1.6	1.82	1.94	1.84	**2**
*prss1**	3.43	**6.01**	3.08	1.6	1.89	2.13
tmprss8	**4.49**	2.96	1.71	-	-	2.08
tmprss11	1.61	**2.11**	1.7	1.7	1.68	-
traf6	1.75	**2.32**	1.99	2.27	1.66	2.25
prtb	1.5	-	**2.69**	1.89	1.79	1.54
*prss2**	1.59	**3.08**	1.97	1.71	1.66	1.48
aprss2	2.93	**3.5**	2.49	2.01	1.86	1.89
parss1	3.85	**5.43**	2.58	2.54	2.11	2.5
*prss3**	1.86	2	2.52	1.69	2.03	**3.59**
prtn	**2.64**	2.42	1.73	-	1.62	-
ovch1	**5.92**	2.72	2.55	-	2.74	-
prss12	1.81	-	1.74	-	1.82	1.57
mmel2	2.95	**3.03**	1.89	-	1.55	-
mmel1	2.2	3.1	1.95	1.6	-	-
*mmp9**	2.84	2.3	2.7	1.88	**4.73**	3.48
mmp14	**1.87**	1.76	1.72	1.61	-	-
klkb1	1.63	1.61	1.52	1.52	1.52	**1.69**
intg	1.93	1.66	2.04	**2.31**	2.01	1.9
hdmb	**3.5**	2.98	2.27	1.54	1.79	1.56
qptc	1.78	-	-	1.5	-	-
eprss	**2.9**	2.74	2.2	1.67	1.68	1.39
tmprss15	2.49	-	**3.04**	-	2.42	-
dpp4	1.63	**2.76**	-	1.85	-	1.65
cndp	1.98	1.91	-	-	-	-
mmp14	**5.75**	2.92	2.83	-	2.87	-
f9	**5.85**	2.82	2.76	-	2.81	-
*ctba**	4.75	**4.77**	-	2.56	-	3.23
*ctsk**	3.64	**6.16**	1.56	1.8	1.58	-
*cpbd**	1.6	**2.26**	1.69	1.67	1.61	1.7
*cpb1**	2.73	**4.14**	2.28	-	1.82	-
pryz1	**4.2**	3.14	2.06	1.5	1.8	1.7

Table 5. Expression profiles of genes enriched in the category oxidative reduction in Atlantic-, coho-, or sockeye-fed *L. salmonis*.

The highest fold change among Atlantic, coho or sockeye is in bold. FC ≤ 1.5 indicated by a hyphen.

	Atlantic-fed		Coho-fed		Sockeye-fed	
Gene	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
tmp195	-	1.55	-	2.06	1.45	**2.24**
retsat	-	**1.55**	-	-	-	-
oxnad1	-	**2.02**	-	-	-	-
dhr	2.06	**2.81**	2.45	2.3	1.97	2.01
adh	-	1.63	-	1.63	-	-
cyp3a9	1.63	-	-	1.58	-	**1.75**
aldhb1	-	1.58	-	-	-	-
aldha1	-	**1.98**	1.54	1.74	-	-
oxred	**2.02**	-	-	1.6	1.88	1.71
tdo2	**1.76**	-	-	1.59	-	1.63
sdr16c5	2.06	**2.81**	2.45	2.3	1.97	2.01
gpx	**9.36**	5.52	4.65	-	2.44	2.08
mt-nd5	1.67	**2.21**	1.86	2.17	1.95	1.83
lao	-	3.38	1.64	3.98	1.7	**4.02**
ifi30	4.04	**4.16**	2.75	2.86	2.3	2.73
grd	1.75	1.83	1.81	**2.11**	1.51	1.64
gpx4	**5.98**	4.26	3.4	1.64	2.22	2.16
ftl	1.99	**3.05**	1.68	1.71	1.51	1.57
dhrs1	**2.22**	1.5	-	-	1.79	-
cyp2l1	**3.07**	**3.07**	1.81	1.85	1.81	2.03
cyp2j2	**3.4**	2.91	2.1	1.56	2.08	-
cyp2a9	1.6	-	-	1.69	-	**1.81**
cox3	8.62	**37.1**	-	5.55	-	1.54
cox2	15	**59.6**	4.86	14.6	15.8	33.66
cyb	13.7	**37.2**	-	1.88	-	-
aspdh	-	1.83	-	**2.04**	1.62	1.77
aldh8a1	-	**1.98**	1.54	1.74	-	-

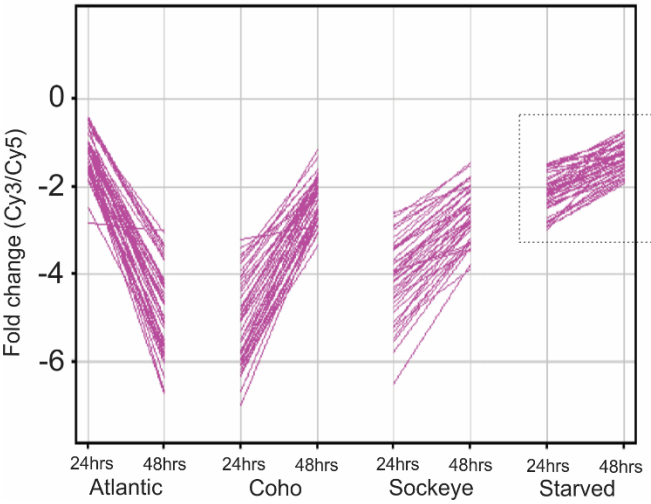
As the number of DEGs was higher in Atlantic-fed compared to either coho- or sockeye-fed lice, we compared the response of *L. salmonis* on Atlantic directly to either coho or sockeye. Species comparisons revealed a more transcriptionally active response by lice feeding on Atlantic salmon compared to either coho or sockeye salmon (Table 6). The number of DEGs after 24 hr on Atlantic salmon compared to either coho or sockeye salmon was 201 and 245, respectively, which was greater than the number of DEGs of lice on coho (61) or sockeye (39) compared to Atlantic salmon. This trend continued at 48 hr with lice feeding on Atlantic salmon compared to either coho or sockeye salmon expressing 224 and 284 DEGs, respectively, while on coho or sockeye compared to Atlantic, there were only 23 and 98 DEGs, respectively. Comparing the response of *L. salmonis* feeding on coho to that of sockeye revealed 98 and 45 DEGs in coho-fed lice, while in sockeye-attached lice there were only 40 and 33 DEGs at 24 and 48 hr, respectively. Thus the initial analysis suggests a more transcriptionally active response when lice are attached to Atlantic salmon compared to either coho or sockeye salmon. Further, the response of *L. salmonis* was least vigorous when attached to sockeye salmon.

Table 6. Differentially expressed transcripts in *L. salmonis* as a comparison among species of salmon.

	24hr	**48hr**
Atlantic	201	224
Coho	61	23
Atlantic	245	284
Sockeye	39	98
Coho	98	45
Sockeye	40	33

K-means clustering analysis comparing the response of *L. salmonis* feeding on Atlantic, coho or sockeye salmon resolved 5 clusters, one of which contained 45 features associated with stress (e.g. heat shock proteins, chaperones etc.). Expression profiles within this cluster were up-regulated in starved lice or coho- and sockeye-fed lice while these genes were down-regulated in Atlantic-fed lice (Figure 29).

Enrichment analysis of DEGs at 24 hr in Atlantic-fed lice compared to either coho- or sockeye-fed lice revealed nine GO categories significantly over-represented compared to coho-fed lice, and five GO categories significantly over-represented compared to sockeye-fed lice. There were two GO categories enriched in sockeye-fed lice, while in coho-fed lice there were four GO categories enriched compared to the response of Atlantic-fed lice. The most significantly enriched biological process categories while lice were feeding on Atlantic salmon compared to coho salmon were proteolysis, hatching, and female pregnancy and compared to sockeye salmon were proteolysis, digestion, and hatching. The most significantly enriched categories of genes in lice feeding on sockeye were skeletal system development and sensory organ development; whereas, on coho salmon the most significantly enriched categories were cytoskeleton organization, actin cytoskeleton organization, actin-filament based processes (Table 7). At 48hrs, there was no functional enrichment of genes in either coho- or sockeye-fed lice, whereas in Atlantic-fed lice there was significant over-representation of genes involved in proteolysis, digestion and hatching (compared to coho-fed lice), and proteolysis, oxidation reduction, and hatching (compared to sockeye-fed lice) (data not shown). Expression levels of genes from the three most populated and/or significantly enriched GO categories (proteolysis, oxidation reduction and hatching/pregnancy) at 24 and 48 hr feeding on Atlantic salmon are shown in Table 8.



Upregulated in coho- and sockeye-fed and starved *L. salmonis*.

Gene	Uniprot#
40S ribosomal protein S9	Q8SRW6
Elongation factor 1-alpha	Q8SS29
Elongation factor 2	Q8SQT7
Heat shock protein 90	Q8SSE8
Heat shock protein homolog ECU03_0520	Q8SSB1
Heat shock protein SSA1	P10591
Probable phenylalanyl-tRNA synthetase alpha chain	Q8SRG9
T-complex protein 1 subunit alpha	Q8SSC9
Tubulin alpha chain	P36220
Vitellogenin-2	P05690

Figure 29. Expression analysis for the transcripts in one cluster that were up-regulated in coho- or sockeye-fed and starved *L. salmonis*.

A total of 45 transcripts (FC ≥ 1.5, $p \leq 0.05$) associated with stress clustered together. Genetic profiles showed these genes were up-regulated in coho- or sockeye-fed or starved *L. salmonis* but down-regulated in Atlantic-fed *L. salmonis*. A small subset of these genes are described in the figure.

Table 7. Gene Ontology enrichment of DEGs as a comparison among species after 24 hrs.

Up-regulation in Atlantic-fed lice (compared to coho) after 24h				
GO term	**Biological function**	**# genes**	**p-value**	**FE[a]**
GO:0006508	Proteolysis	13	6.5E-04	2.95
GO:0035188	Hatching	3	9.3E-04	57.56
GO:0007565	Female Pregnancy	3	0.007	23.03
GO:0001824	Blastocyst Development	3	0.011	17.71
GO:0022904	Respiratory Electron Transport Chain	3	0.034	10.01
GO:0001835	Blastocyst Hatching	2	0.038	51.17
GO:0055114	Oxidation Reduction	8	0.049	2.29
GO:0007566	Embryo Implantation	2	0.050	38.38
GO:0050679	Positive Regulation Of Epithelial Cell Proliferation	2	0.050	38.38
Up-regulation in Atlantic-fed lice (compared to sockeye) after 24h				
GO term	**Biological function**	**# genes**	**p-value**	**FE[a]**
GO:0006508	Proteolysis	21	3.6E-09	4.33
GO:0007586	Digestion	4	3.9E-04	25.37
GO:0035188	Hatching	3	0.001	52.33
GO:0007565	Female Pregnancy	3	0.008	20.93
GO:0001835	Blastocyst Hatching	2	0.041	46.52
Up-regulation in sockeye-fed lice (compared to Atlantic) after 24h				
GO term	**Biological function**	**# genes**	**p-value**	**FE[a]**
GO:0001501	Skeletal system development	3	0.006	22.57
GO:0007423	Sensory organ development	3	0.030	9.84
Up-regulation in coho-fed lice (compared to Atlantic) after 24h				
GO term	**Biological function**	**# genes**	**p-value**	**FE**
GO:0007010	Cytoskeleton organization	4	0.013	7.11
GO:0030036	Actin cytoskeleton organization	3	0.021	11.81
GO:0030029	Actin filament-based process	3	0.022	11.63
GO:0040018	Positive regulation of multicellular organism growth	2	0.039	46.52

[a]Fold enrichment

Table 8. Genetic profiles of transcripts enriched in proteolysis, oxidative reduction, and reproduction categories in *L. salmonis* feeding on Atlantic salmon compared to coho or sockeye salmon.

Fold change of transcripts in over-represented biological function categories in *L. salmonis* feeding on Atlantic salmon compared to either coho or sockeye salmon.

Proteolysis	**vs. coho**		**vs. sockeye**	
Gene	**24h**	**48h**	**24h**	**48h**
prss	-	1.86	2.5	2.1
prss1	-	1.82	2.2	2.19
mmel2	1.56	1.81	2.36	2.54
mmel1	-	1.54	1.94	2.37
cpa1	1.8	2.08	2.32	2
oryz1	2.04	2.33	2.09	1.85
f9	2.11	2.07	1.95	2.03
mmp1	2.03	2	2.02	2.18
ovch1	2.32	2.17	2.04	2.17
prtn	1.53	1.63	1.66	1.97
eprss	-	1.73	1.64	1.97
prss2	-	1.57	1.74	1.85
hdmb	1.53	1.68	1.95	1.78
cndp2	-	1.52	1.74	1.66
prss36	-	1.52	-	1.54
tmprss8	2.63	3.13	2.04	-
ctrb2	3.32	3.31	1.89	-
prss36	2.12	2.55	4.54	2.91
cpb1	1.5	2.12	3.01	3.02
ctsk	2.34	2.3	3.49	4.68

Oxidative Reduction	**vs. coho**		**vs. sockeye**	
Gene	**24h**	**48h**	**24h**	**48h**
cox3	6.08	11.8	7.31	33.24
cyb	16.5	14.7	19.79	41.76
dhrs1	1.69	-	1.52	1.65
cyp2l1	1.7	1.69	1.6	1.66
cyp2j2	1.62	1.63	1.87	2.31
gpx	2.01	3.83	3.75	2.66
cox2	7.33	-	8.44	1.44

Reproduction	**vs. coho**		**vs. sockeye**	
Gene	**24h**	**48h**	**24h**	**48h**
ncer	2.03	2.25	2.16	2.17
grn7	1.75	1.96	2.23	2
pp11	-	1.52	-	1.54
gas1	-	-	-	1.55

Evidence of up-regulation over time in *L. salmonis* suggests a favorable response. Thus amplification of a response specific to feeding on a particular host species indicates this host provides a desirable environment for the parasite, and we hypothesize that such amplification is associated with a susceptible phenotype. Therefore we analyzed transcriptomic responses from lice feeding on all three species for evidence of temporal activation (e.g. higher response at 48hrs compared to 24hrs, or vice versa) by performing enrichment of DEGs in Atlantic, coho and sockeye salmon that either increased or decreased over time. On Atlantic salmon, the overall response increased over time while on coho or sockeye salmon, the response either decreased or remained the same. There was significant over-representation in 9 GO categories of up-regulated genes in *L. salmonis* feeding on Atlantic salmon. Enrichment of down-regulated transcripts was observed in coho-fed *L. salmonis*, and there was no enrichment for up- or down-regulation over time in sockeye-fed lice (Table 9).

Table 9. Gene Ontology of DEGs of transcripts enriched over time in *L. salmonis* feeding on different species.

Enrichment of DEGs increasing over time was only observed in Atlantic-fed *L. salmonis*, and enrichment of DEGs decreasing over time was only observed in coho-fed *L. salmonis*.

Increasing over time - Atlantic-fed *L. salmonis*				
GO Term	**Biological function**	**# genes**	**p-value**	**FE[a]**
GO:0055114	Oxidation reduction	9	0.005	3.12
GO:0019748	Secondary metabolic process	4	0.005	10.63
GO:0006769	Nicotinamide metabolic process	3	0.023	12.13
GO:0009820	Alkaloid metabolic process	3	0.023	12.13
GO:0010817	Regulation of hormone levels	3	0.023	12.13
GO:0019362	Pyridine nucleotide metabolic process	3	0.025	11.63
GO:0001501	Skeletal system development	3	0.048	8.21
GO:0019563	Glycerol catabolic process	2	0.021	93.03
GO:0046168	Glycerol-3-phosphate catabolic process	2	0.021	93.03

Decreasing over time - Coho-fed *L. salmonis*				
GO Term	**Biological function**	**# genes**	**p-value**	**FE[a]**
GO:0006979	Response to oxidative stress	3	0.009	18.80
GO:0006508	Proteolysis	5	0.046	3.24

5.4.3 Validation of the microarray

To confirm findings from microarray analyses, transcript abundance was analyzed for a sub-set of significantly DEGs using RT-qPCR. Genes with potential significance to feeding and energy production in *L. salmonis* as well as genes differentially expressed by the array were chosen for validation. All genes tested had the same direction of fold change as predicted from microarray analysis. There was high correlation between the qPCR and microarray data (n = 14 gene comparisons, Figure 30). With the exception of one gene (*pcd4*), the direction of regulation was the same using both methods. Additionally, temporal trends observed in the genes from Atlantic salmon-fed lice by microarray analysis were also detected by qPCR analysis which showed significant up-regulation of proteases (e.g., *ctsk*, *prss-1*) and mitochondrial enzymes (e.g., *cyb*, *cox2*) (Figure 31).

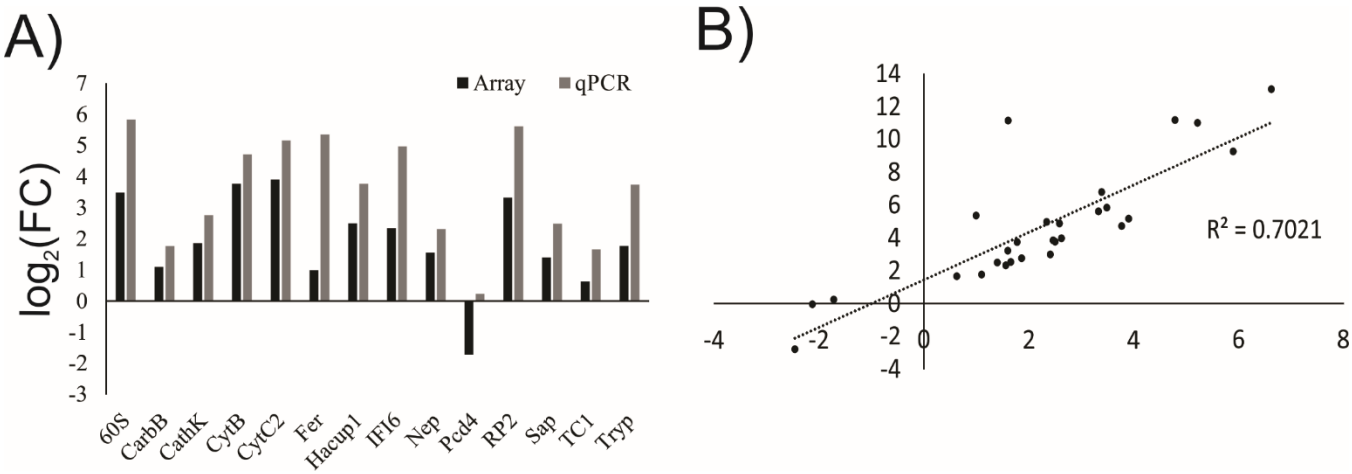


Figure 30. Correlation between RT-qPCR and microarray.

The genetic profiles of a select subset of genes were compared using RT-qPCR and microarray. With the exception of *pcd4*, all genetic profiles had the same direction of regulation (A). There was a high degree of correlation between the two methods (B; $R^2 = 0.70$).

5.4.4 Acclimation

Two divergent hypotheses explain the elevated transcriptomic responses in Atlantic salmon-fed lice. Firstly, as the lice had been sourced from Atlantic salmon, they may have been acclimated to that species (acclimation hypothesis). Alternatively, the response observed on Atlantic salmon resulted from host-specific factors (host effect hypothesis), such as higher nutritive status or lower immunocompetence. The host effect hypothesis is based on the notion that *L. salmonis* prefers a more susceptible host species, in contrast to a species (i.e., coho or pink salmon), likely to mount an aggressive defense response.

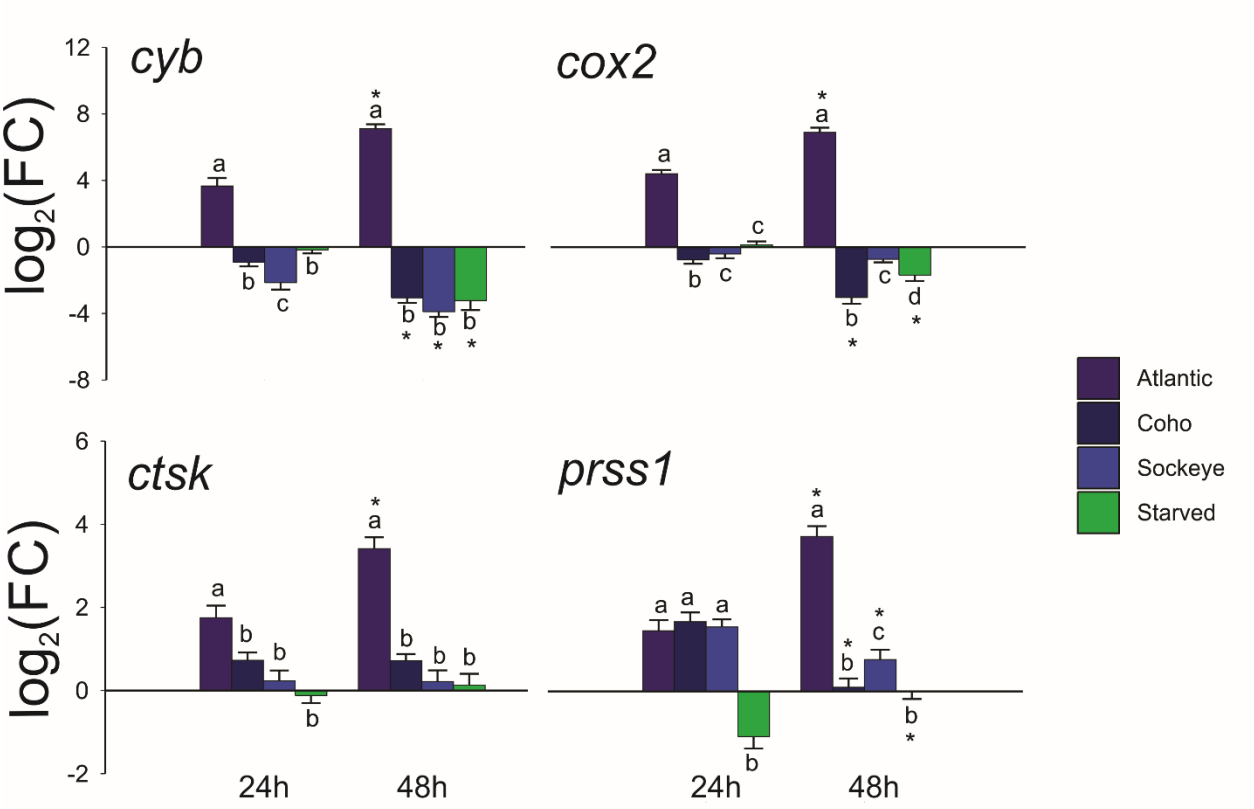


Figure 31. RT-qPCR profiles of mitochondrial enzymes (*cyb, cox2*) and digestive enzymes (*ctsk, prss1*) in Atlantic-fed, coho-fed, sockeye-fed or starved *L. salmonis*.

A subset of genes found differentially expressed in the microarray experiment are shown as the average of $\log_2$-transformed FC (n = 8) ± SEM. Differences between groups (denoted by lower case letters) and between 24 and 48 hr (*) were determined by a two-way ANOVA ($p \leq 0.05$) followed by Holm-Sidak post hoc comparisons.

To test the acclimation hypothesis, we used qRT-PCR to assess mRNA levels of a subset of *L. salmonis* genes observed to be differentially regulated during feeding. Sea lice were sourced from Atlantic salmon during a commercial harvest, and from sockeye salmon during a wild fish test-fishery survey. For acclimation, we predicted more robust responses in sea lice attached to salmon belonging to homologous host species. Results from this analysis indicated that the homologous host species was less influential on the louse response than the current host species: expression of digestive and oxidative reduction enzymes was significantly higher on Atlantic-fed lice, irrespective of their source. Thus, the expression of genes involved in proteolysis/digestion (e.g., *ctsk, prss-1*) was higher in lice feeding on Atlantic salmon compared to sockeye salmon (Figure 32). This was also observed for oxidative reduction enzymes (*cox2, cyb*, Figure 32) and protein synthesis genes (*rpl2,* data not shown). These results did not support the acclimation hypothesis and provided supporting evidence for a host-effect on the responses of *L. salmonis*. Thus divergent louse responses observed in the array study were due to the differences among host species.

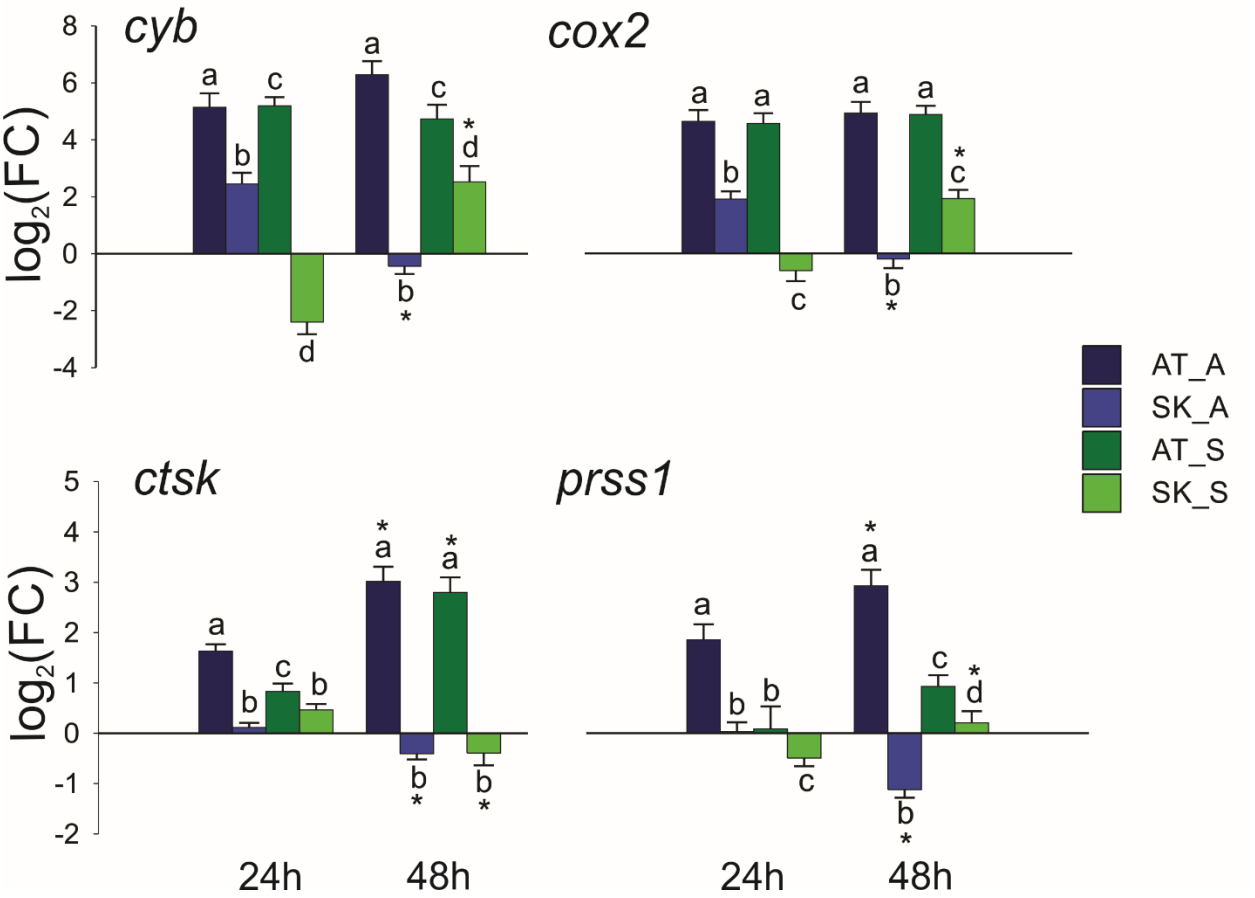


Figure 32. RT-qPCR profiles of genes in *L. salmonis* after the acclimation experiment.

L. salmonis sourced from Atlantic salmon or sockeye salmon were collected after feeding on Atlantic salmon (AT_A, SK_A) or sockeye salmon (AT_S, SK_S) after 24 and 48 hr, respectively. Expression of mitochondrial enzymes (*cyb, cox2*) and digestive enzymes (*ctsk, prss1*) are shown as the average of $\log_2$-transformed FC (n = 8) ± SEM. Differences between groups (denoted by lower case letters) and between 24 and 48 hr (*) were determined by a two-way ANOVA ($p \leq 0.05$) followed by Holm-Sidak post hoc comparisons.

5.5 Discussion

Many parasites have developed host selection and feeding strategies based on host resistance and nutritive value to exploit the host which most optimizes parasite fitness (Bize *et al.* 2008; Heylen *et al.* 2012). *Lepeophtheirus salmonis* is a salmonid specialist which displays host-seeking behavior suggesting a preference towards Atlantic salmon (Mordue Luntz & Birkett 2009). In addition, the parasite grows faster on Atlantic salmon, than on the resistant coho salmon (Fast *et al*. 2002), and produces more proteases when exposed to mucus from Atlantic salmon compared to that of coho salmon (Fast *et al.* 2002b). Together these data support the notion that the Atlantic salmon is a more desirable host environment for *L. salmonis*. Here we provide transcriptomic evidence that for the Pacific subspecies *L. salmonis oncorhynchi*, Atlantic salmon is a favored host compared with either coho or sockeye salmon, and that the feeding responses of *L. salmonis* on coho or sockeye salmon are more benign and in some cases resemble those of starvation.

5.5.1 Starvation as a stress response of *L. salmonis*

We observed a stress response in *L. salmonis* following a starvation period of 48 hrs which was characterized by up-regulation of genes involved in endocytosis, membrane invagination and negative regulation of transcription, metabolic and catabolic processes which are similar to the survival responses in starved *Caenorhabditis elegans* (Cui *et al.* 2013). Interestingly, starvation was also associated with up-regulation of genes involved in muscle-associated processes such as *troponin C1*, *myosin heavy chain* and *tropomyosin*. Up-regulation of tropomyosin and calmodulin during starvation was unexpected as these genes are associated with smooth muscle contraction surrounding the mid-gut during feeding (Sodja *et al.* 2007). However, during starvation muscle contraction may optimize the uptake of nutrients which may be an important adaptation for survival off the host. After initial attachment by the copepodid, *L. salmonis* is

restricted to the fish host, however, un-attached lice can survive in chilled seawater for up to 2 weeks (L. Braden, unpublished observation); thus, like other arthropods, *L. salmonis* appears to possess a tolerance for starvation (Burgess *et al.* 2012). In ticks, starvation is characterized by autophagy, which acts to compensate for the lack of host-derived nutrients (Kawano *et al.* 2011), and although we did not detect up-regulation of *ATG*, a hallmark of autophagy, ontology enrichment of closely related processes such as endocytosis and membrane invagination suggests a similar process occurs in *L. salmonis.* The up-regulation of proteolytic enzymes in starved *L. salmonis* as described here had previously been associated with preparation to feed in starved mites (*Psoroptes ovis*) (Burgess *et al.* 2012). Similar to *P. ovis*, identification of stress-related gene targets in parasitic copepods may hold high value for potential targets of prophylactic and chemotherapeutant control. Recent work has identified several such targets specific to abiotic chemical (Sutherland *et al.* 2014b) and environmental stressors such as temperature and salinity (Sutherland *et al.* 2012) and biological stressors such as molting and reproduction (Eichner *et al.* 2008).

5.5.2 The feeding response of *L. salmonis*

To our knowledge, this is the first transcriptomic analysis of the feeding response of *L. salmonis*. We hypothesized that the feeding response of sea lice would be stronger on susceptible hosts (Atlantic and sockeye salmon) compared to resistant hosts (coho salmon). As expected, enrichment analysis revealed transcripts associated with feeding belonged to pathways associated with proteolysis, digestion, energy metabolism, response to nutrients, reproduction and hemostasis. Under the auspices of the immuno-ecological view previously described (Roulin *et al.* 2003), we assessed whether the response of *L. salmonis* feeding on different host species was related to the susceptibility status of the host fish. If true, then the response of sea lice on

susceptible Atlantic and sockeye salmon would be indicative of a host environment that is more conducive to increased parasite fitness (Roulin *et al.* 2003; Bize *et al.* 2008), in contrast to that predicted on a resistant host, such as coho salmon.

Blood-feeding ectoparasites utilize strategies which suppress or alter the host immune response in order to maximize survival (Wikel 1999). Host blood is a major dietary component of the adult female louse (Brandal *et al.* 1976); therefore, like other hematophagous ectoparasites, *L. salmonis* must produce enzymes capable of blood digestion as well as provide anti-coagulants to encourage and maintain a free flow of blood to the feeding site. Proteases comprise the largest number of putative expressed genes associated with blood-meal digestion in hematophagous ectoparasites, and these can be categorized into four major groups: serine, aspartic, cysteine and metalloproteases. Cysteine proteases contribute to essential biological functions in parasites but are also frequently described in the excretory/secretory products of parasites and may act to disrupt host responses by killing cellular mediators of inflammation and destroying the extracellular matrix (Tovy *et al.* 2011; Radulović *et al.* 2014; Shareef & Abidi 2014). *Lepeophtheirus salmonis* produces several proteases (Cunningham *et al.* 2010), and in the present paper, we observed up-regulated expression of *cathepsin K* during feeding, with the most pronounced expression while feeding on Atlantic salmon. The involvement of cathepsin K in the catabolism of elastin, collagen and gelatin, remodeling of bone (Robinson *et al.* 2008), and suppression of host immune responses suggests this enzyme assists *L. salmonis* in digestion of collagen-rich host skin during feeding. Interestingly, resistance to *L. salmonis* in coho salmon is associated with exaggerated inflammation and tissue remodeling (Braden *et al.* 2015) and in the present study, coho-fed lice expressed the lowest levels of *cathepsin K,* while pronounced expression of *cathepsin K* was observed among lice feeding on susceptible Atlantic salmon

(Johnson & Albright 1992a; Fast *et al.* 2002a; Braden *et al.* 2012; Sutherland *et al.* 2014a). The expression of legumain was elevated in Atlantic-fed *L. salmonis*. Legumain is a cysteine protease that is important in blood digestion in ticks (Shareef & Abidi 2014). In *Shistosoma mansoni*, legumain is responsible for activation of cathepsin B (Sajid & McKerrow 2002; Abdul Alim *et al.* 2007), and the expression of *legumain* observed in the present study may represent a similar virulence factor for sea lice. A neutral endopeptidase, *neprilysin-1* was up-regulated in Atlantic-fed lice. This enzyme is highly conserved and in some invertebrates there are more than 20 copies of the gene present (Ottaviani *et al.* 2012). Expression of *neprilysin-1* in *L. salmonis* feeding on Atlantic salmon was significantly higher than on either coho or sockeye salmon. *Neprilysin* expression in mouse intestine during nematode infection is associated with regulating inflammation (Barbara *et al.* 2003), and therefore production of this enzyme may represent a novel sea louse virulence factor that acts to regulate the inflammatory response in the skin. Zinc-metalloproteinases are involved in a variety of physiological functions ranging from hatching to tissue remodeling (Yan *et al.* 2002). Members of this family are widespread among hematophagous organisms (Yan *et al.* 2002) and in the present study *zinc metalloproteinase* nas-7 was expressed by *L. salmonis* feeding on all three species; however, expression on Atlantic salmon was pronounced and observed to increase over time. Results from this study support the importance of proteases as effector molecules during the host-parasite interaction.

In addition to proteases, protease inhibitors have also been reported in tick saliva and are hypothesized to assist in evasion of host immune responses (Prevot *et al.* 2006; Corral-Rodríguez *et al.* 2009). Tick saliva contains serine protease inhibitors (serpins), cysteine protease inhibitors, carboxypeptidase inhibitors, and ATPase inhibitors (Radulović *et al.* 2014). *Serpin z-10* was up-regulated in *L. salmonis* following feeding on all species; however the expression was only

significant in sockeye-fed lice. As with ticks, we expected a large suite of serpins to be expressed during feeding (Prevot *et al.* 2006). However, the lack of representation may be the result of a largely unannotated *L. salmonis* genome (Table 10).

Table 10. Annotated vs. unannotated transcripts in the number of differentially expressed genes detected using the 38k oligonucleotide array.

	Host vs. Starved		Host comparisons		Species over time
Differentially expressed genes	**24h**	**48h**	**24h**	**48h**	**24 → 48h**
Annotated	237	282	590	718	224
Unannotated	191	179	417	489	211
Total	428	467	1007	1507	435

Prevention of the clotting cascade is important for blood-feeding parasites. Anti-coagulant compounds released into the attachment site limits blood loss while preventing coagulation. In teleosts, the clotting cascade is similar to that of higher vertebrates with an extrinsic pathway activated by cutaneous factors, and intrinsic factors stimulated by thrombocytes (Manship *et al.* 2012). Expression of metalloproteases (MMPs) and carboxypeptidases likely help prevent coagulation. These compounds have been observed in tick saliva (Anderson *et al.* 2008; Wang *et al.* 2009; Oliveira *et al.* 2013), and in the present study up-regulation of several metalloproteases was observed in feeding *L. salmonis* and the highest expression was observed in Atlantic-fed lice with the exception of *matrix metalloproteinase 9,* which was only associated with sockeye-fed lice. In higher vertebrates, carboxypeptidase B is a negative regulator of fibrinolysis in blood coagulation (Sakharov *et al.* 1997) and has been characterized in the mid-gut of mosquitoes (Sodja *et al.* 2007), ticks (Motobu *et al.* 2007; Anderson *et al.* 2008) and mites (Burgess *et al.* 2012). We observed up-regulation of *carboxypeptidase B* in feeding *L. salmonis*, and this expression was highest in lice feeding on

Atlantic salmon compared to either coho or sockeye salmon. It is likely that this enzyme acts by preventing the extrinsic clotting cascade of the fish host thus aiding blood-feeding by the louse.

In ticks, blood-feeding causes elevated levels of iron in the hemolymph as hemoglobin is digested. Ferritin and other molecules that act to limit oxidative stress must be produced to protect from oxidative damage (Anderson *et al.* 2008; Radulović *et al.* 2014). *Ferritin*-like genes were up-regulated in feeding *L. salmonis* regardless of the species of host; however Atlantic-fed lice had the highest expression compared to coho or sockeye-fed lice suggesting higher exposure to oxidative stress while on Atlantic salmon. An interesting finding specific to Atlantic-fed lice was significant up-regulation of *high-affinity copper-uptake protein-1 (ctr1).* Although toxic in excess of cellular requirements, trace amounts of copper ions are critical as cofactors for enzymes involved in many physiological processes including oxidative phosphorylation and mobilization of iron (Burmester 2004; Robinson & Winge 2010). In crustaceans, the most frequently utilized copper-utilizing protein (cuproprotein) is the mitochondrial enzyme cytochrome c oxidase (cox1) (Ahearn *et al.* 2004). The transfer of available copper to these cuproproteins is accomplished by uptake proteins and cellular copper homeostasis is tightly regulated to prevent cytotoxicity. High-affinity copper-uptake proteins are highly conserved membrane proteins involved in cellular copper entry, and represent a components of cellular copper homeostasis (Molloy & Kaplan 2009). Thus high expression of *crt1* would facilitate the increased requirement for copper as co-factors for up-regulated *cox1*. Indeed, pathways of oxidative reduction and respiratory electron chain synthesis were highly enriched in Atlantic-fed lice compared to coho- or sockeye-fed lice. Moreover, genetic profiles of the genes involved in mitochondrial functions (*cox1*, *cyb*) were over-expressed when feeding on Atlantic salmon

providing a mechanistic explanation for concomitant expression of *crt1* in Atlantic-fed *L. salmonis*.

The capacity of a parasite to modulate the host immune system may be a factor in determining host preference and species range. *L. salmonis* is restricted to salmonids, although infections on non-salmonid species have been observed (Jones *et al.* 2006; Pert *et al.* 2009). The existence of a variable host response among species of salmon during infection with *L. salmonis* suggests there may be a host-preference for susceptible species (Atlantic salmon), which may be thought of as a more desirable host species. Exposure of *L. salmonis* to mucus from Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) resulted in production of enzymes that differed significantly from enzymes produced by *L. salmonis* exposed to coho salmon or winter flounder (*Pseudopleuronectes americanus*) mucus (Fast *et al.* 2003), which supports the susceptibility status of the different species. It would seem extremely likely that host-location mechanisms in *L. salmonis* would involve species-specific cues that would elicit behavioral responses by the louse, and further, that these cues would be related to the susceptibility status of the host.

Global comparison of responses among host species revealed that after 24 hrs, lice attached to Atlantic salmon displayed a response characterized by significant enrichment of pathways involved in proteolysis, hemostasis, reproduction, and oxidative reduction. Conversely, lice attached to either coho or sockeye salmon had distinctly different patterns of expression characterized by enrichment of genes involved in skeletal system and sensory system development (sockeye salmon), cytoskeletal organization, regulation of growth and nucleosome organization (coho salmon). Further, after 48 hrs, there was no significant enrichment of biological processes in lice feeding on coho- or sockeye-fed lice, whereas in Atlantic-fed lice, enriched pathways remained abundant and highly populated. Hierarchical and K-means

clustering of the overall transcriptomic responses indicated the response of sockeye and coho-fed lice were more similar to the starvation response and distinct from the response elicited by feeding on Atlantic salmon, including up-regulation of genes associated with starvation in coho- or sockeye-fed lice but down-regulated in Atlantic-fed lice. Thus the feeding response of *L. salmonis* indicated that Atlantic salmon is the most desirable host compared coho or sockeye salmon.

Physiological differences among Atlantic or Pacific salmon species may partially explain the observed differences in parasite fitness. For example, compared with rainbow trout (*O. mykiss*) and coho salmon, the skin of Atlantic salmon is thinner and less populated with mucocytes (Fast *et al.* 2002b). Variability in such cutaneous parameters may be related to parasite rejection in resistance species, and with respect to *L. salmonis*, may be driving host preference towards species with less of these cells. The present study suggests that the Atlantic salmon is a preferred host to the salmon louse which is likely because it presents a more favorable ratio of nutritional value to immunological status. Balancing host immunity with nutritive value is a driver of parasite host-specificity and has been observed in many different host-parasite relationships (Heylen & Matthysen 2011; Heylen *et al.* 2012), therefore it is also a likely key determinant in the observed host preference between *L. salmonis* and Atlantic salmon.

The present transcriptomic analysis suggests that Atlantic salmon presents a more desirable host environment to *L. salmonis* compared to either coho or sockeye salmon, as shown by pronounced expression of important virulence factors, reproductive enzymes and energy metabolism when attached to Atlantic salmon. Enhanced parasite fitness has been observed by more rapid larval development and higher parasite abundances in Atlantic salmon compared to either chinook (*O. tshawytscha*) (Johnson 1993) or coho salmon (Fast *et al.* 2002a). We observed

enrichment of genes associated with reproduction in Atlantic-fed compared to either coho- or sockeye-fed *L. salmonis*. High energy metabolism is correlated with high reproductive output, and as such parasites have evolved to prefer hosts in which they are able to extract the highest available energy and thus produce the most offspring (Bize *et al.* 2008). By definition, energy metabolism and reproductive output may be used as a proxy to define the relative nutritional value of the host in the absence of physiological parameters. Data from this present study provides evidence to suggest the nutritional status of Atlantic salmon is higher compared to that of coho or sockeye salmon.

5.5.3 Response to coho and sockeye salmon more similar to starved *L. salmonis*

We hypothesized that resistance status of the host determines the louse response and as such the response to coho salmon was expected to be distinct from that to sockeye salmon. However, compared to Atlantic-fed lice, differential regulation of *L. salmonis* feeding on either coho or sockeye salmon was characterized by a small population of genes that were more similar to that of starved *L. salmonis* including *heat shock protein 90*, *tubulin alpha chain*, and *T-complex protein 1*. These data contradict our hypothesis, as based on the high susceptibility status of sockeye salmon, we would expect to see a lice feeding on sockeye respond in a similar manner as Atlantic salmon.

After 24 hrs on coho and sockeye salmon, there was over-representation of genes in pathways of actin-associated processes. In *Anopheles gambiae*, blood-meal digestion is characterized by significant up-regulation in actin production which supports the changing shape of the mid-gut during feeding (Sodja *et al.* 2007). A similar mechanism in coho- and sockeye-fed *L. salmonis* may explain the up-regulation of actin and skeletal processes signifying a physiological response to feeding by the louse. However, enrichment of those genes was not

accompanied by enrichment of proteolytic enzymes required for digestion. Specifically there was down-regulation over time in expression of proteolytic-associated genes in coho-fed lice. Interestingly, up-regulation of actin and skeletal processes were also associated with starved *L. salmonis*, which indicates that these processes were activated both in starvation as well as during feeding on coho or sockeye salmon.

The Pacific subspecies of *L. salmonis* has been coevolving with Pacific salmonids for the last several million years (Yazawa *et al.* 2008) and as such, the host-parasite relationship between the various *Oncorhynchus* spp. and *L. salmonis oncorhynchi* is a product of divergent evolutionary adaptations that have resulted in some species having a more efficient immunological response during infection as observed in other host-parasite systems (Price 1980), and as a function of life history trade-offs (Zuk & Stoehr 2002). For example, coho salmon exhibits heightened resistance towards other ectoparasitic copepods (Gonzalez *et al.* 2000). With respect to *L. salmonis*, comparative laboratory exposures demonstrate that chum and sockeye salmon support the highest infections and exhibit weakened cellular and humoral inflammatory responses at the louse attachment site, compared to that of coho or pink salmon (Fast *et al.* 2002a; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012, 2015). Transcriptomic evidence from the present study fails to corroborate the hypothesis that susceptibility of sockeye salmon is associated with a heightened feeding response by the louse. Rather, these results suggest that responses elicited during feeding on *Oncorhynchus* spp. (irrespective of susceptibility status), are governed by co-evolutionary adaptation between host and parasite leading to a tightly controlled parasite-host interaction. Consequently, the observed variation in disease susceptibility among members of the genus *Oncorhynchus* must be related to the inherent immunological competence

of the individual species which is likely governed by different life history strategies (Norris & Evans 2000; Zuk & Stoehr 2002; Pecquerie *et al.* 2011).

5.5.4 Supporting evidence for a host-specific response by *L. salmonis*

Results from this study indicated a strong host-specific response by *L. salmonis* on Atlantic salmon; however, the parasites were obtained from Atlantic salmon. Significant divergence in feeding responses by *L. salmonis* on Atlantic salmon compared to coho or sockeye salmon might be explained by local acclimation of the parasite (Raeymaekers *et al.* 2011). To address this possibility, we exposed lice sourced from sockeye salmon to Atlantic and sockeye salmon, and lice sourced from Atlantic salmon to sockeye and Atlantic salmon. If acclimation occurred, then we would expect the parasite to respond better on the same species they initially infected. On the contrary, we observed *L. salmonis* to respond more robustly to Atlantic salmon, irrespective of the original host species. These results corroborate the host-effect hypothesis and show that Atlantic salmon provides a more desirable environment for *L. salmonis*, permitting exaggerated expression of virulence factors which results in a susceptible phenotype. A similar phenomenon is observed in ticks (*Rhipicephalus microplus*) feeding on cattle (*Bos taurus*) or white-tailed deer (*Odocoileus virginianus*). Although white-tailed deer are physiological suitable hosts for *R. microplus*, parasite engorgement and reproductive fitness are reduced when feeding on cattle and this difference in virulence is attributed to differential expression of proteins in the saliva of *B. microplus* (Popara *et al.* 2013). A similar approach should be conducted to compare the constituents of *L. salmonis* secretions to determine if host-specific virulence is reflected in the secretome.

5.6 Conclusion

Understanding the feeding response of *L. salmonis* may help explain the variable response of different species of salmon, such as the delayed or muted inflammatory response that is associated with susceptibility in Atlantic, chum and sockeye salmon (Johnson *et al.* 1996; Fast *et al.* 2006; Skugor *et al.* 2008; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012, 2015). We hypothesized that the feeding response of *L. salmonis* is predictive of host susceptibility: the feeding response on a susceptible salmon is characterized by a robust transcriptomic response; whereas, the feeding response on a resistant salmon is a more benign. However, an exaggerated feeding response was only detected when attached to Atlantic salmon. As the local tissue response of Atlantic salmon to attachment of *L. salmonis* is characterized by a weak inflammatory reaction and heightened susceptibility, this data presents evidence for an explanatory mechanism. When feeding on Atlantic salmon there is marked expression of virulence factors (i.e., serine proteases, cysteine proteases, metalloproteases) that act to dampen the inflammatory response at the attachment site. This response by *L. salmonis* appears to be specific to *Salmo* sp. as we did not detect similar expression by the susceptible sockeye salmon. Therefore, this work suggests that there may be two independent pathways of susceptibility towards infection with *L. salmonis*. Firstly, lice feeding on *Salmo* spp. differ from that of *Oncorhynchus* spp., in that genetic responses are more robust on Atlantic salmon and characteristic of a more desirable host environment, including enhanced production of energy metabolism, reproduction, and virulence factors. We suggest that this difference is related to co-evolutionary adaptation between *Oncorhynchus* spp. and the local parasite (*L. salmonis onchorhynci*), and that Atlantic salmon provides a naïve environment that is easier to exploit without an accompanying immune response and has a larger nutritional value-immune response ratio, which would be more beneficial for parasitic exploitation and thus present a more “tasty

fish" (Sheldon & Verhulst 1996; Norris & Evans 2000). The second pathway of susceptibility describes the variable host response among *Oncorhynchus* spp., whereby certain species (coho or pink salmon) respond to infection with a more efficient immune response that results in heightened resistance and is a product of variable life histories among the species. An earlier study attempted to capture potential inherent immunological differences in juvenile chum and pink salmon after an intraperitoneal vaccine injection focusing on a small subset of innate immune targets (Fast *et al.* 2007b); however, a more comprehensive model examining functional as well as genetic profiles will greatly enhance our understanding of the immunological capabilities of different salmonid species at a fundamental level.

The secretory components of *L. salmonis* were shown to contain serine proteases (Firth *et al.* 2000; Johnson *et al.* 2002; Kvamme *et al.* 2004) and prostaglandin E_2 (Fast *et al.* 2004), suggesting sea lice are able to modulate host immune functions. Although there has been some efforts to quantify the effects of sea lice secretions on salmon (Lewis *et al.* 2014), the mechanism and extent of immuno-suppression has not yet been elucidated. Results from the present study indicate that there is a much larger suite of molecules expressed by sea lice from what was previously thought (Fast *et al.* 2007a), and in ticks, they are implicated as virulence factors in the host-parasite interactions. Profiling the proteomic response of *L. salmonis* is necessary to determine the transcripts that are secreted at the louse-salmon interface, and further, would elucidate the proteomic response of *L. salmonis* on resistant or susceptible hosts.

5.7 Chapter acknowledgements

This research was funded by Genome BC, the Province of BC, the Department of Fisheries and Oceans Canada (DFO), NSERC, the University of Victoria, and Marine Harvest. Thanks to Holly Hicklin (DFO) for assistance with fish culture. We are grateful to Dr. Chris Foote and the Chase River Hatchery for supplying coho salmon.

Chapter 6: The excretory/secretory proteome of *Lepeophtheirus salmonis*

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LMB conceived of the study, prepared protein samples for mass spectroscopy, interpreted the data and wrote the manuscript.

BFK assisted with interpretation and edited the manuscript.

SRMJ conceived of the study, assisted with interpretation and wrote the manuscript.

6.1 Abstract

The salmon louse, *Lepeophtheirus salmonis*, is a serious concern for salmon aquaculture in the Northern hemisphere but the host-parasite relationship between sea lice and salmon is poorly understood. Earlier work identified a few immunosuppressive molecules in the secretions of *L. salmonis* that may be important in determining susceptibility during infection. To further describe the secretome of sea lice, one dimensional gel electrophoresis was used to separate proteins from the dopamine-elicited secretory/excretory fractions of an adult female Pacific *L. salmonis*. Protein size fractions were subjected to trypsin digestion and analyzed by liquid chromatography followed by MS/MS analysis. Following feeding on Atlantic salmon, a total of 52 proteins were detected. Ontology analysis determined the highest enrichment of proteins to belong to the categories of proteolysis, organ development, and protein metabolism. Of proteins included in the proteolysis category, we detected 18 different proteases with functions of tissue digestion, anti-hemostasis and fibrinolysis. We further characterized the proteins present in the three most common protein size fractions (~200, 30, and 10 kDa) and of the 31 proteins detected, 15 were proteases. This study provides a much more detailed catalogue of proteins present in the secretome of *L. salmonis*.

6.2 Introduction

By definition, parasites feed on their hosts (Price 1980). The relative harm or virulence caused by the parasite is influenced by the amount of nutrients extracted as well as the counter-acting defense responses of the host (Poulin & Combes 1999). Distribution of parasites among host populations is characterized by a few individuals that harbor many parasites while others have few to none, indicating that some hosts are more profitable than others (Shaw & Dobson 1995). Host nutritive status is correlated with good condition factor, and in some parasites, reproductive success is increased when hosts are supplemented with additional feed (Christe *et al.* 2003; Tseng 2006; Tschirren *et al.* 2007). A consequence of good host condition is the ability to mount vigorous immune defenses, which is costly to the parasite. Therefore, by exploiting hosts with the highest nutritional value and lowest immune status, parasites will be the most successful and exhibit the highest fitness (Price 1980; Sheldon & Verhulst 1996; Bize *et al.* 2008). To maximize fitness, parasites will often exhibit behavioral preferences for certain host species, which correlates with host susceptibility status (Sears *et al.* 2012).

Heavy infestations of Atlantic salmon (*Salmo salar*) by the sea louse, *Lepeophtheirus salmonis*, results in a cost to the salmon farming industry of over $500 million USD annually, most of which is associated with chemotherapeutant treatments and losses due to lower production and mortality (Johnson *et al.* 2004; Costello 2009). Initial infection of a suitable host is accomplished by host-seeking behavior of copepodids and is driven in part by chemical cues from the host (Devine *et al.* 2000; Boxaspen 2006; Mordue Luntz & Birkett 2009). The majority of damage to the fish is due to grazing of the epithelial and mucosal skin layers by mobile pre-adult and adult lice (Pike & Wadsworth 1999). Host blood also comprises an important part of the diet in adult *L. salmonis* (Brandal *et al.* 1976). In pink and coho salmon, which are more resistant species, damage to the epidermis invokes a pronounced inflammation and infiltration of

cellular effectors at the attachment site (Sutherland *et al.* 2011; Braden *et al.* 2012, 2015). This response is substantially weaker or absent in susceptible hosts (Atlantic, sockeye, chum salmon) due to various factors from both the host and the parasite (Sutherland *et al.* 2011, 2014; Braden *et al.* 2012, 2015; Braden *et al.* 2015, *in prep*).

Production of molecules that suppress host immune responses is well documented among ticks (Bowman *et al.* 1996; Kopecký *et al.* 1999; Gwakisa *et al.* 2001; Aljamali *et al.* 2002; Oliveira *et al.* 2013), mites (Richards *et al.* 2011; Burgess *et al.* 2012; Bartley *et al.* 2012), and mosquitoes (Volz *et al.* 2005; Sodja *et al.* 2007; Takken & Verhulst 2013). These virulence factors facilitate exploitation of host nutrients by the parasite while limiting negative host-associated defense responses. The secretory response of *L. salmonis* during feeding is poorly understood. Earlier investigation of the dopamine-induced secretory/excretory products (SEPs) of *L. salmonis* revealed the presence of prostaglandin E_2 (PGE_2) (Fast *et al.* 2004) and trypsin-like proteases (Firth *et al.* 2000; Johnson *et al.* 2002; Kvamme *et al.* 2004). Recently we analyzed the transcriptomic response of *L. salmonis* during feeding on susceptible Atlantic (*Salmo salar*), sockeye (*Oncorhynchus nerka*), and resistant coho (*O. kisutch*) salmon (Braden *et al*. 2015, *in prep*). Gene expression profiling revealed an exaggerated expression of several virulence factors during feeding on Atlantic salmon compared to coho or sockeye salmon, suggesting that the Atlantic salmon is a more desirable host. Importantly, greater annotation of the *L. salmonis* transcriptome (Yazawa *et al.* 2008; Yasuike *et al.* 2012) enables more comprehensive proteomic studies to be pursued.

Secretagogues have been used to study secretomes from ticks (Kaufman 1978; Ribeiro *et al.* 1992; Bowman *et al.* 1997; Fezza *et al.* 2003; Oliveira *et al.* 2013) and sea lice (Fast *et al.* 2004, 2007a; Lewis *et al.* 2014). Although less well defined in *L. salmonis*, sympathomimetic

(dopamine and epinephrine) and cholinomimetic (pilocarpine, carbachol and acetylcholine) secretagogues provoke secretion by salivary glands in ticks by the excitation of cholinergic and catecholaminergic receptors on secretory nerves (Kaufman 1978). A comparative study of the efficacy of pilocarpine (PC) or dopamine (DA) for the collection of *Rhipicephalus sanguineus* saliva found that proteins present in DA-elicited saliva were absent in PC-elicited saliva, but that the former formed more diffuse bands after 1D gel-electrophoresis (Oliveira *et al.* 2013).

The purpose of this work was to generate a more comprehensive inventory of proteins present in the dopamine-elicited *L. salmonis* secretory response.

6.3 Materials & Methods

6.3.1 Fish culture and parasite challenges

All procedures involving the handling and use of fish in this study were approved by the Canadian Council of Animal Care (CCAC) prior to initiation. Atlantic salmon (*Salmo salar*) parr were obtained from a commercial salmon hatchery on Vancouver Island, British Columbia. Fish were reared on brackish water (15 ppm) until smolting, after which they were maintained on ultraviolet-treated salt water in single-pass flow-through tank systems on a 12:12 hr light:dark cycle and fed 1% total biomass daily. Fish were randomly divided among twelve 330 L tanks with 2 tanks for each species (2X infection tanks), acclimated for approximately 7 days and starved at least 24 hr prior to any manipulation.

Adult female *L. salmonis* were collected during harvest of Atlantic salmon at a commercial aquaculture site on Vancouver Island, British Columbia, Canada. After collection, the lice were rinsed in sea-water and transported back to the Pacific Biological Station (Nanaimo, British Columbia, Canada) in 8°C aerated previously sterilised sea water. Only lice firmly attached to the collection vessel were included in the study. The time between collection of the sea lice and initial infection time was < 24 hr.

6.3.2 Collection of excretory-secretory (ES) products

Excretory/secretory (ES) products were obtained from Pacific *L. salmonis* collected from Atlantic salmon after a 24 hr experimental challenge with Atlantic salmon.

Sea lice were incubated in 0.25 mM dopamine-sterile sea water (2 lice/mL) for 45 minutes at 8°C in 50 mL Falcon® tubes as previously described (Fast *et al.* 2004, 2007a; Lewis *et al.* 2014). After incubation, the lice were removed from the tubes and the resulting excretory/secretory products (ES products) was centrifuged using 3 kDa molecular weight cut-off columns (Pall) at 4°C for 30 minutes at 3,000 x g. A protease inhibitor cocktail (Sigma) was added to each sample (1:100) before immediate storage at -80°C.

6.3.3 Protein separation, detection and identification

Soluble ES products from *L. salmonis* were precipitated using ice-cold acetone (4:1 ratio of acetone to sample) overnight at -20°C. After centrifugation, pelleted samples were re-suspended in 2X Laemmli sample buffer and boiled in 2X Laemmli sample buffer (BioRad) for 10 min and 20 µl of each sample was resolved on an Any-kD 4-20% Bis-Tris precast gel (BioRad) at 80 volts for 70 minutes and visualized by colloidal Coomassie blue staining as described (Candiano *et al.* 2004). A lane from the resulting gel was sliced into 15 equal pieces and gel fragments were destained using 50% acetonitrile in 25 mM NH_4HCO_3, pH 8.4, and vacuum dried. Molecular-grade porcine trypsin (Sigma) was added and the samples were incubated overnight at 37°C. The peptide digests (15µL) were bound to a C18 StageTip, washed with 25µL 3% acetonitrile/water/0.5% formic acid and eluted with 40µL 80% acetonitrile/0.5% formic acid. The samples were speed-vac centrifuged and re-hydrated to 10 µL with 2% acetonitrile/0.1% formic acid prior to LC-MS/MS analysis.

The peptide mixtures (3µL of 10µL) were separated by on-line reverse phase chromatography using an EASY-nLC 1000 system (Thermo Fisher Scientific) with a reversed-

phase pre-column Magic C18-AQ (100µm I.D., 2 cm length, 5µm, 100Å, and an in-house prepared reverse phase nano-analytical column Magic C-18AQ (75µm I.D., 15 cm length, 5µm, 100Å, Michrom BioResources Inc, Auburn, CA), at a flow rate of 300 nl/min. The chromatography system was coupled on-line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray Flex NG source (Thermo Fisher Scientific). Solvents were A: 2% acetonitrile, 0.1% formic acid; B: 90% acetonitrile, 0.1% formic acid. After a 300 bar (~ 12µL) pre-column equilibration and 300 bar (~ 3µL) nanocolumn equilibration, samples were separated by a 55 minute gradient (0 min: 0%B; 45 min: 45%B; 2 min: 100%B; hold 8min: 100%B). The Orbitrap Fusion instrument parameters were as follows: nano-electrospray ion source with spray voltage 2.4kV, capillary temperature 275 ℃. Three analyses were performed: OT-IT (CID). The survey MS1 scan m/z range 400-2000 profile mode, resolution 120,000 FWHM@200m/z one microscan with maximum inject time 200 ms. The Siloxane mass 445.120024 was used as lock mass for internal calibration. Data-dependent acquisition Orbitrap survey spectra were scheduled at least every 3 seconds, with the software determining "Top-speed" number of MS/MS acquisitions during this period. The automatic gain control (AGC) target values for FTMS was 400,000. The most intense ions charge state 2-6 exceeding 50,000 counts were selected for CID MSMS fragmentation in the ion routing multipole. Monoisotopic Precursor Selection (MIPS) was enabled and Dynamic exclusion settings were: repeat count: 2; repeat duration: 15 seconds; exclusion duration: 60 seconds with a 5ppm mass window. The data dependent (ddMS2) IT CID scan used a quadrupole isolation window of 2 Da; Iontrap rapid scan rate, auto normal m/z range, centroid detection, 1 microscan, 35ms maximum injection time, AGC target 10,000 and normalized CID collision energy of 30%.

6.3.4 Proteomics data analysis of *L. salmonis* ES products

Raw files were created by XCalibur 3.0.63 (Thermo Scientific) software and analysed with Proteome Discoverer 1.4.0.228 software suite (Thermo Scientific). Parameters for the spectrum selection to generate peak lists of the CID spectra were: activation type CID; s/n cut-off: 1.5; total intensity threshold: 0; minimum peak count: 1; precursor mass: 350-5000 Da). The peak lists were submitted to an in-house Mascot 2.4.1 server against database Uniprot-Swissprot 20130530 (540,261 sequences; 191,876,607 residues) and an in-house annotated *L. salmonis* EST/RNA-seq database.

Database search parameters were as follows: precursor tolerance 8 ppm; MS/MS tolerance 0.8 Da; Trypsin enzyme 1 missed cleavages; ESI-TRAP instrument type; fixed modification: carbamidomethylation (C); variable modifications: oxidation (M), propionamide (C), and deamidated (N,Q). The Decoy database Percolator settings: Max delta Cn 0.05; Target FDR strict 0.01, Target FDR relaxed 0.05 with validation based on q-Value.

Statistical analyses of the Proteome Discover result files were performed with the Scaffold Q+S software package (Proteome Software, Inc, Portland, OR).

6.3.5 Protein ontology

Functional data for each identified protein were obtained from Uniprot and included gene ontology (GO) annotations, EC number, and Interpro motifs. Assignment of GO terms to identified proteins was done by Blast2GO software (version 3.0) in three main steps: blasting to find homologous sequences, mapping to collect GO terms associated with blast hits, and annotation to assign functional terms to query sequences from the pool of GO terms collected in the mapping step.

6.4 Results

6.4.1 Identification of louse proteins in the whole excretory/secretory (ES) product of *L. salmonis* feeding on Atlantic salmon

After tryptic digestion of dopamine-elicited ES fractions from Atlantic salmon-fed *L. salmonis,* 52 louse proteins were identified (Figure 33, Table 11). The proteins were assigned to functional categories on the basis of InterPro domains and/or GO categories. The group of proteins with the highest representation in the category of biological process was proteolysis (34.6%). The remaining proteins involved in carbohydrate metabolism, cell adhesion, cell proliferation, cell signaling, chitin metabolism, cytoskeleton organization, development and pregnancy, oxidation reduction and protein folding. In proteolysis there were 18 proteases identified including trypsins, cathepsins, neprilysins, zinc metalloproteases, collagenases, endopeptidases, coagulation factors, and aminopeptidases (Table 12**)**. Proteases identified in *L. salmonis* ES fractions were compared with that of published literature from tick-host systems to reveal probable function.

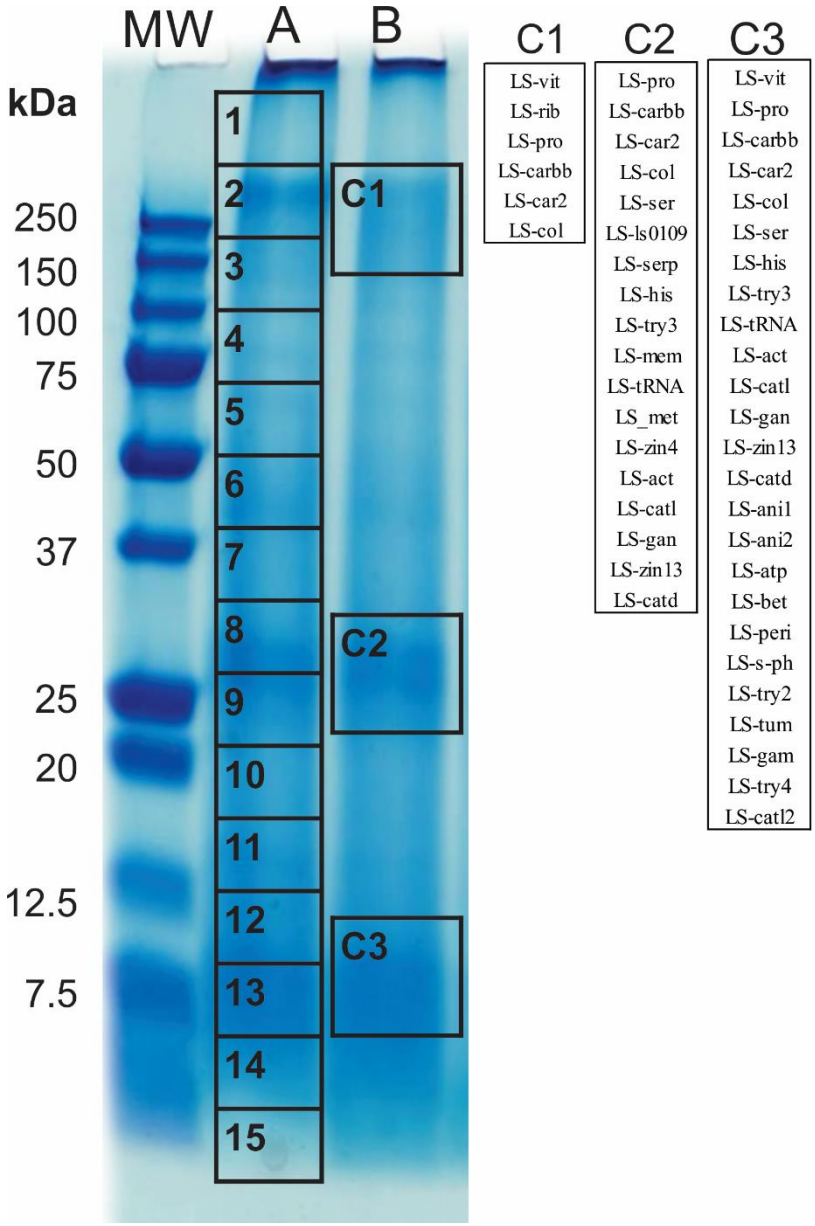

Figure 33. One dimensional gel electrophoresis of *L. salmonis* ES products.

ES products were collected as described and separated under reducing conditions at 80 V for 70 minutes before analysis by LC-MS/MS. There was a consistent banding pattern with a cluster of bands at ~150 kDa, ~30 kDa and ~10 kDa. One lane was sliced into 15 equal pieces (A) a second was targeted to identify proteins in the three major clusters of bands (B). Proteins identified in each cluster (C1, C2, C3) are listed. For description of protein labels see Table 13. MW = molecular weight ladder.

Table 11. Proteins identified by LC-MS/MS in the whole ES product of *L. salmonis*.

M.W.[a]	Description	Mascot[b]	Coverage[c]	Accession #	BP[d]
22.4	Enolase	42.9	5.1	P15007.2	Carbohydrate metabolism
35.5	Glycoside catalytic core	52.3	11.22	n/a	Carbohydrate metabolism
30.3	Neural-cadherin	61.1	3.6	O15943.2	Cell adhesion
62.8	Transforming growth factor-beta-induced protein ig-h3	191.2	9.31	Q15582.1	Cell adhesion
44.9	Protein arginine N-methyltransferase 5	35.2	2.46	Q8CIG8.3	Cell proliferation
23.6	Polyubiquitin-A	164.5	19.8	P0CG71.1	Cell signalling
108.5	Pecanex-like protein 1	63.3	2.77	Q98UF7.1	Cellular component membrane
25.1	Chitin deacetylase 5 isoform x6	53.2	9.43	n/a	Chitin metabolic process
81.4	Chitooligosaccharidolytic beta-N-acetylglucosaminidase	49.5	4.39	P49010.1	Chitin metabolic process
18.6	Hemocytin	54.2	7.27	P98092.1	Chitin metabolic process
26.8	Histone H4	116.2	12.12	Q6WV74.3	Chromatin organization
34.1	Actin 5C	840.1	27.87	P10987.4	Cytoskeleton organization
16.9	Actin, plasmodial isoform	170.8	25	P02576.2	Cytoskeleton organization
24.1	Cofilin/actin-depolymerizing factor homolog	44.8	5.74	P45594.1	Cytoskeleton organization
67	Armadillo segment polarity protein	53.9	2.73	P18824.1	Developmental process
117.1	Pregnancy zone protein	117.8	4.15	Q03626.1	Female pregnancy
45.8	Glyceraldehyde-3-phosphate dehydrogenase	72.9	11.19	A3FKF7.1	Glycolytic pathway
35.7	Triosephosphate isomerase B	74.0	14.15	Q90XG0.1	Glycolytic pathway
16.8	Pantetheinase hydrolase	73.2	8.55	Q58CQ9.1	Innate immune response
26.3	Ganglioside gm2 activator	132.1	10.74	n/a	Lipid storage
104.5	Vitellogenin-like protein	2828	20	ABU41136.3	Lipid transport
32.5	hypothetical protein	64.4	8.56	n/a	n/a
28.9	Phospholipid hydroperoxide glutathione peroxidase	44.3	8.24	Q32QL6.2	Oxidation reduction
28.9	Thioredoxin peroxidase	104.9	4.31	Q26695.1	Oxidation reduction
46.5	Thioredoxin-2	184.5	11.22	Q6XHI1.1	Oxidation reduction
97.7	Endoplasmin	30.1	1.42	P41148.1	Protein folding
72.6	Protein disulfide-isomerase 2	214.3	6.19	Q17770.1	Protein folding
64.9	Importin-9	31.0	4.27	Q96P70.3	Protein import into nucleus
63.8	Intracellular protein transport protein USO1	31.6	6.43	P25386.2	Protein transport
17.6	72 kDa type IV collagenase	147.5	10.97	Q90611.1	Proteolysis
38.7	Anionic trypsin-2	28.8	7.83	P00763.2	Proteolysis
48.5	Cathepsin D	60.0	7.74	P18242.1	Proteolysis
42.1	Cathepsin L	93.1	7.95	Q95029.2	Proteolysis
30.5	Chymotrypsin BI	370.4	23.83	Q00871.1	Proteolysis
58.8	Coagulation factor XII	421.4	9.85	Q04962.1	Proteolysis
48.5	Matrix metalloproteinase 9	255.8	27.91	P41245.2	Proteolysis
17.7	Membrane metallo-endopeptidase like 14	90.6	7.43	Q495T6.2	Proteolysis
17.6	Membrane metallo-endopeptidase like 2	69.9	24.84	Q495T6.2	Proteolysis
15.6	Neprilysin-2	26.4	12.03	O16796.2	Proteolysis
23.2	Ovochymase-1	55.1	3.81	Q7RTY7.2	Proteolysis
62.8	Probable cytosol aminopeptidase	174.6	12.54	Q9PP04.1	Proteolysis
25.7	Probable methionine--tRNA ligase	41.9	4.1	Q9ZTS1.2	Proteolysis
56	Serine protease	385.6	9.52	Q8VIF2.1	Proteolysis
36.2	Thymus-specific serine protease 16	62.6	6.58	Q9QXE5.1	Proteolysis
31.1	Trypsin-1	98.2	6.62	P00765.1	Proteolysis
44.8	Zinc metalloproteinase nas-15	97.1	8.38	P55115.2	Proteolysis
36	Zinc metalloproteinase nas-4	207.0	23.1	P55112.4	Proteolysis
93.7	Papilin	144.2	7.76	Q868Z9.2	Reg. of peptidase activity
75.8	Heat shock 70 kDa protein 8	45.2	3.89	P63018.1	Response to stress
17.6	Saposin B domain-containing protein	88.9	7.14	BT078242.1	Sphingolipid metabolism
64.2	Elongation factor 1-alpha	105.1	4.84	P02993.2	Translation elongation
28.8	Gamma crystallin	114.3	15.54	n/a	Visual perception

[a] Expected molecular weight

[b] Mascot score

[c] Percent coverage of the protein by the peptide

[d] Biological process as determined by protein ontology analysis

Table 12. Putative proteases identified in the ES products of *L. salmonis*.

MW (kDa)	Protease	Accession #	Probable function[a]
17.6	72 kDa type IV collagenase	Q90611.1	Anti-hemostasis
38.7	Anionic trypsin-2	P00763.2	Tissue digestion
48.5	Cathepsin D	P18242.1	Tissue digestion
42.1	Cathepsin L	Q95029.2	Tissue digestion
30.5	Chymotrypsin BI	Q00871.1	Tissue digestion
58.8	Coagulation factor XII	Q04962.1	Anti-hemostasis
17.6	Saposin B domain-containing protein	BT078242.1	Fibrinolysis
48.5	Matrix metalloproteinase 9	P41245.2	Anti-hemostasis
17.7	Membrane metallo-endopeptidase like 14	Q495T6.2	Anti-hemostasis
17.6	Membrane metallo-endopeptidase like 2	Q495T6.2	Anti-hemostasis
15.6	Neprilysin-2	O16796.2	Fibrinolysis
23.2	Ovochymase-1	Q7RTY7.2	Fibrinolysis
62.8	Probable cytosol aminopeptidase	Q9PP04.1	Tissue digestion
56	Serine protease	Q8VIF2.1	Tissue digestion
36.2	Thymus-specific serine protease 16	Q9QXE5.1	Tissue digestion
31.1	Trypsin-1	P00765.1	Tissue digestion
44.8	Zinc metalloproteinase nas-15	P55115.2	Fibrinolysis
36	Zinc metalloproteinase nas-4	P55112.4	Fibrinolysis

[a]Based on Anderson *et al.* 2008; Hu *et al.* 2013; Oliveira *et al.* 2013; Radulović *et al.* 2014

6.4.2 Characterization of proteins in the prominent protein bands from *L. salmonis* ES products

We observed three clusters of intense banding (~200 kDa, ~30 kDa, and ~10 kDa) present after 1D gel-electrophoresis of *L. salmonis* ES products. To characterize these bands further we excised each of the three bands and analyzed them by in-gel tryptic digestion and LC-MS/MS. Using Scaffold 4 we identified 31 proteins with at least 2 peptides at a 99.0% probability and an estimated false discovery rate of less than 0.1%, (Figure 33, Table 13). Among the 31 proteins there was overlap in proteins present in the 200 kDa fraction (cluster 1), 30 kDa fraction (cluster 2), and the 10 kDa fraction (cluster 3) shown by the number of peptides observed for each protein (Figure 33, Table 13). For example, LS-vit which has an expected size of ~ 100 kDa was observed in all three clusters. Several other proteins were also represented in clusters that were not their expected size (ES) including LS-pro (ES ~ 58 kDa), -carbb (ES ~ 34 kDa), -car2 (ES ~ 30 kDa), -col (ES ~ 30 kDa), -ser (ES ~56 kDa), -his (ES ~ 26 kDa), -try3 (ES ~ 30 kDa), -act (ES ~ 34 kDa), -catl (ES ~ 42 kDa), -gan (ES ~ 26 kDa), -zin13 (ES ~ 40 kDa), and -catd (ES ~ 48.5 kDa).

6.4.3 Characterization of salmonid proteins in the whole ES fraction of *L. salmonis*

We detected a number of host proteins (9) in the ES products of *L. salmonis* (Table 14). These proteins included host blood proteins hemoglobin subunit beta and serotransferrin-1 as well as proteins associated with immunity such as alpha-2-macroglobulin-like protein 1 and leukocyte elastase inhibitor.

Table 13. Proteins identified by LC-MS/MS in the three main clusters of proteins in *L. salmonis* ES fractions

Fraction → peptides[a]	Protein	Symbol[b]	MW[c] (kDa)	Accession	BP[d]
1→132|**2**→80|**3**→77|	Vitellogenin	LS-vit	104.5	EF490956.1	Lipid metabolism
1→2|	Ribonuclease UK114	LS-rib	187	BT078006.1	Reg. of translation
1→2|**2**→10|**3**→7|	Prophenoloxidase activating factor	LS-pro	102	n/a	Blood coagulation
1→4|**2**→24|**3**→19|	Carboxypeptidase B	LS-carbb	34	BT121391.1	Proteolysis
1→6|**2**→5|**3**→8|	Carbonic anhydrase 2	LS-car2	30	BT078283.1	Response to pH
1→7|**2**→8|**3**→18|	Collagenase	LS-col	30	BT121975.1	Proteolysis
2→10|**3**→6|	Serine protease	LS-ser	56	BT121759.1	Proteolysis
2→2|	LS0109 unknown protein	LS-ls0109	61	EF490960.1	Unknown
2→2|	Serpin B11	LS-serp	50	BT121344.1	Reg. of proteolysis
2→2|**3**→7|	Histone H2A	LS-his	14	BT121344.1	Nucleosome assembly
2→2|**3**→8|	Trypsin 3	LS-try3	25	AY522439.1	Proteolysis
2→3|	Neprilysin	LS-mem	110	n/a	Proteolysis
2→3|**3**→11|	tRNA-dihydrouridine synthase 4-like	LS-tRNA	121	BT077769.1	tRNA processing
2→4|	Metalloproteinase	LS_met	70	EF490868.1	Proteolysis
2→4|	Zinc metalloproteinase nas-4-like	LS-zin4	40	EF490868.1	Proteolysis
2→4|**3**→4|	Actin	LS-act	42	BT121502.1	Cytoskeletal org.
2→4|**3**→4|	Cathepsin L	LS-catl	35	EF490928.1	Proteolysis
2→4|**3**→5|	Ganglioside gm2 activator	LS-gan	53	EF490924.1	n/a
2→4|**3**→5|	Zinc metalloproteinase nas-13-like	LS-zin13	50	n/a	Proteolysis
2→6|**3**→3|	Cathepsin D	LS-catd	34	n/a	Proteolysis
3→2|	Anionic trypsin 1	LS-ani1	30	BT121215.1	Proteolysis
3→2|	Anionic trypsin 2	LS-ani2	30	BT121199.1	Proteolysis
3→2|	ATP synthase subunit beta	LS-atp	57	BT077801.1	ATP transport
3→2|	Beta crystallin A1	LS-bet	70	BT121181.1	Visual perception
3→2|	Peritrophic membrane chitin binding protein	LS-peri	45	BT121534.1	Chitin metabolism
3→2|	S-phase kinase-associated protein 1	LS-s-ph	19	BT078596.1	Phosphorylation
3→3|	Trypsin 2	LS-try2	30	AY522438.1	Proteolysis
3→3|	Tumor-associated calcium signal transducer 2	LS-tum	36	BT076565.1	Cell proliferation
3→6|	Gamma crystallin A	LS-gam	98	BT121359.1	Visual perception
3→7|	Trypsin 4	LS-try4	77	AY522440.1	Proteolysis
3→9|	Cathepsin L2 precursor	LS-catl2	37	HM439290.1	Proteolysis

[a] Fraction number → number of peptides identified from LC-MS/MS

[b] Symbol of protein in Figure 33

[c] Expected molecular weight

[d] Biological function as determined by protein ontology analysis

Table 14. Host proteins identified by LC-MS/MS in the whole ES fraction of *L. salmonis*

Fraction number → number of peptides[a]	Protein	MW (kDa)	Accession #	BP[b]
1→11|**2**→6|**3**→5|	Alpha-2-macroglobulin 1	446	n/a	Reg. of peptidase activity
3→2|	Annexin A2	19	BT048446.1	Fibrinolysis
3→3|	Annexin A5	110	NM_001141036.1	Fibrinolysis
3→2|	Gamma-interferon-inducible lysosomal thiol reductase	48	n/a	Antigen presentation
1→4|**2**→2|**3**→4|	Hemoglobin subunit beta	43	BT058629.1	Oxygen transport
3→4|	Keratin 13	36	NM_00112376.1	Cytoskeletal structure
2→3|**3**→12|	Leukocyte elastase inhibitor	127	BT044977.1	Reg. of peptidase activity
1→4|**2**→5|**3**→5|	Serotransferrin-1	30	BT045182.1	Iron homeostasis
1→8|**2**→9|**3**→9|	Type II keratin	166	NM_001124743.1	Cytoskeletal structure

[a] Fraction number → number of peptides

[b] Biological function as determined by protein ontology analysis

6.5 Discussion

The primary goal for this work was to generate a more comprehensive inventory of the secretory proteome of *L. salmonis*. Analysis of the DA-elicited ES fractions revealed the presence of 52 different proteins which enhances our understanding of sea lice secretions as a complex biological mixture. Earlier work identified the presence of trypsin-like proteases (Fast *et al.* 2003) and prostaglandin E_2 (Fast *et al.* 2004) in the secretions of *L. salmonis*. A full characterization by these authors was impeded by the absence of an annotated *L. salmonis* transcriptome. Here we have characterized a complex mixture of bioactive molecules in the secretions of *L. salmonis* that includes proteases, house-keeping proteins, structural proteins and proteins involved in reproduction. The highest represented biological category was proteolysis. Of the proteins in this category were several classes of proteases that are also virulence factors of ticks and mites, such as cysteine, serine and metalloproteases (Sajid & McKerrow 2002; Ahearn *et al.* 2004; Radulović *et al.* 2014). We then analyzed three clusters of protein bands that were strongly represented after 1D gel electrophoresis to provide higher resolution of those bands. Of the 31 proteins identified in the three clusters, ~ 48% (15/31) were proteases and included cysteine proteases, carboxypeptidases, metalloproteases, and serine proteases.

Among the proteases in the *L. salmonis* secretome, there was high representation of cysteine proteases cathepsin D and L, which are important virulence factors in other parasitic secretomes (Robinson *et al.* 2008; Horn *et al.* 2009; Bartley *et al.* 2012; Cantacessi *et al.* 2012; Popara *et al.* 2013). The protease is thought to function by disrupting tissue repair responses and killing cellular mediators of inflammation (Sajid & McKerrow 2002). The presence of this protein in the secretome of *L. salmonis* suggests a similar process occurs in the skin of salmon. Indeed, inhibition of tissue repair and a weakened inflammatory response are characteristics of the susceptible phenotype observed in Atlantic, chum and sockeye salmon (Skugor *et al.* 2008;

Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012, 2015), and may be related to suppressive effects of louse-derived proteases such as cathepsin L.

Among cathepsins identified by proteomic analysis, the corresponding protein for cathepsin K, which was highly differentially regulated in the *L. salmonis* transcriptome (Braden *et al.* 2015, *in prep*), was not detected in the ES proteome. It is possible that cathepsin K was produced at a level of expression below the detection limit of the mass spectrometer as discrepancies between predicted secreted proteins from transcriptomic data and that of proteins identified by MS/MS have been reported for a liver fluke (*Fascioloides magna*) (Cantacessi *et al.* 2012). Another possible explanation for the difference between proteins identified by mass spectroscopy and those predicted from transcriptomic data is that cathepsin K may be an endogenous protease that is involved in physiological pathways and not involved in exogenous digestion (Sajid & McKerrow 2002; Robinson *et al.* 2008).

Several proteases were identified in the secretome of *L. salmonis* that may act to suppress inflammation. For example, neprilysin was detected in the whole ES fraction of *L. salmonis* corroborating an earlier finding of elevated expression of *neprilysin-2* in *L. salmonis* feeding on Atlantic salmon (Braden *et al.* 2015, *in prep*). It is possible that this molecule represents a louse-virulence factor with a possible role in immunoregulation as suggested for murine inflammatory models (Ottaviani *et al.* 2012).

Approximately 500 serpin-like proteins have been identified in plants, animals and viruses. Members of this superfamily are involved in fundamental biological processes including blood coagulation, inflammation, fibrinolysis and angiogenesis (Prevot *et al.* 2006). Several serpins have been isolated from hard ticks, and are involved in hemostasis and immunosuppression (Prevot *et al.* 2006; Oliveira *et al.* 2013). In *Ixodes ricinus*, a serpin was

characterized to have similar properties to leukocyte elastase inhibitors that interfered with the coagulation and fibrinolysis pathways (Prevot *et al.* 2006). In the present study we identified a serpin from *L. salmonis* after feeding on Atlantic salmon. Anti-inflammatory mediators such as serpins may be involved in suppressing the inflammatory response of the salmon host at the site of attachment such as what is observed in susceptible hosts (Braden *et al.* 2012).

Recent transcriptomic analysis of the feeding response of *L. salmonis* identified expression of metalloproteases and carboxypeptidases (Braden *et al.* 2015, *in prep*) and in concordance with that study, we detected several metalloproteinases as well as carboxypeptidase B (CPB) in the ES proteome. Metalloprotease fragments have been found in the sialoproteome of hard and soft ticks (Yan *et al.* 2002; Francischetti *et al.* 2003, 2011; Wang *et al.* 2009; Mulenga & Erikson 2011; Cantacessi *et al.* 2012; Radulović *et al.* 2014). These proteins have been shown to play a critical role in anti-fibrinolysis in *Ixodes scapularis* (Francischetti *et al.* 2003) and similar proteins are major hemorrhagic proteases of snake venom (Takeda *et al.* 2012). Five metalloproteases were detected in the ES proteome of *L. salmonis* including matrix metalloproteases, zinc-metalloproteases, and metallo-endopeptidases. Blood is an important component of the *L. salmonis* diet (Brandal *et al.* 1976), so the high representation of anti-fibrinolytic enzymes is expected. There is little information describing the action of carboxypeptidases during blood feeding; however, the conservation of these proteins in the secretome of several parasitic genera supports an important role during the host-parasite interaction (Yan *et al.* 2002; Motobu *et al.* 2007; Radulović *et al.* 2014). In mouse models of rheumatoid arthritis, CPB was shown to regulate inflammation by degrading C5a and bradykinin (Song *et al.* 2011), and cathepsin A (a serine carboxypeptidase) is shown to have vasodilatory effects (Radulović *et al.* 2014). Therefore we may conclude that CPB contributes to the feeding

response of *L. salmonis* in a similar manner at the louse-skin interface by potentially interfering with complement and reducing host awareness at the cutaneous interface (Wikel 1999).

Hematophagous parasites limit the oxidative damage from reactive oxygen species and iron which are released by the digestion of blood. The presence of antioxidant proteins including fatty-acid-binding proteins, glutathione hydroxide peroxidases and thioredoxins were detected in the *L. salmonis* secretome. These molecules may also play a role in immune evasion as previously described (Cantacessi *et al.* 2012), and our observations are in concordance with up-regulated expression of antioxidant-associated transcripts during feeding in *L. salmonis* (Braden *et al*. 2015, *in prep*).

Salmonid proteins were detected in the *L. salmonis* secretome, including hemoglobin, serotransferrin, keratin, actin, and annexins. Similarly, analysis of the secretory proteome of *Hyalomma marginatum rufipes* revealed the presence of 77 host-specific proteins (Francischetti *et al.* 2011) and 58 rabbit proteins were identified in the pilocarpine-induced secretome of the rabbit parasite, *Rhipicephalus sanguineus* (Oliveira *et al.* 2013). It is possible that like other ectoparasites, *L. salmonis* recycles host proteins from the hemolymph that are then secreted back onto the host during feeding (Jeffers & Michael Roe 2008). The appearance of host proteins in those studies may be an artifact of contamination from the tick gut, and we cannot exclude this as a likely source of salmon proteins in the present work. On the other hand, the cholineminergic action of dopamine on the nervous system of *L. salmonis* has not been well described, thus in addition to stimulation of salivary glands, regurgitation of the stomach contents may also be occurring.

House-keeping proteins involved in physiological processes (ATP synthase) and cytoskeleton organization (actin) were detected in the secretome of *L. salmonis*, may be

explained by the indiscriminate stimulation of exocrine glands by dopamine. However, it is possible these proteins may also function in the host-parasite relationship, as was suggested for *R. sanguineus* (Oliveira *et al.* 2013).

The host-parasite relationship between sea lice and salmon involves responses by both parasite and host, and quantitative (e.g., high expression of inflammatory cytokines by the host) or qualitative (e.g., different molecules secreted by the louse) differences in the response by either player likely contributes to the variable host response observed among juvenile species. Heightened inflammation and infiltration of cellular effectors in the skin of coho (Johnson & Albright 1992a; Braden *et al.* 2015) and pink salmon (Jones *et al.* 2008a; Sutherland *et al.* 2011; Braden *et al.* 2012) suggests a weakened or absent secretory response of the parasite on these host species. In contrast, diminished inflammatory processes in the skin of susceptible Atlantic (Skugor *et al.* 2008; Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012), sockeye (Braden *et al.* 2015) and chum (Sutherland *et al.* 2011, 2014a; Braden *et al.* 2012) salmon, may be explained by exaggerated secretion of virulence factors at the attachment site. We have demonstrated the presence of several known virulence factors in the secretome of *L. salmonis* after feeding on Atlantic salmon, a susceptible species. Potential differential regulation of the secretome of *L. salmonis* while feeding on susceptible and resistant hosts needs to be investigated.

6.6 Conclusions

Until now, there has been limited research on the secretomes of fish ectoparasites. Trypsin inhibitors were identified in recently-hatched *Paragnathia formica* juveniles (Manship *et al.* 2012), and earlier work found trypsin-like proteases in *L. salmonis* (Fast *et al.* 2003); however, the full characterization of the *L. salmonis* secretome was impeded by limited molecular resources. The availability of the entire genome of *L. salmonis* has provided opportunities for

genomic, transcriptomic and now proteomic analysis of the sea louse. From the secretions of *L. salmonis* we have identified the presence of 52 proteins. Of these, 18 were proteases including cathepsins, carboxypeptidases, trypsins, collagenases, metallopeptidases and metalloproteinases, all known virulence factors in tick host-parasite relationships. We provide a detailed inventory of proteins present in the secretome of *L. salmonis*, which represents an important component of the louse-salmon relationship and may be used to further explain observed variation in host susceptibility.

6.7 Chapter acknowledgements

This research was funded by Genome BC and Fisheries & Oceans Canada`s Program for Aquaculture Regulatory Research. Thanks to Derek Smith at the University of Victoria Proteomics Centre for mass spectroscopy workflow. Thanks to Dr. Terry Pearson, Richard Yip, and Dr. Morty Razavi for valuable assistance with proteomics. We gratefully acknowledge cooperation by Brad Boyce (Marine Harvest Canada) in collection of Pacific *L. salmonis*. LMB was funded by an NSERC Alexander Graham Bell Canada Graduate Scholarship.

Chapter 7: General discussion

7.1 The sea louse-salmon relationship

The importance of the cutaneous mucosa as an immunologically active organ in teleost fishes has been thoroughly described (Subramanian *et al.* 2007; Zhao *et al.* 2008; Salinas *et al.* 2011; Esteban 2012). The current work emphasizes that responses between the louse and salmon occurring at the skin attachment site are key in determining successful settlement of *L. salmonis*. Comparative laboratory experiments among resistant and susceptible species allow for evaluation of the pathways and molecular mechanisms involved in either phenotype, and it is clear that variable and complex species-specific responses occur at the louse-skin interface (Chapters 2 & 3). Characteristics of responses by the host fish (immunity, defense) and by the parasite (feeding/immunomodulation, attack) at the attachment site determine whether a susceptible or resistant host phenotype will result. Understanding molecular mechanisms that direct these responses may provide powerful tools for enhancing the resistance of susceptible species for salmon aquaculture.

Using a combination of transcriptomic, proteomic and functional immunological techniques, the data presented herein significantly advance our understanding of the louse-salmon relationship at the skin interface both in the context of responses by the salmon host as well as by louse.

7.1.1 Cutaneous responses by the salmon host

Among species of Pacific salmon, juvenile pink and coho are the most resistant to *L. salmonis*. Chapters 2 and 3 showed that in the skin, this resistance was characterized by exaggerated expression of inflammatory mediators (e.g., *IL-1β, IL-6, TNF-α, COX-2*), acute-phase proteins (e.g., *CRP, SAA*), infiltration of cellular effectors (e.g., *IL-8, MH II)* and tissue remodelling enzymes (eg. *MMP9, MMP13*). These data are supported by earlier transcriptomic studies that

showed juvenile pink salmon respond to infection with *L. salmonis* by up-regulation of innate inflammatory genes, tissue remodelling, and iron regulatory pathways (Sutherland *et al.* 2011, 2014a).

Although informative at the mRNA level, gene expression studies are limited in descriptive power due to post-translational modification, alternative splicing, and various protein isoforms. Thus, to provide deeper resolution of the host response to sea lice at the attachment site I applied cell-specific markers to infected skin tissue (Chapter 3) and revealed significant infiltration of cellular responders such as IL-1β^+, TNF-α^+ and MHII$^+$ cells in the skin of resistant species. Exaggerated expression of *MH II* in pink salmon (Chapter 2) at 24 hours post infection compared to either Atlantic or chum salmon suggested this marker was involved in resistance against infection with *L. salmonis*; however, the cellular localization of MH II production was unclear. The concomitant infiltration of MHII$^+$ cells and up-regulation of *MH II* in the skin of resistant coho salmon in Chapter 3 confirmed this molecule as a biomarker for resistance.

Two cellular morphologies were detected by α-MHIIβ antibodies: a small lymphocyte-like cell and a larger more diffuse dendritic-like cell. The dendritic-like cell had a morphology similar to dendritic cells found in rainbow trout spleen (Bassity & Clark 2012) and therefore provided evidence for a mucosal counterpart in the skin of salmon that is recruited to the attachment site in resistant species. Further characterization of this cell using antibodies for other dendritic-like markers such as CD209 or CD83 will aid in determining if this cellular effector is fact a teleost mucosal dendritic cell.

The presence of regulatory immune pathways are typically associated with parasitic infections as they act to dampen the effects of sustained inflammation and permit wound healing and tissue remodelling (Allen & Wynn 2011). Until now, a T_H2-type response was thought to be

characteristic of susceptible species (Skugor *et al.* 2008; Tadiso *et al.* 2011; Krasnov *et al.* 2012). However, the early and exaggerated expression of a major T_H2-type cytokine, *interleukin-4*, in the skin of resistant coho salmon during *L. salmonis* infection (Chapter 3) suggests that this pathway may be more pertinent than previously thought. This result suggests that not only is immediate inflammation an important anti-louse response, but that this inflammation needs to be controlled and switched to a regulated response permissive of wound healing and tissue remodelling (Figure 35).

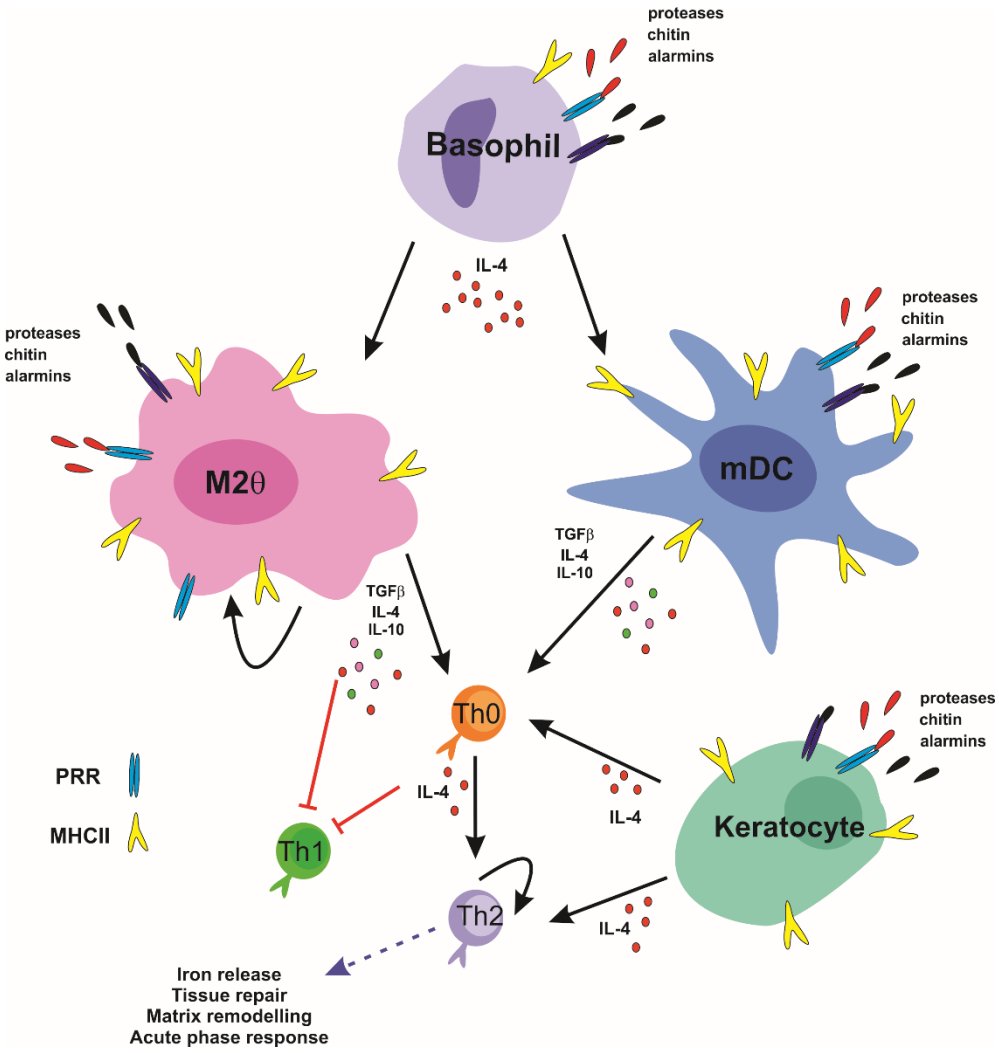

Figure 34. Potential cellular sources of interleukin-4 in the skin of salmon.

In this hypothetical schematic resident basophils would be activated to produce IL-4 by binding of parasite-associated molecules such as proteases or chitin-like molecules or damage-associated molecular patterns (DAMPs) such as alarmins to pathogen recognition receptors (PRRs). The production of IL-4 would activate pro-inflammatory macrophages to differentiate into regulatory (M2θ) macrophages which would start to produce regulatory, anti-inflammatory cytokines such as TGF-β, IL-10, and IL-4. Proteases, chitin-like molecules or alarmins would also activate resident mucosal dendritic cells or keratocytes to produce regulatory cytokines. In turn, the local environment would be polarized from an inflammatory T_H1-like response to a T_H2-like response, with the dominant population of CD4+ T lymphocytes differentiating from T_H1 to T_H2 cells. This environment would be conducive for tissue remodelling and wound healing, which is associated with resistance in *L. salmonis* infections.

The cellular source of IL-4 in salmon skin is unknown. Among the T_H2-type cytokines, IL-4 expression is important for clearing multicellular parasites such as helminthes (Maddur *et al.* 2010). In mammalian systems, basophils are well known for their role as effector cells of the T_H2 response that can be activated by allergens and parasite-derived proteases (Phillips *et al.* 2003; Sokol & Medzhitov 2010). Up-regulation of MH class II molecules is observed on basophils after stimulation by papain proteases and their role as antigen-presenting cells is well-documented (Mikhak & Luster 2009; Sokol *et al.* 2009; Maddur *et al.* 2010). The importance of basophils during resistance to ectoparasite infection was shown in guinea pigs (Allen 1973; Brown *et al.* 1982) and cattle (Brown *et al.* 1984). More recent work has demonstrated basophils are important effector cells that are activated by parasite-derived molecules to produce T_H2-type cytokines and to shift immunity towards regulatory and anti-inflammatory pathways (Maddur *et al.* 2010; Karasuyama *et al.* 2011). Considering the importance of these cells in the skin during tick-host relationships, it is plausible that a comparable cell is activated in teleosts. Exaggerated expression of *IL-4* in coho salmon skin during infection with *L. salmonis* (chapter 3) suggests a T_H2 response is an important biomarker for resistance to sea lice. In the absence of a cellular source of IL-4 in salmon skin, we can postulate that a basophil-type cell may be involved. However, in the skin of rainbow trout a T_H2-response was not associated with the presence of MHII+ cells during infection with *Ichthyobodo necator* (Chettri *et al.* 2014). This discrepancy may be related to species- and pathogen-specific differences.

7.1.2 Species-specific cutaneous responses to ectoparasites

Comparative studies that examine responses by resistant and susceptible species or strains of hosts are informative as to the pathways that contribute to species-specific responses to pathogens. Furthermore, these studies provide valuable information on the divergent host

responses to different pathogens. For example, up-regulation of *inducible nitric oxide synthase* (*iNOS*) is commonly associated with ectoparasitic infection in teleosts (Sigh *et al.* 2004b; Bridle *et al.* 2006a; Gonzalez *et al.* 2007a; Forlenza *et al.* 2008; Heinecke & Buchmann 2013). Gene expression studies (chapters 2 & 3) failed to associate the production of *iNOS* with infection by *L. salmonis* in *Oncorhynchus* spp. (chum, pink, coho or sockeye salmon), but in Atlantic salmon there was signficant up-regulation of *iNOS* during louse infection. Sustained levels of iNOS production can cause immunopathology, and as Atlantic salmon are susceptible to infection with *L. salmonis*, this may be an example of a genus-specific pathway contributing to susceptibility.

A molecule associated with susceptible Atlantic and sockeye salmon was the host defense molecule cathelicidin. Compared to coho salmon, *cathelicidin* was up-regulated in the skin of Atlantic and sockeye salmon during infection with *L. salmonis* (Chapter 3). High induction of *cathelicidin* was also reported in rainbow trout infected with *Ichthyophthirius multifiliis* (Heinecke & Buchmann 2013) or *Ichthyobodo necator* (Chettri *et al.* 2014). This molecule has been shown to act both as a pro-inflammatory and anti-inflammatory molecule, depending on complex pathways regulated by extra-cellular and cellular signals (Choi & Mookherjee 2012). Aberrant regulation in Atlantic and sockeye salmon during louse attachment may be contributing to skewing the local tissue response in a way that is insufficient to result in parasite clearance. Inappropriate activation of cathelicidin is associated with chronic autoimmune diseases in mammals by preventing NF-κB translocation and subsequent production of inflammatory mediators (Choi & Mookherjee 2012). Localization of this potent molecule to a particular cell in salmon skin would help elucidate its role during ectoparasite infection.

7.1.2 Responses by the louse

I present the first transcriptomic dataset of *L. salmonis* feeding on resistant or susceptible species (Chapter 5), and also add resolution to the existing literature on the proteomic secretions of *L. salmonis* (Chapter 6). Specifically, when feeding on Atlantic salmon, the sea louse responds more vigorously in the expression of proteolytic, energetic and reproductive genes. It is accepted that parasites prefer hosts with high nutritive value and low immune-competence (Christe *et al.* 1998, 2003; Roulin *et al.* 2003; Bize *et al.* 2008; Bize & Roulin 2009; Heylen & Matthysen 2011). The sea louse is known to exhibit host preference towards Atlantic salmon, and thus it follows this species provides a more profitable environment. Following host-parasite theory as described by Price (1980), this preference by the sea louse must result in an advantage for the parasite, either by higher nutritive value, or by lower immune exposure. While feeding on Atlantic salmon, there is heightened expression of several immunomodulatory molecules at the transcriptomic and proteomic level. Concomitant weakened tissue responses observed in this species may be explained by these louse-derived effects. Secretion of immunomodulatory molecules is common in hematophagous arthropods such as ticks, mosquitoes, and mites. Earlier analysis of *L. salmonis* secretions identified PGE_2 (Fast *et al.* 2004) and trypsin proteases (Firth *et al.* 2000). Results from LC-MS/MS analysis of *L. salmonis* secretions in the present work include the identification of 52 proteins, demonstrating the louse secretome is a complex biological mixture. The existence of a host-specific sea lice secretome is not known; however, in *Boophilus microplus*, the proteomic feeding response was enhanced when parasitizing the more preferred host (*Bos taurus*) compared to an incidental host (*Odocoileus virginianus*) which was correlated with immunosuppression in *B. taurus* (Popara *et al.* 2013). It is plausible that a similar phenomenon exists in *L. salmonis* feeding on the resistant or susceptible hosts. Based on the exaggerated transcriptomic expression of proteolytic genes in *L. salmonis* feeding on Atlantic

salmon, we would expect this expression to be observed at the proteomic level. A comparative analysis of secretions from *L. salmonis* feeding on susceptible and resistant species would clarify the role of immunomodulatory secretions in host susceptibility. Characterization of the nutritive value of skin, mucus and blood components among resistant and susceptible species of salmon will help elucidate the mechanisms of host preference.

7.2 Resistance and tolerance as strategic defense against *L. salmonis*

Selective pressure from parasites is important in shaping life history, and resistance to disease is associated with increased fitness (Zuk & Stoehr 2002). However, despite the advantage of resistance to infectious diseases, susceptibility among host species is prevalent (Coustau *et al.* 2000; Rigby *et al.* 2002; Svensson & Raberg 2010; Medzhitov *et al.* 2012), and is the result of trade-offs between immunity and other important characteristics such as growth rate or development time (Roff 1992). Thus, among closely related species or individuals of the same species, there are variations in resistance that reflect differences in life histories, ages, reproductive history or habitats (Norris & Evans 2000; Zuk & Stoehr 2002). As resistance to disease is a costly trait, the evolution of optimal functioning in immune systems is driven by the specific ecological constraints and energetic requirements of the organism.

7.2.1 Pathways of resistance to the salmon louse

With respect to Pacific salmon, there exists a large variability in response to infections with sea lice, which may be thought of in the context of life history adaptations. As mentioned in chapter 1, the evolution of physiological traits such as fecundity, growth rate, size at maturation, niche exploitation, and immunocompetence are all under the constraints of bioenergetic allocations. Thus, resistance in pink and coho salmon is likely a product of greater allocation of resources to a more functional inflammatory response. As these fish have the shortest ocean-phase and are

less fecund than either sockeye or chum salmon (susceptible species), it is likely that these life history trade-offs are correlated with disease resistance. There appears to be two different pathways of susceptibility towards infection with *L. salmonis*: the first involving differences in evolutionary adaptation between host and parasite, and the second involving differences in life history adaptations among *Oncorhynchus* spp.

Host-preference studies indicate that *L. salmonis* prefers Atlantic salmon compared to Pacific species, and secretes more proteolytic enzymes when exposed to Atlantic salmon mucus compared to that of coho salmon (Fast *et al.* 2003). In addition, reproduction *of L. salmonis* is heightened while parasitizing Atlantic salmon (Fast *et al.* 2002a), and during co-habitation studies Atlantic salmon retain higher parasite numbers compared to resistant pink salmon (Sutherland *et al.* 2014a). This data implied that Atlantic salmon is the preferred host to *L. salmonis*. Assessing the transcriptomic response of the parasite while feeding on Atlantic, coho or sockeye salmon corroborated this hypothesis. Specifically, expression of genes involved in reproduction, feeding, and energy metabolism were all exaggerated on Atlantic salmon compared to either coho or sockeye salmon. Thus, the data from behavioral, physiological and now molecular studies supports the Atlantic salmon as a more beneficial host to the sea louse. Atlantic salmon is a naïve host to the waters of the Pacific Northwest, and as such, there has been no co-evolutionary interaction with *L. salmonis*. Therefore, the parasite is able to exploit this host more efficiently.

The second pathway of susceptibility involves the variable host response observed among species of Pacific salmon and likely is related to differences in life history adaptations (Zuk & Stoehr 2002). Juvenile coho and pink salmon are resistant to infection with *L. salmonis*, while chinook, chum and sockeye salmon are more susceptible. This variability among species may be

explained by the costs associated with immune defenses (Sheldon & Verhulst 1996). The divergent evolution of *Oncorhynchus* spp. resulted in life history differences in growth rate, habitat, fresh water residence, size of maturation, and reproductive output (Burgner 1991; Healey 1991; Heard 1991; Salo 1991; Sandercock 1991; Montgomery 2000). Variations in these parameters influence the resources available for allocation towards energetically demanding processes, and thus resistance would only be amenable for hosts that could afford to divert energy away from other costly traits such as growth or reproduction (Zuk & Stoehr 2002). However, bioenergetic requirements may shift during an organism's life cycle. For example, despite resistance of juvenile pink salmon, adult migrating pink salmon support heavy loads *of L. salmonis* (Nagasawa 2001; Beamish *et al.* 2005). Potential mechanisms for the switch in resistance during maturation was investigated (chapter 4) by comparing the cutaneous response of juvenile and mature pink salmon. I detected significant reductions in *MH II* transcript abundance and MHIIβ^{+} cells in the skin of mature pink salmon, which further implicate this protein as an important biomarker for resistance. Intuitively, smaller juvenile pink salmon would need heightened protection from an ectoparasite, while in larger adult pink salmon, the effects of parasitism are reduced and energy requirements are better allocated towards maturation and development of secondary sexual characteristics (Figure 35). Therefore, understanding the basis for resistance to the sea louse needs to be in the context of developmental and ecological constraints that may shift from resistance to tolerance.

The ability for a host to reduce the damage caused by parasites without the cost of an immune response is also selected for (Svensson & Raberg 2010), and may explain the seemingly higher susceptibility of chinook, chum and sockeye salmon. Tolerance does not reduce parasite load, but rather ameliorates tissue damage. Juvenile chinook and sockeye salmon enter the ocean

after a longer fresh-water residence (Burgner 1991; Healey 1991), and as such are larger when encountering infectious *L. salmonis* larvae. Perhaps these larger juveniles can afford to support higher louse burdens, and choose instead to limit the collateral effects of inflammation from aggressive immune defenses. This has been viewed as a negative selection pressure in the adoption of resistance mechanisms as the organism strives to avoid immunopathology (Bonneaud *et al.* 2003; Sorci & Faivre 2009).

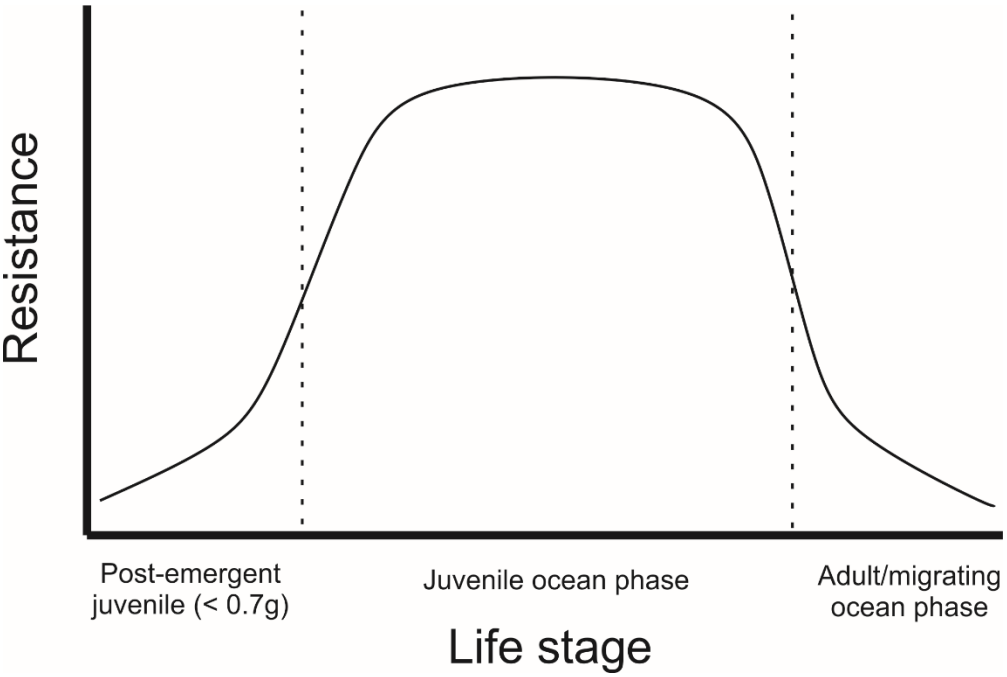

Figure 35. Resistance to *L. salmonis* as a function of life stage.
Pink salmon juveniles enter the ocean at a very small size and lack scales. With growth and development of scales and an immune defense resistance, resistance to infection by *L. salmonis* increases. Sexual maturation is accompanied by growth and development of gonads and secondary sexual characteristics. Energy originally used for the maintenance of costly immune defenses is shifted towards reproduction, which is coincident with decreasing resistance towards the sea louse.

7.3 Targeting mucosal immunity to enhance disease resistance in aquaculture

The mucosal immune system of teleosts has been the focus of many recent studies, and the gut skin and gills share many characteristics with type I mucosal surfaces of mammals (Iwasaki 2007). Because of this similarity, teleost mucosa offer unique models to study many unresolved aspects of mammalian immunity, however more importantly this common theme among vertebrates presents a novel stratum to target against aquatic infectious diseases (Bienenstock *et al.* 1978; Meeusen 2011; McGhee & Fujihashi 2012). As the microbiome is known to control the development and function of the mucosal immune system, understanding the biological composition of teleost microbiomes is of particular importance; however, the diverse microbiota of various teleost species have not been fully characterized (Gomez *et al.* 2013). Manipulation of salmonid microbial communities may hold tremendous promise in mariculture for enhancing overall fish health, which necessitates a comprehensive understanding of the microbial communities of fish mucosa. One way to augment mucosal microbiota is through the use of probiotics (Nayak 2010; Martínez Cruz *et al.* 2012; Newaj-Fyzul *et al.* 2014), or through the application of immunostimulants (Magnadottir 2010; Covello *et al.* 2012; Sheikhzadeh *et al.* 2012; Igboeli *et al.* 2013), which act to enrich commensal microbiota and thus act directly on the teleost microbiome to enhance immunological function (Gomez *et al.* 2013).

Teleost skin represents a dynamic mucosal immune organ that is densely populated with many cellular effectors of both innate and adaptive immune functions. An important cellular effector that is yet to be identified in teleost skin is the mucosal dendritic cell (mDC). In mammals mDCs are critical as both innate and adaptive effectors, and also act to regulate inflammation and induce tissue remodelling after injury (Iwasaki 2007). The identification of a $MHII^{+}$ cell with dendritic-like morphology in the skin of *L. salmonis*-resistant salmon (Chapter 3) implies mDCs may also be important effectors in teleost mucosal immunity. An $MHII^{+}$ cell

was observed in Moreover, as these cells were reduced in susceptible species (Atlantic, chum or sockeye salmon) or absent during sexual maturation (mature pink salmon), mDCs may represent a cellular effector important during resistance to *L. salmonis.* Inducible cell populations in the skin of resistant species during louse infection could be viewed as targets for mucosal augmentation. The interconnected nature of mucosal interfaces results in sensitization of distant mucosal surfaces by targeting another (Bienenstock *et al.* 1978), which may explain the decrease in *L. salmonis* prevalence on Atlantic salmon fed immunostimulants (Covello *et al.* 2012; Purcell *et al.* 2013). In one study, increased protection against *L. salmonis* was accompanied by heightened expression of pro-inflammatory cytokine *IL-1β* and the collagenase *MMP9* (Covello *et al.* 2012). Matrix metalloproteinases are important for wound repair and tissue remodelling (Chadzinska *et al.* 2008), and are associated with resistance during *L. salmonis* infections (Skugor *et al.* 2008; Sutherland *et al.* 2011, 2014; Chapters 2 & 3).

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Appendix A: Primers used in qPCR experiments

All primers that were used for salmon and *L. salmonis* qPCR components in preceding chapters are collected here.

Table 15. Salmon specific primers used in RT-qPCR assays.

Showing accession number, reference, forward and reverse sequence, and amplicon size. Superscript numbers represent the chapters for each primer set.

Gene target	Accession #	Reference	Forward (5-3')	Reverse (5-3')	Amplicon
Interleukin 1β-1[2,3,4]	AY617117	Fast et al., 2004	CGTCACATTGCCAACCTCAT	ACTGTGATGTACTGCTGAAC	200
Interleukin 4[3]	AB574339	Primer3	TGCATCGTTGTGAAGAGCCA	AAGTCTCCTCAGCTCCACCT	63
Interleukin 6[2,3]	DQ866150	Braden et al., 2012	CTCTCGGCGCTCGTGGTGTT	CACCGTCTGTCCCGAGCTCCT	106
Interleukin 8[2,3,4]	NM_001140710.2	Braden et al., 2012	GAATGTCAGCCAGCCTTGTC	TCCAGACAAATCTCCTGACCG	226
Tumor necrosis factor α-1[2,3,4]	AJ278085.1	Fast et al 2004	GGCGAGCATACCACTCCTCT	TCGGACTCAGCATCACCGTA	124
Interleukin 10[2,3]	EF165028	Skugor et al 2008	ATGAGGCTAATGACGAGCTGGAGA	GGTGTAGAATGCCTTCGTCCAACA	54
Transforming growth factor β[2,3]	DQ834851.1	Primer3	TAAGGACCTGGGCTGGAAGT	GGCCGATGCAGTAGTTAGCA	65
Interferon-induced GTP-binding protein[3]	CB516446	Purcell et al., 2013	GGTTGTGCCATGCAACGTT	GGCTTGGTCAGGATGCCTAAT	112
Immunoglobulin M[3]	S48652.1	Primer3	TCATTAAGATCACCCCGCCG	TTCCTCGACATCGCACACAA	77
Inducible nitric oxide[2,3]	AJ300555.1	Braden et al., 2012	AACGAGAGCCAACAGGTGTC	GGTGCAGCATGTCTTTGAGA	81
Arginase-2[3]	BT058927.1	Primer3	TCGTCTTCTACGGACGCAAC	TTTGGGTCCATGTTCCACCC	118
Major histocompatibility class II β-chain[2,3,4]	X70166	Braden et al., 2012	AAGGCTTGAAGACACGTTGC	CAGTCCAGCAGTAACGTCCA	106
CD8α[3]	AY693391	Skugor et al 2008	CGTCTACAGCTGTGCATCAATCAA	GGCTGTGGTCATTGGTGTAGTC	266
Cyclooxygenase 2[2,3,4]	AY848944.1	Fast et al 2004	CAGTGCTCCCAGATGCCAAG	GCGAAGAAGGCGAACATGAG	99
Prostaglandin D synthase[2,3]	CA352578	Braden et al., 2012	CCTACACCAACCTGAACGCTGATG	ACGCTGGCTGGTGAAGGTGAAG	98
C-reactive protein[2,3,4]	NM_001140668	Braden et al., 2012	GAAAGACCCAGAAGAGAGCTCGAGT	TCAGAACCAGCAGAAACAGCAGC	115
Serum amyloid-P[2,3]	NM_001123671.1	Braden et al., 2012	ACTCAATCTAATGGCGAAGCTGGTG	TGAGGTGCTTGTCGCCATTGGG	115
Serum amyloid-A[2,3]	NM_001146565.1	Primer3	CTCGGGGCAACTATGATGCTA	AGTCCTCATGTCCTCGACCA	114
Hepcidin-1[3]	NM_001140849.1	Sutherland et al., 2011	TTCAGGTTCAAGCGTCAGAG	AGGTCCTCAGAATTTGCAGC	125
Thioredoxin[3]	CA043161.1	Primer3	TAAATGGTTGGTGGTGGCGT	GGAGGAGAAGGTCAACGCTC	95
Cathelicidin[3]	NM_001123573.1	Primer3	GACAAGAAGAGGCAAGCCCA	AATGGAGGAGCCAAACCCAG	121
Transferrin[3]	CX144614	Primer3	GTCTGGTTGAAGGTGCTGGA	CTTTGTGACTGGCTGGGTAGT	152
Matrix metalloproteinase 9[3]	CA342769	Skugor et al 2008	ACTCTACGGTAGCAGCAATGAAGGC	CGTCAAAGGTCTGGTAGGAGCGTAT	72
Matrix metalloproteinase 13[2,3,4]	DW539943	Skugor et al 2008	CCAAAAAGAGGGCACCAGATGG	CCAAAAAGAGGGCACCAGATGG	53
p38 Mitogen activated protein kinase[3]	EF123661.1	Holen et al 2011	GGCACACAGACGATGAGATG	ACAGCGTTCTGCCAGTGAG	150
Pax-5[3]	EU147491	Zwollo et al 2005	ACGGAGATCGGATGTTCCTCTG	GATGCCGCGCTGTAGTAGTAC	136
CCAAT-enhancer binding protein β[2,3]	EU668996.1	Skugor et al 2008	CGCGTGGAGCAGCTGTCAAGA	TGGGCACTCCGGTGTGGCTA	84
Interleukin-1 receptor-associated kinase 1[3]	CA378286.1	Primer3	AGCGTGATCGTTCTTACCGT	ACAAGGGAGAAGTCGCACAG	156
Elongation factor 1-α[2,3,4]	NM_001141909.1	Jones et al 2007	GTGGAGACTGGAACCCTGAA	CTTGACGGACACGTTCTTGA	155
Ribosomal subunit 18S[2,3,4]	AF427629	Fast et al 2006	TCGCCGAGAGGCGTGGGTAA	GCGACGGGCGGTGTGTACAA	150
Eukaryotic translation initiation factor 3 [2,3,4]	CX040383	Skugor et al 2008	GTCGCCGTACCAGCAGGTGATT	CGTGGGCCATCTTCTTCTCGA	92
β-actin[2,3,4]	AF012125	Jones et al 2007	CAACTGGGACGACATGGAGA	AGTGAGCAGGACTGGGTGCT	88
Glyceraldehyde phosphate dehydrogenase[2,3,4]	BU693999	Braden et al., 2012	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTGATG	96

Table 16. Louse-specific oligonucleotide primers used in real time qPCR experiments in the Chapter 5, with sense and anti-sense sequences, accession numbers and amplicon size.

Gene target	Name	Accession #	Reference	Forward (5-3')	Reverse (5-3')	Amplicon
60S ribosomal protein L7	*rpl7*	Q5ZJ56	Primer3	TCAACGGTGTCAGTCCCAAG	GTACCCCCAAGCGATGTAGG	140
Carboxypeptidase B	*cbpb*	P04069	Primer3	TGGTATATGGGCGTCCTTGG	TCGTAAATGCAGCCCAGAGC	130
Cathepsin K	*ctsk*	O35186	Primer3	AACTCATGGGGGAACAGTTGG	CCGATAGGGTAGCTTGTGGT	179
Cytochrome b	*cyb*	Q35925	Primer3	AATTGGGTGAGTGGGCGAAA	GCTCTATTCGCACCCAACCT	242
Cytochrome c oxidase subunit 2	*cox2*	Q37677	Primer3	GAATCTCCAATCCGCGTCCT	TACTCCAGGTCGAGAGGCAA	135
Ferritin	*fer*	P41822	Primer3	TAATCAAACAGGCCAAAGCCC	TGCTAAAGAAGAGGCCAAGGA	72
Neprilysin 2	*nep2*	O16796	Primer3	GAATGGGTGGACTCGGCTTT	GGACTCTGCCACAAGTTCCTT	131
High affinity copper uptake protein 1	*ctsr1*	Q8WNR0	Primer3	CTACAAATCCCACTGAATGCC	AATTGAAGGACGTGCAGAGC	178
Peroxinectin	*perox*	JP326607.1	Sutherland et al., 2014	TGGGCTTTGGCCGCTCCAAA	GGCTGTGTCCGAATCGAAAGGCA	104
Programmed cell death protein 4	*pcd4*	Q9JID1	Sutherland et al., 2012	TCAATCGTAAGATGCCGTCC	CCAGTATTCCTTGAATCGGC	77
Ribosomal protein P2	*rp2*	BT078447.1	Primer3	CTAATCAAACAGGCCAAAGCCC	CTGCTAAAGAAGAGGCCAAGGA	74
Saposin	*sap*	BT078242.1	Primer3	TGACTTGATCGGCGTTGTCT	CCGCCACTGTCTCTAAGGAT	117
Troponin C1	*tc1*	P21797	Primer3	CTACCCAGTGTGCCGTGTAA	CACCACGGATCAACTTCGAGA	180
Trypsin-1	*prss1*	P00765	Primer3	CCTTGGCCCTCAACGAGAAA	CGGACACAACAACATCACCG	81
Structural ribosomal protein S20	*rps20*	BT121430.1	Frost & Nilsen 2003	GTCACCTCAACCTCCACTCC	TGACTTGCCTCAAAGTGAGC	274
Elongation factor 1α	*ef1a*	BT121243.1	Carmichael et al .,2013	CCAAATTAAGGAAAAGGTCGACAGACGTACTG	TGCCGGCATCACCAGACTTGA	86
RMD5 homolog A	*rmd5a*	ACO15319.1	Carmichael et al .,2013	TCTCCTTATGCCCACTTGCT	GAGTTCCGTCCTTTGCATTC	220
HGPRT	*hgprt*	ACO14905.1	Carmichael et al .,2013	GCAGCAAACATCGAATCTCA	TCTTTGCACGAACAAACTGC	187
Tubulin beta chain	*tub*	BT077612.1	Sutherland et al., 2012	TGCGGCTATATTTAGAGGGC	AGGTGGAATGTCACAAACGG	136

Appendix B: Antibodies used in immunohistochemistry experiments

All antibodies that were used in immunohistochemical experiments in the preceding chapters are listed below and include the source, concentration, antigen retrieval method and target.

Table 17. Salmon-specific antibodies used in immunohistochemistry assays.

The peptide target, source, antigen retrieval method and concentration for each antibody is included. The conditions and species specificity for each antibody was determined empirically prior to actual assays.

Antibody	Target	Source/Reference	Antigen Retrieval	Concentration
S.sa MHIIβ[3,4]	Beta 2 domain of MHC II β chain in *Salmo salar*	Hetland *et al.*, 2010	HIER, Tris/EDTA pH 9, 25 min	1/200
S.sa mIgM[3,4]	Heavy chain of membrane bound IgM in *Salmo salar*	Jorgenson *et al.*, 2010	HIER, Tris/EDTA pH 9, 25 min	1/25
O.my Pax5[3]	Paired domain of vertebrate Pax5	Zwollo *et al.*, 1998	PIER, 1% protK, 10 min	1/200
O.my IL1β[3,4]	aas 208-225 *O. mykiss* IL1-β1 (Accession# CAA11684.1)	Zwollo *et al.*, 2014	HIER, Tris/EDTA pH 9, 25 min	1/6000
O.my TNFα[3]	aas 224-239 O. mykiss TNF-α (Accession# CAB92316.1)	Braden *et al.*, 2015	HIER, Tris/EDTA pH 9, 25 min	1/3000

Superscript represents the chapters in which each antibody was used