Comparative responses of salmon to sea lice *Lepeophtheirus salmonis* infections, and lice responses to chemical and environmental stressors

by

Ben James Gerard Sutherland
B.Sc., Thompson Rivers University, 2008

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Supervisory Committee

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Abstract

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Systems biology methods can provide novel insight into the responses of an organism to a suboptimal environment, an infection or exposure to a xenobiotic. In the interaction of salmon and salmon lice, there are several areas requiring further research. These include the impacts of lice infection on wild salmon, response mechanisms of different salmon species or life stages to lice infections, effects of environmental conditions on lice stress, and mechanisms underlying the emergence of resistance to important parasiticidal chemicals. Here, I combine global gene expression analyses with phenotypic and physiological responses of salmon or salmon lice to further our understanding of these topics. In the first chapter, I introduce the work by discussing relevant background material on the current knowledge of salmon and salmon lice interactions, salmon immunity, the state of salmon and louse genomics and the emerging field of ecological genomics. I also discuss how these approaches are applied to the study of non-model organisms and sustainable aquaculture development and fisheries conservation. In the second chapter, I present the first large-scale transcriptome profiling of a Pacific salmon to a salmon lice infection, identifying transcript signatures associated with an infection in a sensitive life stage of pink salmon *Oncorhynchus gorbuscha*. In the third chapter, I present the results of multiple co-habitation infections of three species of Pacific and

Atlantic salmon to compare physiological and transcriptomic responses at the local (skin) and systemic levels (anterior kidney). In the fourth chapter, I explore louse transcriptome functioning during temperature and salinity perturbations to characterize the molecular stress response and coping strategies of lice, as well as provide stressor context to response genes. In the fifth chapter, I evaluate sensitive Pacific and resistant and sensitive Atlantic lice responses to emamectin benzoate, an important compound for louse control which has recently been evaded by the louse through resistance development in multiple regions worldwide. In the sixth and final chapter, I conclude with a synthesis of what was learned about knowledge gaps discussed above and how to best apply this information by providing some approaches for future research to address remaining challenges.

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Dedication

Dedicated to my Family. Thank you for inspiring my curiosity, creativity and deep-rooted love of the natural world.

-BJGS

Chapter 1: Introduction

1.1 Overview and objectives

The data chapters of this dissertation aim to refine our understanding of the host parasite interaction of salmon and the salmon louse *Lepeophtheirus salmonis* in several ways, including by comparing successful or unsuccessful host responses to lice infections, investigating effects of abiotic environmental factors capable of influencing louse epidemiology, and increasing our understanding of resistance mechanisms of salmon lice to important chemical control agents. These objectives will aid predictions of the impacts of lice infections on wild salmon and of environmental conditions on lice populations, will facilitate control methods and selection efforts for less susceptible farmed salmon, and will improve our understanding of the selective pressures shaping resistance to parasiticides in aquaculture as well as improve resistance monitoring.

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

1) Pink salmon *Oncorhynchus gorbuscha* gene expression responses can provide insight on mechanisms related to sensitivity to lice infection prior to manifestation of physiological damage or mortality
2) Comparing physiological and global gene expression responses at the local and systemic level in salmon hosts of differing sensitivity to lice infections will indicate what comprises a successful host response
3) Stress in salmon lice can be identified at the level of the transcriptome, and stress-specific markers can be developed to evaluate stress levels
4) Analyzing molecular responses of populations of lice differing in sensitivity to the parasiticide emamectin benzoate will help us understand mechanisms related to resistance development

1.2 Pacific and Atlantic salmon

1.2.1 Biology, ecology, food sustainability and research

The salmonids (Family Salmonidae) are a highly studied group of fish with massive ecological, economic and cultural significance living in fresh and saltwater environments. Numerous fascinating aspects of salmonid biology have attracted research, for example the phenotypic plasticity of life history traits (e.g., variation in anadromy propensity and timing in steelhead *Oncorhynchus mykiss*; Nichols *et al.* 2008), migratory fidelity to natal streams and navigation abilities (Putman *et al.* 2014), or adaptive radiation of salmon to many unique niches, among many others (Groot & Margolis 1991). The importance of salmon aquaculture has led to research with translational outcomes, for example identifying quantitative trait loci related to tolerance of crowding (Rexroad *et al.* 2012) or elevated temperature (Somorjai *et al.* 2003; Quinn *et al.* 2011), or related to rapid growth (Wringe *et al.* 2010). Production or husbandry methods will benefit from this research, for example by determining the genetic sex of individuals for efficient aquaculture propagation through improved characterization of the sex-determination mechanism in rainbow trout *Oncorhynchus mykiss* (Yano *et al.* 2012). Characterization of essential nutrients in feed has improved recently, allowing for formulation of protein substitutions to improve sustainability of aquaculture development (Hasan 2001). New research is also improving our understanding of salmon disease through the study of wild sockeye salmon *O. nerka* in British Columbia (Miller *et al.* 2011). Furthermore, the genetics of salmon in particular has been highly studied due to a relatively recent whole genome duplication in the common ancestor of the lineage (~65 million years ago; Allendorf & Thorgaard 1984; Taylor *et al.* 2003; Davidson *et al.* 2010). The resulting re-diploidization from the tetraploid genome provides a model for studying the evolution of duplicated genes (Koop *et al.* 2008; Leong *et al.* 2010).

Even with the rich history of salmon research, many aspects of salmon biology important for conservation and rearing remain unknown. In the past ten years, advances have occurred in the study of juvenile wild salmon in the Pacific Ocean (Trudel & Hertz 2013). Some existing unknowns are currently being explored using genomic-enabled tools, such as our understanding of disease dynamics in nature and of countermeasures in hosts. Increased understanding of these topics will greatly improve our ability to monitor impacts of infections on wild populations, to estimate impacts of various biotic and abiotic factors on host immunocompetence, and to identify priorities for effectively reducing anthropogenic influences on salmon populations.

Salmon are a highly valued and nutritious food source. The economic value of salmon in Canada originates mainly from sports fisheries, commercial fisheries and aquaculture production. Beyond economics, the cultural and ecological importance of salmon for Canada is immeasurable. Continued increases in demand for salmon globally have resulted in increases in salmon farming through the development of intensive, large-scale, commercial aquaculture operations, the global yield of which surpasses catch fisheries (FAO 2008). However, large-scale aquaculture is criticized for many practices. Some issues like antibiotic overuse are being addressed in several countries, but other issues remain. Examples include nutrient loading, ecosystem disturbances (Burridge *et al.* 2010), escapees surviving in non-endemic environments and potentially interbreeding with wild salmon (Volpe *et al.* 2000; Bourret *et al.* 2011), and parasite or pathogen transfer (Krkošek *et al.* 2008). Parasite or pathogen transfer is one of the largest issues facing salmon aquaculture with impacts locally and globally on ecosystem dynamics (Costello 2009b). However, with a growing population, demand is likely to be met through aquaculture production (Walsh 2011). Government, industry, and other aquaculture

proponents look to improve the industry through policy changes and increased standards for sustainability; this has driven research to assess or circumvent problems.

The study of genomics is increasing our understanding of the salmonids. Multiple projects have been conducted to obtain salmon gene sequences for use in functional genomics studies (e.g., cGRASP (Koop *et al.* 2008); TRAITS-SGP (Taggart *et al.* 2008); STARS (Krasnov *et al.* 2011a)). The culmination of efforts of the International Collaboration to Sequence the Atlantic Salmon Genome (ICSASG) brings additional tools for research and development (Davidson *et al.* 2010). Extensive work has also been conducted to create maps for other salmonids, such as rainbow trout *O. mykiss*, with genome sequencing projects underway (Palti *et al.* 2011). Among the many successes of these projects, cGRASP tools have enabled studies on sockeye salmon spawning and disease exposure in natural populations (e.g., FishManOmics; Miller *et al.* 2011), environmental toxicology (e.g., estrogen effects; Gunnarsson *et al.* 2007), impacts of hybridization of domesticated and wild salmonids (Roberge *et al.* 2008; White *et al.* 2013), impacts of selection (Sauvage *et al.* 2010) and of transgenics (Devlin *et al.* 2009), effects of nutritional substitutes in aquaculture (e.g., fishmeal substituted with soybean meal; Sahlmann *et al.* 2013), salmon development (Jantzen *et al.* 2011a) and ovary maturation (von Schalburg *et al.* 2005a), vaccine development (Bridle *et al.* 2012), and many others. With threats to global fish populations (Worm *et al.* 2009), new advances are currently needed to conserve these irreplaceable resources.

1.2.2 Health, immunity and disease

Living in both freshwater and saltwater at different life stages, salmon are exposed to a diverse range of endemic infectious agents including viruses, bacteria, fungi, myxosporidians, ectoparasitic copepods, and others. These do not always occur in isolation, and the presence of

one may facilitate the colonization of another. For example, salmon lice may act as a mechanical vector for bacteria or viruses (Barker *et al.* 2009; Jakob *et al.* 2011). New detection techniques have resulted in the identification of potential threats previously not considered in our understanding of disease dynamics of wild salmon (Miller *et al.* 2011). Aquaculture animals stocked at high densities held in a constant location are exposed to many of these same pathogens. Alongside husbandry improvements it is important that we learn how salmon disease dynamics work when the two systems are open and are able to exchange pathogens or parasites. However, in some cases parasites can be an indicator of a healthy ecosystem (Hudson *et al.* 2006) and do not always cause disease. The health of the ecosystem can be disturbed by anthropogenic removal of natural boundaries between susceptible animals and parasites (Krkošek *et al.* 2007). The interaction of salmon and parasites or viruses merits further study, and results will be important for sustainable aquaculture development.

The salmon immune system is multi-faceted and efficient, with the potential to protect the fish from many threats. However, when the immune system is modulated by pathogens (Fast *et al.* 2007a), by low nutrition or stress (Bonga 1997), or when the fish is undergoing other energetically-intensive activities (e.g., smoltification; Maule *et al.* 1987), pathogens can evade this system. Crucial to salmon defence is innate immunity (Jones 2001; Whyte 2007). As poikilotherms, immunity is temperature dependent and the innate system has a more rapid response (Magnadottir 2006). Other mechanisms may also play important roles in defense, such as nutritional immunity (i.e. withholding nutrients from pathogens; Hammer & Skaar 2011; Hood & Skaar 2012). Other important innate response components include inflammation and tissue remodelling (e.g., induced by matrix metalloproteinase-9; Chadzinska *et al.* 2008; Skugor *et al.* 2008), the acute phase response (Bayne & Gerwick 2001), cytokine activity (Secombes *et al.*

2001) and the complement system (Magnadottir 2006). The immune system of bony fish (Superclass: Osteichthyes) has greater similarity to higher vertebrate immune systems than do jawless fish (Superclass: Agnatha), however the system still has many differences from that of mammals (Tort *et al.* 2003). For example, fish do not have bone marrow, and instead use the anterior kidney and spleen for primary and secondary hematopoiesis, respectively (Zapata *et al.* 1996). Similar to other animals, fish skin is an important immunological organ as the first line of defense against invaders. However, in fish this has a mucosal layer capable of producing many protective effector molecules (Ángeles Esteban 2012). The study of the immune system of fish is important for both theoretical and practical application.

Linking response genes to potential pathogens may aid in selective breeding methods (Jones *et al.* 2002) and provide markers indicative of pathogen presence (Miller *et al.* 2011). Characterization of response genes and pathways have been conducted for viruses (Krasnov *et al.* 2011b; Rise *et al.* 2012) and for salmon lice infecting Atlantic salmon (Skugor *et al.* 2008; Tadiso *et al.* 2011; Krasnov *et al.* 2012), although more work is needed to understand these complex interactions. Evidence of selection on important immune genes has been identified and in some cases related to pathogen presence in an ecological context (e.g., Bernatchez & Landry 2003; Tonteri *et al.* 2010). These types of approaches can also identify genes not traditionally considered involved in immunity (Tonteri *et al.* 2010) opening new areas for research. Conservation in gene family and pathway function has been important for functional genomics studies of non-model organisms (Primmer *et al.* 2013), enabling much of this work.

1.2.3 Parasitic copepods of salmon

Effects of infections with the ectoparasitic salmon louse *Lepeophtheirus salmonis* on wild and farmed salmon has been an area of active research in the past quarter century (Pike & Wadsworth

1999; Torrissen *et al.* 2013). Salmon and salmon lice have co-evolved over millions of years, and observations of lice infecting salmon have been documented as early as the 1600s (Boxaspen 2006). However, in the absence of sufficient lice control, open net-pen systems of modern intensive salmon aquaculture can change the dynamics of this host-parasite interaction. The system can provide a reservoir for louse infection; farmed fish can be infected with free-swimming copepodids or motile stages transferring from wild hosts, and then lice propagate until chemical intervention or harvest of salmon (Costello 2006). Lice from farmed salmon can then transfer to wild fish, sometimes at a density or during a life stage of which would not typically occur in nature (e.g., disrupting migratory allopatry between juveniles and adults; Krkošek *et al.* 2007) leading to concerns of local extirpation of pink salmon (Krkošek *et al.* 2008).

Policy changes to protect juvenile pink salmon *O. gorbuscha* during outmigration suggest coordination of chemical treatments at a specific time of year (Jones & Hargreaves 2009). This currently appears to be protecting pink salmon from population level impacts of lice infections (Peacock *et al.* 2013). Although a debate exists regarding the impact of lice from farms on wild populations (Marty *et al.* 2010; Krkošek *et al.* 2011), if resistance were to develop in Western Canada then new control methods would be needed. Beyond pink salmon, population effects on other salmon species are less understood, although experimental exposures do provide some insights (Johnson & Albright 1992; Jones *et al.* 2007). It was recently discovered that sockeye salmon are susceptible to lice infection (Jakob *et al.* 2013). However, more work remains to be done.

The biology, pathology and ecology of the salmon louse has been the subject of several reviews (e.g., Pike & Wadsworth 1999; Boxaspen 2006; Igboeli *et al.* 2013a; Torrissen *et al.* 2013; Fast 2014) and relevant details are discussed in the following chapters. In brief, the

stenohaline ectoparasitic copepod *L. salmonis* has eight moult stages comprised of two nauplius stages (free-swimming), one copepodid stage (infective), two chalimus stages (attached, feeding), two pre-adult stages and an adult stage (motile, browsing; Johnson & Albright 1991; Hamre *et al.* 2013). The most pathogenic stages are the motile stages (Grimnes & Jakobsen 1996), probably due to the larger size and more aggressive feeding. Feeding behaviour results in the most host damage and stress (Wagner *et al.* 2008). Salmon lice mainly browse on skin and mucus, but also blood in some stages (e.g., adult female; Bron *et al.* 1993; Kvamme *et al.* 2004). Substantial differences in susceptibility to infection has been identified among salmonid genera and species (Johnson & Albright 1992). Negative impacts of infection can include skin damage and osmotic imbalance (Wagner *et al.* 2008), secondary infections, immunomodulation (Fast *et al.* 2007a), and the death of the host in some cases of high infection density (Boxaspen 2006). Salmonids defend primarily with an innate response in the rejection of parasites (Jones 2001). Specifically, neutrophil infiltration and local inflammation correlate with a refractory response (Johnson & Albright 1992). The details of successful responses are still being characterized (Igboeli *et al.* 2013a), and our understanding of the responses are evolving with the new tools available. Novel insights have been provided by gene expression and transcriptomic approaches (Fast *et al.* 2007b; Jones *et al.* 2007; Skugor *et al.* 2008; Tadiso *et al.* 2011; Braden *et al.* 2012; Krasnov *et al.* 2012). The adaptive immune response does not appear to play a main role, and vaccine development attempts have not yet been successful (Raynard *et al.* 2002), although efforts continue. Semiochemical research (Mordue & Birkett 2009) in understanding attractants that can lead to different infection levels (Fast *et al.* 2003) will also be important to continue pursuing.

Many approaches to controlling salmon lice on farms have been attempted, but the most frequently-used control methods are mainly chemical (reviewed in Igboeli *et al.* 2013a). A

relatively limited range of mechanisms of action of chemical treatments (Denholm *et al.* 2002), and methods that may allow sub-therapeutic dosing of parasiticides (i.e. differential medicated feed ingestion in individual fish) has increased the risk of resistance development to commonly used agents in controlling lice (Igboeli *et al.* 2013a). Resistance in *L. salmonis* to emamectin benzoate (SLICE™, Merck) has been reported in Atlantic Canada (Jones *et al.* 2012a; Igboeli *et al.* 2013b; Jones *et al.* 2013), in Scotland (Carmichael *et al.* 2013) and Norway (Espedal *et al.* 2013), as well as in *Caligus rogercresseyi* in Chile (Bravo *et al.* 2008). Due to cost, new control compounds for fish are slow to develop and to reach market (Denholm *et al.* 2002). Currently, there is a need for improved methods of monitoring development of resistance (Carmichael *et al.* 2013; Igboeli *et al.* 2013a), or detecting stress signatures that may help identify biotic or abiotic factors stressful to the louse (Sutherland *et al.* 2012).

Other potential avenues of louse control include cleaner wrasse (SEARCH 2006), push-pull methods using semiochemicals (i.e. push parasite away from fish, pull to traps; Mordue & Birkett 2009), selective breeding for louse resistance (Jones *et al.* 2002), immunostimulation (Covello *et al.* 2012; Purcell *et al.* 2013), leaving farms to fallow, reducing synthetic light, selecting sites with high water velocity (Brooks, 2009), and using methods applied in integrated multitrophic aquaculture, such as the inclusion of filter feeders to remove parasite larvae (IMTA; Webb *et al.* 2013). The need to improve detection and evasion of pathogenic agents in farm-wild interactions will continue as long as aquaculture occurs in an open system in nature. While lice are an important disease agent, there are also pathogenic viruses and bacteria that require monitoring in aquaculture, and disease continues to put pressure on the industry.

1.3 Ecological genomics and fish biology

1.3.1 Ecological genomics as a systems biology approach

Advances in high-throughput sequencing are being incorporated into many biological fields. A new emerging field, termed ecological genomics, is specifically applying the study of genes and genomes in ecological settings (Feder & Mitchell-Olds 2003; Landry & Aubin-Horth 2014) and is an offshoot of a systems biology approach. At the root of systems biology is the combination of genomics-based discovery science (i.e. collecting and inventorying data) and derived hypothesis-driven science, with the aim to study biological systems through comprehensive profiling of responses to stimuli (Ideker *et al.* 2001). One of the important aspects of systems biology is the holistic nature of the approach, attempting to comprehensively understand complexity in a biological system by studying not only individual components and their connections (structure), but also the dynamics of modules in response to stimuli, and formulating models to predict these responses (Kitano 2002). Using systems biology approaches to study organisms in natural settings is likely to be challenged by confounding variables and increased noise relative to that in a model species or a strain in a laboratory. However, the environmental context may bring unexpected interactions and behaviours of the system. An end-goal of systems biology is to be able to predict behaviour of biological systems (Ideker *et al.* 2001), which is also a goal of ecological studies of disease or other environmental- and conservation-related studies (e.g., response to temperature perturbation). Simultaneous profiling of many molecular responses is a characteristic of this approach, and can result in previously unexpected responses (e.g., Tonteri *et al.* 2010).

Genomics provides information on system components and sequence variation that can be related to phenotypic variation, transcriptomics can provide information on the state of the system and responses to perturbation, and transcriptomic correlation and RNA interference can provide

insight on function and structure of network modules. Most of these approaches are now available for use in any species (Wang *et al.* 2009) especially through sequence similarity-based functional categorization (i.e. Gene Ontology, KEGG pathways; Primmer *et al.* 2013). Progress has been made linking ecologically-relevant genes or genotypes to phenotypes and fitness variation in natural populations and will continue with increased genomic accessibility to non-model organisms. Mechanistic and integrative approaches will also aid in the effort to interpret the impact of sequence variation on the organism (Dalziel *et al.* 2009). These ecological genomics approaches require substantial computational resources and interdisciplinary expertise. Similar to other omics-based fields, it is important to ensure open access to data, and that authors follow benchmark requirements, such as those set forth for microarray experiments (minimum information on a microarray experiment (MIAME); Brazma *et al.* 2001), qPCR experiments (minimum information for a qPCR experiment (MIQE); Bustin *et al.* 2009) and those proposed for microbiome research (Kuczynski *et al.* 2012). The developments resulting from advances in cancer genomics and high throughput sequencing are being applied to species important to conservation and ecology, such as in the i5k initiative (Robinson *et al.* 2011) and the 1000 fish transcriptomes projects (BGI 2013). The effective application of these high-throughput methods, through maintaining objectives and documenting outcomes will be important in moving forward; roadmap papers are helping to guide the way (Allendorf *et al.* 2010; Andrew *et al.* 2013).

1.3.2 Insights into fish biology and immunology from ecological transcriptomics

Genomics has been applied to aquaculture and fish conservation in recent years. With genomic characterization of an organism comes an understanding of encoded genes in that organism, and the ability to profile the expression of these genes in different environmental contexts (i.e. transcriptomics). As described above, transcriptomics can connect genes to physiological traits

such as growth and maturation for broodstock development, and can identify impacts of selective pressures that may not occur in coding regions of a gene but that have an effect on the expression of that gene (Nielsen & Pavey 2010).

The application of ecological transcriptomics has affected many different fields. Research on adaptation and speciation of fishes has advanced using transcriptomics (e.g., Schulte 2001; St-Cyr *et al.* 2008; Mavarez *et al.* 2009; Meier *et al.* 2014), as this can identify mechanisms of population divergence or local adaptation. This can provide information on the adaptation potential of populations (the ability of a population to respond to environmental change) which is of particular importance for fisheries management (Nielsen & Pavey 2010). As discussed above, aquaculture advancement has also utilized transcriptomics, for example through investigating effects of reduced nutrition on immunity (Li *et al.* 2014) or in determining mechanisms inducing intestinal inflammation from feed replacements (e.g., soybean meal; Sahlmann *et al.* 2013). Environmental toxicology is another area expanding through transcriptomic advancement (ecotoxicogenomics), for example to investigate effects on fish health of the oil spill in the Gulf of Mexico (Whitehead *et al.* 2012; Dubansky *et al.* 2013), heavy metal contaminants (e.g., yellow perch; Bougas *et al.* 2013), or endocrine-disrupting hormones (Marlatt *et al.* 2012; Lado *et al.* 2013). These studies are instrumental in advancing our understanding of the impacts of anthropogenic environmental changes on organisms. With these approaches, ecotoxicological assessment of anthropogenic impacts can be even more sensitive. For this to work, first it is necessary to be able to consistently and reliably measure these features in important sentinel species. *Daphnia pulex* is an emerging model to be used for studying ecotoxicology, and this may be where some of these advances are to be first applied (Colbourne *et al.* 2011).

A major benefit of using transcriptomics to study impacts of various biotic or abiotic stimuli is the ability to characterize trade-offs that may occur during responses. Instead of only profiling several targets that represent different components of the immune system, other important processes can be simultaneously measured (e.g., metabolism or growth). This approach will be highly informative for fields such as ecological immunology, which aims to investigate immune responses in an ecological context, investigating the trade-offs that can occur due to the mounting of an immune response (Rolff & Siva-Jothy 2003; Martin *et al.* 2011).

1.3.3 Copepod genomics

Copepods (Phylum: Arthropoda; Class: Maxillopoda; Subclass: Copepoda) are a highly diverse group of organisms, but our genomic understanding of this group is very limited, especially considering the biological innovations and important ecological roles of this group (Bron *et al.* 2011). Some initial work has been done on the harpacticoid copepod *Tigriopus japonicus* an emerging model for ecotoxicology and environmental genomics (Raisuddin *et al.* 2007) and much of the work in copepod genomics has been conducted on important ectoparasitic copepods of aquaculture species (e.g., *L. salmonis*, *Caligus rogercresseyi* and others). Information transfer from model organisms is limited due to the evolutionary distance and thus the abundance of unknown genes in copepods (Bron *et al.* 2011). A recently proposed approach for handling the large number of unannotated genes in non-model species is to build a new system similar to the Gene Ontology but through annotation with ecological associations (Pavey *et al.* 2012). This may help meet the challenge of the large number of unknown genes in copepods. Although this would only make associations to terms, and not provide definitive functions for unknown genes, recent advancement of RNA interference in salmon lice (Campbell *et al.* 2009; Dalvin *et al.* 2009) may be able to provide functions for some of the most interesting targets identified. The continued

expansion of our understanding of stress-associated transcripts, or genes involved in pathogenicity or drug resistance is important for aquaculture development and fish conservation.

1.4 Topics of dissertation

In relation to the challenges and advances described in this introductory chapter, with the work presented here I will try to meet several gaps in our understanding of the host-parasite interaction of salmon and the salmon louse *L. salmonis*, an important interaction for both ecological and aquacultural sustainability. Stated here for clarity, these topics will be explored sequentially in the following chapters:

1) Why are some developmental stages of juvenile pink salmon susceptible to salmon lice infections and some are not?
2) What leads to infection susceptibility differences among salmon species?
3) What molecular responses occur in salmon lice from short-term temperature or salinity changes?
4) How do salmon lice develop resistance to the parasiticide emamectin benzoate, and what are the comparative responses in Pacific and Atlantic lice in response to this chemical?

Chapter 2: Differentiating size-dependent responses of juvenile pink salmon (*Oncorhynchus gorbuscha*) to sea lice (*Lepeophtheirus salmonis*) infections

Adapted from: Ben J. G. Sutherland[1], Stuart G. Jantzen[1], Dan S. Sanderson[1], Ben F. Koop[1], Simon R. M. Jones[1,2]. *Comp Biochem Physiol, Part D* (2011), **6**(2): 213-223.

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SGJ assisted in analysis and edited the manuscript.

DSS assisted in microarray work and edited the manuscript.

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

SRMJ conceived of the study and wrote the manuscript.

2.1 Abstract

Salmon infected with an ectoparasitic marine copepod, the salmon louse *Lepeophtheirus salmonis*, incur a wide variety of consequences depending upon host sensitivity. Juvenile pink salmon (*Oncorhynchus gorbuscha*) migrate from natal freshwater systems to the ocean at a young age relative to other Pacific salmon, and require rapid development of appropriate defenses against marine pathogens. We analyzed the early transcriptomic responses of naïve juvenile pink salmon of sizes 0.3 g (no scales), 0.7 g (mid-scale development) and 2.4 g (scales fully developed) six days after a low-level laboratory exposure to *L. salmonis* copepodids. All infected size groups exhibited unique transcriptional profiles. Inflammation and inhibition of cell proliferation were identified in the smallest size class (0.3 g), while increased glucose absorption and retention was identified in the middle size class (0.7 g). Tissue-remodeling genes were also up-regulated in both the 0.3 g and 0.7 g size groups. Profiles of the 2.4 g size class indicated increased cell-mediated immunity and possibly parasite-induced growth augmentation. Understanding a size-based threshold of resistance to *L. salmonis* is important for fisheries management. This work characterizes molecular responses reflecting the gradual development of innate immunity to *L. salmonis* between the susceptible (0.3 g) and refractory (2.4 g) pink salmon size classes.

2.2 Introduction

The salmon louse *Lepeophtheirus salmonis* (Copepoda: Caligidae) is an ectoparasitic marine copepod that infects wild and farmed salmonids in the Northern Hemisphere (Nagasawa *et al.* 1993; Jones 2009). The life cycle consists of two free-swimming larval stages (nauplius I, II) an infectious copepodid stage and seven parasitic stages (chalimus I–IV, pre-adult I–II and adult; Johnson & Albright 1991). Parasitic stages predominantly feed upon host epidermis and mucus, and occasionally blood (Johnson & Albright 1991; Bron *et al.* 1993). Chalimus stages are relatively small and tethered to the host by a frontal filament, whereas later pre-adult and adult stages are larger, motile, and more damaging to the host (Mackinnon 1993; Grimnes & Jakobsen 1996). *L. salmonis* infections have serious economic and ecological implications among valuable salmon populations (Costello 2006).

In the susceptible Atlantic salmon (*Salmo salar*) the effects of *L. salmonis* infection include changes in plasma cortisol, glucose and ion concentration (Grimnes & Jakobsen 1996; Bowers *et al.* 2000; Finstad *et al.* 2000; Wagner *et al.* 2003), changes in mucus lysozyme and alkaline phosphatase presence and activity (Fast *et al.* 2002; Easy & Ross 2009), skin damage, reduced growth and food conversion, behavioral changes, and stress-induced mortality (reviewed in Costello 2006; Wagner *et al.* 2008). Stress responses can be attributed to both feeding mechanisms and immune-modulatory salivary secretions of the louse (Fast *et al.* 2005; Firth *et al.* 2000). Molecular evidence of tissue remodeling without accompanying wound healing indicates the infections in Atlantic salmon are chronic (Skugor *et al.* 2008).

Variation in susceptibility to *L. salmonis* occurs among salmon species (Johnson & Albright 1992; Nagasawa *et al.* 1993; Fast *et al.* 2002; Jones *et al.* 2007). Therefore it is important to understand the interactions of *L. salmonis* with a variety of host species (Costello

2006; Wagner *et al.* 2008). In laboratory exposures, coho salmon (*Oncorhynchus kisutch*) exhibit the greatest resistance to louse infection (Fast *et al.* 2002), a response attributed to early inflammation (Johnson & Albright 1992).

A size-dependent sensitivity to *L. salmonis* was identified in juvenile pink salmon (Jones *et al.* 2008). In this laboratory exposure of pink salmon, the average intensity of infection at 6 days post exposure (dpe) was similar, with 4.9, 3.0, and 2.8 lice per fish (lpf) among 0.3 g, 0.7 g, and 2.4 g size classes, respectively. However, by 37 dpe the infection prevalence was much higher in the 0.3 g and 0.7 g groups (36.4% and 35%, respectively) than in the 2.4 g size class (5%). Furthermore, significant mortality occurred solely in the 0.3 g size class and mostly occurred after 13 dpe (0.3 g mortality ~ 37%; 0.7 g mortality ~ 5%; 2.4 g no mortality). The pattern of louse development was similar on all size groups, with mainly copepodids present at 6 dpe (one chalimus I was identified on a 2.4 g fish), and chalimus I and II present at 12 dpe. In this exposure, 80.9% of lice on dead fish were chalimus IV stage or earlier. The absence of significant mortality in the 0.7 g and 2.4 g pink salmon indicates an onset of protection between the 0.3 g and 0.7 g size classes (Jones *et al.* 2008). To further understand the mechanisms behind these variable responses, we have profiled transcriptomes of the three size classes of juvenile pink salmon from the aforementioned exposure trials (Jones *et al.* 2008). Using both a 32K cDNA microarray and qRT-PCR, we investigated the responses at 6 dpe, before significant mortality occurs, to investigate the primary host responses to early stages of lice.

2.3 Materials and methods

2.3.1 Animals

A complete description of the source and maintenance of animals was previously reported (Jones *et al.* 2008). Briefly, juvenile pink salmon were derived from a naturally spawned, gravel-reared population and collected with in-river rotary screw traps from the Glendale River on the central

coast of British Columbia (BC) in April 2007. Fry were transported to and maintained at Pacific Biological Station (Nanaimo, BC, Canada) in 400 L stock tanks with a mixture of flow-through dechlorinated fresh water and sand-filtered sea water. Salmon were fed a crumble (0 and 1) ration (Ewos, Canada Ltd., Surrey, BC, Canada) at an average daily rate of 1.2% body weight. Salmon were acclimatized to seawater for one week prior to experimental challenges.

Gravid lice were obtained from Atlantic salmon from commercial net pens near Vancouver Island. Egg strings were dissected, hatched at 32.5‰ salinity and 8.9 °C, and incubated for one week at which time an inoculum containing a known number of copepodids was created by pooling incubation beakers.

2.3.2 Louse exposure and tissue extraction

A complete description of exposure methodology was previously reported (Jones *et al.* 2008). Briefly, three separate trials with different size groups of salmon were conducted. The mean weights (± SE) of salmon at the beginning of each trial were 0.25 ± 0.01 g, 0.69 ± 0.02 g, and 2.37 ± 0.04 g. In each trial, fish were exposed at a rate of 100 copepodids per fish. The exposure was performed by halting water flow, reducing volume to 3 or 4 L with supplemental aeration, and sedating fish with 0.07 mg/L metomidate.HCl. The copepodid inoculum was added, tanks were then kept dark for 2 h, and then water flow was resumed. Fish were then maintained at 12 h light then 12 h dark photoperiods. For all trials, mean temperature and salinity were 8.9 °C (range, 7.7–9.6 °C) and 32.5 ‰ (range, 28–34 ‰), respectively. Control fish with the same history were treated the same as the experimental fish, but without the addition of copepodids. All treatments and control groups were maintained and challenged in duplicate tanks.

At 6 dpe fish were sedated and 10 uninfected and 10 infected fish were killed in an overdose of MS-222 (Syndel). Each fish was rapidly processed (mass and length measured, louse

number and molt stage recorded, flash frozen in liquid nitrogen and stored at − 80 °C) to preserve RNA quality (Jones *et al.* 2008).

2.3.3 RNA preparation

Tissue cross-sections of approximately 2 mm thick were obtained from frozen fish by making parallel bisections anterior to the dorsal fin. The still-frozen tissue was placed directly into TRIzol® and homogenized with a *TissueLyser II* (Qiagen, Valencia, CA, USA). Total RNA was extracted as per manufacturer's instructions (Invitrogen), and purified through RNeasy spin columns, as per manufacturer's instructions (Qiagen). Total RNA was quality-checked with agarose gel electrophoresis, quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA), and stored at − 80 °C.

2.3.4 Synthesis of experimental channel (cDNA) and reference channel (aRNA) samples

Six individuals from each condition were randomly selected for cDNA synthesis (three per tank, 12 per size group, 36 total). Using a Superscript™ Indirect cDNA Labeling System (Invitrogen), cDNA was synthesized as previously reported (von Schalburg *et al.* 2005b). In brief, 10 μg total RNA was primed with oligo$(dT)_{20}$ primers and reverse transcribed to incorporate amino-allyl-modified nucleotides. Modified cDNA was labeled with Mono-Reactive Cy5™ dye in a 2 h reaction in coupling buffer (Amersham Biosciences), purified using S.N.A.P. columns (Invitrogen), quantified through spectrophotometry (NanoDrop Technologies), and kept dark and cool until hybridization.

An aRNA reference pool was synthesized from total RNA obtained from juvenile pink salmon representing a variety of size and infection conditions. Reference aRNA was synthesized using the Amino-Allyl MessageAmp™ aRNA kit (Applied Biosystems, Austin, TX, USA) as per manufacturer's instructions. In brief, 2 μg total RNA was reverse-transcribed with T7 oligo$(dT)_{20}$

primers and reverse transcriptase. Second strand cDNA was synthesized using DNA polymerase, then purified using cDNA Filter Cartridges. aRNA was then synthesized through *in vitro* T7 transcription with amino-allyl modified UTPs, purified with aRNA Filter Cartridges, and quantified using spectrophotometry (NanoDrop Technologies). Equimolar amounts of aRNA from each of the eight samples were pooled, aliquoted, and stored at − 80 °C until labeling. Labeling of the aRNA was the same as described above for the cDNA, except with Mono-Reactive Cy3™ dye. Labeled cDNA (250 ng) and aRNA (500 ng) were combined and brought up to 23 µl with RNase-free water (Invitrogen) and kept dark at 4 °C.

2.3.5 Microarray hybridization, scanning, and spot quantification

Samples were hybridized to a single batch of cGRASP salmonid 32K cDNA microarrays (*http://web.uvic.ca/grasp/microarray/array.html*) using a Tecan Pro HS 4800™ Hybridization Station (Tecan Group Ltd., Männedorf, Switzerland). The cGRASP microarray has been fully described (Koop *et al.* 2008). The array was designed to consist of 27,917 Atlantic salmon and 4065 rainbow trout cDNA elements, and can be used for hybridizations of all 68 members of *Salmonidae*, including pink salmon (Koop *et al.* 2008).

The microarray hybridization protocol was adapted from previous work (Koop *et al.* 2008). All slides were pre-washed with 0.1X SSC, 0.2% SDS three times for 30 s at 23 °C, 0.2X SSC two times for 60 s at 23 °C and once with 5X SSC, 0.01% SDS, 0.2% BSA for 60 s at 49 °C. The final pre-wash solution was incubated for 1 h at medium agitation to block non-specific binding of the array. Slides were then washed twice with 2X SSC, 0.014% SDS for 60 s at 49 °C. To each sample, 2 µl LNA dT blocker (Genisphere LLC., Hatfield, PA, USA) and 100 µl hybridization buffer at 65 °C (Applied Biosystems,) were added and heated to 80 °C for 10 min, then kept at 65 °C until sample loading. Hybridization occurred over 16 h with periodical hourly

temperature oscillations of 49 °C and 53 °C. Following incubation, slides were washed with 2X SSC, 0.014% SDS for 60 s at 49 °C, incubated for 3 min, washed at 49 °C for 60 s, at 39 °C for 20 s, and at 30 °C for 20 s. Slides were finally washed with 1X SSC for 60 s at 23 °C and with 0.2X SSC for 30 s at 23 °C, dried with 255 kPa nitrogen gas and kept dark in a low-ozone environment (ozone ≤ 0.005 ppm) and scanned as soon as possible with a ScanArray® Express (PerkinElmer, Inc., Waltham, MA; 5 μm resolution; PMTs: Cy5:74, Cy3:76; ozone ≤ 0.016 ppm). Fluorescence intensity data and quality measures were extracted using ImaGene® 8.0 (BioDiscovery, El Segundo, CA, USA).

Array element identification and annotation was assigned by the cGRASP consortium (*http://web.uvic.ca/cbr/grasp*) and has previously been reported (von Schalburg *et al.* 2005b; Koop *et al.* 2008). The annotation file can be found at *http://web.uvic.ca/grasp/microarray/array.html*.

2.3.6 Microarray normalization, filtering, and analysis

Data normalization and analysis was performed with GeneSpring™ GX11 (Agilent). Raw signal was transformed to a threshold of 1.0. Arrays were normalized using a per-slide, per-block intensity-dependent *Lowess* normalization and a per-sample, per-gene baseline to median normalization. Data files were deposited in NCBI's Gene Expression Omnibus under the accession GSE27528 (*http://www.ncbi.nlm.nih.gov/geo/*). Due to technical limitations for comparing expression differences across size groups, direct comparisons were only made within a size class. For each size class, normalized spot values, or entities, were filtered to only retain those in which at least 65% of the samples in at least one of the two conditions (exposed or control) had raw signal greater than or equal to 500. Entities were then filtered using volcano plot (Mann–Whitney $p \leq 0.05$, no multiple test correction, and fold change (FC) ≥ 2).

Functions over-represented in each size group's differential gene list were investigated using Gene Ontology and pathway annotation. Differentially expressed entity lists ($FC \geq 2$, $p \leq 0.05$) were used as an input into the GX11 GO browser (Agilent), and enriched categories were retained ($p \leq 0.05$, no multiple test correction). GO Trimming was performed on the significantly enriched GO lists in order to reduce redundancy in displayed tables. 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.* 2011b), and does not change enrichment values of terms, but rather just systematically selects a subset of terms to be discussed.

For each size group, differentially expressed genes ($FC \geq 2$, $p \leq 0.05$) with Gene ID annotation were used for Find Significant Pathways analysis (Agilent) and enriched pathways ($p \leq 0.05$) were retained.

2.3.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

The same individual RNA samples included in the microarray analysis were used for qRT-PCR. Briefly, cDNA was synthesized using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen), as per manufacturer's instructions, and as described for first strand modified cDNA but with unmodified dNTPs. Genes of interest (GOI) were selected from microarray results due to high fold change, involvement in enriched GO categories from either the present investigation or a previous microarray investigation (*data unpublished*), or to compare to trends identified in the response of Atlantic salmon to sea lice infection (Skugor *et al.* 2008). Primers used are shown in Table 19, and were designed using Primer3 (Rozen & Skaletsky 2000) and AlleleID®7.0 (PREMIER Biosoft International, Palo Alto, CA, USA) based on conserved

sequences from available data for salmonid species, including *S. salar*, *Oncorhynchus mykiss*, *Oncorhynchus nerka* and in some cases *Danio rerio*. The original sequence used as the search query was the contiguous sequence (contig) of the cDNA element of interest on the microarray. All related sequences were obtained from NCBI or the cGRASP database (cGRASP; *http://lucy.ceh.uvic.ca/contigs/cbr_contig_viewer.py*).

Equimolar amounts of all samples from all conditions investigated (n = 36) were pooled, diluted two-fold, and then used as a starting point for a five point, five-fold standard dilution series to be used as a standard curve for testing primer amplification efficiencies (Appendix A Table 19). This series was also run in duplicate as a positive control during each GOI plate run. Experimental samples, −RT and no template control (NTC) were also run in duplicate on a single plate for each GOI. qRT-PCR was performed in 20 µl reactions using SYBR GreenER™ qPCR SuperMix Universal master mix as per manufacturer's instructions (Invitrogen) in an Mx3000P™ thermal cycler (Agilent). The following thermal regime was used for all samples: Segment 1, 95 °C for 120 s, 1 cycle; Segment 2, 95 °C for 15 s, 55 °C for 30 s (fluorescence read at end), 72 °C for 20 s, 40 cycles; and Segment 3, 95 °C for 60 s, 55 °C for 30 s, and then ramp up to 95 °C for 30 s (fluorescence read each 0.5 °C increment).

Quality control of duplicate wells permitted a standard deviation of less than one Ct. In order to confirm amplicon identity and singularity, melt curve analysis and amplicon sequencing were performed. All GOIs reported displayed a single product. Each amplicon was purified post-qRT-PCR with SureClean™, as per manufacturers' instructions (Bioline), and sequenced bi-directionally by BigDye® Terminator sequencing as per manufacturer's instructions (Applied Biosystems) in 5 µl reactions with 1 µl of 3.2 µM gene-specific forward or reverse primer, BigDye®, and 2.5 ng of template cDNA. The sequencing reaction thermal regime was as follows:

Segment 1, 95 °C for 60 s, 1 cycle; Segment 2, 95 °C for 30 s, 50 °C for 15 s, 60 °C for 90 s, 35 cycles; Segment 3, 60 °C for 5 min, 1 cycle. PCR products were then ethanol precipitated and run on a 3730 DNA Analyzer (Applied Biosystems), as per manufacturer's instructions, with an injection time of 15 s. Trace files were interpreted using a short-read analysis algorithm (Applied Biosystems). All sequences corresponded to the expected amplicon.

A representative sample from each group was included as a negative reverse transcriptase (− RT) control. − RT samples were tested with primer pairs for sestrin-1 and ubiquitin and amplified by PCR with GoTaq® (Promega, Madison, WI, USA) with the thermal regime used for qRT-PCR. PCR products were viewed on a 1% agarose gel, and the samples with the most product after 40 cycles were included in each qRT-PCR run as a − RT control. The difference between the − RT and the + RT sample was greater than 6 Ct (Qiagen), and the NTC remained clean.

Normalizer gene candidates were selected based on static expression in microarray results: *eukaryotic translation initiation factor 4H* (*eif4h*), *actin cytoplasmic 1* (*actb*), *ubiquitin* and *plastin-1*. Transcript expression stability was investigated using geNORM algorithms (Vandesompele *et al.* 2002). After removal of the least stable normalizer candidates, *eif4h* and *actb* had an M value of 0.4279 and CV of 14.92%, values within necessary criteria for stable normalizer genes (Vandesompele *et al.* 2002; Pérez *et al.* 2008). Normalization of experimental samples was performed with the geometric mean of these two normalizers (Vandesompele *et al.* 2002). Relative quantities were determined using primer-specific amplification efficiencies (Table 19) in REST© v.2.0.13 (Qiagen), and statistical significance was determined by a Pair Wise Fixed Reallocation Randomization Test© (Pfaffl *et al.* 2002).

2.4 Results

2.4.1 Microarray size-dependent expression profiles

The use of the cGRASP 32K salmonid cDNA array enabled the investigation of global gene expression changes of the early response of three size groups of post-smolt pink salmon (0.3 g, 0.7 g, and 2.4 g) to a low-level lice infection. Due to the large quantity of changed expression (Table 1), further analytical methods were applied to the gene list and these guided the interpretation of gene profiles.

Table 1. Infection intensity and genes differentially expressed at 6 dpe for 0.3g, 0.7g, and 2.4g pink salmon

Size (g)	Avg (range) lice per fish at 6 dpe	Total entities up-regulated (FC > 2; p ≤ 0.05)	Total entities down-regulated (FC > 2; p ≤ 0.05)
0.3	4.9 (1-8)	281	308
0.7	3.0 (1-5)	1282	1843
2.4	2.8 (1-8)	296	174

Infection density data from Jones *et al.,* 2008

Shared differentially expressed genes were rare among the size groups (Figure 1). Less than ten entities were common to all size classes in either the up or down-regulated direction, and the greatest similarity was found in the intersect of the 0.3 g and 0.7 g gene lists. Differentially regulated genes with the highest up- or down-regulated fold change (FC) from each size group are presented in Table 2, Table 3, Table 4.

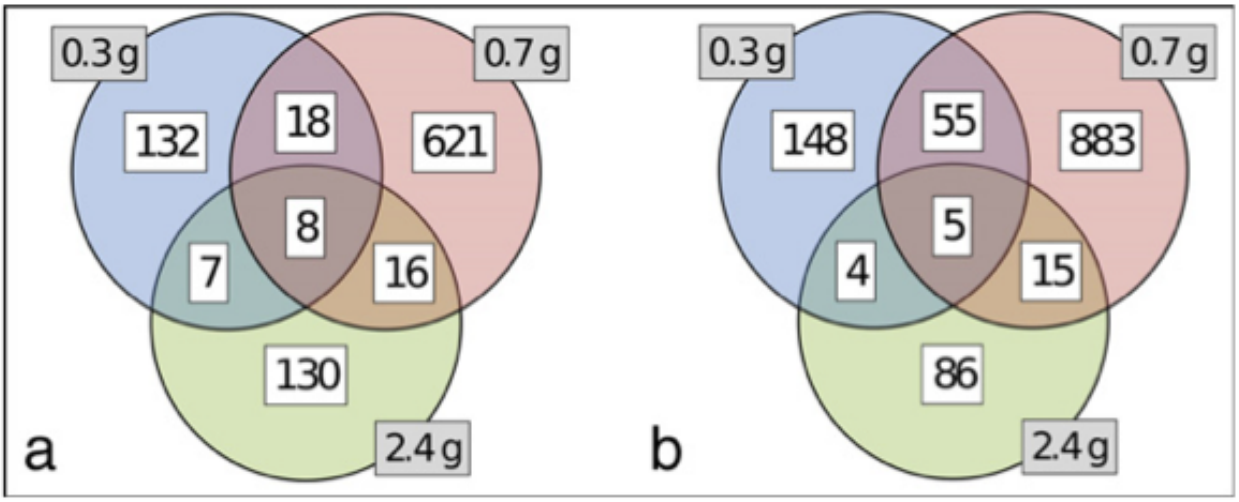

Figure 1. Differentially expressed genes unique or common among size groups of infected pink salmon

Diagram displays the numbers of differentially expressed transcripts (FC ≥ 2; p ≤ 0.05) shared among size groups in the a) up- or b) down-regulated directions.

2.4.2 0.3 g salmon

Up-regulated genes with the highest fold change (FC > 3; Table 2) included 20S proteasome subunit alpha type-1, and sterile alpha motif domain-containing protein 9, which is involved in *in vitro* cell proliferation inhibition (Li *et al.* 2007).

Several down-regulated genes with the highest fold change (FC > 3; Table 2) included exportin-1 (FC = 20.7) and FK506-binding protein 1A, both of which have been identified as up-regulated during cell-cycle progression (Kudo *et al.* 1997). Additionally, hemicentin-1, a conserved matrix protein with involvement in epidermis development in *Caenorhabditis elegans* (Vogel & Hedgecock 2001), was down-regulated.

2.4.3 0.7 g salmon

Many genes displayed high fold change expression changes in the 0.7 g list, and those with the highest are displayed in Table 3 (FC > 7). Both *sodium/glucose co-transporter 1* (*sglt1*) and *2*

(*sglt2*) were up-regulated (FC > 4.5, p < 0.02), and are involved in transporting sugar from the gut lumen (*sglt1*), or re-absorbing from the glomerular filtrate (*sglt2*; Wright & Turk 2004). Other transcripts up-regulated are involved in cell motility, including *talin-1* and *intraflagellar transport protein 46* (Burridge *et al.* 1988; Hynes 1992; Hou *et al.* 2007).

Table 2. Up- or down-regulated genes in the 0.3g infected pink salmon with highest fold change

Gene Description	Up-reg FC	p-value	GenBank
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	5.44	0.004	CB507773
SAPS domain family member 3	4.72	0.010	DY732411
VAR1 Mitochondrial ribosomal protein	4.67	0.016	CA063979
Vang-like protein 1	4.36	0.010	DW583738
Proteasome subunit alpha type-1	4.23	0.025	CA043404
Sterile alpha motif domain-containing protein 9	4.22	0.025	EG758275
LYR motif-containing protein 1	3.95	0.010	EG920165
Alpha-protein kinase 1	3.89	0.025	CB518092
Beta-2-glycoprotein 1 precursor	3.80	0.037	CA037450
Palatin-like phospholipase domain containing protein-4	3.72	0.010	CA045263

Gene Description	Down-reg FC	p-value	GenBank
Exportin-1	20.74	0.037	CB487200
Ubiquitin carboxyl-terminal hydrolase 5	6.35	0.016	CB496544
Protein FRA10AC1	6.33	0.025	EG835604
AF-4 proto-oncoprotein	6.20	0.016	CX027714
RNA-binding protein EWS	5.67	0.037	CB515604
Peroxiredoxin-5, mitochondrial precursor	4.83	0.004	CB493194
Glycylpeptide N-tetradecanoyltransferase 1	4.05	0.025	CB494396
FK506-binding protein 1A	4.04	0.010	CA049957
Ezrin	4.02	0.010	CA043385
Hemicentin-1 precursor	3.89	0.010	CB498739

Genes discussed in text have bold font

Genes down-regulated with high fold change are displayed in Table 3 (FC > 4). *Isotocin*, the oxytocin homologue in bony fishes was down-regulated (FC = 5.9), as was *titin*, a major component of vertebrate striated muscle (Labeit & Kolmerer 1995). *Myosin-9* (FC = 5.5) and *tubulin alpha-1B chain* (FC = 5.1, p < 0.02) were down-regulated, and are involved in cell structure.

Table 3. Up- or down-regulated genes in the 0.7g infected pink salmon with highest fold change

Gene Description	Up-reg FC	p-value	GenBank
Slime mold cyclic AMP receptor	13.39	0.016	CB507773
Radial spoke head 1 homolog	12.67	0.006	DY732411
Oxysterol-binding protein-related protein 6	9.89	0.037	CA063979
Forkhead box protein J3	9.64	0.010	DW583738
Sodium/glucose cotransporter 1	9.13	0.016	CA043404
Snurportin-1	8.34	0.006	EG758275
DNA topoisomerase 1	8.10	0.004	EG920165
Intraflagellar transport protein 46	7.99	0.037	CB518092
Mitochondrial ribosomal protein (VAR1)	7.58	0.010	CA037450
Talin-1	7.54	0.004	CA045263

Gene Description	Down-reg FC	p-value	GenBank
Titin	10.55	0.006	EG795798
26S proteasome non-ATPase regulatory subunit 12	9.12	0.037	CK990611
Copia protein	7.03	0.006	DY719895
Apolipoprotein A-I precursor	6.51	0.004	CB510585
Titin	6.11	0.004	EG868655
Syntaxin-18	6.10	0.010	DW539041
Nucleoside diphosphate kinase, mitochondrial precursor	5.99	0.037	CB510514
IT-I gene for isotocin	5.90	0.016	CA050111
Fatty acid-binding protein, adipocyte	5.81	0.037	CK990220
Intestinal mucin-like protein	5.79	0.004	CB510438

Genes discussed in text have bold font

2.4.4 2.4 g salmon

Genes with the highest up-regulated fold change in the 2.4 g size class are displayed in Table 4 (FC > 3). The transcript with the highest fold change was *acidic mammalian chitinase* (*AMCase*) precursor, involved in both chitin degradation and immune responses (FC = 8.6). Stress suppression-related transcripts *protein phosphatase 1L*, involved in the regulation of cytotoxic stress-induced apoptosis (Saito *et al.* 2007) and *Cdc37*, a co-chaperone of Hsp90 involved in the promotion of cell growth (Hunter & Poon 1997) were up-regulated (FC > 3.5).

Genes with the highest down-regulated fold change in the 2.4 g size class are displayed in Table 4 (FC > 3). Transcripts involved in cell motility were down-regulated, such as *adhesion-related integrin beta-7 precursor*, involved in mucosal lymphocyte localization (Parker *et al.*

1992), and *collagen alpha-3(VI) chain precursor*, involved in connective tissue cell anchoring (FC > 3, p = 0.01). *Metalloproteinase inhibitor 2 (timp2)* was also down-regulated.

Table 4. Up- or down-regulated genes in the 2.4g infected pink salmon with highest fold change

Gene Description	Up-reg FC	p-value	GenBank
Acidic mammalian chitinase precursor	8.58	0.025	CB511226
Retroviral integration site protein Fli-1 homolog	5.10	0.037	DW536407
ATP-binding cassette sub-family D member 3	5.02	0.016	CA056730
VAR1 Mitochondrial ribosomal protein	4.93	0.025	CK991139
Heterogeneous nuclear ribonucleoprotein L-like	4.11	0.004	DY693285
Pepsin A precursor	4.08	0.025	CB503148
Ligatin	4.00	0.016	DW556429
Protein phosphatase 1L	3.97	0.016	DW554038
Ecto-ADP-ribosyltransferase 5 precursor	3.92	0.037	EG909433
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform	3.73	0.037	CB506378

Gene Description	Down-reg FC	p-value	GenBank
Alpha-actinin-1	4.77	0.037	DW558198
Condensin-2 complex subunit D3	4.65	0.010	CA062925
Hemoglobin subunit alpha-4	3.97	0.016	CA770045
TGF-beta receptor type-1 precursor	3.76	0.037	CB499379
Histone H2AV	3.71	0.025	CB491527
Similar to Apolipoprotein C-I precursor	3.63	0.006	CA047039
Nuclear receptor interaction protein	3.60	0.025	CA050283
Ras-related GTP-binding protein A	3.41	0.016	CA057387
Chloride channel protein ClC-Kb	3.38	0.025	DW583374
Metalloproteinase inhibitor 2 precursor	3.27	0.010	CA059039

Genes discussed in text have bold font

2.4.5 Microarray functional analysis

Gene lists were further analyzed through Gene Ontology (GO) and pathway analysis to interpret differential gene lists. Trimmed significantly enriched GO category lists ($p \leq 0.05$) are presented in Table 5, Table 6 and Table 7, for the 0.3 g, 0.7 g and 2.4 g size groups, respectively.

Functions indicated in the GO analysis of the 0.3 g group included cell structure, immune response, ion homeostasis, and negative regulation of cellular metabolism (Table 5). The category negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process was enriched (seven entities) and is a daughter group of the enriched category negative

regulation of cellular metabolic process (p = 0.01; nine entities). Enriched cell structure-related categories included cytoskeleton part and endoplasmic reticulum. Inflammation and ion transport categories were also enriched.

Table 5. Trimmed Gene Ontology categories significantly enriched in the infected 0.3g pink salmon bi-directional differentially expressed gene list

GO category	Count in Selection	Count in Total	p-value	GO Accession
cytoskeletal part	14	295	0.001	0044430
transcription factor binding	9	173	0.005	0008134
inflammatory response	5	63	0.006	0006954
multicellular organismal process	18	517	0.008	0032501
ion transporter activity	7	154	0.025	0015075
endoplasmic reticulum	7	158	0.029	0005783
ion transport	5	94	0.031	0006811
negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	7	173	0.044	0045934

Categories discussed in the text are in bold font

The 0.7 g differential gene list contained more enriched categories than the other two size groups, reflective of the larger input gene list. Functions in this list included catabolism, immune response, cell structure and motility, and iron handling (Table 6). Catabolic categories included digestion, hydrolase activity, and chitin catabolic process. Immune response and lysozyme activity were also enriched. Cell structure and motility-related enriched categories included ruffle, cell projection, and actin cytoskeleton organization and biogenesis. Related to iron handling, ferroxidase activity was enriched; all 17 differentially expressed entities (16 down-regulated) in the category were ferritin subunits.

Table 6. Trimmed Gene Ontology categories significantly enriched in the infected 0.7g pink salmon bi-directional differentially expressed gene list

GO category	Count in Selection	Count in Total	p-value	GO Accession
response to acid	10	12	3.78E-09	0001101
lysozyme activity	10	12	3.78E-09	0003796
cell wall chitin metabolic process	10	12	3.78E-09	0006037
response to fungus	10	12	3.78E-09	0009620
chitinase activity	10	13	1.50E-08	0004568
chitin catabolic process	10	13	1.50E-08	0006032
chitin binding	10	13	1.50E-08	0008061
ferroxidase activity	17	46	5.74E-07	0004322
structural constituent of ribosome	28	118	5.42E-06	0003735
sugar binding	14	39	8.53E-06	0005529
structural molecule activity	53	299	8.66E-06	0005198
digestion	11	26	1.29E-05	0007586
carbohydrate binding	18	68	5.55E-05	0030246
hydrolase activity, hydrolyzing O-glycosyl compounds	16	57	6.61E-05	0004553
translation	26	121	7.28E-05	0006412
macromolecule biosynthetic process	34	196	5.28E-04	0009059
serine-type endopeptidase activity	18	86	0.0013	0004252
nuclear matrix	9	30	0.0016	0016363
hydrolase activity	111	887	0.0021	0016787
actin cytoskeleton reorganization	5	12	0.0037	0031532
RNA binding	44	303	0.0039	0003723
carbohydrate metabolic process	29	181	0.0045	0005975
translational elongation	6	18	0.0054	0006414
stress fiber	7	24	0.0062	0001725
extracellular space	21	122	0.0063	0005615
endopeptidase activity	31	203	0.0069	0004175
phosphoinositide binding	6	19	0.0073	0035091
translation elongation factor activity	5	14	0.0080	0003746
cell projection	21	129	0.0119	0042995
actin cytoskeleton organization and biogenesis	21	132	0.0153	0030036
COPI vesicle coat	6	22	0.0156	0030126
ruffle	8	35	0.0165	0001726
extracellular region part	34	243	0.0176	0044421
heart morphogenesis	5	17	0.0193	0003007
trypsin activity	6	24	0.0238	0004295
muscle myosin complex	6	24	0.0238	0005859
structural constituent of cytoskeleton	11	59	0.0244	0005200

Arp2/3 protein complex	5	18	0.0247	0005885
embryonic development	11	60	0.0273	0009790
amine metabolic process	17	110	0.0349	0009308
multicellular organismal development	8	40	0.0351	0007275
response to biotic stimulus	15	94	0.0357	0009607
phosphoric monoester hydrolase activity	18	119	0.0369	0016791
biopolymer catabolic process	17	112	0.0406	0043285
myofibril assembly	6	27	0.0408	0030239
immune response	20	138	0.0430	0006955
cellular macromolecule catabolic process	18	122	0.0456	0044265

Categories discussed in the text are in bold font

Functions in the 2.4 g enriched GO categories were related to cell motility and other cellular activities (Table 7). Cell motility-related categories included extracellular region part and cell projection. Other enriched categories included metal ion binding, enzyme activator activity, transferase activity, and receptor binding.

Table 7. Trimmed Gene Ontology categories significantly enriched in the infected 2.4g pink salmon bi-directional differentially expressed gene list

GO category	Count in Selection	Count in Total	p-value	GO Accession
extracellular region part	12	243	3.23E-04	0044421
protein dimerization activity	7	152	0.0085	0046983
transferase activity	22	861	0.0106	0016740
metal ion binding	9	243	0.0118	0046872
transferase activity, transferring hexosyl groups	5	91	0.0125	0016758
enzyme activator activity	5	108	0.0244	0008047
cation binding	7	188	0.0247	0043169
receptor binding	7	192	0.0273	0005102
extracellular space	5	122	0.0384	0005615
cell projection	5	129	0.0469	0042995
organelle organization and biogenesis	12	459	0.0470	0006996

Categories discussed in the text are in bold font

As was viewed among differential gene lists, GO categories were also distinct among the different size groups. Commonalities were identified between the categories enriched in the 0.7 g

and 2.4 g groups, such as the enrichment of categories involved in cell structure and motility, including the category cell projection.

Pathway analysis was used to find functional patterns in a gene list by investigating for the enrichment of a pathway in a gene list. Only the differential gene list of the 2.4 g infected pink salmon ($FC \geq 2$; $p \leq 0.05$) contained significantly enriched pathways, and included cell adhesion molecules (CAMs), tryptophan metabolism, glycosphingolipid biosynthesis (lacto and neolacto series), and aminosugar metabolism. None of the other size classes tested produced significant pathways.

2.4.6 Real-time quantitative polymerase chain reaction

After quality control, a total of 21 GOIs were included in the analysis of all size classes. Calculated relative expression ratios of GOIs normalized by *actb* and *eif4h* are presented in Figure 2. Most GOIs are separated into the functional categories of proliferation inhibition, ROS and immunity, and remodeling and motility.

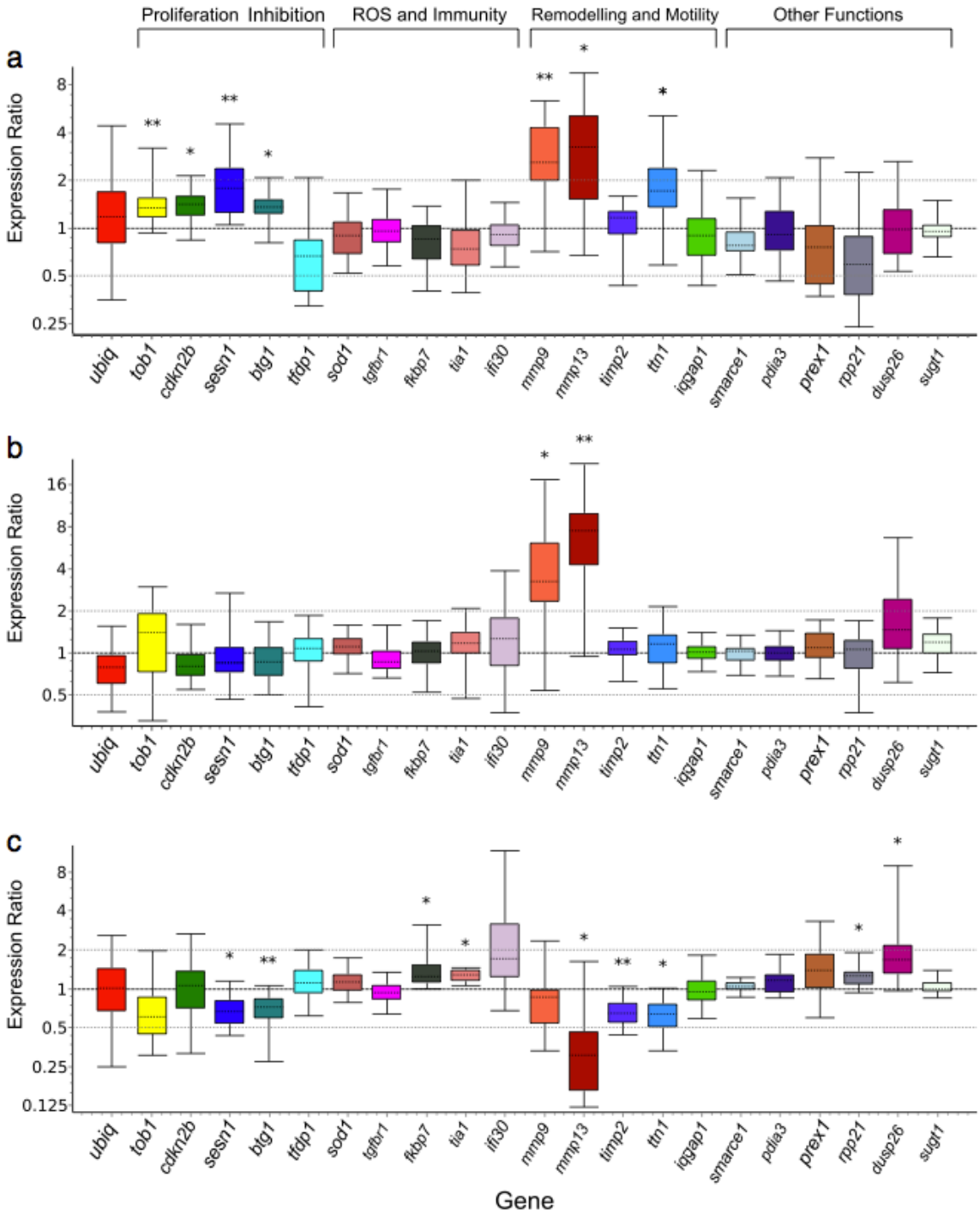

Figure 2. qRT-PCR gene expression ratios in statistical whisker-box plots of genes of interest responding to infection in the a) 0.3 g, b) 0.7 g and c) 2.4 g size groups

Expression ratios are relative to control samples and are normalized by the geometric mean of expression levels of *eif4h* and *actb*. *Ubiquitin* is included on the graph as an example of a potential normalizer gene, that did not end up being used to normalize. Box area, whiskers and

hatched line in box display interquartile range, upper or lower quartiles, and median, respectively. Y-axis scales are different for the different size groups (a–c), and each size group should be considered individually. Significance was determined by Pair Wise Fixed Reallocation Randomization Test©, *denotes $p \leq 0.05$ **denotes $p \leq 0.01$.

Expression profiles of stress-related anti-proliferative genes further confirmed the general trends in the microarray data among salmon size classes. These transcripts, including *sestrin-1* (*sesn1*), *transducer of erbB-2 1* (*tob1*), *cyclin-dependent kinase 4 inhibitor B* (*cdkn2b*), and *B-cell translocation gene 1 protein* (*btg1*) were only up-regulated in the 0.3 g pink salmon. Additionally, *transcription factor DP-1* (*tfdp1*), involved in integrating cell cycle events with transcriptional apparatus (Bandara *et al.* 1993) was close to passing significance testing (FC = 1.6 down; $p = 0.064$). All of these transcripts remained unchanged due to infection in the 0.7 g size group. In the 2.4 g size group, those that were differentially regulated showed inverse expression profiles to the 0.3 g infected pink salmon (Figure 2). Furthermore, in the 2.4 g size group, protein synthesis-related transcripts were up-regulated, including *peptidyl-prolyl cis-trans isomerase* (*fkbp7*), a member of a gene family involved in protein folding, and *ribonuclease P protein subunit p21* (*rpp21*), which encodes a tRNA processor. Additionally, *dual specificity protein phosphatase 26* (*dusp26*), involved in promoting cell proliferation by inhibiting apoptosis in human tumors (Yu *et al.* 2007), was also up-regulated in the 2.4 g group.

Tissue remodeling, previously implicated in the sensitivity of Atlantic salmon to *L. salmonis* (Skugor *et al.* 2008) requires cell motility, an identified function in our Gene Ontology analysis (Table 6). The qRT-PCR analysis confirmed involvement of the tissue remodeling function in the response to louse infection; matrix metalloproteinase family members *mmp9* and *mmp13* were up-regulated due to infection in the 0.3 g group by 2.5 fold and 2.9 fold ($p < 0.02$),

respectively, and in the 0.7 g group by 3.3 fold ($p < 0.02$) and 6.4 fold ($p < 0.01$), respectively (Figure 2). *Tissue inhibitor of metalloproteinase-2* (*timp2*) did not show differential regulation in these size groups. However, consistent with microarray results, *timp2* was down-regulated in the 2.4 g group (FC = 1.5; $p < 0.01$), as was *mmp13* (FC = 3.4; $p < 0.02$). The similar expression of the matrix metalloproteinases in the 0.3 g and 0.7 g groups are one of the only strong similarities between the responses among these two size classes identified in this investigation.

Immune-relevant transcripts were differentially regulated only in the 2.4 g pink salmon (Figure 2c). *Nucleolysin* (*tia1*), involved in cell-mediated killing, was up-regulated (FC = 1.3; $p < 0.01$). *Gamma-interferon-inducible lysosomal thiol reductase* (*ifi30*) was selected from microarray results as a possible Th2 response gene. This transcript showed a high level of individual variation in the qRT-PCR results (Figure 2c), and did not pass significance testing, although it was close ($p = 0.056$). *Superoxide dismutase-1* (*sod1*), a destroyer of toxic cellular radicals was selected to investigate the role of reactive oxygen species. This transcript remained unchanged in response to infection in all size groups.

2.5 Discussion

2.5.1 Transcriptome response overview and comparison

L. salmonis infections resulted in large transcriptome perturbations in all size classes of pink salmon investigated (Table 1). At 6 dpe, primary responses of the different size classes to the lice infection were largely different when compared to each other, as identified in differentially expressed gene lists (Figure 1), enriched GO functional categories (Table 5, Table 6, Table 7), and qRT-PCR expression profiles (Figure 2). This emphasizes the importance of considering each size class independently for louse sensitivity, including the 0.7 g and 2.4 g size classes, which did not show significant variation in mortality rates (Jones *et al.* 2008). Similarities among responses, although rare, also provide insight into the gradual development of resistance to the

parasite as the salmon develops from sensitive (0.3 g) to competent (2.4 g). Limitations in the experimental design included the absence of the 2.4 g size class in the reference channel and multi-tissue sampling, which may have allowed the capture of varying ratios of tissues among the different size groups. These limitations could confound cross-size class comparisons, however should not hinder within-size class comparisons. Additionally, one of the problems with both microarray and PCR analyses is the potential for cross-hybridization, or cross-reactivity, with other gene family members or duplicates, of particular relevance in the pseudotetraploid salmonids. For example, expression profiles of *titin* did not correspond completely to those identified in microarray results, which likely occurred due to alternate forms of the transcript cross-hybridizing to the microarray. Although many precautions were taken to eliminate this problem in qRT-PCR methods, such as the use of direct amplicon sequencing and qRT-PCR melt curve analysis, the complete genome is not yet known and we cannot fully eliminate the possible influence of this confounding factor.

Transcriptome profiling enabled the identification of potential detrimental effects in the 0.3 g infected pink salmon during the first week of infection that were therefore attributable to the attached copepodid stages of *L. salmonis*. This early time point of sampling was selected to investigate the primary responses to the infection, and should capture primary innate immune responses such as those identified early in the infection period in other Pacific salmon (Johnson & Albright 1992). Additionally, the selection of this early time point allowed for the sampling of a broad range of responders, and thus prevented the confounding variable of only profiling the more robust surviving fish, which would have been more probable at later time points, particularly in the smallest size class.

Response trends in the sensitive 0.3 g pink salmon included inhibition of cell proliferation through the integration of cell stress signals accompanying inflammation and tissue remodeling. Main trends in the response of the competent 2.4 g pink salmon included growth and cell motility, with some immune response. The 0.7 g size class shared tissue remodeling with the 0.3 g pink salmon, although without inhibited cell proliferation, and shared cell motility with the 2.4 g pink salmon.

2.5.2 Sensitivity and growth

Juvenile 0.3 g pink salmon are sensitive to low-level, attached *L. salmonis* infections with indicators of stress-associated transcriptional profiles identifiable within a week of infection initiation (6 dpe). This stress is mainly identified by cell proliferation inhibition, and is followed later by mortality and decreased weight gain, which become evident between 13 and 37 dpe (Jones *et al.* 2008). The expression profiles of transcripts related to cell cycle progression and Gene Ontology enriched categories (Table 3 and Table 5) indicate decreased proliferation. Profiles of up-regulated cell proliferation inhibition-related transcripts in the qRT-PCR analysis of the 0.3 g size class provide further evidence for this function (Figure 2). These genes include up-regulation of *sesn1*, which can occur during *in vitro* cell starvation (Velasco-Miguel *et al.* 1999), and up-regulation of the *in vitro* cell proliferation suppressor *tob1*, which can indicate unresponsive T-cells (Maekawa *et al.* 2002; Tzachanis et al., 2001). Additionally, up-regulation of *btg1*, a member of the same anti-proliferative family as *tob1*, has been shown to be up-regulated at G0/G1 halting and down-regulated as the cells move through G1 (Rouault *et al.* 1992). CDKN2b binds and inhibits Cdk2, an important G1/S transition protein, and the up-regulation of its transcript can indicate cell cycle arrest induced by interferon-α (Sangfelt *et al.* 1999) or by TGF-β in keratinocytes (Reynisdóttir *et al.* 1995). Differential expression of such a

diverse range of transcripts towards a function of anti-proliferation may be indicating that cell cycle events are being integrated with the transcriptional machinery in the juvenile infected pink salmon. Integration for cell cycle proliferation induction can be conducted by transcription factors (TF), such as DRTF1/E2F, which can be activated by synergistic interactions of TFDP1 and E2F-1 (Bandara *et al.* 1993). We investigated the expression of *tfdp1*, as a reduction of this signal could potentially occur through reduction of these interacting factors, but the expression was not significantly different from control (Figure 2a; FC > 1.5; $p = 0.064$). Transcription changes in the 0.3 g juvenile pink salmon indicate cell stress, which is not evident in the larger size groups tested.

Cell stress and decreased proliferation in the 0.3 g juvenile pink salmon may be derived from parasite-induced nutrient diversion, and therefore the sensitivity in this size group could be related to a lack of nutrients during a life stage of rapid growth (Heard 1991). From the results presented here, it is not possible to detect if nutrients are being directly lost to the parasite or are being indirectly lost due to host responses to the parasite. Both are possible, as parasitism can directly reduce growth (Fernandez & Esch 1991), as can host responses to parasitism, such as long term cortisol exposure and chronic inflammation (Barton *et al.* 1987; DeBenedetti *et al.* 1997). Nutrient limitation or inappropriate nutrient partitioning at a key growth stage may be contributing to the sensitivity of this size group.

Inappropriate nutrient partitioning may occur via increased inflammation or tissue remodeling. These processes are indicated in the 0.3 g size group by enriched Gene Ontology categories, and by the up-regulation of *mmp9* and *mmp13* (Figure 2). In the susceptible Atlantic salmon, *L. salmonis* infections may become chronic due to increased inflammation and matrix metalloproteinase-dependent tissue remodeling without accompanying cell proliferation (Skugor

et al. 2008). *mmp9* has been shown to have a role in both the initiation and resolution of inflammation in teleosts (Chadzinska *et al.* 2008). Increased expression of *mmp9* and *mmp13* in the 0.3 g infected pink salmon accompanies the aforementioned cell proliferation inhibition (Figure 2a). Furthermore, although these two transcripts were up-regulated to a greater extent in the 0.7 g infected pink salmon, the accompanying cell proliferation inhibition was absent (Figure 2b), as was the mortality and weight gain (Jones *et al.* 2008). Inflammation with inhibited cell proliferation appears to be a distinguishing feature of the incompetent response to *L. salmonis* in the susceptible 0.3 g size class of pink salmon.

Metabolism and growth in the 0.7 g and 2.4 g size classes do not mirror the responses of the younger 0.3 g size group in response to the *L. salmonis* infection. The infected 0.7 g pink salmon display changes to nutrient processing transcripts, with enriched Gene Ontology categories digestion and hydrolase activity (Table 6), and stable expression of qRT-PCR-investigated cell proliferation-related transcripts (Figure 2b). The differential transcriptome of the 2.4 g infected pink salmon included genes involved in protein stabilization and cell proliferation. For example, up-regulation of *protein phosphatase 1L* and *Cdc37*, and down-regulation of *timp2* were identified with high fold change (Table 4). Up-regulation of *Cdc37*, a co-chaperone of Hsp90, is important for cell growth (Hunter & Poon 1997). *timp2* up-regulation can block EGF-mediated mitogenic signaling (Hoegy *et al.* 2001), and it is therefore interesting to note the down-regulation in the 2.4 g salmon (Figure 2c). Additionally, the 2.4 g infected salmon showed up-regulation of protein folding family member *fkbp7*, cell proliferation promoter *dusp26*, and tRNA processor *rpp21* (Figure 2c). Cell proliferation inhibition transcripts identified from the 0.3 g infected salmon were either static, or down-regulated in the 2.4 g infected salmon, including down-regulation of both *sesn1* and *btg1* (Figure 2c). *sesn1* down-regulation can occur when

androgen receptors are activated by the ligands testosterone or dihydrotestosterone (Wang *et al.* 2006), and progression into G1 phase requires down-regulation of *btg1* (Rouault *et al.* 1992).

In addition to the differential expression of the aforementioned growth-related transcripts, one population of infected 2.4 g pink salmon was found to have a slight, but significant increase in weight gain at the end of the 37 day infection trial (Jones *et al.* 2008). More work would need to be done to confirm this apparent increased growth. All pink salmon from this exposure were planktivorous, and would feed on copepods in their natural environment (Heard 1991). The 2.4 g size class may have gained nutrients by feeding on lice introduced to the tank during the exposure, although the salmon were sedated at the time of the exposure and no filter was in place to keep lice in the tank after flow rates were resumed. The activity of the aforementioned growth-related transcripts in the 2.4 g salmon six days after the exposure indicates that this growth increase is continuing throughout the exposure, after all unattached copepodids were presumably removed from the tank by flow rates. Grazing of mobile *L. salmonis* by cleaner fish, such as threespine stickleback (*Gasterosteus aculeatus*) has been documented (Losos *et al.* 2010), but we are not aware of any cases of salmon grazing *L. salmonis* off of each other. Additionally, the lice present at 6 dpe are attached copepodids, thus much smaller than those in the aforementioned stickleback study.

Alternately, the infection with *L. salmonis* after a size threshold may be inducing a parasite-induced growth in the larger juvenile pink salmon. Low-level parasite infections have been shown to increase growth rates of immature hosts (reviewed in Phares 1996), including threespine stickleback infected experimentally with a cestode parasite (Arnott *et al.* 2000), and naturally-infected developing whitefish (*Coregonus laveratus*; Pulkkinen & Valtonen 1999). In host-castrating systems, increased host growth only occurs in instances of infection prior to

reproductive maturity (Keas & Esch 1997). In non-castrating systems, low-level infections and sympatric host-parasite systems result in the fastest parasite-induced host growth (Ballabeni 1994). Interestingly, the 2.4 g pink salmon in this study meet all of the above criteria necessary for parasite-induced growth. Furthermore, in other host-parasite systems, changes to host growth can occur through parasite-derived secreted growth hormone receptor agonists (Phares 1996). As some bioactive substances have already been identified and characterized in *L. salmonis* secretions (Fast *et al.* 2007a), this could be an avenue of further research if other work supports this association between infection and increased growth. It would also be useful to investigate relative host reproductive success in the previously-infected and control individuals upon reaching reproductive maturity, as parasite-induced growth can occur at a cost to reproductive fitness (Baudoin 1975). Additionally, in louse exposure experiments utilizing lethal sampling, gut content analysis would be helpful to understand the extent of the differential feeding of these fish on the infecting ectoparasite.

Increased growth is not observed in the 0.7 g infected pink salmon. The response of the 0.7 g size class is less apparent, but appears to be a transitional stage between the reduced growth (0.3 g) and the increased growth (2.4 g) stages. The transcriptomic responses of the 0.7 g and 2.4 g pink salmon do not indicate systemic stress, as is suggested from the 0.3 g infected pink salmon.

2.5.3 Immunological responses

Salmon immune-modulation has been documented *in vitro* resulting from *L. salmonis* secretions (Fast *et al.* 2007a). The identified up-regulation of matrix metalloproteinase expression in the 0.3 g and 0.7 g pink salmon may be due to immune modulation caused by parasite secretions. Prostaglandin E_2 (PGE_2), a component of louse secretions (Fast *et al.* 2004) induces increased

mmp9 expression in mice (Yen *et al.* 2008). Louse secretions are elicited by host skin mucus and are variable depending on host species (Fast *et al.* 2003). If the release of PGE_2 is equal among members of the same host species, smaller fish are potentially subjected to higher plasma concentrations and increased downstream effects. However, to conclusively say if this was a factor, more work would need to be done.

The identification of *ferritin* up-regulation in the 0.7 g infected pink salmon may be indicative of iron sequestering to prevent parasitic acquisition. Previous work has shown an increase in *transferrin* in adult Atlantic salmon in response to the louse infection, and it was hypothesized as a method of reducing iron availability to the parasite (Easy & Ross 2009).

Cellular motility and immune response appear to be affected by the infection in the 0.7 g and 2.4 g pink salmon, possibly indicating immune cell migration, wound healing or both. Although similarities between host responses of the different size classes are rare, differentially expressed cell motility transcripts were identified in both the 0.7 g and 2.4 g groups. Additionally, Gene Ontology categories involving cell motility were enriched in both the 0.7 g and the 2.4 g infected salmon (Table 6, Table 7), and one of the pathways enriched in the 2.4 g transcriptome profile included cell adhesion molecules (CAMs), involved in anchoring migrating cells to a target.

Although cell motility activity was present in the transcriptome response, few direct effectors of immune responses were identified. Of the immune-related genes investigated with qRT-PCR, most had high variation among individuals, including *IFN-γ-induced lysosomal thioreductase* (*ifi30*) and did not pass significance testing (Figure 2; FC > 2; $p = 0.056$). IFI30 denatures proteins by reducing disulfide bonds, and may have a role in antigen processing in antigen presenting cells (Arunachalam *et al.* 2000). Significantly up-regulated in the 2.4 g

infected salmon was *nucleolysin* (*tia1*), an indicator of neutrophil nucleolytic activity and of the induction of apoptosis in cytotoxic T lymphocyte target cells (Tian *et al.* 1991). Neutrophils have a role in the defense responses of coho salmon to sea lice (Johnson & Albright 1992).

2.6 Conclusions

Pink salmon size classes responded differently to *L. salmonis* infections, and susceptibility markers in the 0.3 g salmon appeared early in the response to the smaller and less damaging attached stages of lice. Susceptibility may be related to nutrient diversion at a rapidly growing life stage, and energy may be diverted to accommodate incompetent immune responses and tissue remodeling. Although 0.5 g pink salmon develop a protective response after vaccination with bacterial antigens (Johnson *et al.* 1982), immune responses of the 0.3 g infected fish did not parallel those measured in the larger size groups; inflammation and tissue remodeling may be exacerbating the effects of infection. Host immune modulation by bioactive agents present in sea louse saliva may also play a role in the susceptibility, as the smaller fish may experience higher concentrations due to size ratios. Important roles of cell motility and nucleolytic activity were evident in the responses of the larger size classes of pink salmon. Additionally, *L. salmonis* infection may result in an increased growth rate of the juvenile pink salmon after they pass a certain size threshold, as was observed in the 2.4 g pink salmon. We have generated and analyzed large transcriptome datasets reflective of juvenile pink salmon responses to *L. salmonis* infections, and in doing so, have increased our understanding of the complexity of this ecologically-relevant host–parasite system.

2.7 Chapter acknowledgements

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2.8 Online material

Supplementary data to this chapter can be found online at doi:10.1016/j.cbd.2011.04.001

Chapter 3: Comparative transcriptomics of Atlantic *Salmo salar*, chum *Oncorhynchus keta* and pink salmon *O. gorbuscha* during infections with salmon lice *Lepeophtheirus salmonis*

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SRMJ conceived of the study, designed the experiment, assisted in analysis, collected and cultured lice and fish, conducted exposures, collected tissues, and measured hematocrit.

BFK conceived of the study, designed the experiment and assisted in analyses.

3.1 Abstract

Salmon species vary in susceptibility to infections with the salmon louse (*Lepeophtheirus salmonis*). Comparing mechanisms underlying responses in susceptible and resistant species is important for estimating impacts of infections on wild salmon, selective breeding of farmed salmon, and expanding our knowledge of fish immune responses to ectoparasites. Herein we report three *L. salmonis* experimental infection trials of co-habited Atlantic *Salmo salar*, chum *Oncorhynchus keta* and pink salmon *O. gorbuscha*, profiling hematocrit, blood cortisol concentrations, and transcriptomic responses of the anterior kidney and skin to the infection.

In all trials, infection densities (lice per host weight (g)) were consistently highest on chum salmon, followed by Atlantic salmon, and lowest in pink salmon. At 43 days post-exposure, all lice had developed to motile stages, and infection density was uniformly low among species. Hematocrit was reduced in infected Atlantic and chum salmon, and cortisol was elevated in infected chum salmon. Systemic transcriptomic responses were profiled in all species and large differences in response functions were identified between Atlantic and Pacific (chum and pink) salmon. Pink and chum salmon up-regulated acute phase response genes, including complement and coagulation components, and down-regulated antiviral immune response genes. The pink salmon response involved the largest and most diverse iron sequestration and homeostasis mechanisms. Pattern recognition receptors were up-regulated in all species but the active components were often species-specific. *C-type lectin domain family 4 member M* and *acidic mammalian chitinase* were specifically up-regulated in the resistant pink salmon.

Experimental exposures consistently indicated increased susceptibility in chum and Atlantic salmon, and resistance in pink salmon, with differences in infection density occurring within the first three days of infection. Transcriptomic analysis suggested candidate resistance functions including local inflammation with cytokines, specific innate pattern recognition

receptors, and iron homeostasis. Suppressed antiviral immunity in both susceptible and resistant species indicates the importance of future work investigating co-infections of viral pathogens and lice.

3.2 Introduction

The global salmon aquaculture industry is challenged by infections with endemic ectoparasitic sea lice such as *Lepeophtheirus salmonis*, *Caligus clemensi*, *C. rogercresseyi* and others. In the Northern Hemisphere, the salmon louse *L. salmonis* has the largest impact (Johnson *et al.* 2004) and must be properly managed to prevent excessive infections and possible damage to wild salmon populations (Costello 2009b). Lice disperse as free-swimming nauplii and molt to infective copepodids which attach to a host, develop through later stages and feed on skin and mucus (Pike & Wadsworth 1999). Motile pre-adult/adult stages are the most damaging to tissues due to large size and aggressive feeding (Grimnes & Jakobsen 1996). While lice infections occur regularly on wild salmon (Nagasawa *et al.* 1993; Beamish *et al.* 2005; Beamish *et al.* 2009) disease can occur at higher parasite intensities (Wagner *et al.* 2008) or when hosts are at a sensitive life stage (Jones *et al.* 2008; Sutherland *et al.* 2011). During infection, the feeding louse elicits a cortisol response in the host (Pickering & Pottinger 1989; Bowers *et al.* 2000; Bjørn *et al.* 2001). Experimental cortisol implants reduce inflammation and increase susceptibility of otherwise resistant coho salmon *Oncorhynchus kisutch* (Johnson & Albright 1992) and reduce wound repair of Atlantic salmon *Salmo salar* (Krasnov *et al.* 2012). Furthermore, louse-derived compounds secreted at the site of attachment can be immunomodulatory (e.g., trypsin-like proteases and prostaglandin E_2; Fast *et al.* 2004; Fast *et al.* 2005; Fast *et al.* 2006; Fast *et al.* 2007a) and may facilitate secondary infections.

Salmon lice display increased rates of attraction to and settlement onto susceptible hosts, and are rejected less throughout the infection (Jones 2001). The host may incur reduced growth and/or mortality (Jones *et al.* 2008). Susceptibility varies among salmon genera and species, and occurs through host (e.g., behavioral, physiological, immunological) and parasite factors (e.g.,

physiological, host preference). Coho salmon are considered resistant and rapidly reject lice by innate local inflammation with neutrophils (Johnson & Albright 1992). Also considered resistant are pink salmon *Oncorhynchus gorbuscha* in which early rejection correlates with pro-inflammatory cytokine expression, whereas chum salmon *O. keta* are considered susceptible based on the delay or absence of rejection of *L. salmonis* following laboratory infections (Jones *et al.* 2007). Atlantic salmon are also considered susceptible to infection and responses to the parasite in this host favour a Th2 subset with limited inflammation, leading to chronic infection (Skugor *et al.* 2008; Tadiso *et al.* 2011). Although important for parasite rejection in the resistant host, inflammation and Th1 cellular responses can be costly and lead to self-damage (Rolff & Siva-Jothy 2003; Luger *et al.* 2008). Balancing resistance with tolerance (e.g., Medzhitov *et al.* 2012) may play an important role in competent responses to lice.

Heritable variation in susceptibility to *L. salmonis* and *C. elongatus* has been identified in populations of sea trout and Atlantic salmon (Mustafa & MacKinnon 1999; Glover *et al.* 2003; Glover *et al.* 2004) indicating the potential for selective breeding towards increased resistance in farmed fish (Gjerde *et al.* 2011) and thus reducing requirements for chemical treatments (Jones *et al.* 2002). Identifying genes or pathways involved in competent responses will be important for this process. Variation in the response profiles of candidate cytokines and other immune genes to adult *L. salmonis* was reported in the skin of Atlantic, chum, and pink salmon (Braden *et al.* 2012) confirming the importance of skin as an immunological tissue of fish (Ángeles Esteban 2012). Here we report a series of controlled exposure trials in which the relative susceptibility of juvenile Atlantic, chum, and pink salmon is confirmed and their physiological responses partially characterised throughout the development cycle of the parasite. In each species, transcriptome profiling of skin and anterior kidney using a recently developed microarray (Jantzen *et al.* 2011a)

assessed mechanisms elicited over nine days following exposure to the parasite to better understand processes associated with resistance and susceptibility.

3.3 Methods

3.3.1 Animals and Exposure

Pink and chum salmon were obtained as swim-up fry (<0.5 g) from the Quinsam River and Nanaimo River hatcheries, respectively, on Vancouver Island, British Columbia. Atlantic salmon (20 - 35 g) were obtained from a commercial freshwater hatchery on Vancouver Island. Prior to experimentation, fish were reared in 400 L tanks in flowing water that was an equal mixture of aerated freshwater and seawater and fed a diet of commercial salmon pellets at a daily rate of 1.0% biomass. The photoperiod was regulated to mimic seasonal variation, ranging from 16 light: 8 dark in summer to 8: 16 in winter. Seawater used for fish maintenance and experimentation was pumped from Departure Bay and sand-filtered to approximately 30 μm with a mean salinity of 29.5 ± 0.5‰ and mean dissolved oxygen of 9.5 ± 0.5 mg/L. The seawater temperature displayed seasonal variation as indicated below. Ovigerous *Lepeophtheirus salmonis* were collected from adult Atlantic salmon following harvest from a farm near Vancouver Island and transported in ice cold aerated seawater to Nanaimo. Dissected egg strings were incubated in filtered and ultraviolet irradiated seawater at 9.5 ± 1.0 C and 29.5 ± 0.5‰ salinity, with supplemental aeration, as described previously (Jones *et al.* 2006). Cultured lice were monitored by daily microscopic examination of triplicate samples and an inoculum containing a known number of copepodids was prepared when the ratio of copepodid to nauplius II stages was greatest.

Three trials were conducted to characterize the infection over the life cycle of *L. salmonis*. In Trials 1-3, the mean seawater temperature was 10.5, 11.5 and 8.5 °C, respectively, reflecting ambient conditions in early November (Trials 1 and 2) and from mid-January to late February (Trial 3). All fish were acclimated to full-strength seawater a minimum of 10 days prior to

exposure to *L. salmonis*. In Trial 1, 10 individuals from each species (approx. 45-70g) were randomly allocated to each of eight seawater tanks. A total of 5014 copepodids (167/fish) were added to each of four tanks using the metomidate hydrochloride (Aquacalm, Syndel Laboratories Ltd.) sedation exposure method described previously (Jones *et al.* 2006). In Trial 2, 15 individuals of each species (approx. 40-70g) were randomly allocated to 4 tanks. A total of 7,335 copepodids (163/fish) were added to each of two tanks as described above. In Trial 3, 12-15 individuals of each species (approx. 50-80g) were randomly allocated to each of four tanks. A total of 8,900 copepodids (199/fish) were added to each of two tanks as described above. In each trial, salmon co-habiting in control tanks were treated the same as exposed fish without the addition of copepodids. In Trial 1, all fish were sampled from one exposed and one control tank at three, six, nine and 12 days post-exposure (dpe). For sampling, salmon were sedated with 0.5 mg/L metomidate, immersed in 200 mg/L MS-222 until immobile and killed with a blow to the head, as previously described (Jones *et al.* 2007). In subsequent trials, fish were sampled as above, but at seven and 14 dpe (Trial 2) and at 28 and 43 dpe (Trial 3). All processing was performed rapidly: each fish was measured for fork length, weight, and lice count and lice were stored in 95% ethanol for later assessment of development stage (Jones *et al.* 2006). Blood was collected from the caudal peduncle into heparinated tubes. In Trial 1, the left pectoral fin and approximately 7 mm of the anterior kidney were rapidly dissected from each fish, flash frozen separately in liquid nitrogen, then stored at -80 °C until RNA extraction. In Trial 1, blood was centrifuged (3,000 RPM, 20 minutes) and plasma collected and stored at -80 °C for cortisol quantification. For Trial 2 and 3, blood was centrifuged for 3 minutes (11,700 RPM, Autocrit Ultra 3, Becton Dickinson) and hematocrit measured immediately. Use of research animals

complied with Fisheries and Oceans Canada Pacific Region Animal Care Committee protocol numbers 06-004 and 09-001.

Total RNA was extracted from fin and kidney samples in Trial 1 using Trizol (Invitrogen), as per manufacturer's instructions, and purified using RNeasy spin columns (QIAGEN), by manufacturer's instructions with the on-column DNase I digestion. The RNA was quality checked by agarose gel electrophoresis and quantified by spectrometry (NanoDrop-1000).

3.3.2 Cortisol, weight and hematocrit analyses

Cortisol levels in plasma were tested by immunoassay of 20 µl samples (Parameter™, R&D Systems). Samples were run in duplicate, and a standard curve and interplate calibrator sample was run on each plate. All samples were within the high range of the standard curve and the reported minimum detectable limit of the kit (R&D Systems).

For each species, data analysis of cortisol concentration, fish weight, and hematocrit levels were performed using a linear models in the statistical environment R (v2.14.1; R Development Core Team 2012) using day and exposure (with interactions) as explanatory variables. Significance between groups was tested using post-hoc Tukey's HSD tests between conditions of interest.

3.3.3 cDNA synthesis and microarray preparation

Total RNA samples were randomized and 200 ng total RNA of each sample was reverse transcribed to cDNA and amplified to labelled-cRNA using Low Input Quick Amp labelling kits as per manufacturer's instructions (Agilent). A reference pool was synthesized using equimolar amounts of Cy3-cRNA from each species/day/infection class condition to hybridize alongside experimental samples to control for hybridization difference in a common reference design (Churchill 2002; 19 samples used in reference pool). Experimental samples (labeled with Cy5)

included 9-11 biological replicates for the infected individuals and 9-10 biological replicates for time-matched controls. Anterior kidney samples for Atlantic and pink salmon were compared at days 3, 6 and 9 post infection, and for chum salmon at day 6 only (total number of infected or control samples at all days = 57, 20, and 60 for Atlantic, chum, and pink, respectively). Skin samples were profiled only at day 6 post infection (total number of samples for both infected and time-matched controls = 18, 20 and 19 for Atlantic, chum, and pink, respectively). Samples were hybridized to randomized-order cGRASP 4x44k salmonid arrays (Koop *et al.* 2008; Jantzen *et al.* 2011a; Agilent eArray AMADID: 025055) as per manufacturer's instructions and slides were washed using stabilization solution to minimize ozone-related problems (Agilent; Sutherland *et al.* 2012). Slides were kept in the dark in a low ozone atmosphere and scanned on a ScanArray Express (Perkin Elmer) at constant PMT settings to produce saturated median values for ~1% of spots (Cy3:80; Cy5:75). Images were quantified using Imagene (v8; BioDiscovery) and poor or empty spots were flagged. Per block, a background correction for all probes was performed by subtracting the block's average signal median value for negative control spots from each probe. Sample files were then imported into GeneSpring (v11; Agilent), and normalized by intensity-dependent *Lowess* normalization (Agilent).

For each species and tissue experiment, (e.g., Atlantic salmon, anterior kidney) filters were applied to retain probes for which 65% or more of all samples within at least one condition had background-corrected raw expression values ≥500 in both channels and flag values for each channel as 'present'. For statistical tests, a probe was deemed differentially expressed if it passed a Benjamini-Hochberg multiple test corrected p-value ≤ 0.05 and fold change ≥ 1.5. In experiments with a time component (e.g., Atlantic or pink salmon head kidney), a 2-way ANOVA was used to detect probes with a significant effect of infection and those with a time-

infection interaction effect. Probes with a main effect but no interaction effect were filtered to retain only those that varied by 1.5 fold between control and experimental for at least one of the three time points. Probes with a significant time by infection interaction were filtered at each time point (FC >= 1.5). Principal component analysis of samples based on gene expression levels was performed in GeneSpring using all quality control filtered probes (Agilent). Enrichment analysis of up- or down-regulated gene lists was performed using the DAVID bioinformatics platform (Huang *et al.* 2009) using a background list specific to each species (all entities passing quality control filter for each experiment). Overlap between differential lists was evaluated using VENNY (Oliveros 2007).

3.3.4 Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Purified total RNA used for the microarray experiments was also used to generate cDNA for reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer's instructions. Each cDNA sample was diluted 20-fold. To ensure efficiency in all species and tissues, a standard curve was generated for each species and tissue (n = 6 dilution series) using pooled equimolar amounts from three samples from each condition, diluting the pool 10-fold and then producing a 5-fold dilution series (six points). All primers had efficiency values within the range of 90-110% for all three species. qPCR amplification was performed using SsoFastTM EvaGreen® (Bio-Rad) in 20µL reactions in an MX3000P (Agilent) as previously described (Sutherland *et al.* 2012), with the exception of running triplicate technical replicates. Genes of interest were selected based on relevance to the study system, presence in enriched functional categories, high significance or fold change, and relevance to multiple tissues. Reference candidates were selected based on other studies, unchanging expression in infected/control individuals in microarray analysis and

moderate expression levels in all three species and tissues. Primers were designed in Primer3 (Rozen & Skaletsky 2000) selecting amplicon sizes of 80-150 bases. Amplicons were checked for single products by melt curve analysis, and were sequenced to confirm identity as previously described (Sutherland *et al.* 2011).

Data analysis was performed using qbasePLUS (Biogazelle) and reference gene stability was tested using geNorm (Vandesompele *et al.* 2002). The three most stable reference genes chosen for the current analysis in all species and all tissues were *dynein light chain 1 cytoplasmic*, *U6 snRNA-associated Sm-like protein lsm8*, and *mRNA turnover protein 4 homolog* with collective M (and CV) values for Atlantic, chum, and pink anterior kidney and skin of 0.321(0.129) and 0.349(0.141), 0.413(0.175) and 0.421(0.455), and 0.254(0.101) and 0.280(0.111), respectively. These values are within the range typically observed for stably expressed reference genes in heterogeneous samples (Hellemans *et al.* 2007). *Eukaryotic translation initiation factor 4h* was also tested but was less stable than the above selected genes and therefore not used. A minimum of 2 technical replicates were found to be within 0.5 Ct for all samples. The interplate calibrator used to compare across plates within a gene had a <0.5 Ct difference for all genes within each species. NTC and -RT controls showed no amplification. Significance for Atlantic and pink salmon anterior kidney was determined by two-way ANOVA, and for all other infected/control comparisons with only one time point by t-test in R (R Development Core Team 2012). All normalized relative expression values were log10 transformed prior to statistical analysis. Correlation between methods were checked using linear best fit lines of log2 expression values for samples measured by RT-qPCR against microarray (using the microarray probe corresponding to the contig used for primer design).

Several immune system genes not present on the array, but identified as louse response genes (Fast *et al.* 2007b; Braden *et al.* 2012) were included in an additional qPCR analysis including *interleukin-1beta*, *interleukin-8*, *prostaglandin D synthase* and *tumour necrosis factor alpha*. For these immune genes, a randomly selected subset of the samples used for the full study were used to test for expression differences (n = 5-7 samples per condition). Expression was relative to the geometric mean of *dynein light chain 1 cytoplasmic* and *eukaryotic translation initiation factor 4H*. Each primer was evaluated for each species individually as described above except that only one tissue was tested for efficiency, and standard curves were approximately in the range of the sample values. All technical replicates were within 0.5 Ct for 224/228 combinations. Primer thermal regimes were reported previously (Braden *et al.* 2012).

3.4 Results

3.4.1 Infection density and louse development

Infection density (lice per host weight (g)) in Trial 1 was highest in chum salmon, followed by Atlantic salmon and lowest in pink salmon ($p < 0.00001$) (Figure 3A). Trials 2 and 3 also showed this relative difference, with chum having the highest infection density on day 7 and 28, and chum and Atlantic salmon having equally high infection densities on day 14 (Figure 3B). By day 43 all lice were motile (Figure 4) and the infection density was reduced and equalized among species. Infection intensity (lice per fish) is also reported in Figure 3, and follows the same trend as the infection density.

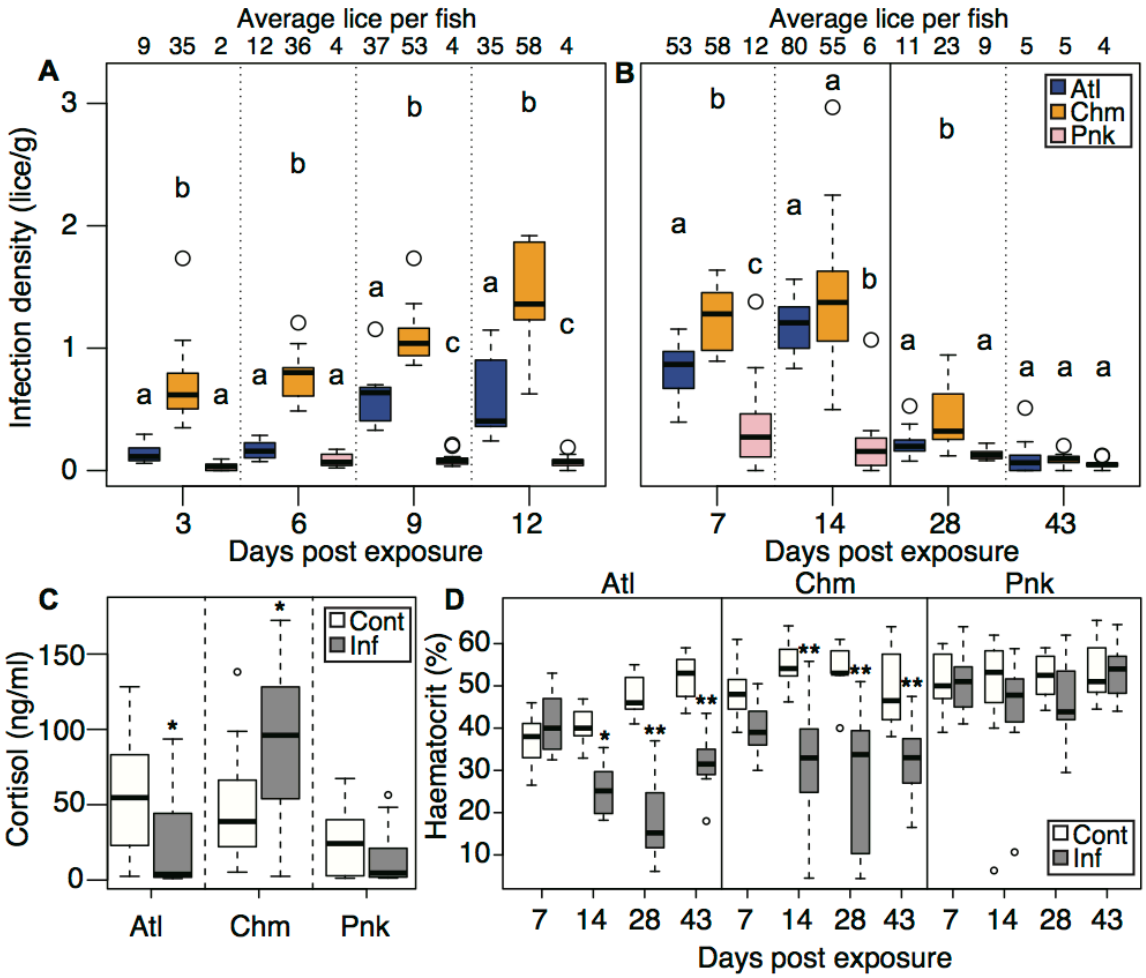

Figure 3. Infection densities and blood parameters

Co-habiting Atlantic, chum, and pink salmon were exposed to copepodids in three experimental trials ((A) Trial 1, (B) Trial 2 and 3), resulting in highest infection density (lice per host weight (g)) in chum, followed by Atlantic, and lowest in pink salmon. Average lice per fish (LPF) for each condition are presented above the boxplot. Conditions within a day that do not share a letter are significantly different from each other. (C) Plasma cortisol (ng/ml) levels in Trial 1 (pooled for days three, six and nine post exposure) indicated elevated cortisol for chum salmon. (D) Hematocrit percentages for exposed Atlantic and chum salmon were reduced compared to controls at days 14, 28 and 43, and did not vary for pink salmon. Boxplot displays median and interquartile range, and circles are outliers. *denotes $p < 0.05$; **denotes $p < 0.001$

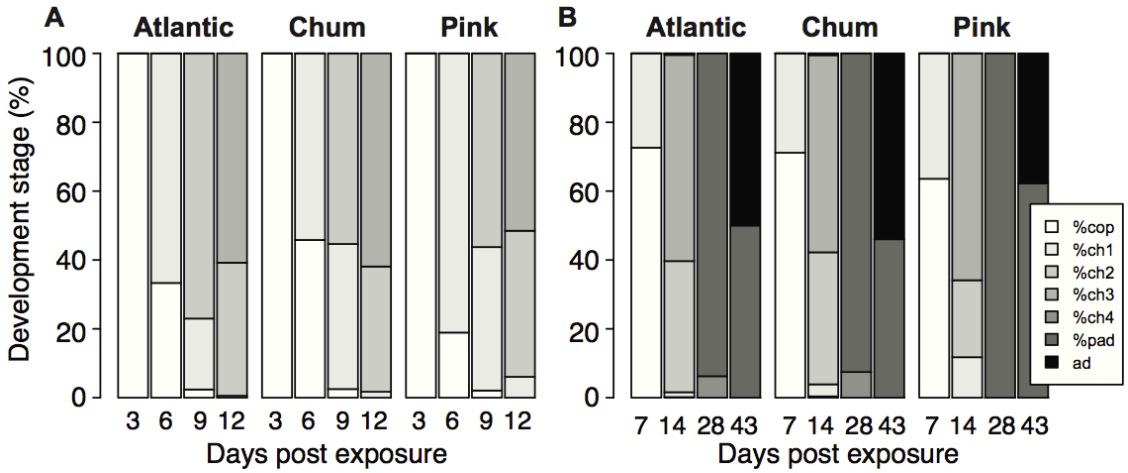

Figure 4. Louse development rates on all species

Development stages of lice as a percentage of the total lice found per day on each species for Trial 1 (A), and Trials 2 and 3 (B)

3.4.2 Fish weights, cortisol, and hematocrit

There was a reduction in weight gain in infected chum salmon relative to controls in Trial 1 ($p = 0.012$), but no differences were identified in Trials 2 and 3. No significant differences from controls in weight gain were identified for pink or Atlantic salmon in any trials.

No significant temporal effect was noted in the cortisol response, and so data was pooled for all days and compared between infected and control fish for each species (Trial 1; Figure 3C). Plasma cortisol was elevated in infected chum salmon relative to controls (1.75-fold; $p = 0.01$). Cortisol was not elevated in infected pink or Atlantic salmon relative to control individuals, although a reduction in cortisol occurred in infected Atlantic salmon compared to controls ($p < 0.01$). This reduction was largely driven by an elevation in control Atlantic salmon cortisol at 9 days post exposure (dpe).

Hematocrit was reduced in Trial 2 and 3 at 14, 28 and 43 dpe in infected Atlantic and chum salmon ($p < 0.01$; Figure 3D). Infected pink salmon hematocrit did not differ significantly from control individuals.

3.4.3 Multiple species utility of microarray

Initial normalization of anterior kidney data from all species indicated the largest difference in transcriptome profiles occurred at the genus and species level (principal components analysis PC1 = 63.85% and PC2 = 17.51%; Figure 5A), which would include species-specific differences in basal gene expression and probe hybridization efficiency. As a result, all species and tissues were separately normalized and comparisons between species were indirect (analysis was performed within a species then results compared across species). Normalized histograms (not shown) and the number of probes passing quality control filters for each species were similar (18096, 16716, and 16458 for Atlantic, chum, and pink salmon skin, respectively). Most of the uniquely annotated genes expressed in one species were detected in all three species (Figure 5B). However, to confirm species differences in expression profiles, qPCR was used to validate hybridization results (Chain *et al.* 2008) by using primers with approximately equal efficiency for all three species (Table 20).

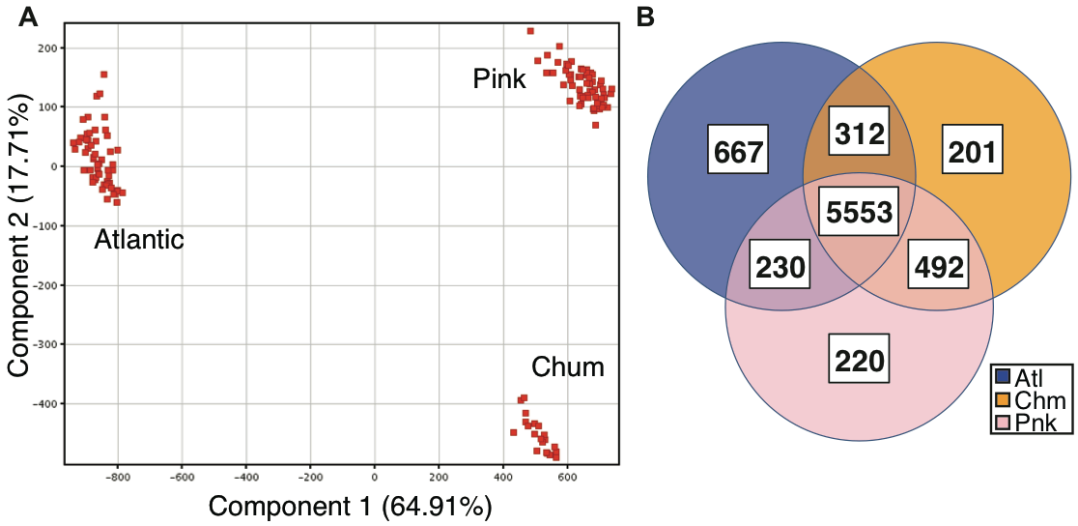

Figure 5. Multiple species utility of microarray

(A) When all species are normalized together, principal components analysis (PCA) indicates the largest variance between genus *Salmo* (PC1+) and *Oncorhynchus* (PC1-), and the second largest variance between species *O. keta* (PC2-) and *O. gorbuscha* (PC2+). The basal expression

differences captured by the PCA are due to both true biological differences and technical differences in probe hybridization efficiency between species. (B) When each species is normalized individually (6 dpe only) a similar quantity and identity passed quality control thresholds in all three species, with 5553 uniquely annotated transcripts present in all three species (union set of the Venn diagram). During differential expression testing, each species was therefore normalized separately, and indirectly compared. *Data shown*: *anterior kidney*

3.4.4 Anterior kidney transcriptomics: systemic responses of Atlantic, chum, and pink salmon

The louse infection affected gene expression in the anterior kidney of all species (Figure 6). Atlantic and pink salmon responses were profiled over nine days at three time points (3, 6, 9 dpe), but chum salmon were only profiled at 6 dpe. For each species, infection class (control or infected), and day combination, 9-11 individuals were profiled (i.e. total Atlantic, chum, and pink salmon anterior kidney samples profiled = 57, 20, 60, respectively). To keep sample numbers similar among species, the initial analysis was restricted to 6 dpe for all species. A similar number of uniquely annotated genes were differentially expressed at 6 dpe, and these were largely species-specific although some similarities were identified between pink and chum salmon (Figure 6A-B).

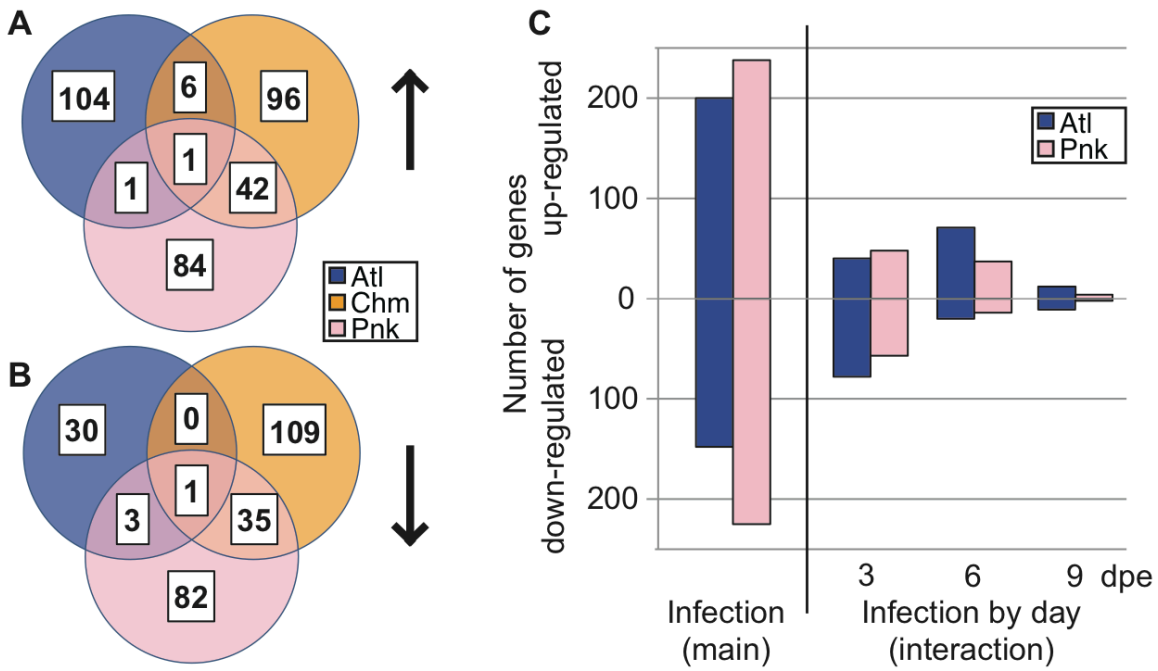

Figure 6. Anterior kidney transcriptome responses

At six days post exposure, anterior kidney responses varied depending on host species in either the (A) up-regulated or (B) down-regulated gene lists. Consistently more genes were shared between chum and pink salmon than with Atlantic salmon, including up-regulation of *hepcidin-1*, *prostaglandin E synthase 3* and down-regulation of antiviral response genes. (C) Most genes were identified with a main effect of infection (response independent of day post exposure). Of the genes responding with a time by infection interaction (response dependent on day) were mainly identified early in the response, at day three or six.

Time course data for Atlantic and pink salmon anterior kidney indicated the majority of differentially expressed genes responded similarly across the first nine days of infection (main effect infection), while a smaller subset responded differently depending on day (time by infection interaction; Figure 6C). Uniquely annotated genes responding in a similar manner across all days included 200 up- and 148 down-regulated genes in Atlantic salmon, and 238 up- and 225 down-regulated genes in pink salmon. For both species, most time-dependent genes were

specific to the early days of the infection (Figure 6C) and these genes were almost entirely exclusive to each species.

The protein folding response was up-regulated in the anterior kidney of all species ($p < 0.01$; Table 8). Unfolded proteins are typically an indicator of cellular stress (see Kültz 2005). Other up-regulated indicators of cellular stress included *stress-induced phosphoprotein 1* (Atlantic and pink), *damage inducible transcript 4-like* and *stress-associated ER protein 1* (chum), *growth arrest and DNA-damage induced protein gadd45 beta* (pink), *programmed cell death protein 10* (pink), *apoptosis induced factor 2* (pink), *stress-70 protein* (Atlantic) (Figure 7). Cyclin-dependent kinase inhibitors promote cell cycle arrest at G1 phase (Sherr & Roberts 1995). In Atlantic salmon, *cyclin-dependent kinase 4 inhibitor b* (*cdkn2b*) was highly up-regulated at day 6 and 9, and *cyclin-dependent kinase inhibitor 1c* was also up-regulated. *cdkn2b* induces cell cycle arrest in response to TGF-β (Hannon & Beach 1994). These genes were not differentially expressed in chum salmon, although the Pacific salmon specifically up-regulated *cyclin-dependent kinase inhibitor 1*, albeit not to the same extent as *cdkn2b* in Atlantic salmon (Figure 7). Energetic costs of the infection, whether from rejection or tolerance mechanisms are reflected in the enrichment of energy usage (ATP-binding $p < 0.05$; Table 8) in Atlantic and chum salmon up-regulated lists.

Table 8. Gene Ontology enrichment of systemic responses to lice infection

Selected Gene Ontology categories enriched in responses occurring generally over the nine days of infection in Atlantic, chum, and pink salmon anterior kidney.

		GO Term	No. Genes	p-value	Fold Enrich.
Atl up	BP	protein folding	13	1.39E-05	4.7
	MF	ATP binding	36	3.37E-04	1.8
	MF	metallopeptidase activity	7	1.00E-02	3.8
Atl down	BP	amine metabolic process	13	6.58E-05	4.0
	MF	enzyme inhibitor activity	7	2.70E-03	4.9
Chm up	BP	amine metabolic process	14	2.20E-05	4.2
	BP	protein folding	8	4.10E-03	3.9
	MF	ATP binding	22	2.62E-02	1.6
Chm down	BP	immune response	11	3.36E-04	4.0
	BP	response to virus	5	3.50E-03	7.7
	BP	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	4	3.08E-04	27.1
	MF	carbohydrate binding	8	4.10E-03	3.9
Pnk up	BP	protein folding	13	7.90E-05	4.0
Pnk down	BP	nitrogen compound biosynthetic process	15	8.42E-05	3.5
	BP	heme biosynthetic process	4	1.30E-03	16.9
	BP	erythrocyte development	4	1.30E-03	16.9
	BP	response to virus	5	2.05E-02	4.7
	BP	immune system process	16	3.37E-02	1.8

BP, biological process; MF, molecular function

		Atlantic			Chum	Pink		
FUNCTION	GENE	D3	D6	D9	D6	D3	D6	D9
Cellular stress and apoptosis	Programmed cell death protein 10	-	-	-	-	1.65	-1.02	1.21
	Stress-induced-phosphoprotein 1	2.11	-1.04	1.54	-	2.30	1.53	1.25
	Growth arrest and DNA-damage-inducible protein GADD45 alpha	-	-	-	-	1.46	1.51	1.13
	DNA-damage-inducible transcript 4-like protein	-	-	-	**9.27**	1.05	2.43	1.40
	Apoptosis-inducing factor 2	-	-	-	-	-1.08*	1.62*	1.08*
	TP53-regulated inhibitor of apoptosis 1	-	-	-	-	*-1.22**	*1.69**	*1.02**
	Stress-associated endoplasmic reticulum protein 1	-	-	-	2.09	-	-	-
	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	-1.05*	3.13*	1.55*	-	-	-	-
	Stress-70 protein, mitochondrial precursor	1.54	1.15	1.10	-	-	-	-
	Growth arrest-specific protein 8	-1.24	-1.22	-1.62	-	-	-	-
	p53 apoptosis effector related to PMP-22	-1.27	-1.30	-1.75	-	-	-	-
	Apoptosis inhibitor 5	1.58	1.08	1.10	-	-	-	-
	Cyclin-dependent kinase 4 inhibitor B	1.00*	4.15*	1.90*	-	x	x	x
	Cyclin-dependent kinase inhibitor 1C	*-1.44**	*1.72**	*1.16**	-	x	x	x
	Cyclin-dependent kinase inhibitor 1	x	x	x	1.61	*-1.55**	*1.52**	*1.20**
	G1/S-specific cyclin-D1	-	-	-	-	1.00	-1.70	-1.58
Prostaglandins and leukotrienes	Prostaglandin E synthase 3	2.12	1.18	1.16	1.83	**1.34**	**1.75**	**1.21**
	15-hydroxyprostaglandin dehydrogenase [NAD+]	-	-	-	-1.56	**-2.29***	**-1.54***	**-1.06***
	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	-	-	-	-	-1.17	2.17	1.48
	Leukotriene B4 receptor 1	1.83	1.41	-1.02	-1.61	x	x	x
FK506-binding	FK506-binding protein 2	-	-	-	**1.66**	-	-	-
	FK506-binding protein 5	2.28	1.78	1.05	**4.81**	2.41*	1.66*	-1.44*
Coagulation	Plasminogen activator inhibitor 1	1.71	1.36	1.48	-	1.86	2.1	1.35
	Tissue-type plasminogen activator	1.51*	3.33*	1.17*	-	-	-	-
	Tissue factor pathway inhibitor 2	-	-	-	-	1.55	1.37	1.05
	Plasminogen	1.7	1.46	2.29	-	-	-	-
	Coagulation factor X	-	-	-	-1.73	-	-	-
	Platelet-activating factor acetylhydrolase	-	-	-	**-2.06**	-1.36	-1.59	-1.24
	Alpha-2-macroglobulin	-	-	-	-	-1.14	-1.66	-1.05
	CD9 antigen	-	-	-	-1.65	-2.18	-1.37	-1.21
Iron-related, hemopoesis, and other related functions	Putative ferric-chelate reductase 1	-1.33	-2.78	-2.64	-	-	-	-
	Ferritin, middle subunit	*-1.73**	*3.98**	*-1.25**	-	-	-	-
	Iron/zinc purple acid phosphatase-like protein	-1.86	-1.13	-1.71	-	-	-	-
	Iron-sulfur cluster assembly 2 homolog, mitochondrial	*-1.51**	*1.28**	*-1.30**	x	-	-	-
	T-cell acute lymphocytic leukemia protein 1 homolog	*-1.14**	*2.26**	*1.14**	-	-1.17	-1.45	-1.57
	Interleukin-20 receptor alpha chain	-	-	-	-1.98	**-1.74**	**-1.71**	**-2.24**
	Peroxisomal proliferator-activated receptor A-interacting complex 285 kDa protein	-	-	-	**-3.79**	**-1.89**	**-2.02**	**-1.26**
	S-antigen protein	-	-	-	x	1.05	2.62	1.74
	Interferon-stimulated 20 kDa exonuclease-like 1	*1.09**	*1.56**	*-1.08**	-	-	-	-
	TRAF and TNF receptor-associated protein homolog	-	-	-	-1.83	**-1.70**	**-1.67**	**-1.31**
	Nuclear factor interleukin-3-regulated protein	-	-	-	-	2.10	1.04	1.11

Figure 7. Differentially expressed cellular stress, prostaglandin, coagulation and other related genes

Differentially expressed genes involved in response to cellular stress, prostaglandin metabolism, FK506-binding, coagulation and other related functions displayed with linear fold change values for each day (D3-D9) and colored by fold change (FC) relative to controls (green = down-regulated; red = up-regulated). Bold values indicate highly significant main effect of infection ($p < 0.0001$), asterisks indicate significant time by infection interaction, and italics indicates no significant main effect (significant interaction only). A hyphen indicates no significant difference identified and an 'x' indicates no probe passing quality control for the species.

While expression of the acute phase protein *serum amyloid A* was increased in all species, pink salmon in particular and to a lesser extent chum salmon up-regulated other components of the acute phase response, including *CCAAT/enhancer binding proteins*, and complement genes including *complement component c7* (pink and chum) and *complement component c3* (pink only; Figure 8). All three species also showed differential expression of components of the coagulation cascade (Figure 7), although the genes involved differed among the species.

Iron regulation was induced alongside up-regulation of complement/acute phase response in pink salmon. The main regulator of iron homeostasis, *hepcidin-1* was highly up-regulated in both chum and pink salmon (Figure 8 and Figure 9A). In pink salmon this induction was specific to 3 and 6 dpe, with expression returning to baseline by 9 dpe. Genes involved in scavenging iron from blood and sequestering in tissues including *serotransferrin-2* and *haptoglobin* were up-regulated in pink and chum salmon, respectively. Pink salmon suppressed heme biosynthesis through suppression of six of the seven enzymes in the pathway (Table 8; Figure 9B). Pink salmon up-regulated the heme-recycling *heme oxygenase* specifically at 3 and 6 dpe, and down-regulated several hemoglobin subunits (n = 5), as well as *mitoferrin-1* and *heme binding protein 2* (Figure 8). Both chum and pink salmon induced iron regulatory mechanisms, although some components were specific to pink salmon (e.g., suppression of heme biosynthesis).

		Atlantic			Chum	Pink		
FUNCTION	GENE	D3	D6	D9	D6	D3	D6	D9
Acute Phase Response & Inflammation	Serum amyloid A protein	1.87	1.23	3.53	**5.28**	**1.67**	**3.83**	**5.85**
	CCAAT/enhancer-binding protein beta	-	-	-	-	1.29	1.84	1.04
	CCAAT/enhancer-binding protein delta	-	-	-	1.51	*1.59**	*1.06**	*-1.45**
	Metalloreductase STEAP4	-	-	-	3.04	-	-	-
	Mast cell immunoreceptor signal transducer	-	-	-	-	*1.91**	*-1.04**	*-1.31**
Iron Regulation and Binding	Hepcidin-1	-	-	-	14.24	**8.3 ***	**7.02***	**-1.13***
	Serotransferrin-2	-	-	-	x	1.05	4.23	3.46
	Heme oxygenase	-	-	-	-	*1.59**	*1.62**	*-1.58**
	Haptoglobin	-	-	-	2.65	x	x	x
	Iron-responsive element-binding protein 2	*-1.79**	*1.34**	*1.06**	-	-1.88	-1.22	-1.12
	Mitoferrin-1	-	-	-	-	**-1.92**	**-2.32**	**-2.1**
	Mitoferrin-2	*1.60**	*1.15**	*-1.21**	-	x	x	x
	Heme-binding protein 2	-	-	-	-	-2.75	-2.02	1.01
	Hemoglobin subunit alpha	-	-	-	-1.83	**-1.35**	**-2.17**	**-1.82**
	Hemoglobin subunit alpha-4	-	-	-	-	-1.11	-1.72	-1.5
	Hemoglobin subunit alpha-D	-1.07*	3.04*	1.18*	-	-1.54	-1.39	-1.24
	Hemoglobin subunit beta	-	-	-	-	**-1.72**	**-2.69**	**-2.39**
	Hemoglobin subunit beta-2	x	x	x	-	-1.81	-3.47	-1.31
Complement	Complement C3-1	-	-	-	-	-1.02	1.67	2.76
	Complement component C7	-	-	-	1.83	1.09	1.67	1.72
	Complement component 1 Q subcomponent-binding protein, mitochondrial	1.92	1.6	1.07	1.65	1.46	1.73	1.11
	Complement C1q subcomponent subunit C	*-1.68**	*-1.05**	*1.05**	-1.52	-	-	-
	Complement C1q-like protein 2	-	-	-	5.74	1.51	3.03	1.94
Proteinases	Collagenase 3	3.48	2.35	1.35	-	**3.03**	**6.56**	**1.58**
	Arginase-1	2.91	2.55	1.42	-	-	-	-
	Arginase-2, mitochondrial	2.31	1.42	1.33	x	**2.04**	**2.24**	**1.5**
	Matrix metalloproteinase-9	1.72	1.76	1.19	-	-	-	-
	Metalloproteinase inhibitor 2	1.58	1.09	1.26	1.68	**1.54**	**1.86**	**1.16**
Pattern Recognition	C-type lectin domain family 4 member M	-	-	-	-1.59	**1.81**	**2.53**	**1.87**
	C-type lectin domain family 10 member A	-	-	-	-1.62	-	-	-
	Mannose-binding protein C	-	-	-	1.87	-	-	-
	Beta-galactoside binding lectin	-	-	-	1.78	*-1.27**	*1.6**	*1.33**
	CD209 antigen-like protein D	-	-	-	-1.75	-	-	-
	CD209 antigen-like protein E	-1.62	-1.13	-1.37	-	-	-	-
	Acidic mammalian chitinase	-	-	-	-	1.03	2.13	1.98
	Polymeric Ig receptor	1.79	1.38	1.11	-1.79	1.61	2.00	1.02

Figure 8. Comparative gene expression in key functional groups

Differentially expressed genes in the anterior kidney involved in the acute phase response, iron regulation, complement activity, proteinase activity, or pattern recognition are displayed with linear fold change values for each day (D3-D9) and colored by fold change relative to controls (green = down-regulated; red = up-regulated). Bold values indicate highly significant main effect of infection ($p < 0.0001$), asterisks indicate significant time by infection interaction, and italics indicates no significant main effect (significant interaction only). A hyphen indicates no significant difference identified and an 'x' indicates no probe passing quality control for the species.

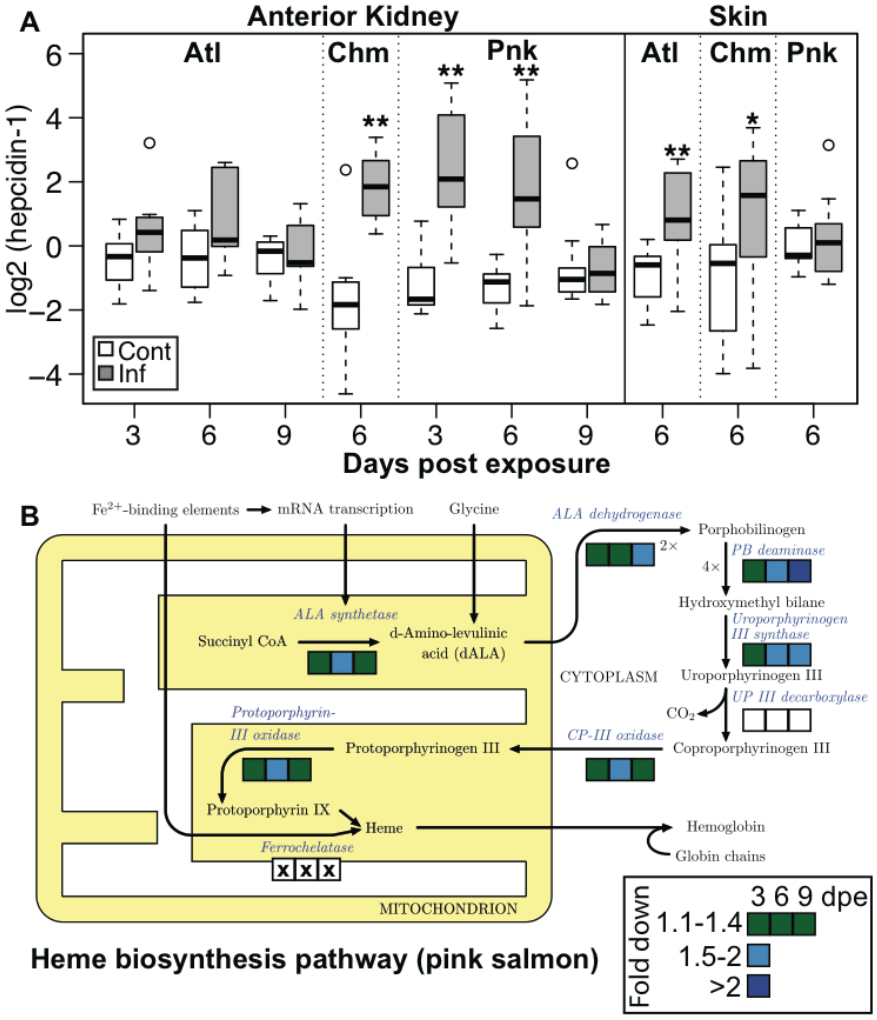

Figure 9. Species and tissue expression of iron regulation mechanisms

(A) *hepcidin-1* was highly up-regulated in the anterior kidney of pink salmon early in the infection period (day three and six only). Chum salmon highly increased *hepcidin-1* expression in the anterior kidney and more moderately in the skin. Atlantic salmon up-regulated *hepcidin-1* in the skin but not the anterior kidney (*hepcidin-1* data shown is from qPCR). Boxplot displays median and interquartile range, and circles are outliers. *denotes $p < 0.05$ **denotes $p < 0.001$. *Hepcidin-1* induction was a general response to the infection, whereas other iron homeostasis mechanisms, such as (B) reduction of expression of the heme biosynthesis pathway, were specific to pink salmon. Boxes indicate fold change for day 3, 6 and 9 post infection; an x indicates no probe for analysis, and an empty box indicates no significant difference in expression (heme

biosynthesis transcripts shown are from microarray data). Image adapted from: *Wikimedia Commons "Heme synthesis" Creative Commons Attribution-ShareAlike 3.0 Unported.*

Innate pattern recognition receptors may be involved in recognizing the parasite or cell damages, and subsequently inducing appropriate response mechanisms. Pattern recognition receptors were induced in all species but the active components were species-specific (Figure 8). Up-regulation of *C-type lectin domain family 4 member M* occurred only in pink salmon ($p < 0.0001$), whereas up-regulation of *mannose-binding protein C* occurred only in chum salmon. *Beta-galactoside binding lectin* up-regulation occurred in pink and chum salmon at 6 dpe. As identified previously (Tadiso *et al.* 2011), *polymeric Ig receptor* increased in expression for Atlantic salmon. Here, pink salmon also up-regulated this transcript, whereas expression was down-regulated in chum salmon (Figure 8). However, an additional *polymeric Ig receptor* probe indicated down-regulation in pink salmon (*data not shown*). Specific to pink salmon was the induction of *acidic mammalian chitinase*, previously identified as one of the highest up-regulated genes in juvenile pink salmon responding to salmon lice (Sutherland *et al.* 2011). The protein encoded by this gene has chitinase activity (Boot *et al.* 2001), and plays a role in allergic inflammation (Zhu *et al.* 2004).

Suppression of antiviral response gene expression was characteristic of the anterior kidney of both pink and chum salmon. Pink salmon down-regulated seven interferon-induced genes such as *interferon-induced GTP-binding protein Mx*, *interferon regulatory factor 1*, *3*, and *7*, three tripartite motif-containing genes and *signal transducer and activator of transcription 1* (Figure 10). Many of these genes were also suppressed in chum salmon. Enrichment was found in the down-regulated lists of both chum and pink salmon for response to virus ($p < 0.05$; Table 8).

Atlantic and chum salmon both down-regulated several chains of the MHC class II antigen presentation machinery (Figure 10).

		Atlantic			Chum	Pink		
FUNCTION	GENE	D3	D6	D9	D6	D3	D6	D9
Antiviral	Galectin-3-binding protein	-	-	-	-1.86	**-1.8**	**-1.58**	**-1.55**
	Interleukin-1 receptor-associated kinase 3	-1.25	-1.67	-1.16	-	-	-	-
	Interferon regulatory factor 1	-	-	-	-	**-1.54**	**-1.65**	**-1.21**
	Interferon regulatory factor 3	-	-	-	-1.77	-1.36	-1.57	-1.28
	Interferon regulatory factor 7	-	-	-	-1.81	**-2.50**	**-2.05**	**-1.75**
	Interferon-induced GTP-binding protein Mx	-	-	-	-3.65	**-1.95**	**-2.09**	**-1.48**
	Interferon-induced guanylate-binding protein 1	-	-	-	-	**-1.57**	**-1.48**	**-1.61**
	Interferon-induced guanylate-binding protein 2	x	x	x	-	-1.30	-1.50	-1.08
	Interferon-induced 35 kDa protein homolog	-	-	-	-	**-1.62**	**-1.30**	**-1.35**
	Interferon-induced protein 44	1.95	1.22	3.82	-4.47	**-2.15**	**-2.54**	**-1.63**
	Interferon-induced protein with tetratricopeptide repeats 5	-	-	-	-1.89	-	-	-
	Interferon-induced very large GTPase 1	-	-	-	-2.41	-1.88	-1.65	-1.26
	Interferon-induced, double-stranded RNA-activated protein kinase	-	-	-	-2.01	-1.69	-1.45	-1.33
	Interleukin-1 receptor type II	-	-	-	x	1.54	1.35	-1.14
	Tripartite motif-containing protein 16	-	-	-	-2.30	-1.45	-1.63	-1.18
	Tripartite motif-containing protein 25	-	-	-	-2.16	-1.40	-1.55	-1.21
	Tripartite motif-containing protein 29	-	-	-	-1.71	-	-	-
	Tripartite motif-containing protein 39	-	-	-	-	**-1.84**	**-1.48**	**-1.43**
	Sacsin	-	-	-	**-3.45**	-1.64	-1.84	-1.26
	Signal transducer and activator of transcription 1	-	-	-	-2.13	**-2.20**	**-2.74**	**-1.74**
	Signal transducer and activator of transcription 1-alpha/beta	-	-	-	-1.67	**-2.17**	**-1.44**	**-1.51**
	Barrier-to-autointegration factor	*-1.51**	*1.17**	*1.14**	-2.16	-	-	-
Antigen presentation	H-2 class II histocompatibility antigen gamma chain	-	-	-	-1.84	-	-	-
	H-2 class II histocompatibility antigen, A-K beta chain	-3.36	1.11	-1.49	-1.64	-	-	-
	H-2 class II histocompatibility antigen, E-S beta chain	-2.30*	1.07*	-1.07*	**-1.58**	-	-	-
	H-2 class II histocompatibility antigen, I-A beta chain	*-2.52**	*1.23**	*-1.18**	-	-	-	-
	HLA class II histocompatibility antigen, DP alpha chain	-	-	-	-1.58	-	-	-
	RT1 class II histocompatibility antigen, B alpha chain	-1.72	-1.26	-1.31	x	x	x	x
	Gamma-interferon-inducible lysosomal thiol reductase	-1.65	-1.11	-1.75	-	-	-	-
Cell-mediated immunity	Beta-2-microglobulin	-	-	-	**-1.72**	-	-	-
	Ig mu chain C region membrane-bound form	-	-	-	2.27	-	-	-
	Interleukin enhancer-binding factor 2 homolog	-	-	-	-	1.13*	1.50*	1.05*
	T-cell immunoglobulin and mucin domain-containing protein 4	-	-	-	-1.72	-	-	-
	T-cell surface glycoprotein CD3 zeta chain	-	-	-	-	-1.20	-1.85	1.01
	CD83 antigen	-	-	-	-1.72	-	-	-
	CD97 antigen	-	-	-	-1.84	-	-	-
	CD276 antigen	-	-	-	1.72	-	-	-
Chemoattraction	Leukocyte cell-derived chemotaxin 2	-	-	-	-	**-2.26***	**-1.01***	**-1.3***
	High affinity interleukin-8 receptor B	1.72	2.38	1.22	-	-	-	-
	Galectin-9	-	-	-	-1.91	-1.52	-1.26	-1.16

Figure 10. Differentially expressed immunity genes

Differentially expressed genes involved in antiviral response, and other immune related functions. Colors and formats are as described in Figure 7.

Considering the important immunomodulatory role of prostaglandin E_2 in the louse-salmon interaction (Fast *et al.* 2007a), it is interesting to note that *prostaglandin E synthase 3* was up-regulated in all species (Figure 8). However, the role of this transcript is unclear because in

addition to generating prostaglandin E_2, this enzyme is a co-chaperone of HSP90 and the unfolded protein response is activated in all species (Table 8). In addition, a prostaglandin inactivator, *15-hydroxyprostaglandin dehydrogenase [NAD+]* was suppressed at 3 and 6 dpe in pink salmon, and at 6 dpe in chum salmon (Figure 7).

Differential expression of several components of cell-mediated immunity was evident in Atlantic salmon responses, including the up-regulation of the highly inflammatory *leukotriene B4 receptor* and *high affinity interleukin-8 receptor B*, both specific to Atlantic salmon (Figure 7 and Figure 10). Chum salmon increased expression of the *Ig mu chain region membrane bound form*, and *CD276 antigen* (Figure 10).

Metalloproteinase expression is typically induced in response to salmon lice (Skugor *et al.* 2008; Sutherland *et al.* 2011; Tadiso *et al.* 2011). Atlantic salmon in the present study up-regulated several metalloproteinases: *collagenase-3* (*mmp13*), *matrix metalloproteinase-9*, and *arginase-1* and *-2* (Figure 8). Only *mmp13* and *arginase-2* were up-regulated in pink salmon, and *mmp13* was one of the highest up-regulated genes for pink salmon anterior kidney (Figure 8). Interestingly, chum salmon did not increase expression of any of these metalloproteinases, although *metalloproteinase inhibitor 2* was up-regulated in all species.

3.4.5 Local transcriptomic responses of Atlantic, chum, and pink salmon

In the microarray analysis of the skin (by sampling pectoral fin), all species were profiled at six days post exposure, with 9 or 10 individuals used for each species and infection class combination (i.e. total Atlantic, chum, and pink skin samples = 18, 20, 19, respectively). Differential expression was mainly identified in chum salmon, with 44 up-regulated genes, and 86 down-regulated genes. There were only four probes differentially expressed in pink salmon

skin (two probes without annotation, *suppressor of fused homolog* and *guanidinoacetate N-methyltransferase*) and no differential expression was found in Atlantic salmon skin.

Genes up-regulated in chum skin were involved in cell death (6 genes; $p = 0.012$) and those down-regulated were involved in immune response (9 genes; $p < 0.001$). The *complement component C7* gene was up-regulated (Figure 11) as in the anterior kidney. Expression of interleukin-20 receptor alpha chain was down-regulated. IL-20 signalling through signal transducer and activator of transcription-3 generates potent cutaneous inflammation (Rich 2003). Cell proliferative genes were also up-regulated, such as *fibroblast growth factor-binding protein 1*, a keratinocyte mitogen up-regulated after skin injury in epithelial cells (Beer *et al.* 2005) and *adseverin*, a regulator of chondrocyte proliferation and differentiation (Nurminsky *et al.* 2007) (Figure 11). However, also up-regulated was *growth arrest and dna-damage-inducible protein gadd45 beta*, which is induced by genotoxic agents or apoptotic cytokines and has a role in reducing proliferation (Vairapandi *et al.* 2002). Furthermore, induction of *thioredoxin* was identified, which is involved in protection from reactive oxygen species-induced stress. Interestingly, the highest up-regulated annotated gene was *FK506-binding protein 5*, which was also up-regulated in the anterior kidney of all species (Figure 7). Similar to the anterior kidney of pink and chum salmon, many antiviral components were suppressed in chum salmon skin (Figure 11). The local and systemic responses of chum salmon indicated some consistencies between tissues, and consistencies were more frequently observed for down-regulated genes (38 of 86 in the anterior kidney) than for up-regulated genes (7 of 43 in the anterior kidney).

FUNCTION	GENE	FC
Immunity	Complement component C7	1.89
Proliferation and differentiation	Growth hormone receptor	1.75
	Adseverin	2.63
	Fibroblast growth factor-binding protein 1	2.58
Other Functions	Breast cancer anti-estrogen resistance protein 1	2.10
	FK506-binding protein 5	4.31
	Growth arrest and DNA-damage-inducible protein GADD45 beta	2.88
	P2X purinoceptor 1	1.95
	Thioredoxin	2.73
Antiviral	Barrier-to-autointegration factor	-1.98
	Beta-2-microglobulin	-2.34
	Galectin-3-binding protein	-2.03
	H-2 class II histocompatibility antigen, A-K beta chain	-1.75
	H-2 class II histocompatibility antigen, E-S beta chain	-2.16
	HLA class II histocompatibility antigen, DP alpha chain	-2.28
	Interferon regulatory factor 3	-1.89
	Interferon regulatory factor 7	-2.09
	Interferon-induced 35 kDa protein homolog	-1.65
	Interferon-induced GTP-binding protein Mx	-2.61
	Interferon-induced guanylate-binding protein 1	-2.53
	Interferon-induced guanylate-binding protein 1	-2.27
	Interferon-induced protein 44	-4.02
	Interferon-induced very large GTPase 1	-2.55
	Lymphocyte cytosolic protein 2	-1.70
	Radical S-adenosyl methionine domain-containing protein 2	-4.42
	Sacsin	-3.66
	Signal transducer and activator of transcription 1	-2.55
	T-cell receptor alpha chain V region HPB-MLT	-1.80
	Tripartite motif-containing protein 16	-1.84
	Tripartite motif-containing protein 25	-1.95
Other Functions	Interleukin-20 receptor alpha chain	-4.33
	Peroxisomal proliferator-activated receptor A-interacting complex 285 kDa protein	-3.33
	Probable E3 ubiquitin-protein ligase HERC4	-2.33
	Thrombospondin-2	-1.84

Figure 11. Differentially expressed genes in chum salmon skin

Selected differentially expressed genes in the skin of chum salmon at 6 days post exposure involved in immunity, proliferation, and other functions. Antiviral genes are suppressed as is seen in the anterior kidney of both Pacific salmon. Colors and formats are as described in Figure 7.

3.4.6 Microarray validation and cytokine exploration by quantitative PCR

All genes tested with quantitative PCR (qPCR) had the same direction of fold change as was found differentially expressed in microarray analysis. Correlation of qPCR and microarray data

indicated reliability of estimates for each species: the average R squared ± standard deviation for anterior kidney genes was 0.648 ± 0.224 (n = 9 gene/species comparisons; Figure 12). The trends identified for *hepcidin-1*, *collagenase-3*, and *15-hydroxyprostaglandin dehydrogenase [NAD+]* in the anterior kidney of all three species were confirmed with qPCR, including the unchanging expression of *collagenase-3* in chum salmon (Figure 12, Figure 13A).

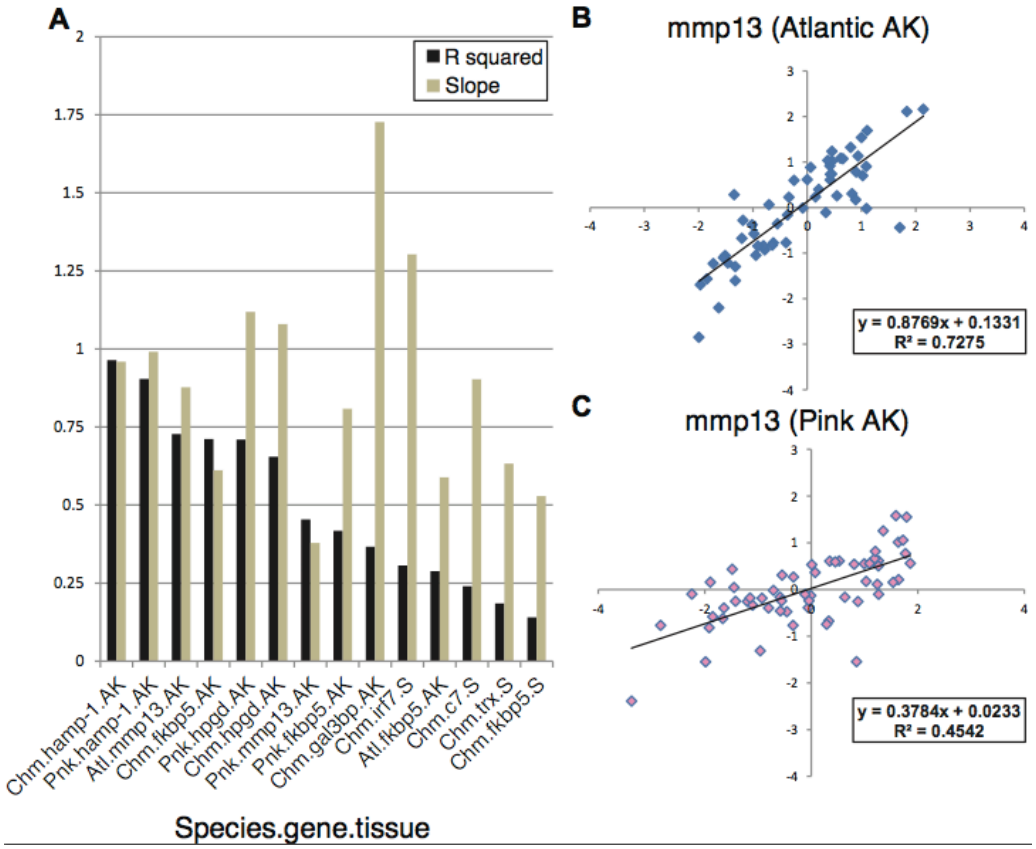

Figure 12. qPCR microarray $\log_2$ expression correlation

(A) Microarray and qPCR expression levels correlated well for all significantly differentially expressed genes in the anterior kidney for all three species. Skin sample correlation was lower, but still always identified the correct direction of fold change. Primers were designed to ensure equal amplification for all species to ensure correct estimates of expression levels, as shown for *collagenase-3* $\log_2$(qPCR) against $\log_2$(microarray) shown for (B) Atlantic and (C) pink salmon. chm = chum; pnk = pink; atl = Atlantic; AK = anterior kidney; S = skin; gene acronyms are as per the primer table (Table 20).

Occasionally, differential expression of certain genes was indicated by qPCR but not by the microarray analysis, presumably because of the multiple test correction applied to the microarray. Measured by qPCR, up-regulation of *hepcidin-1* occurred in Atlantic and chum salmon skin (Figure 9A). Also, *complement C7* up-regulation occurred in Atlantic salmon skin (Figure 14A) but not in pink salmon, despite up-regulation in pink salmon anterior kidney. Additionally, *interferon response factor 7* was identified as down-regulated by qPCR in skin of all species including Atlantic salmon (Figure 14A). qPCR identified down-regulation of *galectin-3-binding protein* and up-regulation of *thioredoxin* in the skin of pink salmon, whereas these genes did not pass significance testing in chum salmon ($p = 0.06$). When tested with qPCR, *15-hydroxyprostaglandin dehydrogenase [NAD+]* was found to be suppressed early in all species (3 or 6 dpe; Figure 13B), not just in pink and chum salmon as identified with the microarray. Use of qPCR to validate the microarray confirmed that the trends identified in the array analysis were largely correct and not confounded by species differences in probe hybridization efficiencies

Exploratory qPCR of targets not on the microarray identified up-regulation in pink salmon skin of pro-inflammatory cytokine *interleukin-1 beta* (2.6 fold; $p = 0.001$), as well as a slight elevation in *tumor necrosis factor alpha* (1.3 fold; $p < 0.05$; Figure 14B). These genes were not differentially expressed in the other species. *Interleukin 8* was not differentially expressed in skin of any species. Increased expression of *prostaglandin D synthase* occurred in the skin of Atlantic (FC = 1.6) and chum salmon (FC = 2.6), but not pink salmon. None of these genes were up-regulated in the anterior kidney of any species, although *tumor necrosis factor alpha* was down-regulated in chum salmon anterior kidney (FC = 1.8; $p < 0.007$), and *prostaglandin D synthase* was down-regulated in Atlantic salmon anterior kidney (FC = 1.7; $p < 0.002$).

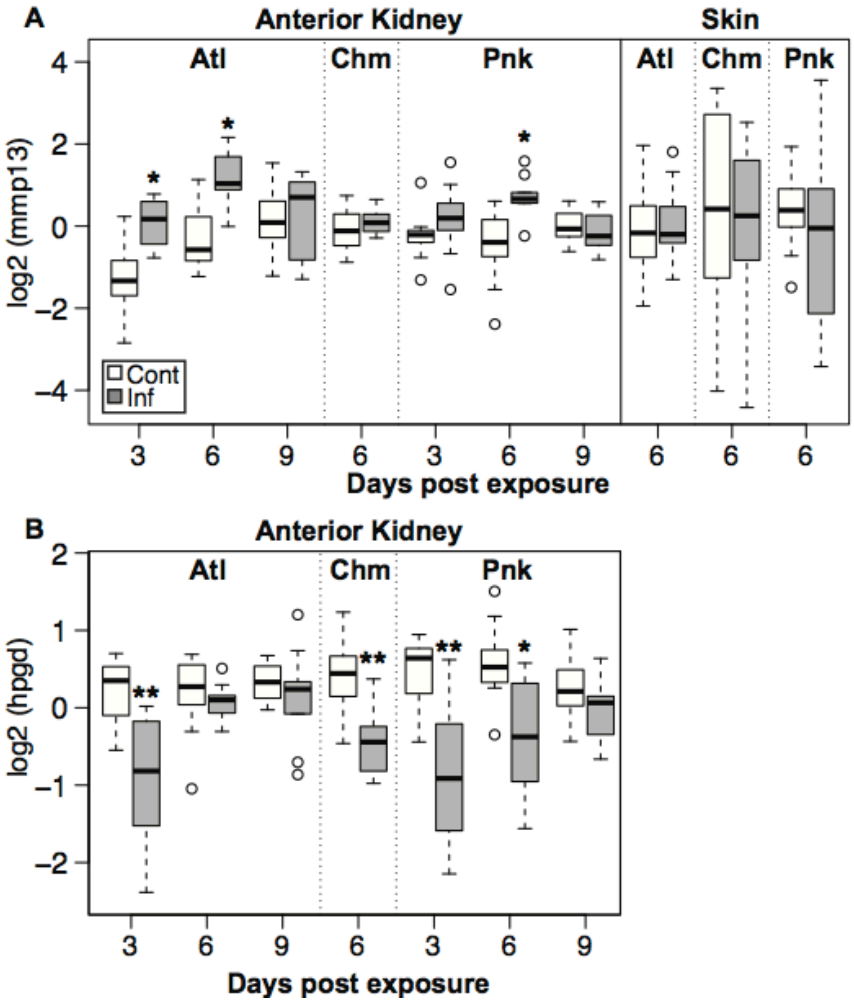

Figure 13. Expression of collagenase-3 and 15-hydroxyprostaglandin dehydrogenase by qPCR
(A) Collagenase-3 expression in the anterior kidney evaluated by qPCR. (B) Expression of the prostaglandin E2 inactivator *15-hydroxyprostaglandin dehydrogenase* was suppressed relative to the control in the anterior kidney of all three species early in the infection. Boxplot displays median and interquartile range, and circles are outliers. *denotes $p < 0.05$ **denotes $p < 0.001$

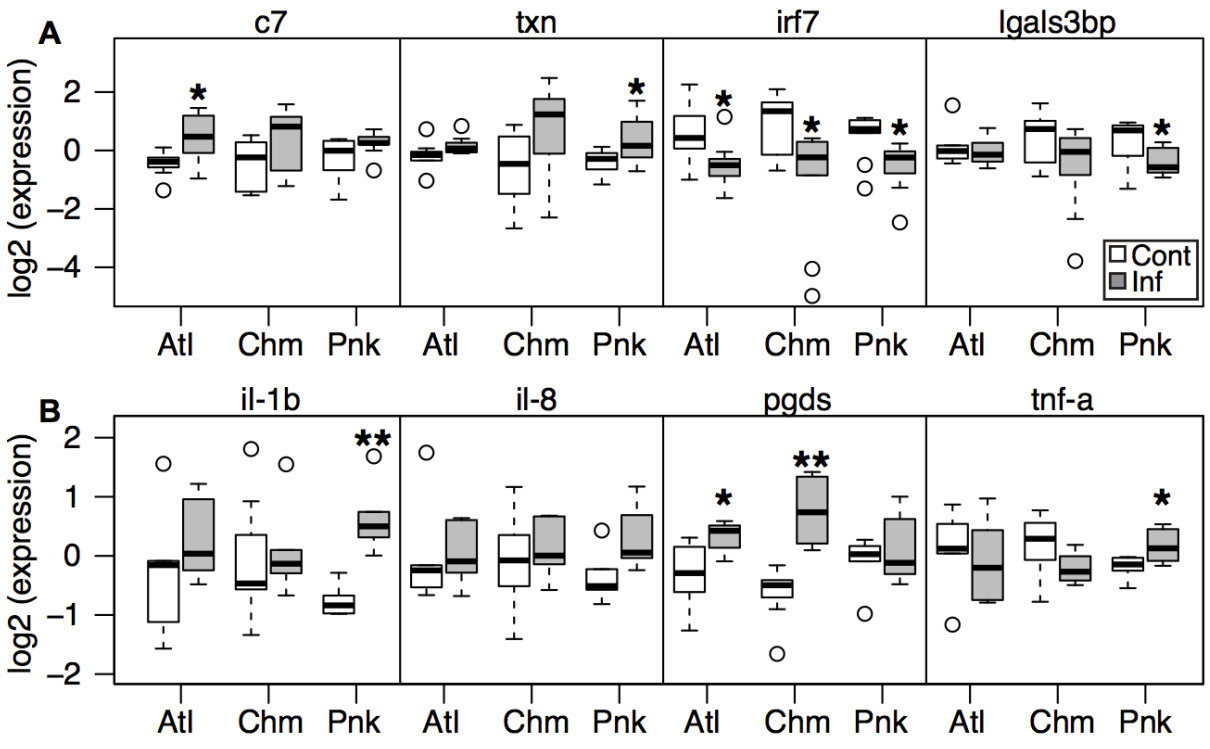

Figure 14. Local expression of immune genes in fin by qPCR

Local tissue expression was profiled in all three species at six days post exposure. Genes displayed in (A) were selected based on microarray analysis, and in (B) were selected based on previous analyses. As per the microarray analysis, all genes were normalized within a species and therefore the only valid comparison to make is between control (white) and infected (grey) within a species. In all three species, *interferon response factor 7* (irf7) was down-regulated. Pink salmon up-regulated *thioredoxin* (txn) and down-regulated *galectin 3-binding protein* (lgals3bp), and Atlantic salmon up-regulated *complement C7* (c7). Both Atlantic and chum salmon up-regulated *prostaglandin D synthase* (pgds), and pink salmon was the only species to induce pro-inflammatory cytokine *interleukin-1 beta* (il-1b), and slightly *tumor necrosis factor alpha* (tnf-a), although the fold change was low (1.3-fold). Boxplot displays median and interquartile range, and circles are outliers. *denotes $p < 0.05$ **denotes $p < 0.001$

3.5 Discussion

The data presented in this chapter show that when co-habited and subjected to identical copepodid exposures, chum salmon become infected with higher densities (lice per host weight

(g)) of salmon lice than do Atlantic or pink salmon. The higher infection density on chum compared to pink salmon was previously identified (Jones *et al.* 2007) and the inclusion of Atlantic salmon here provides more information on the susceptibility spectrum of Pacific and Atlantic salmon. We conclude that juvenile pink salmon are resistant whereas juvenile Atlantic and particularly chum salmon are susceptible. This comparative infection system permitted the analyses of hematological parameters in addition to local and systemic transcriptomic responses to identify mechanisms driving this susceptibility variation.

Differences in infection density among species were observed three days post exposure indicating either a) the rapid onset of an innate effector mechanism in pink salmon or b) greater affinity of infective copepodids for chum and Atlantic salmon through behavioral or chemical cues (e.g., Nagasawa *et al.* 1993; Fast *et al.* 2002; Mordue & Birkett 2009) or c) a combination of these processes. While further research is required to better understand the relative affinity of *L. salmonis* for Pacific salmon species, it is understood that pathology occurs throughout the infection, with most damage occurring after the lice molt to adult stages (Grimnes & Jakobsen 1996). Here, the consequences of elevated infection densities on chum and Atlantic salmon were reflected in elevated plasma cortisol (chum), reduced weight gain (chum), and reduced hematocrit (chum and Atlantic). Hematocrit reduction in exposed chum and Atlantic salmon and no significant effect in pink salmon confirms previous observations in these species, and this reduction was also noted in sea trout *S. trutta*, and in sockeye salmon *O. nerka* infected by lice (Grimnes & Jakobsen 1996; Bjørn & Finstad 1997; Jones *et al.* 2007; Jakob *et al.* 2013). In these studies, the reduced hematocrit was related to infection intensity and possibly indicative of a microcytic anemia induced by lesions in the skin caused by feeding parasites, leading to fluid loss. Elevated plasma chloride levels, frequently reported during *L. salmonis* infections, are

associated with altered osmoregulatory capacity caused by feeding behavior of the larger and more aggressive motile stages (Grimnes & Jakobsen 1996; Bjørn & Finstad 1997; Finstad *et al.* 2000). Plasma cortisol was elevated in chum salmon infected with chalimus stages, confirming an earlier report for chum salmon infected with motile *L. salmonis* stages (Jones *et al.* 2007). Similarly, other studies have identified elevated plasma cortisol in Atlantic salmon coincident with the first appearance of motile *L. salmonis* stages (Bowers *et al.* 2000; Fast *et al.* 2002). It is possible that the earlier induction of cortisol in the present study as well as elevated cortisol in control Atlantic salmon could be due to stresses of co-habitation with mixed species. The apparent increase in infection density in all three species at day 9 and 12 in Trial 1 was due to the shedding into the anaesthetic bucket of copepodids that were incompletely attached via frontal filament on days 3 and 6, as observed previously (Johnson & Albright 1992). This comparative laboratory infection model has provided a reliable tool with which to explore the transcriptomic basis of host responses to *L. salmonis* among salmon species displaying resistant and susceptible phenotypes.

Cytokine profiling and functional analysis of gene lists indicated that inflammation and the acute phase response (APR) were important response mechanisms following exposure to *L. salmonis*. The pro-inflammatory cytokines IL-1β and TNF-α were induced only in the skin of pink salmon. IL-1β promotes the T helper 17 (Th17) cell response, further indicating the importance of this function in responses to salmon lice (e.g., Skugor *et al.* 2008). Th17 responses induce inflammation during host defense against bacterial or fungal infection, but can also play a role in tissue pathology and autoimmunity (Waite & Skokos 2012). In the present work, the APR was recognized in all species by the increased expression of *serum amyloid A* during infections (Trey & Kushner 1995). Other identified acute phase proteins were induced in pink and chum

salmon, including common and species-specific responses. In pink salmon, with the exception of *serum amyloid A*, the APR decreased by day 9, whereas in Atlantic salmon, the onset of *serum amyloid A* expression appeared delayed. Atlantic salmon previously have been shown to respond to lice after one to three dpi with induction of genes involved in the acute phase response (Tadiso *et al.* 2011; Braden *et al.* 2012). Up-regulation of complement components was also identified as a general response in all three species. Complement plays a role in chemotaxis, opsonization and vascular permeability, and can be induced alongside acute phase responses (Trey & Kushner 1995). The up-regulation of *c3* solely in pink salmon indicated increased capacity for innate immunity through complement activation via classical, alternative and lectin pathways (Jones 2001). Coagulation is an important first step of tissue repair following injury (Midwood *et al.* 2004) and the identification of these functions mainly in pink and chum salmon suggested they are part of a general response to the infection. Infections with *L. salmonis* are known to elicit inflammation at attachment sites on the skin and that these reactions differ considerably among host species. Reactions to *L. salmonis* are minimal in the skin of Atlantic salmon and pronounced in coho salmon (Johnson & Albright 1992; Fast *et al.* 2002). It has therefore been postulated that the capacity to mount an inflammatory response at the site of parasite attachment is an indicator of resistance and more explicitly, that inflammation is an important defence mechanism in promoting early rejection of parasites (Jones *et al.* 2007; Wagner *et al.* 2008). Our data confirm the occurrence of general and species-specific indicators of cutaneous and systemic inflammation following exposure to *L. salmonis*. Furthermore in pink salmon, the cutaneous production of proinflammatory cytokines, systemic APR and enhanced capacity for complement function may help explain the low levels of infections compared with those on chum and Atlantic salmon.

Early infection with *L. salmonis* was associated with changes in the expression of genes associated with iron regulation and binding. The affected pathways tended to be species-specific: *haptoglobin* was only up-regulated in chum salmon and the majority of dysregulated genes was observed in pink salmon. The fold change of *hepcidin-1* up-regulation in pink and chum salmon was the highest of all genes measured in this study. *Hepcidin-1* regulates iron homeostasis by preventing export of iron from cells into the blood (Nemeth & Ganz 2006) and is induced by *interleukin-6* during inflammation (Nemeth *et al.* 2004), by endoplasmic reticulum stress (Wessling-Resnick 2010) or as part of a type II acute phase response (Nemeth *et al.* 2003). Both the antimicrobial and iron regulatory roles of *hepcidin-1* are evolutionarily conserved in a broad range of fish species (for review see Shi & Camus 2006; Robertson 2011). For example *hepcidin-1* was induced in the anterior kidney of barramundi *Lates calcarifer* after intraperitoneal injection with lipopolysaccharide (Barnes *et al.* 2011), in the anterior kidney of miiuy croaker *Miichthys miiuy* after injection with *Vibrio anguillarum* (Xu *et al.* 2012) and in the liver of sea bass *Dicentrarchus labrax* from both iron overload and bacterial infection (Rodrigues *et al.* 2006). Here, *hepcidin-1* expression was induced in the anterior kidney of both pink and chum salmon by the louse infection. By day 9 however, hepcidin-1 expression was back to baseline in pink salmon kidney, coincident with the highest *serum amyloid A* up-regulation. *Hepcidin-1* was also induced in the skin of Atlantic and chum salmon, the most heavily infected species. However, other iron homeostatic components were specific to pink salmon, including up-regulation of heme recycling *heme oxygenase* and iron scavenging *serotransferrin-2*, and down-regulation of hemoglobin subunits and the heme biosynthesis pathway. This suggests that nutritional immunity (Wessling-Resnick 2010; Hammer & Skaar 2011; Hood & Skaar 2012), the sequestration of host nutrients from pathogens may have a role in defence against salmon lice. A highly anemic state is likely

not the end result of this protective mechanism, as in Trials 2 and 3 only Atlantic and chum salmon showed significant hematocrit reduction, likely due to breaches in the circulatory system as discussed above. Alternatively, anemia of inflammation is often mild and accompanies changes in iron handling and erythrocyte production and lifespan (Roy 2010). Both the necessity of iron in the salmon louse diet and the role for sequestration of iron during the host-parasite interaction merit further study.

Tolerance of infection can also be an adaptive alternative to inflammation-based rejection mechanisms by reducing damage to self (Allen & Wynn 2011). Up-regulation of the protein folding response, evident in the anterior kidney of all species during *L. salmonis* infection, was an indication of cellular protection. Previous work also identified up-regulation of protein folding transcripts in the skin of Atlantic salmon infected with lice at 22-33 dpe (Skugor *et al.* 2008). These cellular protective mechanisms in the anterior kidney suggest infection is associated with self-damage induced by reactive oxygen species or other defense mechanisms. Similarly, evidence of increased expression of the antioxidant *thioredoxin* in the skin of pink and chum salmon provided additional support of pro-tolerance mechanisms as overexpression of thioredoxin can protect from oxidative stress induced during infection or inflammation in mammals (Yoshida *et al.* 2005). Enrichment of ATP binding in chum and Atlantic salmon indicates costs are associated with either mechanisms of tolerating infection or responding to infection. We suggest that salmon adopt a species-specific but balanced response to *L. salmonis*, including both resistance and tolerance mechanisms, in which energetic costs are minimized while reducing negative consequences of infection.

Metalloproteinases are important for initiation and resolution of inflammation in teleosts by degrading damaged extracellular matrix prior to tissue remodeling (Chadzinska *et al.* 2008)

and expression of these genes in response to salmon lice has been recognised in both Atlantic and pink salmon (Skugor *et al.* 2008; Sutherland *et al.* 2011; Tadiso *et al.* 2011). In the present study, *collagenase-3* and *arginase-2* were up-regulated in the anterior kidney of both pink and Atlantic salmon throughout the infection. The induction of *arginase-1* was specific to Atlantic salmon. This transcript suppresses Th2 cytokine-driven inflammation, an important mediator of ectoparasite defense (Allen & Wynn 2011). Previously, reduced cell proliferation combined with increased metalloproteinase activity was identified in chronic infections of susceptible Atlantic salmon (Skugor *et al.* 2008) and in *L. salmonis*-sensitive juvenile pink salmon (Sutherland *et al.* 2011). Here, *cyclin-dependent kinase 4 inhibitor b* was highly up-regulated in Atlantic salmon coincident with multiple metalloproteinases, providing further evidence for this combination in susceptible species. Interestingly, metalloproteinase transcripts were not up-regulated in chum salmon, but the effect of this apparent deficiency with respect to louse susceptibility is not known.

Innate pattern recognition molecules such as lectins can relay information about self damage or danger, and can induce appropriate pathways of defense. Unique to pink salmon was the induction of *c-type lectin domain family 4 member M* (*clec4m*) and *acidic mammalian chitinase* (*amcase*). *clec4m* is a transmembrane pattern recognition receptor involved in cell adhesion and capable of recognizing various divergent pathogens, and its role in the response to *L. salmonis* is not known. The Th2 response mediator and chitin degrading enzyme amcase was also one of the most highly up-regulated genes in 0.7g juvenile pink salmon during salmon lice infection (Sutherland *et al.* 2011). We suggest these two pattern recognition molecules play a role in the innate defence of juvenile pink salmon to *L. salmonis*, and that additional research is required to determine more precisely their function.

A striking result in both the susceptible chum salmon and the resistant pink salmon was the suppression of many antiviral response genes, including *interferon response factor 3* and *7* and *signal transducer and activator of transcription 1*. Previous work reported suppression of antiviral response genes in Atlantic salmon skin in response to salmon lice (1-10 dpe; Tadiso *et al.* 2011). We propose that the suppression is due to an inverse relationship to another component of the immune system. The antiviral response may exert a negative effect on the more suitable immune response, could be energetically expensive or may induce further self damage. An inverse correlation between antiviral (type I interferons, IFN-α and IFN-β) and anti-bacterial/anti-parasitic (type II interferons, IFN-γ) has been identified in human anti-mycobacterial responses (Teles *et al.* 2013). Energetic costs of tissue remodeling during louse infection have been identified in sensitive juvenile pink salmon (Sutherland *et al.* 2011). Protection from cellular damage was identified in the protein folding response in the anterior kidney of all species responding to the louse infection. Interestingly, the suppression of antiviral immunity transcripts implies a basal surveillance mechanism exists in healthy fish, and this has been referred to as intrinsic antiviral immunity in mammals (Bieniasz 2004). The inverse relationship between these components of the immune system also raises important questions concerning the influence of *L. salmonis* infection on host susceptibility to viruses and other intracellular pathogens. An alternate hypothesis to the inverse regulation hypothesis is that the suppression is due to parasite-derived immunomodulatory compounds. The presence of the suppression in the resistant pink salmon at the same time as activation of more suitable immune activity suggests this is not the case. Another possibility is that the down-regulation is due to the cells carrying these antiviral transcripts are mobile and move to another tissue, but suppression was identified in both the anterior kidney and the skin, reducing the likelihood of this possibility. Therefore, we propose

that antiviral suppression during a louse infection is a general response to the infection, and is an intrinsic response that occurs from inverse regulation to another component of the immune system.

Few probes were found to be differentially expressed in skin of Atlantic or pink salmon, despite using the same multiple test correction methods applied to the anterior kidney transcripts. This is probably due to the relatively low infection densities on pink and Atlantic salmon and the use of pectoral fin as a surrogate for skin, regardless of the presence of lice. Previous work found differences in host gene expression between the site of attachment and a distant site on the skin of the same fish (Braden *et al.* 2012). Also, the fin sample included multiple tissue types, thus contributing to variation in the data, and reducing the possibility of finding differentially expressed genes with stringent statistical testing. In contrast to Atlantic and pink salmon, the heavier infection of the chum salmon increased the probability of infection on the fin in all samples with a corresponding increase in the transcriptome response.

This study reports the transcriptomic responses of three salmon species over nine days following exposure to *L. salmonis*. It is likely that the response characterized here would change upon louse development to the later, more aggressively feeding stages, as shown earlier in Atlantic salmon (Tadiso *et al.* 2011). Additionally, in the present study some genes changed over time independent of exposure status (control or infected). These changes could have been from the exposure or mock exposure of the fish to *L. salmonis* (i.e. reduced water volume and use of the sedative), and indicate the importance of using time-matched controls. Some consistencies in responses were identified in anterior kidney and skin (e.g., antiviral suppression in all species and increased expression of *fkbp5* and *complement C7* in chum salmon). However, the systemic response contained unique aspects relative to the local response. For example, specific to the

anterior kidney response was the reduction in iron and heme availability, whereas specific to the skin were pro-inflammatory cytokines *IL-1β* and *TNF-α*, as well as the antioxidant *thioredoxin*. The inclusion of both systemic (anterior kidney, blood) and local tissues (pectoral fin) in the present work allowed for additional understanding of the organismal response to lice infections, such as iron sequestration in comparison to local inflammation by pro-inflammatory cytokines. Furthermore, the inclusion of both susceptible and refractory species allowed for the comparative characterization of general, susceptible, and resistant responses to lice infections (Table 9).

3.6 Conclusions

Multiple experimental infections of Atlantic, chum, and pink salmon indicate highest susceptibility in chum salmon (high infection density, reduction in weight gain and hematocrit, and elevated cortisol), followed by Atlantic salmon (high infection density, reduction in hematocrit), and lowest susceptibility in pink salmon. Differences in susceptibility were observed as early as three days post exposure. General systemic response mechanisms were identified, including cellular protection, acute phase response, complement cascades and pattern recognition receptors. Due to susceptibility differences between chum and pink salmon, comparisons within *Oncorhynchus* were important in understanding potential resistance factors, such as systemic iron sequestration, increased expression of pattern recognition receptor *C-type lectin family 4 member M* and *acidic mammalian chitinase*, as well as local induction of pro-inflammatory *interleukin-1 beta* in pink salmon. Furthermore, in both local and systemic responses of Pacific salmon, up-regulation of lice response genes coincided with suppressed antiviral genes, indicating the importance of investigating co-infection dynamics of salmon responding to both lice and viruses.

Table 9. Response functions and relation to susceptibility or resistance.

Summarized response types of susceptible (Atlantic and chum) and resistant (pink salmon) separated by evidence of a general response (Atlantic and/or chum and pink), a susceptible response (Atlantic and/or chum and not pink) or a resistant response (pink and not Atlantic or chum). Functions are also identified as being present as a systemic response or local response.

Function	**Response Type**			
	General [A +/or C] + P	**Susceptible** [A +/or C] no P	**Resistant** P only	**Systemic or Local**
Unfolded protein response	Y			sys
Acute phase response	Y			sys
Prostaglandin production	Y			sys
Stress-induced and apoptosis	Y			sys/loc
Complement and coagulation	Y			sys/loc
Metalloproteinase activity	Y			sys/loc
Antiviral suppression	Y			sys/loc
Antioxidant activity	Y			loc
Antigen presentation suppression		Y		sys
Reduced hematocrit		Y		sys
Elevated cortisol		Y		sys
Iron homeostasis/heme suppression			Y	sys
Innate pattern recognition receptor			Y	sys
Local inflammation/cytokines			Y	loc

3.7 Chapter acknowledgements

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3.8 Online material

Supplementary data to this chapter can be found online at doi:10.1186/1471-2164-15-200.

Chapter 4: Transcriptomics of coping strategies in free-swimming *Lepeophtheirus salmonis* (Copepoda) larvae responding to abiotic stress

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4.1 Abstract

The salmon louse *Lepeophtheirus salmonis* is a marine ectoparasite of wild and farmed salmon in the Northern Hemisphere. Infections of farmed salmon are of economic and ecological concern. Nauplius and copepodid salmon lice larvae are free-swimming and disperse in the water column until they encounter a host. In this study, we characterized the sublethal stress responses of *L. salmonis* copepodid larvae by applying a 38K oligonucleotide microarray to profile transcriptomes following 24 h exposures to suboptimal salinity (30–10 parts per thousand (‰)) or temperature (16–4 °C) environments. Hyposalinity exposure resulted in large-scale gene expression changes relative to those elicited by a thermal gradient. Subsequently, transcriptome responses to a more finely resolved salinity gradient between 30 ‰ and 25 ‰ were profiled. Minimal changes occurred at 29 ‰ or 28 ‰, a threshold of response was identified at 27 ‰, and the largest response was at 25 ‰. Differentially expressed genes were clustered by pattern of expression, and clusters were characterized by functional enrichment analysis. Results indicate larval copepods adopt two distinct coping strategies in response to short-term hyposaline stress: a primary response using molecular chaperones and catabolic processes at 27 ‰; and a secondary response up-regulating ion pumps, transporters, a different suite of chaperones and apoptosis-related transcripts at 26 ‰ and 25 ‰. The results further our understanding of the tolerances of *L. salmonis* copepodids to salinity and temperature gradients and may assist in the development of salmon louse management strategies.

4.2 Introduction

The salmon louse *Lepeophtheirus salmonis* (Copepoda: Caligidae) is an ectoparasite of wild and farmed salmonids (*Salmo* and *Oncorhynchus* spp.) in the Northern Hemisphere (Nagasawa *et al.* 1993; Johnson *et al.* 2004; Beamish *et al.* 2009), although genetically distinct varieties of *L. salmonis* occur in the Atlantic and Pacific Oceans (Yazawa *et al.* 2008). The louse develops through three free-living and nonfeeding stages (nauplii I and II and the infective copepodid) and seven parasitic stages (four nonmotile chalimus, two motile pre-adult stages and one motile adult; Johnson & Albright 1991). In British Columbia, Canada, adult Pacific salmon carry gravid *L. salmonis* when they return from the ocean to spawn (Beamish *et al.* 2005). In addition, farmed salmon in open-net pens and other resident hosts in the area support infections with the parasite (Johnson *et al.* 2004; Morton *et al.* 2004; Beamish *et al.* 2005; Jones 2009). If not properly managed, infections transmitted from farmed salmon can cause epizootics on juvenile wild salmon leading to population-level effects (Krkošek *et al.* 2007). The costs of treatment and management of *L. salmonis* on farmed salmon globally are approximately $400M CAD per annum; infections remain a major obstacle to sustainable industry development (Costello 2009a). There are a limited number of chemical treatment options (Johnson *et al.* 2004), raising concerns for resistance development to commonly used treatments in Scotland, Norway and Atlantic Canada (Jones *et al.* 1992; Denholm *et al.* 2002; Boxaspen 2006; SEARCH 2006; Brooks 2009; Burridge *et al.* 2010; Chang *et al.* 2011). Integrated pest management principles advocate reduction of pesticide reliance to avoid resistance development and minimize environmental residues (Brooks 2009; Burridge *et al.* 2010). Other potential methods of control may include the use of cleaner wrasse, leaving farms to fallow, reducing synthetic light, and ensuring high water velocity at sites (SEARCH 2006).

The biology of the salmon louse is strongly influenced by environmental conditions, and there is an interest in understanding how changes in these conditions affect the propagation dynamics of louse populations (Brooks 2005, 2009; Price *et al.* 2010). For example, temperature influences fecundity and time to hatching (Boxaspen & Næss 2000; Johnson *et al.* 2004; Boxaspen 2006; Costello 2006), and increased temperature during exposure results in increased louse settlement success, development and prevalence over a 10-day experimental infection (Tucker *et al.* 2000). Development and survival of *L. salmonis* are optimal at salinities greater than 26 parts per thousand (‰; Bricknell *et al.* 2006). Without a host, adult female *L. salmonis* can osmoregulate down to 12.5 parts per thousand (‰) salinity (<8 h to death in freshwater), while adult lice attached to the host survive in freshwater from 3 to 7 days, possibly through diet-obtained ions (Hahnenkamp & Fyhn 1985; Connors *et al.* 2008). Experimental infections of Atlantic salmon with copepodids at 34‰ or 24 ‰ consistently resulted in reduced settlement success and slower louse development at 24 ‰ (Tucker *et al.* 2000). In contrast to attached stages, larval lice are more sensitive to low salinity, potentially due to the absence of dietary ions and the increased energetic demands of the hyposaline stress (Bron *et al.* 1993; Bricknell *et al.* 2006). Copepodid development is inhibited at salinities <30 ‰ (Johnson & Albright 1991), although detrimental effects may be transient if exposure is short term (Bricknell *et al.* 2006). Experimental incubations suggest negative effects on copepodids are manifested at salinities <27 ‰: several hours at ~26 ‰ severely compromised survival and infectivity potential; 1 h at 16 ‰ resulted in mortality of approximately 50% of copepodids; and below 12 ‰, death was rapid (Bricknell *et al.* 2006). An improved understanding of the larval *L. salmonis* response to hypo-osmotic environments may allow the incorporation of salinity levels into parasite management strategies (Brooks 2009).

The application of genomics to copepod biology provides ecological, evolutionary and economic insights (Bron *et al.* 2011) and adds to the knowledge base from ecotoxicology studies (Raisuddin *et al.* 2007). Recently, a transcriptomic analysis of hyposaline responses in the euryhaline green crab *Carcinus maenas* has provided new information on the responses of crustaceans to environmental salinity changes (Towle *et al.* 2011). Many gene expression studies of environmental abiotic stressors in marine copepods (temperature, salinity, environmental contaminants) utilize specific gene markers and enzyme isoforms (Lauritano *et al.* 2011), although transcriptomic studies exist (e.g., *Tigriopus japonicus* responses to copper; Ki *et al.* 2009). Collectively, these studies indicate large variations in responses, but identifying stress-specific markers remains a goal (Lauritano *et al.* 2011). Transcriptomics has also been applied to identifying genes involved in *L. salmonis* postmoulting maturation and egg production (Eichner *et al.* 2008). The earlier observations support a hypothesis that *L. salmonis* experiences physiological stress in association with reduced salinity and that this depends on salinity level, development stage and host association. The development of a 38K *L. salmonis* oligonucleotide microarray described herein has provided a platform to test this hypothesis and to characterize the transcriptomic basis of the stress response of free-swimming *L. salmonis* responding to changes in environmental salinity or temperature.

4.3 Methods

4.3.1 Animal preparation, exposures and RNA extraction

Lepeophtheirus salmonis obtained from seawater netpen-reared Atlantic salmon *Salmo salar* in western British Columbia were maintained in cold aerated seawater during transport to the Pacific Biological Station in Nanaimo, BC. Intact and pigmented egg strings were removed and incubated in flasks containing 400 mL of filtered and aerated seawater. The resulting nauplii were maintained at 30 ‰ salinity until a majority moulted to copepodids (Johnson & Albright 1991), at

which time they were pooled and then aliquoted into groups of ~500 lice per beaker. Triplicate flasks were incubated for 24 h at 4, 10 or 16 °C with salinity held constant at 30 ‰. In another experiment, triplicate flasks containing seawater diluted to 30 ‰, 25 ‰, 20 ‰ or 10 ‰ were incubated at 10 °C. These wide-range experiments were repeated once. A single high-resolution salinity experiment was conducted as above, but with six beakers per condition and at salinities of 30 ‰, 29 ‰, 28 ‰, 27 ‰, 26 ‰ and 25 ‰ and a constant temperature of 10 °C.

The lice were recovered onto 47-mm cellulose acetate/cellulose nitrate filter membranes with a pore size of 8.0 μm (EMD Millipore). The membranes were flash-frozen in liquid nitrogen and stored at −80 °C. Frozen filters containing lice were homogenized with a mixer mill (Retsch® MM 301), and RNA was extracted using TRIzol® (Invitrogen), as per manufacturers' instructions, and purified through RNeasy spin columns with an on-column DNase I treatment (QIAGEN) to degrade genomic DNA. Total RNA was then quantified by spectrophotometry (NanoDrop-1000) and quality-checked by electrophoresis on a 1% agarose gel. Samples were then randomized for all downstream nucleic acid manipulations.

4.3.2 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 nucleotide (Perkin Elmer). For each experiment, a reference pool was

generated using equimolar cRNA from each experimental condition. In the wide-range salinity experiment, the 25 ‰ condition was added at a later date, and therefore, this condition was not included in the reference.

4.3.3 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 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: 70; Cy3: 70).

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) and have been uploaded to GEO (GSE37976). 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.

4.3.4 Differential expression and functional analysis

Array probes were tested for significance in each experiment using a one-way ANOVA without equal variance assumption, with a post hoc Tukey's HSD ($P \leq 0.01$). Probes were filtered for fold change difference ≥1.5 from control (10 °C and 30 ‰ in temperature and salinity experiments, respectively). All probes passing significance and fold change filtering in the salinity experiment (high resolution) 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 in DAVID bioinformatics tool (modified Fisher's exact test; Huang *et al.* 2009), using UniProt accession numbers of clustered probes compared with a background list as all probes passing quality control filters for each experiment.

4.3.5 Reverse transcriptase–quantitative polymerase chain reaction (RT–qPCR)

The same RNA samples used for microarrays in the high-resolution salinity experiment were used for RT–qPCR. Synthesis of cDNA was performed with 2 μg total RNA in 20-μL reactions using oligo(dT) primers and SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen), as per manufacturer's instructions. Each cDNA sample was diluted 20-fold. To generate a standard curve, one sample from each of the six conditions was randomly selected and synthesized as described previously. These samples were then pooled and diluted 7-fold. This pool was then used for a serial dilution (5-point, 5-fold each point) for efficiency tests. qPCR amplification was performed using SsoFast™ EvaGreen® (Bio-Rad) in 20-μL reactions with 0.3 μm of each primer using the following thermal regime: segment 1, 95 °C for 30 s, 1 cycle; segment 2, 95 °C for 5 s, 55 °C for 20 s, 40 cycles; segment 3, 95 °C for 10 s; melt curve, ramp from 55 to 95 °C (fluorescence read each 0.5 °C increment). Genes of interest were selected from the microarray results due to biological relevance, high significance level or presence in significantly enriched GO categories. Reference gene candidates were selected from microarray

results indicating stable expression across conditions, consistency across replicate spots and moderate levels of expression as well as from previous literature (Frost & Nilsen 2003). Primers were designed in Primer3 (Rozen & Skaletsky 2000) selecting amplicon sizes of 80-150 base pairs (Table 1; all R2 were ≥0.99). Amplicons were checked for single products by melt curve analysis and were sequenced to confirm identity as previously described (Sutherland *et al.* 2011). RT–qPCR data analysis was performed using qbasePLUS (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* (Frost & Nilsen 2003) and *filamin-A*, with a collective M value of 0.581 and CV of 0.203, a value within the range typically observed for stably expressed reference genes in heterogeneous samples (Hellemans *et al.* 2007). Other tested reference genes that were not used to normalize due to higher variability included the following: *vinculin* and *tubulin beta chain* (data not shown). Technical replicates were within 0.5 Ct for 934/936 sample–target combinations. NTC and RT controls showed no amplification. Statistical significance was identified by one-way ANOVA ($P \leq 0.05$) with pairwise significance determined by means of confidence intervals (Biogazelle). Correlation between methods (RT–qPCR and array) was checked using a linear best fit line of log2 expression values for RT–qPCR samples vs. microarray log2 expression ratios (Cy5/Cy3) for the probe corresponding to the contig used for primer design.

4.4 Results

4.4.1 Broad survey – responses to thermal and hyposalinity exposures

Exposures to a wide range of salinity (10–30 ‰) and temperature (4–16 °C) were used to survey for transcriptome perturbances in *L. salmonis* copepodids. Temperature incubations for 24 h resulted in few genes differentially expressed from the 10 °C control (Figure 15, Table 10). Total gene numbers differentially expressed (Table 10) and the fold change differences from the

control (Figure 15) suggested 16 °C had a greater influence on gene expression than did 4 °C. In general, few differentially expressed genes and low consistency between temperature exposure trials indicate that the temperature range selected does not have a strong effect on copepodid gene expression over a 24 h exposure.

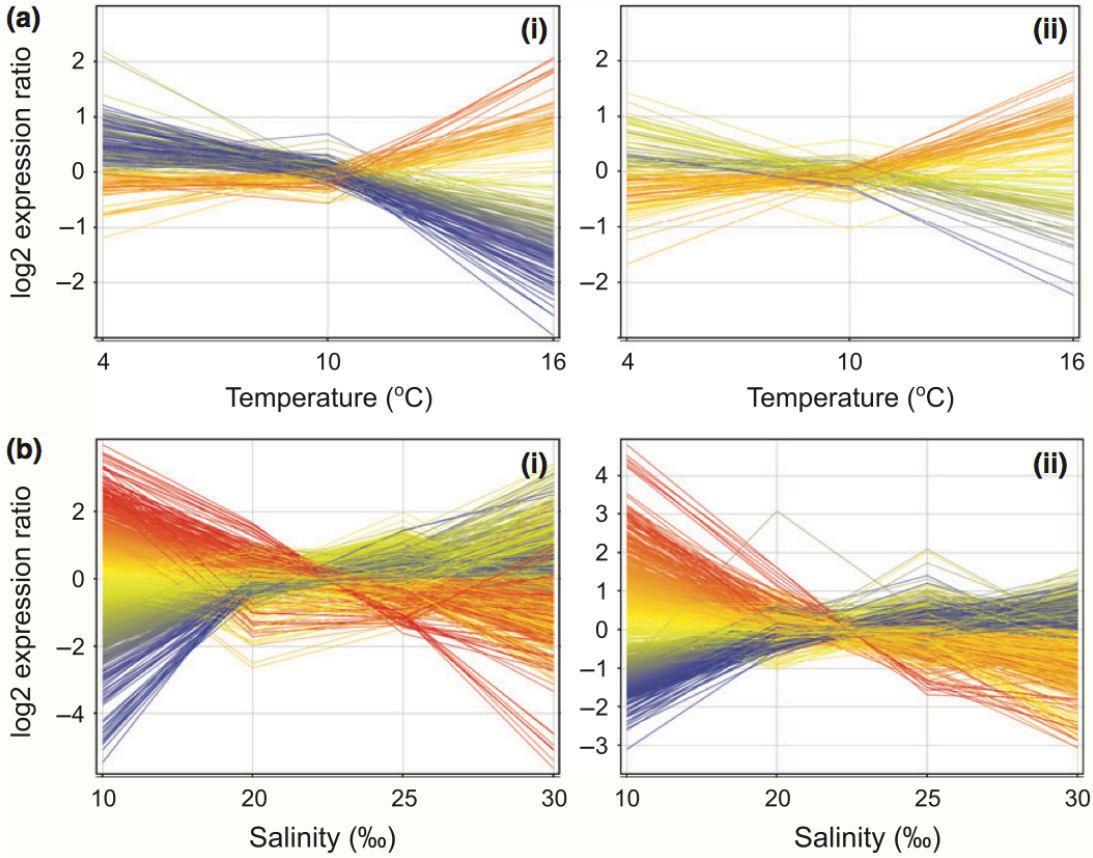

Figure 15. Transcriptome response to 24 hour exposures to changed temperature or reduced salinity A 24 h exposure to changes in temperature (a) affected the expression of fewer genes compared with a similar exposure to hyposalinity (b). Each coloured line displays the average log2 expression ratio (Cy5-sample/Cy3-reference) of a transcript across all conditions. Each transcript is normalized to the median expression level of that transcript across all conditions. Each unit of vertical deflection of the expression ratio corresponds to a 2-fold change in regulation. Lines are coloured according to the magnitude and direction of expression at 10 ‰ or 16 °C. Each plot represents an independent experiment. To be present on a plot, a transcript must be differentially expressed in at least one condition com- pared with the control (one-way ANOVA and Tukey's HSD $P \leq 0.01$; $FC \geq 1.5$).

Table 10. Genes responding to temperature and salinity changes

24-hour exposure to hypo-salinity resulted in a large number of differentially expressed genes, whereas temperature had less of an effect. Trials 1 and 2 represent independent experiments. Genes were tested for differential expression from control by one-way ANOVA and Tukey's HSD ($p \leq 0.01$; $FC \geq 1.5$). Numbers of genes differentially expressed represent genes with unique annotations.

Expt.	Comparison (°C or ‰)	Trial	Direction	Differentially expressed genes	Common bw. trials
Temp (WR)	**4 vs. 10** ºC	1	up	13	1
		2		17	
		1	down	4	0
		2		12	
	16 vs. 10 ºC	1	up	29	1
		2		19	
		1	down	38	5
		2		25	
Salinity (WR)	**25 vs. 30 ‰**	1	up	295	31
		2		59	
		1	down	119	6
		2		24	
	20 vs. 30 ‰	1	up	281	35
		2		97	
		1	down	139	9
		2		32	
	10 vs. 30 ‰	1	up	441	91
		2		209	
		1	down	340	72
		2		183	
Salinity (HR)	**29 vs. 30 ‰**	*n/a*	up	4	*n/a*
			down	8	
	28 vs. 30 ‰	*n/a*	up	16	*n/a*
			down	31	
	27 vs. 30 ‰	*n/a*	up	193	*n/a*
			down	282	
	26 vs. 30 ‰	*n/a*	up	221	*n/a*
			down	138	
	25 vs. 30 ‰	*n/a*	up	464	*n/a*
			down	408	

WR = wide range experiment; HR = high-resolution experiment

Hyposalinity exposures resulted in many genes changing in expression from the 30 ‰ control (Figure 15). At 10 ‰, transcriptome perturbance was largest (Table 10), although many genes had already changed between 30 ‰ and 25 ‰. A larger number of differentially regulated genes were observed in salinity trial 1 relative to trial 2, and this difference may be from different copepod broods being used in each trial (Table 10). However, responding genes common to both trials were identified (see 'Common bw. trials' in Table 10). On average, the proportion of up-regulated genes shared between trials in each trial was approximately 15 % and 44 % for trial 1 and 2, respectively. The magnitude of fold changes for each differentially expressed gene, and the larger number of differentially expressed genes in the short-term hyposalinity exposure contrasts with the results of the short-term temperature exposure and indicates the importance of salinity for free-swimming *L. salmonis*.

Increased transcription of chaperones is often viewed as an indicator of cellular stress (Lauritano et al. 2011). Several chaperone or proteasome genes were up-regulated in both salinity trials including *26S proteasome non-ATPase regulatory subunit 6* (25 ‰), *26S proteasome non-ATPase regulatory subunit 4* (20 ‰, 10 ‰), *proteasome subunit beta type-3* (10 ‰), *60-kDa heat shock protein, mitochondrial* (20 ‰, 10 ‰) and *heat shock 70 kDa protein cognate 4* (20 ‰). *Trypsin-1* was down-regulated in both trials at 25 ‰. *Programmed cell death protein 4* was up-regulated in both trials at 20 ‰ and 10 ‰. Interestingly, several cuticle proteins were up-regulated in both trials at 10 ‰ (*cuticle protein 6*; *cuticle protein CP14.6*; *chitin bind 4*). *Calreticulin* was identified as up-regulated in both trials at 10 ‰. The consistent presence of these chaperone- and apoptosis-related transcripts probably indicates hyposalinity stress in the copepodids.

4.4.2 High-resolution profiling of hyposaline transcriptome responses

To identify a threshold of response, and to capture primary responses to hyposaline stress, a higher-resolution range was used (30–25 ‰, single increment decreases). Relative to the control, the number of transcripts differentially expressed increased rapidly at 27 ‰ compared with changes at 28 ‰ or 29 ‰ (1179 probes differentially expressed by 27 ‰; uniquely annotated genes: 193 up- and 282 down-regulated; Table 10; Figure 16a). This increase in differentially expressed genes may indicate a threshold of response above which reduced salinity does not have a measurable effect on the transcriptome. At 26 ‰, differentially expressed transcripts belonged to a different suite of genes; many genes that responded in the 27 ‰ condition were not up-regulated in the 26 ‰ or 25 ‰ conditions (Figure 16a). The greatest number of differentially expressed transcripts in the high-resolution study occurred at 25 ‰ (2451 probes differentially expressed; uniquely annotated genes: 464 up and 408 down). The differentially expressed transcripts were clustered by similar patterns of expression to resolve several salinity response types (Figure 16b) described as primary, differentially regulated at 27 ‰ (cluster *i* and *ii*); secondary, differentially regulated at lower salinity (26 ‰ and 25 ‰; cluster *iii*); or continual, gradually increasing or decreasing across the exposure conditions (cluster *iv* and *v*). Primary response genes are either at baseline in lower salinity conditions or drop below the baseline. These different clusters are largely composed of different genes and response functions at different salinity levels.

Certain chaperone types were typical of specific salinity responses. For example, *hsp90 alpha*, *hsp70 protein 14*, *protein disulphide isomerase 2*, and several *chaperonin-containing t-complex protein* (*cct*) *subunits* were all up-regulated at the primary peak (Table 11; Figure 16i). However, with the exception of *cct subunit epsilon*, none of these genes were differentially expressed in the 26 ‰ or 25 ‰ response conditions. *Heat shock protein beta-1*, several *hsp70*

isoforms and some *DnaJ homologs* increased at the lower salinities (Table 11). CCT substrate was originally thought to be restricted to tubulin and actin and linked to cell cycle progression; however, it is now known to have broader specificity (Brackley & Grantham 2009). CCT subunits may be up-regulated during proliferation; however, CCT's role in abiotic stress handling was recently identified in cold hardiness of insects during diapause (Rinehart *et al.* 2007).While the precise role of CCT in the present study is not clear, the concerted regulation with the other chaperones suggests a role in the maintenance of cellular function (Table 12). Proteasome activity is also identified within the primary peak (proteasome complex; P = 0.001; Table 11, Table 12).

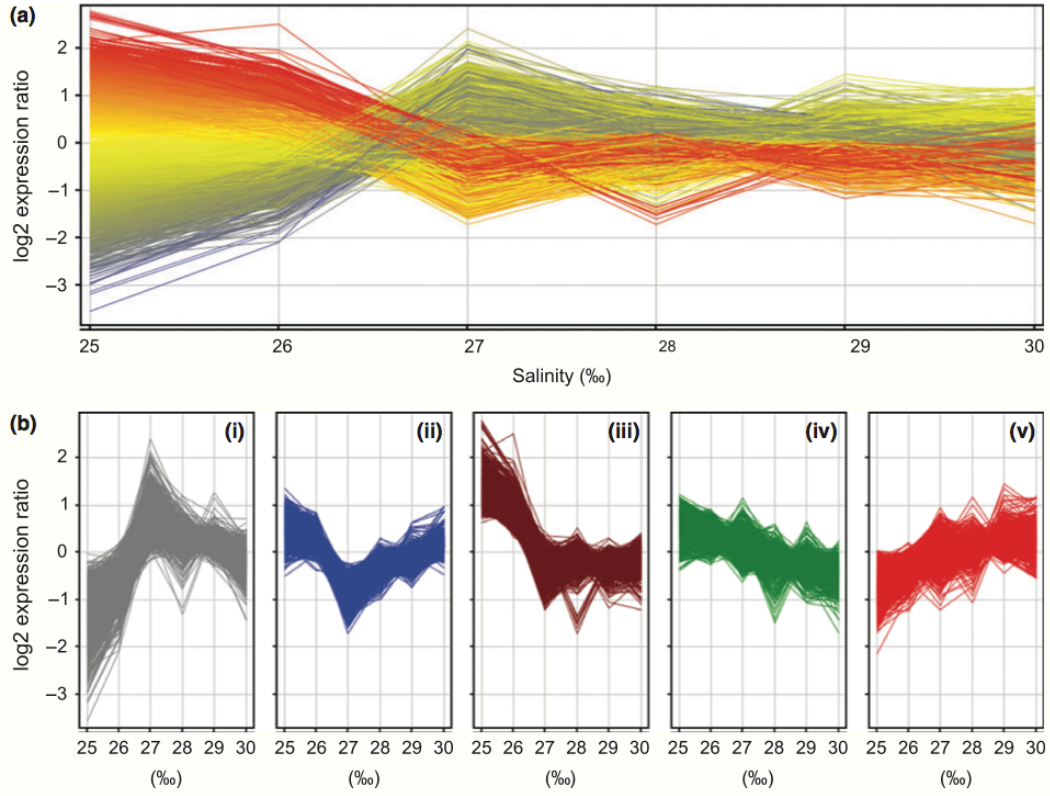

Figure 16. Gene expression changes from single unit changes in salinity between 30 to 25 parts per thousand salinity

Gene expression affected by single unit changes in salinity between 25 ‰ and 30 ‰. (a) Overview of log2 expression ratios (Cy5-sample/Cy3-reference) of all transcripts differentially expressed from the control (in at least one condition) indicates few changes at 29 ‰ or 28 ‰, an

initial response at 27 ‰, and a large secondary response at 26 ‰ and 25 ‰. Each transcript is normalized to the median expression level of that transcript across all conditions. Each unit of vertical deflection of the expression ratio corresponds to a 2-fold change in regulation. (b) Five patterns of expression were identified by cluster analysis, indicating different responses typical of different salinity levels. Differential expression was detected by one-way ANOVA and Tukey's HSD ($P \leq 0.01$; $FC \geq 1.5$).

Table 11. Genes involved in protein folding and degradation were affected by hypo-salinity exposure relative to the 30 part per thousand salinity control

Genes were tested for differential expression from the control by one-way ANOVA and Tukey's HSD ($p \leq 0.01$; $FC \geq 1.5$). Fold change ratios are $\log_2$(experimental) – $\log_2$(control) with standard error. Value of +1 = 2-fold up-regulation. Absent values indicate no significant difference from control.

Gene	*Probe ID*	*Salinity (‰)*				
		25	*26*	*27*	*28*	*29*
Protein Folding – production and maintenance of proper protein conformation						
Heat shock 70 kDa protein 14	C250R106	-	-	1.04 ± 0.19	-	-
Heat shock protein HSP 90-alpha	C252R026	-	-	1.49 ± 0.31	-	-
T-complex protein 1 subunit alpha	C213R139	-	-	1.22 ± 0.29	-	-
T-complex protein 1 subunit beta	C010R138	-	-	2.21 ± 0.54	-	-
T-complex protein 1 subunit delta	C198R114	-	-	1.48 ± 0.29	-	-
T-complex protein 1 subunit epsilon	C170R116	1.20 ± 0.24	-	1.41 ± 0.42	-	-
T-complex protein 1 subunit zeta	C191R120	-	-	1.81 ± 0.41	-	-
T-complex protein 1 subunit eta	C213R160	-	-	2.29 ± 0.51	-	-
Protein disulfide-isomerase 2	C242R105	-	-	0.95 ± 0.22	-	-
Heat shock 70 kDa protein	C150R102	3.02 ± 0.37	2.78 ± 0.42	-	-	-
Heat shock 70 kDa protein 4L	C192R161	1.28 ± 0.31	1.25 ± 0.29	-	-	-
Heat shock 70 kDa protein cognate 4	C219R057	-	1.27 ± 0.23	-	-	-
Heat shock protein beta-1	C172R035	1.25 ± 0.27	1.27 ± 0.41	-	-	-
Heat shock protein homolog	C130R040	-0.96 ± 0.15	-	-	-	-
Protein disulfide-isomerase A4	C124R001	-	-1.38 ± 0.45	-	-	-
Protein disulfide-isomerase A6	C088R134	-1.58 ± 0.43	-	-	-	-
DnaJ homolog subfamily B member 4	C006R133	1.75 ± 0.29	1.45 ± 0.47	-	-	-
DnaJ homolog subfamily B member 6-A	C251R008	1.00 ± 0.23	0.93 ± 0.26	-	-	-
DnaJ homolog subfamily C member 1	C107R150	1.22 ± 0.21	-	-	-	-
DnaJ homolog subfamily C member	C123R120	-1.57 ± 0.23	-	-	-	-

27						
Proteasome – degradation of unneeded or damaged proteins						
26S proteasome non-ATPase regulatory subunit 2	C229R164	-	-	1.77 ± 0.35	-	-
26S proteasome non-ATPase regulatory subunit 4	C262R145	-	-	1.61 ± 0.31	1.19 ± 0.33	-
26S proteasome non-ATPase regulatory subunit 7	C091R061	-1.23 ± 0.44	-	-	-	-
26S proteasome non-ATPase regulatory subunit 8	C060R115	-0.76 ± 0.14	-	-	-	-
26S proteasome non-ATPase regulatory subunit 10	C091R010	1.11 ± 0.20	-	-	-	-
Proteasome activator complex subunit 4	C161R067	0.92 ± 0.21	0.89 ± 0.26	-	-	-
Proteasome subunit alpha type-6	C048R139	-1.11 ± 0.17	-	-	-	-
Proteasome subunit beta type-1	C134R118	-0.65 ± 0.13	-	-	-	-
Proteasome subunit beta type-2	C133R004	-0.75 ± 0.17	-	-	-	-
Proteasome subunit beta type-3	C055R153	-1.08 ± 0.14	-0.79 ± 0.22	-	-	-
Proteasome subunit beta type-4	C155R060	-0.96 ± 0.18	-	-	-	-

Other genes present in the primary response (cluster *i*) are involved in energy acquisition and control (i.e. cell redox homoeostasis, carbohydrate catabolic process) and chromatin binding (Table 12). Chromatin regulation may be involved in the coordination of the responses at different salinities, enabling highly co-regulated suites of genes (Figure 16). Epigenetic and chromatin regulation is probably important for integrating environmental signals and cell stress with transcriptional programmes (Kim *et al.* 2010). Two chromobox homologs were up-regulated at different phases of the response; *cbx1* followed the primary response, whereas the induction of *cbx2* was consistent with the secondary response (Figure 17). *Histone-binding protein rbbp4* was up-regulated with *cbx1* at 27 ‰ (Figure 17). Interestingly, metamorphosis was identified as an enriched GO category in the primary peak cluster, possibly relating to early stages of tissue reorganization in response to hyposaline stress. Heat shock has been shown to induce metamorphosis in some sessile marine invertebrates (Kroiher *et al.* 1992; Gaudette *et al.* 2001). The presence of moulting nauplii in the samples probably contributed to this signature.

Table 12. Selected enriched functional categories in the five hypo-salinity response patterns

The primary peak (i) and secondary response (iii) represent different mechanisms responding to different levels of hypo-salinity. Significance of enrichment was tested by a modified Fisher's exact test. Clusters *i*-*v* are shown in Figure 16b.

Cluster	Type	Gene Ontology term	Genes in cluster	p-value
(i) primary peak	*BP*	cell redox homeostasis	9	0.0052
		carbohydrate catabolic process	10	0.0022
		metamorphosis	8	0.0236
	CC	proteasome complex	10	0.0010
	MF	chromatin binding	6	0.0351
(ii) primary valley	*BP*	retrograde vesicle-mediated transport, Golgi to ER	4	0.0108
		electron transport chain	9	0.0128
	CC	mitochondrion	57	1.29E-07
	MF	structural constituent of ribosome	13	7.31E-04
		N-acetyltransferase activity	5	0.0450
(iii) secondary response	*BP*	transport	45	0.0086
		response to stress	18	0.0378
		small GTPase mediated signal transduction	14	2.51E-06
		wing disc development	6	0.0189
		gamete generation	12	0.0184
	MF	small conjugating protein ligase activity	8	0.0283
(iv) gradual up	*BP*	macromolecule catabolic process	8	0.0141
		proteolysis	9	0.0218
		modification-dependent protein catabolic process	6	0.0412
	MF	GTP binding	5	0.0319
(v) gradual down	*BP*	muscle contraction	6	0.0044
		tRNA processing	6	0.0436
	MF	ion channel activity	9	0.0088
		structural constituent of ribosome	14	0.0127

BP = biological process; CC = cellular component; and MF = molecular function

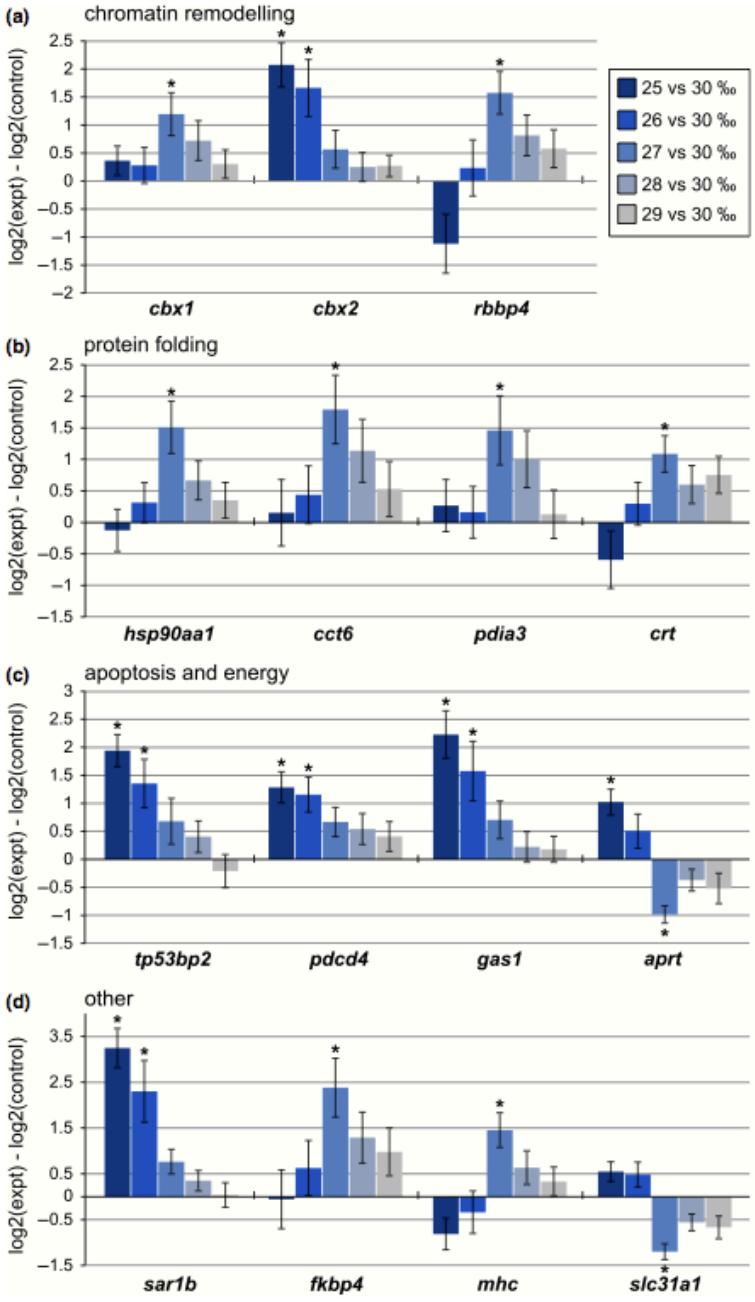

Figure 17. qPCR of selected genes involved in key functions identified by expression clustering

RT–qPCR of selected genes involved in key processes identified by functional enrichment analysis confirms patterns identified in transcript expression clustering. Expression levels are displayed as log2 fold change ± SEM for genes of interest (log2(experimental) – log2(control)). A ratio value of 1 is a 2-fold change, and asterisks denotes significance in difference of condition against control ($P \leq 0.05$).

Genes that are gradually up-regulated and significantly different from control by 26 ‰ or 25 ‰ (cluster *iv*) are involved in macromolecule catabolism (eight genes; $P < 0.014$) and proteolysis (Table 12). These functions may be involved in energy acquisition, the degeneration of peptides to generate free amino acids for hypo-osmotic stress buffering, the degradation of accumulated misfolded proteins or a combination of these. Alternatively, genes that are gradually down-regulated by 26 ‰ and 25 ‰ (cluster *v*) included ion channel functions (9 genes; $P < 0.01$), muscle contraction and ribosomal functions (Table 12). Decreased ribosomal functions (including structural constituents of ribosomes and tRNA processing) may relate to protein translation inhibition for energy preservation and/or halting protein production due to accumulated misfolded proteins. Decreased production of structural proteins may be related to the involvement of muscle contraction, although this could also be due to ionic imbalances from hyposaline stress.

Secondary response genes (cluster *iii* in Figure 16) are involved in transport (Table 12; 45 genes; $P < 0.01$), response to stress (18 genes; $P = 0.04$), small GTPase-mediated signal transduction (14 genes; $P < 0.001$), several remodelling/metamorphosis-related categories and small conjugating protein ligase activity (eight genes; $P = 0.028$). Although several transport proteins were up-regulated at the 27 ‰ response group, such as *Na/K-transporting ATPase subunit alpha* and *sarcoplasmic/endoplasmic reticulum calcium ATPase 1*, the majority of transport proteins are responding at the lower salinities, including down-regulation of several calcium channels and up-regulation of several amino acid transporters and V-type proton ATPase subunits (Table 13). The stress response is probably related to the identified apoptosis-related transcripts up-regulated at 26 ‰ and 25 ‰, such as *apoptosis-stimulating of p53 protein 2* (*tp53bp2*), *programmed cell death protein 4* (*pdcd4*; Figure 17) and *caspase-1 subunit p12* (Table 14). However, this is not clear, as other transcripts such as up-regulated *bax inhibitor 1* and *fas*

apoptotic inhibitory molecule 2 indicate anti-apoptotic activity (Table 14). Small GTPase-mediated signal transduction may relate to vesicular transport. Vesicular transport via COPII vesicles is involved in the unfolded protein response (UPR) maintaining ER homoeostasis by regulating endoplasmic reticulum–associated degradation (ERAD) (Higashio & Kohno 2002; Liu & Chang 2008). *GTP-binding protein sar1b* was up-regulated >8-fold at 25 ‰ (Figure 17) and is an important component of vesicle budding during ER COPII transport (Higashio & Kohno 2002) and cargo proteins transport (Takai *et al.* 2001). Whether the role of this is to alleviate ER stress or to move newly synthesized transport proteins (Table 13) to the cell membrane is unknown. With continual catabolic-related increases (Table 12), down-regulation of protein translation machinery and up-regulation of *growth arrest-specific protein 1* (*gas1*) at 26 ‰ and 25 ‰ (Figure 17) energy may be a constraint in coping with the abiotic stress. The response at 25 ‰ is more indicative of a stress response, of tissue remodelling (including apoptosis) and of longer-term coping mechanisms compared with the potentially transient response at 27 ‰.

Table 13. Hypo-salinity affected the expression of genes for transporters of molecules (e.g., amino acids), ions, or protons

Relative to the 30 ‰ control, at 25 ‰ calcium transporters were down-regulated, whereas amino acid and proton transporters were mainly up-regulated. Differential expression from the control was tested by one-way ANOVA and Tukey's HSD ($p \leq 0.01$; $FC \geq 1.5$). Fold change ratios are $\log_2$(experimental) – $\log_2$(control) with standard error (value of +1 = 2-fold up-regulation). Absent values indicate no significant difference from control.

Function	*Gene*	*Probe ID*	*Salinity (‰)*				
			25	*26*	*27*	*28*	*29*
Ion – Sodium & Potassium	Bumetanide-sensitive sodium-(potassium)-chloride cotransporter	C215R132	1.00 ± 0.15	-	0.88 ± 0.19	-	-
	Sodium/potassium-transporting ATPase subunit alpha	C006R049	-	-	0.59 ± 0.18	-	-
	Sodium/potassium-transporting ATPase subunit alpha-1	C214R147	-1.24 ± 0.20	-	-	-	-

	Trimeric intracellular cation channel type A	C112R134	-1.97 ± 0.34	-1.30 ± 0.44	-	-	-
	Trimeric intracellular cation channel type B	C145R152	-0.62 ± 0.18	-0.83 ± 0.18	-0.76 ± 0.16	-	-
Ion - Calcium	Voltage-dependent calcium channel type D subunit alpha-1	C071R127	-0.76 ± 0.08	-0.91 ± 0.18	-	-	-
	Plasma membrane calcium-transporting ATPase 1	C121R156	-1.59 ± 0.37	-	-	-	-
	Plasma membrane calcium-transporting ATPase 2	C233R020	-2.05 ± 0.21	-1.46 ± 0.51	-	-	-
	Calcium channel flower	C016R093	-0.92 ± 0.17	-	-	-	-
	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	C201R145	-	-	1.28 ± 0.25	-	-
	Calcium-binding protein p22	C229R056	0.99 ± 0.21	1.09 ± 0.29	-	-	-
	Sarcoplasmic calcium-binding protein, beta chain	C263R153	-	0.80 ± 0.15	-	-	-
Ammon-ium	Ammonium transporter Rh type B-B	C057R148	-1.31 ± 0.19	-1.11 ± 0.13	-1.17 ± 0.27	-	-
	Ammonium transporter Rh type C	C065R149	-1.19 ± 0.17	-0.92 ± 0.18	-1.19 ± 0.30	-	-
Amino Acid	Proton-coupled amino acid transporter 4	C203R034	1.62 ± 0.28	1.71 ± 0.38	-	-	-
	Low affinity cationic amino acid transporter 2	C203R001	1.26 ± 0.29	-	-	-	-
	Orphan sodium- and chloride-dependent neurotransmitter transporter NTT73	C094R122	-1.79 ± 0.33	-1.42 ± 0.31	-	-	-
Proton (pH)	V-type proton ATPase 16 kDa proteolipid subunit	C061R055	1.34 ± 0.16	-	-	-	-
	V-type proton ATPase subunit C	C073R064	1.60 ± 0.29	-	-	-	-
	V-type proton ATPase subunit D	C107R139	0.61 ± 0.14	-	-	-	-
	V-type proton ATPase subunit E	C011R121	1.05 ± 0.21	-	-	-	-
	V-type proton ATPase subunit e 2	C046R143	1.43 ± 0.22	-	-	-	-
	V-type proton ATPase subunit F	C190R101	1.59 ± 0.19	1.25 ± 0.32	-	-	-

4.4.3 Correlation between qPCR and microarray

Microarray expression levels correlated well with qPCR expression levels (Figure 18). R^2 values and slope from the best fit line of each sample's log2 expression value from qPCR against microarray are displayed in Figure 18 (average (and median) of R^2 and slope values for genes in Figure 18 are 0.70 (0.68) and 0.82 (0.74), respectively). The clusters were confirmed through the

RT–qPCR analysis, including the primary peak, primary valley and secondary response (Figure 17). Only *aquaporin-9*, *hsp90 co-chaperone cdc37* and *collagen alpha-2 (IV) chain* of 18 tested genes did not show similar patterns (not shown), possibly due to the amplification of paralogs or to false positives from microarray results.

Table 14. Hypo-salinity affected the expression of genes involved in apoptosis (programmed cell death) and acid/base balance and detoxification

Relative to the 30 ‰ control, many of these functions were up-regulated at 25 ‰. Differential expression from the control was tested by one-way ANOVA and Tukey's HSD ($p \leq 0.01$; $FC \geq 1.5$). Fold change ratios are $\log_2$(experimental) – $\log_2$(control) with standard error (value of +1 = 2-fold up-regulated). Absent values indicate no significant change from control.

Function	*Gene*	*Probe ID*	*Salinity (‰)*				
			25	*26*	*27*	*28*	*29*
Apoptosis	Apoptosis-stimulating of p53 protein 2	C179R103	1.04 ± 0.19	0.80 ± 0.21	-	-	-
	Autophagy-related protein 16-1	C037R044	-	-0.86 ± 0.19	-	-	-
	Caspase-1 subunit p12	C225R096	1.45 ± 0.42	1.62 ± 0.38	-	-	-
	Fas apoptotic inhibitory molecule 2	C120R093	1.32 ± 0.20	1.06 ± 0.29	-	-	-
	Programmed cell death protein 4	C168R058	1.63 ± 0.26	1.26 ± 0.30	-	-	-
	Bax inhibitor 1	C233R167	1.30 ± 0.37	-	-	-	-
	Growth arrest-specific protein 1	C222R122	1.50 ± 0.34	-	-	-	-
Acid/base balance & detoxif-ication	Beta carbonic anhydrase 1	C212R074	1.28 ± 0.33	-	-	-	-
	Glutathione S-transferase kappa 1	C038R059	-	-	-0.69 ± 0.16	-	-
	Microsomal glutathione S-transferase 1	C190R083	-	-	-0.84 ± 0.13	-	-
	Glutathione S-transferase DHAR1, mitochondrial	C208R018	1.01 ± 0.13	-	-	-	-
	Glutathione S-transferase Mu 3	C102R105	0.86 ± 0.18	-	-	-	-
	Glutathione S-transferase kappa 1	C038R059	-	-	-0.69 ± 0.16	-	-

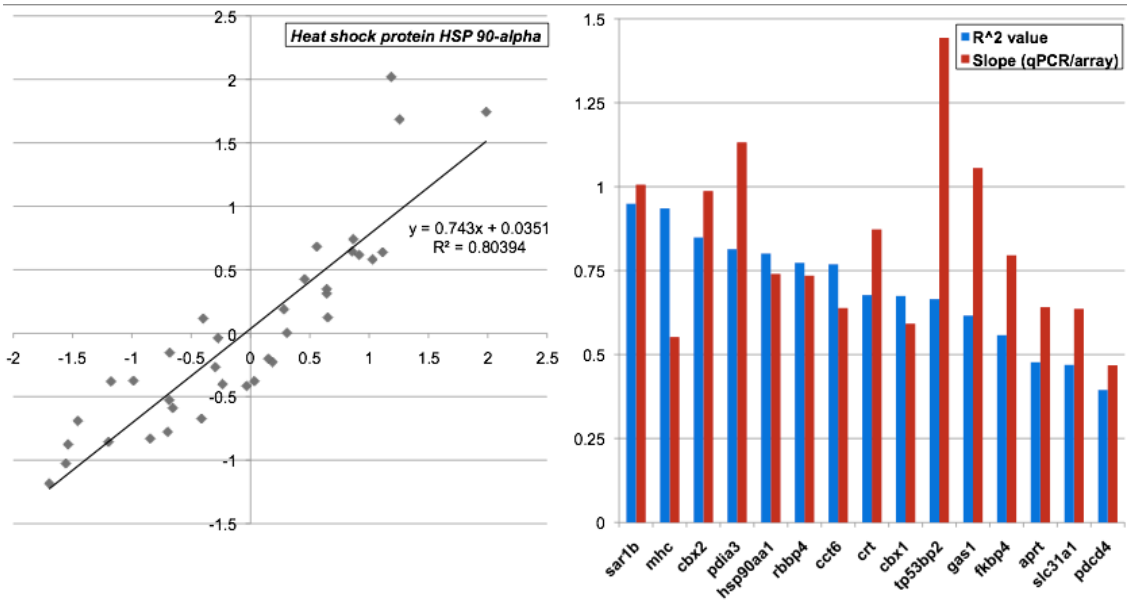

Figure 18. Correlation between log2 qPCR (y-axis) and log2 microarray (x-axis) expression values

4.5 Discussion

A relatively brief hyposaline exposure resulted in large transcriptional changes consistent with distinct stress responses in larval dispersal stages of *L. salmonis*. In contrast, a similarly large effect on transcription was not observed following short-term exposures to hypo- or hyperthermal environments, although some effects were identified at high temperature. It is possible that longer-term exposures (days–weeks) to hypothermal environments would have a larger effect on growth-related functions. Experimental replication with different broods of lice indicated variation in responses which may partly result from differences in energy reserves among individuals (Bricknell *et al.* 2006). Despite this variation, it is clear that hyposaline water causes large-scale changes in gene expression programmes of *L. salmonis* larvae.

Host-seeking behaviour displayed by *L. salmonis* includes movement towards and maintenance at haloclines near river mouths during salmon migrations, and thus, copepodids must be able to cope with short-term salinity fluctuations (Brooks 2005). Coping mechanisms are

expected to minimize effects of suboptimal environments and to minimize costs associated with coping. The transient cellular stress response (CSR) is induced by various stressors through macromolecule damage and can target cell cycle control, protein chaperoning, DNA/chromatin stabilization, removal of damaged proteins and some aspects of metabolism (reviewed in Kültz 2005). The threshold response at 27 ‰ may be an *L. salmonis* CSR, characterized by chaperone and proteasome activity, chromatin binding and redox homoeostasis (Table 11, Table 12; Figure 17). Down-regulated genes at 27 ‰ (cluster ii; Figure 16), including structural components of ribosomes (Table 12), may indicate the down-regulation of other genes during rapid onset of HSPs (Rinehart *et al.* 2007). Proteasome and chaperone activities usually require ATP hydrolysis (Kültz 2005), and therefore, this coping strategy requires energy expenditure. Below 27 ‰, the initial suite of chaperones may not be optimal for the level of stress (possibly due to elevated energy consumption), as the expression of the HSPs responding at 27 ‰ is at baseline in the 26 ‰ condition (Table 11). It is also possible these chaperones are not up-regulated at salinities <27 ‰, because a second suite of chaperones are better suited to the less transient stress (Table 11) or because of anti-apoptotic activity of chaperones (see Kültz 2005 for review).

The coping strategy at 26 ‰ and 25 ‰ involves up-regulation of transporters, apoptosis-related genes, different types of chaperones (Table 11) and genes involved in vesicular transport (Figure 17). A less-rapidly induced programme of cells is the cellular homoeostatic response (CHR), a long-term process that will continue until the stressor is removed (Kültz 2005). Unlike the aforementioned CSR, the CHR is stressor specific, with sensors specific to the environmental change (Kültz 2005). The response at 25 ‰ may be typical of a *L. salmonis* CHR to low salinity. Aspects of this response are similar to osmoregulation. Expression changes of some transporters (Table 13) are similar to those identified in the gills of the euryhaline green crab *Carcinus*

maenas responding to hyposalinity (Towle *et al.* 2011). For example, *Na+/K+-ATPase alpha subunit* and *carbonic anhydrase* were up-regulated in the green crab at 10–15 ‰ (Towle *et al.* 2011) and also in the present study at 27 ‰ and 25 ‰, respectively (Table 13, Table 14). Vesicular transport was identified as an important part of the 25 ‰ response in the present study (Table 12; Figure 17), and a gene involved in regulating plasma membrane protein composition was up-regulated in the green crab in a hypo-osmotic environment (Towle *et al.* 2011).

Movement of transporter proteins to cell membranes is important for cellular osmoregulation to increase activity of certain ion pumps and amino acid transporters for pumping free amino acids out of the cell to buffer the osmotic gradient between the cell and interstitial spaces (Pierce 1982). However, differences between the response of the euryhaline green crab gills and *L. salmonis* copepodids, including stable expression of stress-related transcripts in the green crab gill (e.g., HSPs, proteasome subunits) that were up-regulated in louse copepodids along with several apoptotic transcripts (Table 11, Table 14; Figure 17), may be attributed to the euryhaline nature of the crab (Towle *et al.* 2011) compared with the stenohaline copepod. Differences in expression changes of voltage-gated calcium channels and the stable expression of *V-type H+-ATPase* in the green crab also differed from the present study, in which multiple subunits were found up-regulated at 25 ‰ (Table 13). The *V-type H+-ATPase* was shown to be important for hypo-osmotic regulation in the marine copepod *Eurytemora affinis* (Lee *et al.* 2011). While differences in tissue profiling (crab gill vs. whole copepod) should be noted, the similarities and differences in patterns of gene expression displayed by the green crab and *L. salmonis* copepodids highlight the relative sensitivity of free-swimming lice to a hyposaline stressor.

Although these coping mechanisms appear necessary for survival of *L. salmonis*, the energetic costs are probably significant for nonfeeding life stages. Increased expression of catabolic process transcripts at 25 ‰ suggests the high cost of these long-term coping strategies (Table 12). Highly up-regulated sar1b expression at 25 ‰ (Figure 17) suggests coordination of the unfolded protein response and vesicular transport, alleviating endoplasmic reticulum stress caused by accumulated misfolded proteins (Higashio & Kohno 2002). If stress exceeds tolerance limits, the result of individual cells is growth arrest and apoptosis (Kültz 2005), which may be occurring in *L. salmonis* at 25 ‰ (Table 14; Figure 17c). The alternative of these costly mechanisms, and the ultimate outcome once energy reserves are depleted, is probably organism death, as was viewed in 50% of copepodids (Atlantic) after 1 h at 16 ‰ salinity (Bricknell *et al.* 2006).

The regulation of a multitude of genes is being affected by hyposalinity (Figure 16), and this may be enabled through chromatin remodelling (Table 12; Figure 17a). Plant responses to environmental stress, such as drought, are integrated and coordinated through histone modifications, changes in nucleosome occupancy, DNA methylation changes and other chromatin remodelling methods (Kim *et al.* 2010).

The sensitivity of *L. salmonis* copepodids (Pacific) to hyposalinity is indicated by the increased expression of coping-related transcripts after 24 h at 27 ‰ seawater and by larger changes in expression profiles identified at 26 ‰ and 25 ‰ seawater. It will be important to determine whether these patterns of response to hyposalinity differ between *L. salmonis* varieties occurring in the Pacific and Atlantic Oceans (Yazawa *et al.* 2008). The results of this work may assist in the interpretation of salinity maps of coastal zones by identifying areas in which larval *L. salmonis* are likely to survive or experience hyposalinity-associated stress. Although adult forms

can be more robust to hyposalinity stress, it is important to consider juvenile forms when defining optimal environmental ranges (Lockwood & Somero 2011). Further, as suggested by Brooks (Brooks 2009), if levels of lice are not higher than set thresholds and a freshwater influx is expected, treating after the natural stressor may be best to reduce numbers of chemical treatments to reduce environmental residues and slow down the development of resistance. Although this work may be useful for salmon farm location identification, some areas with large freshwater inputs may not be suitable as aquaculture sites due to the importance of preserving wild salmon migratory routes (Johnson *et al.* 2004; Krkošek *et al.* 2007; Jones *et al.* 2008; Sutherland *et al.* 2011). Regardless of extent of population-level effects, the present work indicates the importance of monitoring salinity around salmon farms.

4.6 Conclusions

A short-term (24 h) exposure to hyposalinity elicited significant changes to the transcriptome of free-swimming larval *Lepeophtheirus salmonis*. These changes were indicative of short- and long-term coping strategies adopted by the copepod that varied according to the extent of hyposaline stress and potentially the energy reserves of the louse. Transient strategies used ATP-dependent molecular chaperones to maintain cellular integrity, whereas longer-term strategies used transporters and channels in combination with different chaperones. Short-term (24 h) temperature exposures between 10 and 4 °C did not result in major changes in transcription. Elevated temperature (16 °C) affected louse transcriptome profiles, although not to the same extent as was viewed in salinity exposures. Despite variable responses among experimental replicates, consistent patterns were identified, and this work provides stressor-level and stressor-type context for ecological response genes.

4.7 Chapter acknowledgements

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4.8 Online material

Supplementary data to this chapter can be found online at doi: 10.1111/mec.12072

Chapter 5: Basal and induced transcriptome differences between Atlantic salmon lice *Lepeophtheirus salmonis* with differing resistance to emamectin benzoate, and comparison to Pacific salmon lice

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BFK conceived of the study, designed experiments, and contributed to analysis and manuscript preparation.

SRMJ conceived of the study, designed experiments and contributed to analysis and manuscript preparation.

5.1 Abstract

Salmon lice *Lepeophtheirus salmonis* are an ecologically and economically important parasite of wild and farmed salmon. Of current concern is the recent development of resistance to a commonly used parasiticide, emamectin benzoate (EMB) in multiple regions globally, including Chile, Scotland, Norway and Eastern Canada. Salmon lice in Western Canada remain sensitive to EMB. New chemical control methods are slow to produce and reach market; delaying or halting resistance development is important. This will be facilitated by early detection and improved monitoring methods, allowing for drug rotation or harvest when necessary and possible. Baseline studies of pre-resistance lice responses and characterization of resistance mechanisms will improve detection efforts. However, resistance mechanisms can be complex, varied and not parallel across populations. Polygenic resistance (i.e. involving multiple and additive gene effects) can be profiled using transcriptomics. Here, we compare the louse responses to EMB bioassays in a Pacific Canada population and two Atlantic Canada populations with differing sensitivity to EMB. In Atlantic populations, male and female responses are also compared, as are basal differences between the different populations and sexes. In Pacific lice, EMB did not induce up-regulation of any genes, however down-regulation of digestive and degradative enzymes was identified at the highest dose (50 ppb). Some genes were found to respond to EMB dose in both Atlantic populations in both sexes, but more genes and higher fold changes were noted in basal differences between populations. In the Atlantic lice, degradative enzymes had higher expression in the resistant population in both sexes, and collagen degradative enzymes had higher basal expression specifically in resistant males. Several genes of note were induced or suppressed from the EMB exposure, including up-regulation of both *alpha-* and *beta-taxilin*, *calcium induced channel slowpoke*, and down-regulation of *kynurenine-3 monooxygenase*. These profiled datasets

will be important for our understanding of resistance mechanisms to EMB and for monitoring emergence of resistance in Pacific Canada.

5.2 Introduction

Salmon lice remain a challenge for the salmon aquaculture industry, with costs associated with louse control, and losses in growth and marketability. For louse control, the salmon aquaculture industry is largely reliant on chemical therapies (Johnson *et al.* 2004; Igboeli *et al.* 2013a) with limited diversity of mechanisms of action, increasing the potential for resistance development (Denholm *et al.* 2002). One of the preferred treatments is emamectin benzoate (EMB; trade name SLICE™, Merck) an in-feed treatment with benefits in comparison to bath treatments including increased ease and safety of administration for farm workers and for the local environment (Telfer *et al.* 2006). In-feed EMB is ingested by the fish over a seven day period, and when administered at 50 μg kg^{-1}, accumulates in flesh and mucus of the salmon at approximately 70-100 ppb (Sevatdal *et al.* 2005). Upon ingestion or absorption by the louse, the macrocyclic lactone avermectin derivative EMB is thought to bind to glutamate-gated chloride channels and causes hyperpolarization in neuromuscular cells, paralysis, and death (Arena *et al.* 1995; Stone *et al.* 1999; Glendinning *et al.* 2011). Although in-feed treatment is preferred for many reasons, unequal ingestion of medicated feed can occur due to individual fish health or hierarchical position in the group, and can lead to sub-therapeutic doses in fish, potentially fostering resistance development (Igboeli *et al.* 2013a). Resistance to EMB has been reported in *L. salmonis* in Norway (Espedal *et al.* 2013), Scotland and Atlantic Canada (Lees *et al.* 2008; Jones *et al.* 2012a; Jones *et al.* 2013) as well as in *Caligus rogercresseyi* in Chile (Bravo *et al.* 2008). Lice in Western Canada remain sensitive (Saksida *et al.* 2013), and continue to be monitored for resistance.

Insecticide resistance can occur through several mechanisms: (1) direct disruption of target site binding; (2) metabolism and detoxification of the active product; or (3) reduction of

drug uptake by cuticle or digestive lining (i.e. penetration resistance; Clark *et al.* 1995; Bonizzoni *et al.* 2012). Detoxification can be further divided into three main components: modification (e.g., cytochromes P450); conjugation (e.g., glutathione-S-transferases); and excretion (e.g., ATP-binding cassette transporters; Lindblom & Dodd 2006). Resistance to ivermectin, another avermectin derivative with a similar mechanism of action to emamectin benzoate, has been reported for the sheep parasite *Haemonchus contortus* (Shoop 1993; Rohrer *et al.* 1994; Le Jambre *et al.* 2000). Ivermectin mechanisms of action and resistance can vary among taxa; within Phylum Nematoda, *H. contortus* and *Caenorhabditis elegans* have differences in gene duplications or losses that may result in differing target sites or resistance factors (Laing *et al.* 2013). Furthermore, resistance mechanisms within a species can differ depending on the selection regime (Gill *et al.* 1998), with different impacts from laboratory-based or field selection (ffrench-Constant *et al.* 2004). It is likely that multiple ivermectin resistance mechanisms exist (Gill *et al.* 1998; Le Jambre *et al.* 2000). Increased selection on the ivermectin target site receptor has been identified in populations of *H. contortus* associated with ivermectin treatment (Blackhall *et al.* 1998b), but resistance can occur without a change in target site binding efficiency (Rohrer *et al.* 1994; reviewed by Kerboeuf *et al.* 2003). A few ivermectin resistant *H. contortus* individuals were collected from a predominantly sensitive population and propagated in a laboratory, a dominant autosomal resistance trait was identified (Le Jambre *et al.* 2000). Even in this case, modifier genes are probably involved in increasing resistance, for example through P-glycoprotein efflux (Blackhall *et al.* 1998a). P-glycoprotein efflux is a main mechanism of ivermectin resistance in *Haemonchus* (reviewed in Kerboeuf *et al.* 2003), where increased transporter basal expression occurs in resistant populations (Xu *et al.* 1998). Additionally, for some drugs, metabolic resistance by esterase breakdown or sequestration may also be involved.

Metabolic resistance is typically polygenic, with many small, but additive effects. This is in contrast to the large effect of a target site mutation (also known as knock-down resistance, *kdr*), for which there has been no identification in the field for ivermectins (ffrench-Constant *et al.* 2004).

Resistance to EMB in *L. salmonis* is an active area of study. In Norway, resistant strains of *L. salmonis* were collected and maintained for four generations, or crossed with a sensitive laboratory strain (Espedal *et al.* 2013). Pure strains remained highly resistant for the four generations, sensitivity of hybrids was intermediate between resistant and sensitive strains, and no evidence of costs associated with resistance was identified. Continued resistance in the absence of selection was also noted in *L. salmonis* from Atlantic Canada over three generations (Igboeli *et al.* 2013b). Intermediate sensitivity in resistant/sensitive hybrids may indicate polygenic resistance mechanisms (Espedal *et al.* 2013); as has been identified in other species to ivermectin (reviewed by Clark *et al.* 1995). An EMB resistance mechanism proposed for *L. salmonis* is P-glycoprotein transporter efflux. Recently, a novel P-glycoprotein was cloned from *L. salmonis* and found to not differ between a resistant and a sensitive strain in basal expression, but to be induced in general by the presence of EMB (Heumann *et al.* 2012). This transporter was also up-regulated in Atlantic Canada *L. salmonis* in response to high doses of EMB, and to increase in archived samples over years coinciding with the development of resistance (Igboeli *et al.* 2012). Further characterization indicated sex-specific up-regulation of this transporter in resistant males in response to 300 and 1000 ppb EMB, up-regulation to a lesser extent in sensitive males, and no differential expression in females (Igboeli *et al.* 2013b). Recently, a transcriptomic analysis of *L. salmonis* populations in Scotland with differing sensitivity to EMB was conducted to identify either induced (200 ppb EMB) or basal gene expression differences between the

populations (Carmichael *et al.* 2013). The largest effect was identified between strains irrespective of EMB presence (359 targets). This included reduced expression of a GABA-gated chloride channel and a neuronal acetylcholine receptor, among others. Transcriptomics in *L. salmonis* is challenging due to evolutionary distance from model organisms and the resultant large number of unknown genes in copepods (Bron *et al.* 2011). Additionally, co-expressed genes in *L. salmonis* can change within a molt stage due to factors such as time since molt, time prior to next molt, or egg production status (females only; Eichner *et al.* 2008).

Microarray profiling can characterize metabolic or penetration resistance mechanisms, but will not identify target site polymorphism. Resistance mechanisms can occur in concert, for example the *kdr* mutation in dichlorodiphenyltrichloroethane (DDT)-resistant mosquitoes does not entirely explain the geographic variation in resistance and other complementary mechanisms are likely (Brooke 2008; Donnelly *et al.* 2009). Advances in genomics and transcriptomics are enabling exploration of complementary resistance mechanisms, such as those occurring in metabolic resistance (ffrench-Constant *et al.* 2004). Transcriptomics is also expanding our knowledge of resistance mechanisms beyond targets typically expected to be involved in drug resistance, and indicates a more complex series of mechanisms than originally expected (Pedra *et al.* 2004; Vontas *et al.* 2005). Polygenic resistance is likely in *L. salmonis* (Espedal *et al.* 2013), and this type of resistance is favoured when selection occurs within the tolerance level of a population, allowing survivors to collectively contribute to minor reduction in sensitivity; when selection occurs outside tolerance, it favours evolution of rarer but more efficacious monogenic resistance (ffrench-Constant *et al.* 2004). Understanding relative contributions of different mechanisms may assist in development of markers for use in resistance monitoring. Improved monitoring may improve management response strategies (e.g., harvest; drug rotation; see Igboeli

et al. 2013a). In-field bioassays to evaluate EC50 values have been developed for *L. salmonis* responses to EMB, however the process is challenging and responses can be variable (Westcott *et al.* 2008). These bioassays are an important method for evaluating resistance, especially when mechanisms are not known (Denholm *et al.* 2002). Analysis of treatment effectiveness using salmon farm data is also useful to detect resistance emergence (Jones *et al.* 2013). Characterization of resistance mechanisms can provide additional reliable means to identify resistance during monitoring with highly sensitive methods (Xu *et al.* 1998).

Here, we apply a 38K oligonucleotide microarray (Sutherland *et al.* 2012; Yasuike *et al.* 2012) to profile gene expression responses of sensitive Pacific Canada lice to EMB, and to compare basal and induced expression differences between two populations of Atlantic Canada lice differing in sensitivity to EMB. Previous work indicates sex-specific differences in EMB tolerance (Westcott *et al.* 2008; Igboeli *et al.* 2013b; Jones *et al.* 2013), and so here we profiled responses in both male and female pre-adult *L. salmonis*. This is the first baseline dataset for transcriptome responses of *L. salmonis* from Western Canada, and provides new insight on functions related to resistance in Atlantic *L. salmonis*.

5.3 Methods

5.3.1 Pacific salmon lice collection and exposure

L. salmonis egg strings were obtained from adult female lice infecting Atlantic salmon in a salmon farm near Campbell River, British Columbia (BC) in March 2009. Eggs were hatched, grown to copepodids, and then grown on Atlantic salmon *Salmo salar* at the Pacific Biological Station in Nanaimo, BC, Canada as previously described (Braden *et al.* 2012). Use of research animals complied with Fisheries and Oceans Canada Pacific Region Animal Care Committee protocol numbers 09-001. Pre-adult lice (n = 777) were removed from salmon sedated with Metomidate hydrochloride (Aquacalm, Syndel Laboratories Ltd.) and held briefly in cold

seawater for less than 1 hour. Approximately 25 pre-adult I and II stage male and female individuals were randomly distributed into each of 24 beakers containing 500 ml filtered seawater (10 ^{o}C; 30 parts per thousand salinity; one air stone per beaker 1.5" by 0.5"). Stock emamectin benzoate (PESTANAL®, Sigma-Aldrich) was prepared to 100 mg/L in methanol, then diluted to working concentrations in seawater. Each of the 24 beakers had been randomly assigned to one of four treatment groups with concentrations of EMB (0, 10, 25, or 50 ppb EMB; n = 6 beakers per condition).

After the 24 hour incubation, lice from each beaker were collected through a mesh filter and flash frozen together (n = 24 pools). Total RNA was extracted from frozen tissue on filters using TRIzol reagent® (Life Technologies) followed by RNEasy column purification as per manufacturers' instructions (Qiagen). Purified total RNA was tested by agarose gel electrophoresis for quality and by spectrophotometry (NanoDrop-1000) for purity and quantity.

5.3.2 Atlantic salmon lice collection and exposure

L. salmonis individuals were obtained from Atlantic salmon farms in Back Bay, New Brunswick, Canada (high EMB resistance) or near Grand Manan, New Brunswick, Canada (low EMB resistance; Jones *et al.* 2012a). Lice collection, egg string harvest, hatching, and infection methods were similar to previous reports (Covello *et al.* 2012; Igboeli *et al.* 2013b). Briefly, lice from the field were grown on fish in house for approximately 80-90 days, after which the third set of egg strings were extruded. Lice hatched from these adults (F1 generation) were grown on Atlantic salmon in house at Atlantic Veterinary College (University of Prince Edward Island) until molt to pre-adult stage. At this point, fish were sedated with Tricaine Methylsulfonate (MS-222) to prevent damage to lice during removal, and lice were collected and placed in Petri dishes with seawater (10 ^{o}C, 33 parts per thousand salinity). Petri dishes were swirled and any lice not

adhering to the side were not used. Bioassays were performed as per standardized protocols (Westcott *et al.* 2008). In brief, pre-adult lice from each population were separated into males and females, and four individuals of each sex and population were randomly distributed to a flask containing 10 ^{o}C seawater. Duplicate flasks were used for each population/sex/concentration combination (n = 40 flasks; Table 15). Dilutions of EMB were prepared as described above (PESTANAL®, Sigma-Aldrich), but to final concentrations of 0, 0.1, 25, 300 or 1000 ppb. These concentrations were selected to include similar concentrations used in Pacific lice exposures (e.g., 25 ppb), as well as concentrations known to cause phenotypic effects in resistant Atlantic lice (1000 ppb). After a 24-hour exposure, lice were flash frozen individually, and then kept at -80 ^{o}C until RNA extraction (n = 77 individuals; Table 15). The use of salmon for this study was approved by Canadian Animal Care Committee protocol #12-016.

Table 15. Experimental design and sample sizes for response of Atlantic and Pacific lice to EMB
For Atlantic lice, each single number is a single louse and for Pacific, each is a pool of 25 lice.

Lice origin	Stage	Sex	EMB-sensitivity	Sample size for each EMB concentration (ppb)				
				0	**0.1**	**25**	**300**	**1000**
Atlantic	pre-adult	female	sensitive	4	4	4	4	4
			resistant	4	4	4	2	4
		male	sensitive	4	4	3	4	4
			resistant	4	4	4	4	4
				0	**10**	**25**	**50**	
Pacific	pre-adult	mixed	sensitive	6	6*	6	6	

**qpcr only*

Total RNA was extracted as described above, but with a TURBO DNase treatment prior to RNEasy cleanup. Purified total RNA was tested by agarose gel and automated electrophoresis (Experion; Bio-Rad).

To confirm EMB resistance differences between the populations, bioassays for EC50 calculations were performed on a subset of the parental collection (F0) and the lice propagated in lab (F1). These bioassays were performed in duplicate flasks with 10 lice per flask per condition (i.e. sex, population, and concentration combination) for F0 lice, and with five and four lice per condition for F1 resistant and sensitive, respectively (F1 EC50 sample sizes were constrained due to the need to reserve lice for bioassays for RNA profiling). After 24 hour incubations (all at 10 ^{o}C and 33 ppt salinity), lice were evaluated as per standards (Westcott *et al.* 2008). EC50 calculations were performed in GraphPad (v6; Prism).

5.3.3 cDNA preparation and microarray hybridization

Labeled cRNA was generated from 825ng total RNA using Low-Input Quick Amp kits (Agilent; v6.5). A Cy3-cRNA reference pool to hybridize alongside samples (Churchill 2002) was created for each of the above experiments, ensuring each experimental condition was included in the pool. Samples were hybridized to oligonucleotide microarrays designed using previously annotated ESTs from both Pacific and Atlantic *L. salmonis* (Yasuike *et al.* 2012) eArray design ID 024389; Agilent), scanned on a ScanArray Express (Perkin Elmer) and quantified on Imagene (v8.1; BioDiscovery) as previously reported (Sutherland *et al.* 2012).

5.3.4 Microarray analysis

For each probe on the microarray, the background median was subtracted from the foreground median. Data analysis was performed in GeneSpring GX11 (Agilent). Each array was normalized using an intensity-dependent *Lowess* normalization (Yang *et al.* 2002). Quality control filters for each of the three experiments retained probes that pass the following criteria in at least 65% of the samples in any one condition: raw signal $\geq$ 500 in both channels and no poor quality flags.

Pacific lice exposures to 0, 25 and 50 ppb (n = 6 per condition; the 10 ppb condition was not included in microarray analysis; Table 15) were tested for differential expression by one-way ANOVA without the assumption of equal variance (Benjamini-Hochberg multiple test corrected $p \leq 0.01$) and post-hoc Tukey HSD between conditions ($p \leq 0.01$). Significantly differentially expressed probes were then filtered by fold change ($FC \geq 1.5$).

Atlantic lice exposures to 0, 0.1, 25, 300 and 1000 ppb in male or female pre-adults from each population (total of 20 conditions; Table 15) were tested for differential expression by three-way ANOVA, using population, sex and EMB concentration as factors and including interaction effects (Benjamini-Hochberg multiple test corrected $p \leq 0.01$). Each condition contained four biological replicates except male sensitive 25 ppb (n = 3) and female resistant 300 ppb (n = 2; Table 15), which were limited due to not enough samples with sufficient high quality RNA. Probes with a three-way interaction effect were removed from other lists and analyzed using k-means clustering based on probe expression (Euclidean distance metric; 4 clusters). Probes with a significant two-way interaction of sex and population were removed from sex or population main effect lists. These probes were subsequently filtered to identify genes with a 1.5-fold change up- or down-regulation between resistant and sensitive populations in one sex and not in the other. Probes with a significant main effect of population were fold change filtered between populations ($FC \geq 1.5$). Probes with a significant main effect of EMB concentration were filtered by fold change against the 0 ppb control ($FC \geq 1.5$). In all cases, probes with a significant interaction effect were removed from main effect lists of factors involved in the interaction.

For both Atlantic and Pacific experiments, Gene Ontology and pathway enrichment was performed in DAVID Bioinformatics using a modified Fishers Exact Test (Huang *et al.* 2009) with $p \leq 0.05$ and at least 4 genes in each category. Identifiers imported to DAVID were Entrez-

IDs assigned to contigs corresponding to the probe (Yasuike *et al.* 2012). Background lists were specific to each experiment (all probes passing quality control for that experiment; Pacific = 12,085 probes; Atlantic = 15,578).

5.3.5 Reverse-transcription quantitative polymerase chain reaction (qPCR) validation and exploration

The Pacific *L. salmonis* samples described above were used for qPCR, and the 10 ppb condition was also included in this analysis. Total RNA was reverse transcribed to cDNA using 2.5 µg input in SuperScript III reactions (Invitrogen) with 50:50 random hexamers to oligo dT primers (total 50 ng), as per manufacturer's instructions. Samples were diluted 20-fold. A standard curve for primer testing was generated by pooling a sample from each condition, diluting seven-fold, and using this as an input for a five-fold, six point serial dilution. All primers were tested for efficiency between 80-110% and a single amplicon using melt curve analysis as well as amplicon purification and sequencing as previously reported (Sutherland *et al.* 2011). qPCR amplification was performed using SsoFast EvaGreen with Low Rox (Bio-Rad) as per manufacturers' instructions for the MX3000P thermocycler (Agilent) using the following thermal regime: 95 ^{o}C for 5 min (1 cycle); 95 ^{o}C for 20s then 55 ^{o}C for 30s (40 cycles); followed by a melt curve (55 ^{o}C to 95 ^{o}C reading fluorescence at 0.5 ^{o}C increments). Samples were run in duplicate, with all technical replicates within 0.5 cycles. A no template control (NTC) and -RT control was run for each gene. Although some contaminating gDNA remained after column purifications, -RT controls for each gene of interest was at least six cycles greater than the most dilute sample for that gene, and thus would have a minimal effect on quantification (Laurell *et al.* 2012).

Pacific salmon lice can carry a microsporidian parasite *Facilispora margolisii* at approximately 50% prevalence (Jones *et al.* 2012b). Since the impact of the microsporidia has not yet been characterized, Pacific lice pools used here were tested for *F. margolisii* using diagnostic

primers (Jones *et al.* 2012b), and all pools tested positive. Additionally, a subset of samples from Atlantic *L. salmonis* was also tested, but no positives were detected, which is consistent with the observation that *F. margolisii* has not been found in Atlantic *L. salmonis* (Simon Jones, *personal observation*).

5.4 Results

5.4.1 Pacific lice transcriptome response to emamectin benzoate (EMB)

The transcriptional response of Pacific lice was minimal until 50 ppb (only 3 probes differential between 25 and 0 ppb). There was no up-regulation at 50 ppb, but 147 probes were down-regulated (43 uniquely annotated genes; Table 16). All of these genes were also down-regulated between 50 and 25 ppb, indicating that the suppression occurred beyond the 25 ppb concentration. These suppressed genes were enriched for peptidase activity (13 genes; p = 8E-11), and protein metabolism (11 genes; p = 2E-2), and included degradative enzymes *trypsin-1*, *carboxylpeptidase B*, *chymotrypsin*, *collagenase*, *acidic mammalian chitinase*, *hypodermin-B* and others. Additionally down-regulated were enzymes *phospholipid hydroperoxide glutathione peroxidase* and *mitochondrial*, *cytochrome P450 2J2*, transport-related *high affinity copper uptake protein 1* and *solute carrier family 15 member 1*, and the calcium-binding neuronal morphogenesis protein, *advillin*.

Table 16. Differentially expressed probes with an effect of EMB dose in the Atlantic and Pacific lice

	Condition (vs. control)	Number probes ≥ 1.5-fold	Number probes ≥ 2-fold
Atlantic	**0.1 ppb**	23	5
	25 ppb	205	41
	300 ppb	571	133
	1000 ppb	485	93
Pacific	**25 ppb**	3	3
	50 ppb	147	144

5.4.2 Atlantic lice differences in sensitivity to EMB

A subset of lice collected from farms (F0 generation) and the F1 generation propagated alongside those being used for RNA profiling were also used for bioassays. For the F0 generation, EC50 values were highest for the Back Bay lice (Bay Management Area (BMA) 1; resistant), with males having the highest EC50 (Table 17). The more sensitive (Grand Manan; BMA 2b) lice had EC50 values that were lower and more similar between males and females. Fewer lice were used from the propagated F1 generation, however the trends viewed for the F0 lice remained. For the F1 generation, all lice were healthy-vigorous at 0, 0.1, and 25 ppb EMB. In the resistant population, at 300 ppb one of the 10 males was moribund (immobile and twitching), and four of 10 females were weak (swimming but not able to attach to beaker), and all others were healthy-vigorous. In the sensitive population at 300 ppb, three of eight females were immobile (no twitching), four were weak (not able to attach to beaker) and one was healthy-vigorous, and in the males, one of eight was immobile, three weak, and four healthy-vigorous. At 1000 ppb EMB all lice in both groups were immobilized after 24 hours.

Table 17. EC50 values for males and females from the two Atlantic populations differing in EMB sensitivity exposed to EMB for 24 hours

	EC50 ppb (95% CI)	
	Males	**Females**
Back Bay (resistant)	840 (614, 1047)	254 (218, 296)
Grand Manan (sensitive)	63 (11, 352)	75 (13, 432)

5.4.3 Atlantic lice transcriptome response to emamectin benzoate (EMB)

Principle components analysis (PCA) indicated the most variation was explained by sex (PC1: 40.1%). Populations clustered individually, and the female resistant population separated along

the gradient of EMB (Figure 19). The main effect lists with the most probes significantly differentially expressed were involved with sex (4684 probes; Table 18), population (3699 probes) or a sex-population interaction effect (8242 probes). Comparisons involving EMB exposure had the fewest probes of these comparisons (1431 probes).

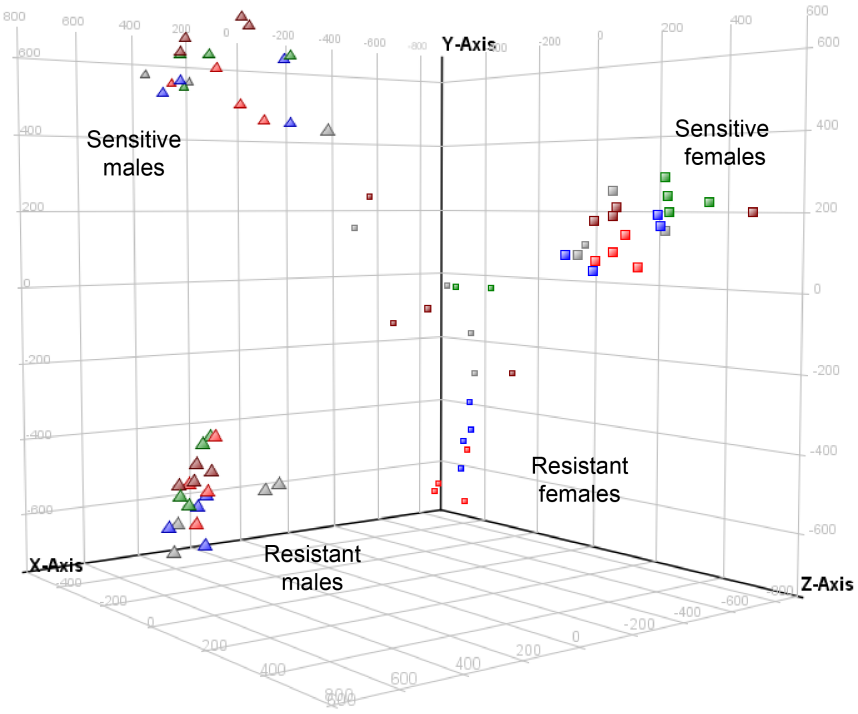

Figure 19. Principal components analysis separates samples by sex and population

The males and females separated along the x-axis (% var), whereas the populations (sensitive or resistant) separated along the y-axis. Interestingly, the only population to show a gradient effect of EMB was the female resistant population. Colors display EMB concentration (red = 0; blue = 0.1; gray = 25; green = 300; brown = 1000), and shape displays the sex (triangle = male; square = female).

Table 18. Results of three-way ANOVA analysis of sex, population and EMB dose on gene expression

Numbers of probes are shown for each significant effect and interaction. Probes with a significant 3-way interaction are not included in significant 2-way interaction or main effect lists, and probes with a significant 2-way interaction are not listed in the significant main effect list involved in the interaction. Numbers shown are prior to any fold change filters.

Comparison	Number of probes*
Sex * Pop * EMB conc	151
Sex * Pop	8242
Sex * EMB conc	26
Pop * EMB conc	19
Sex	4684
Pop	3699
EMB conc	1431

*probes in 3-way interaction were removed from 2-way interaction lists; probes with significant two-way interaction involving the selected factor are removed from that factor's main effect list. Numbers shown are prior to any fold change filters.

5.4.3.1 Effect of EMB exposure, independent of sex or population

The number of genes responding to EMB increased over the doses until 300 ppb, after which the number stayed similar (31 probes at 0.1 ppb, 205 at 25 ppb, 571 at 300 and 485 at 1000 ppb). Even at the highest concentrations, fold changes were generally low, with only 36 probes more than 2.5-fold differentially expressed at 300 ppb. Several of the genes with high fold changes in response to EMB were similar in expression to those with a specific response in females, but the 3-way interaction effect was not significant (e.g., *a disintegrin and metalloproteinase with thrombospondin motifs 12* and *18*, and *venom allergen 3*). Other highly significant up-regulated genes were *ring finger protein nhl-1*, and *alpha-* and *beta-taxilin*. Unchanged at concentrations lower than 1000 ppb, *heat shock protein beta-1* was found to be 2-fold up-regulated at 1000 ppb.

Several ion transport or calcium-related transcripts were up-regulated by EMB including *sarcoplasmic calcium-binding protein* (*beta chain*), *calcium activated potassium channel slowpoke*, *voltage-gated potassium channel subunit beta-2*, *caldesmon*, and *calmodulin* (FC > 1.5). Many of the up-regulated probes with lower fold change (1.5 < FC < 2) were annotated as structural-related genes such as *myosin heavy chain, muscle, titin, troponin C, isoform 1*. Three probes annotated as *sodium- and -chloride-dependent GABA transporter* were differentially expressed, however, two were up-regulated and one was down-regulated at 300 ppb. Also up-regulated were *gamma-aminobutyric acid* (GABA) *receptor subunit beta-like*, and *subunit gamma-2* (FC = 1.6 at 300 ppb). No significant Gene Ontology categories were identified in the genes up-regulated in both 300 and 1000 ppb conditions.

Down-regulated probes included many annotated as degradative enzymes (e.g., *metalloproteinase-9*, *72 kda type IV collagenase*). Also down-regulated were *kynurenine 3-monooxygenase*. Several other transporters were also down-regulated but had lower fold changes, such as *solute carrier family 25 member 38*, *ABC transporter G family member 20*, and *low-affinity cationic amino acid transporter* (FC > 1.5 down-regulated) at 300 and 1000 ppb. In the down-regulated list, response to inorganic substance (4 genes; p = 2.2E-3), system development (9 genes, p = 2.3E-2), and metallopeptidase activity (4 genes; p = 1.9E-2) were enriched.

5.4.3.2 Basal expression differences between populations consistent in males and females

A large number of genes were differentially expressed between the resistant and sensitive populations (Table 18). Resistant lice had higher expression of 466 probes (149 > 2.5-fold) and lower expression of 576 probes (137 > 2.5-fold) than the sensitive population. The resistant population had higher basal expression of genes involved in lipid metabolism (17 genes; p = 3.7E-5), response to chemical stimulus (13 genes; p = 4.7E-3), catalytic activity (60 genes; p =

2E-3), serine-type peptidase activity (7 genes; p = 2.4E-3), and peroxidase activity (4 genes; p = 1.2E-2). The gene with highest fold change was *peroxidasin homolog* (2 probes; > 150 fold). Also overexpressed by at least 5-fold in the more resistant population were *collagenase*, *ovochymase-1*, *trypsin-1*, *phospholipid hydroperoxide glutathione peroxidase* (*mitochondrial*), *cathepsin D*, *carboxypeptidase B*, and several probes annotated as containing *saposin (B) domains.* Other notable overexpressed genes were *cytochrome p450 2J2* (>3-fold), *glutathione peroxidase 2* (2.3-fold), *mucin* (2.5-fold) and *hemicentin-1* (2.4-fold).

The resistant population had lower basal expression of genes involved in system development (21 genes; p = 7E-4), cell differentiation (18 genes; p = 7.5E-4), calcium ion binding (16 genes p = 1.6E-5), microtubule binding (5 genes; p = 2.4E-3), as well as endopeptidase activity (10 genes; 5.7E-3). Genes in the endopeptidase activity category enriched here (e.g., *papilin*, *stubble*, *calpain 11*, and *proteasome subunit alpha type 4* and others) were different than those with high expression in the resistant population. Probes with the highest fold change with lower expression in the resistant population were often not annotated, with six probes > 100-fold different. The annotated probe with the lowest expression in the resistant population was *serine protease inhibitor dipetalogastin* (10-fold down). Both *alpha-* and *beta-taxilin* had lower expression in the resistant population (FC>2.5), as was *glutamate receptor, ionotropic kainite 2*, and *gamma-aminobutyric acid receptor subunit beta-like*. Several structural-related transcripts including *myosin heavy chain, muscle*, *titin*, *troponin c, isoform 1*, *microtubule-actin cross-linking factor 1* also had lower expression in the resistant lice that the sensitive, although fold change for these were lower (FC <2.5 fold). Many unknowns were present in this list (287/576 probes).

Interestingly, more than half of the genes responding to EMB at 300 ppb in all populations and sexes (26 of 41 uniquely annotated genes) also were expressed lower in the resistant population (included 137 uniquely annotated genes). Therefore, for these genes the lowest expression was in the resistant control lice (0 ppb EMB) and the highest expression was in the sensitive lice exposed to high doses of EMB (300 or 1000 ppb). This included *alpha*- and *beta-taxilin*, several structural proteins, and *glutamate receptor ionotropic kainate 2*, among others.

5.4.3.3 Basal expression differences between populations specific to males or females

Some work has indicated differences in sensitivity to EMB between male and female lice (Igboeli *et al.* 2013b; Jones *et al.* 2013). It is therefore of interest to compare responses specific to one sex. Here, many genes had a significant sex by population interaction effect (Table 18). To refine this list further, a fold change filter was applied to identify genes that differ between populations more than 1.5-fold in one sex and not in the other (or not in the same direction in the other sex). Due to the high volume of genes in this list, most of the analysis depended on high fold change or enrichment of functional categories.

Genes with higher basal expression in resistant females compared to sensitive (but not higher in resistant males; 1723 probes) were enriched for development-related functions, such as gland morphogenesis. Some genes were highly differentially expressed, including *tristetraproline* (167-fold), *trypsin-1*, *hemicentin-1*, *trypsin-4*, *tenascin*, and *von Willebrand factor D and EGF domain-containing protein* (FC > 50). Alternately, genes with lower basal expression in resistant females compared to sensitive (but not lower in resistant males; 1332 probes) were enriched for stress response, morphogenesis and neuron morphogenesis, among others (1332 probes). The

highest fold change transcripts were often not annotated, but included *tyrosine aminotransferase*, *72 kda type IV collagenase*, and *histone-lysine N-methyltransferase setd7* (FC > 7).

Genes with higher basal expression in resistant males compared to sensitive (but not higher in resistant females; 1625 probes) were enriched for carbohydrate and collagen metabolism (1625 probes). This included higher expression of *zinc metalloproteinase nas-14*, *nas-6*, and -*nas4* (FC > 50), *glutathione reductase*, *extracellular superoxide dismutase [Cu-Zn]* (FC > 30), and *putative ascorbate peroxidase* (FC > 19). Many of the genes in this list have collagenase functions. Alternately, genes with lower basal expression in resistant males compared to sensitive (but not lower in resistant females; 1820 probes) were involved in development. High fold change was identified for *axoneme associated protein mst101*, and several probes annotated as *DOMON domain-containing proteins*, as well as many unknowns (FC > 400). Many probes annotated as *chitin_bind_4* and *cuticle protein cp14* were also in this list (FC > 50).

5.4.3.4 Genes responding to EMB specifically in one sex and population combination

Genes that responded to the EMB exposure differently depending on sex and population (3-way interaction effect) mainly consisted of genes responding to EMB in the female resistant population only. This is also the condition that showed the separation of concentrations in the PCA (Figure 19). Characterization of genes within this list (151 probes) was enabled by expression clustering into four clusters of co-expressed genes (Figure 20). Cluster 4 response starts with low expression in the controls of both male and female resistant lice, and the female resistant lice increase expression over the doses to the level of the sensitive populations (Cluster 4). This cluster contained *glutenin high molecular weight subunits*, *plasmodium histidine-rich protein*, *a disintegrin and metalloproteinase with thrombospondin motifs 20*, *adhesive plaque matrix protein*, and several unknowns. Other clustered genes start with high expression in

sensitive females, then reduce to a level of the rest of the conditions (Cluster 3), or to the level of the sensitive populations but lower than the resistant males (Cluster 1). Cluster 3 contained transcripts such as *fascilin domain*, *enolase*, *glucosamine-6-phosphate isomerase*, *hemicentin-1*, *protein jagged-1*, *thrombospondin-1*, *transforming growth factor-beta-induced protein igh3* as well as several unknowns.

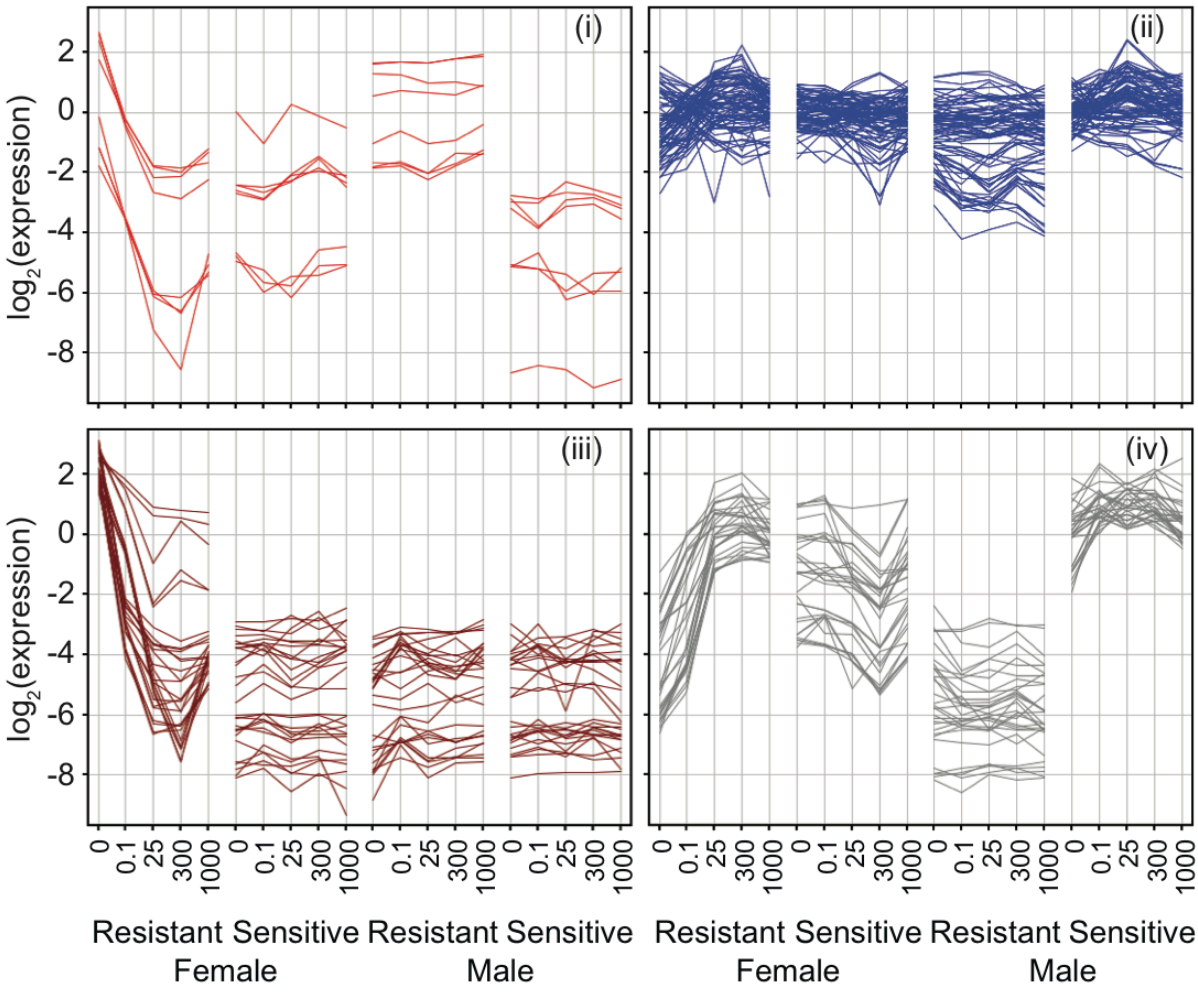

Figure 20. Clustering of probes responding to EMB specifically in one condition

This set of genes (3-way interaction effect $p \leq 0.01$) was mainly comprised of genes responding over the concentration of EMB in the female resistant population. In most clusters the expression of the genes returns to a level more similar to the sensitive populations, or all conditions.

5.4.4 qPCR validation and exploration

The 10 ppb condition was included in the Pacific lice qPCR analysis, in addition to the rest of the samples ran on microarrays. qPCR data confirmed the lack of differential expression until 50 ppb EMB (Figure 21). Additionally, a transporter *aquaporin-9* was also suppressed between 25 and 50 ppb EMB. High correlation was obtained between array and qPCR methods for all genes identified as differentially expressed from the microarray (and *aquaporin-9*) for all significant genes from the microarray, including *carboxypeptidase b* (Pearson r = 0.97), *high affinity copper transport protein* (r = 0.93), *acidic mammalian chitinase* (r = 0.90), and *aquaporin-9* (r = 0.93). Two samples did not correlate well for *trypsin-1*, although the rest of the samples correlated well between the methods for this gene (r = 0.64).

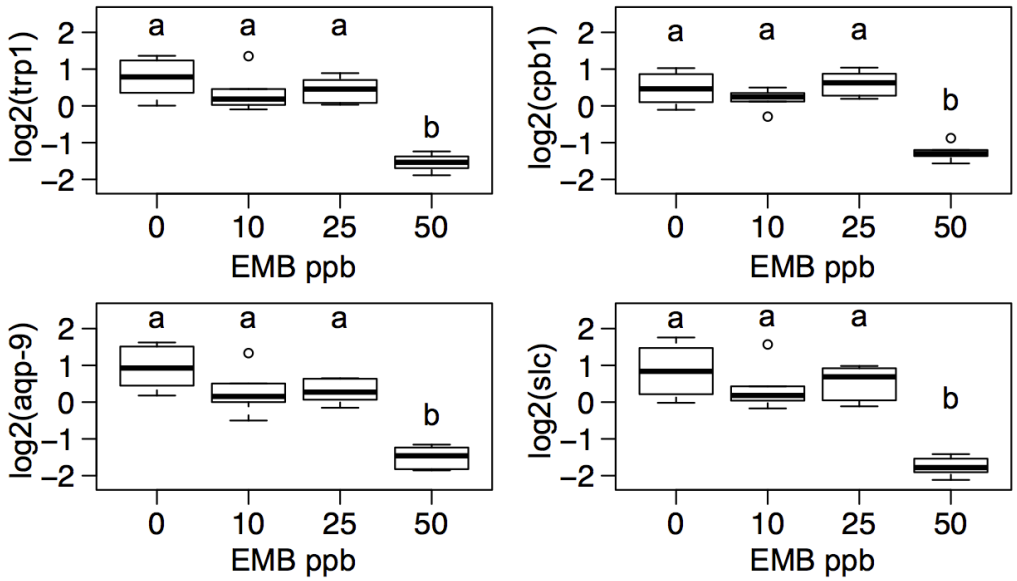

Figure 21. Selected genes down-regulated from EMB profiled by qPCR

Several transporters and peptidase transcripts were found to be suppressed at 50 ppb EMB in the Pacific lice, including *trypsin-1* (trp1), *carboxypeptidase-1* (cpb1), *aquaporin-9* (aqp-9) and *high-affinity copper transport protein* (slc). *Acidic mammalian chitinase* also was found to follow this same expression profile. Conditions that do not share a letter above the boxplot are significantly different from each other (Tukey's HSD $p \leq 0.01$). Boxplot displays median and interquartile range. Data shown = qPCR normalized to *rps20*.

5.5 Discussion

Currently, *Lepeophtheirus salmonis* from Pacific and Atlantic Canada differ in sensitivity to emamectin benzoate (EMB), as resistance is present in Atlantic Canada (Jones *et al.* 2012a) similar to that in Scotland and Norway (Lees *et al.* 2008; Espedal *et al.* 2013), but not in Pacific Canada (Saksida *et al.* 2013). In Atlantic Canada, some areas have been observed to have higher resistance than others, suggesting regional differences in sensitivity (Igboeli *et al.* 2013b). Basal gene expression differences between Atlantic and Pacific *L. salmonis* are due to long term reproductive isolation, but regional differences in Atlantic Canada may be more related to the resistance phenotype. Additionally, potential resistance mechanisms may be inducible in EMB presence (Igboeli *et al.* 2012), and therefore here we can indirectly compare the responses of the Atlantic (male and female pre-adults) and Pacific *L. salmonis* (mixed sex pre-adults) to EMB. Together with previous work characterizing potential resistance mechanisms in response to EMB, this work provides additional insight on resistance to this important compound used in salmon aquaculture, and potentially in polygenic mechanisms not typically considered as part of a resistance response.

In Atlantic lice, population differences in basal expression were larger in number and in fold change than induced responses, in concordance with basal and induced expression differences identified between populations of *L. salmonis* from Scotland with differing EMB sensitivity (Carmichael *et al.* 2013). Even though we exposed the lice to higher doses of EMB in the present study (300 and 1000 ppb compared to 200 ppb (Carmichael *et al.* 2013), and profiled both females and males, we also found the largest effects were not induced by EMB presence. Interestingly, the Pacific lice did not up-regulate genes in response to EMB. It is possible that this could be due to high variance and that an increased sample size may improve estimates, but the down-regulation of genes was identified here at 50 ppb relative to 25 or 0 ppb. In EMB resistant

Caligus rogercresseyi from Chile, any changes in gene expression during EMB treatment of salmon hosts for genes involved in detoxification (e.g., cytochrome P450s) or transport (MRP1; Pgp1) detected by semi-quantitative PCR were moderate (Cárcamo *et al.* 2011). Induced responses to EMB have been noted in previous work, for example in the expression of P-glycoprotein transporters in pre- and post-resistant *L. salmonis* (Igboeli *et al.* 2013a). Interestingly, *heat shock protein-1* was up-regulated in the current work not at 300 ppb but only at 1000 ppb, the condition that immobilized all lice. However, additional evidence of the cellular stress response of the lice was not identified, and was therefore not similar to that induced by salinity stress (Sutherland *et al.* 2012). It is likely that other control compounds with different mechanisms of action could induce different responses than those observed here. Future work will need to be conducted to profile these responses. The 2009 Pacific *L. salmonis* responses profiled here is only one baseline dataset, and considering the variation seen even in phenotypic effects of bioassays (Westcott *et al.* 2008) and of treatment response analysis (Jones *et al.* 2013), it will be important to continue profiling these responses, using markers identified here, and resistance candidates from other work (Carmichael *et al.* 2013; Igboeli *et al.* 2013b), as well as stress response markers identified previously (Sutherland *et al.* 2012). Variability in responses may also reflect the dynamic nature of resistance emergence and multiple methods to assess sensitivity will be useful (Jones *et al.* 2013).

Transcriptomic analyses can aid the characterization of polygenic resistance mechanisms (ffrench-Constant *et al.* 2004). The large number of genes differentially expressed between Atlantic sensitive and resistant lice populations (Table 18) suggests that some of the differential expression could be from other factors not directly related to the resistance phenotype, such as other genetic or epigenetic differences accumulated between these populations, or different

parental contribution to egg development. Additionally, slight differences in timing of clutch development could result in differences in timing since molt, timing until next molt, or state of egg production in females, and these within-molt stage differences can have a large effect on the expression of the louse transcriptome (Eichner *et al.* 2008). The dynamic nature of the transcriptome, involving co-expressed gene sets increases the difficulty of isolating resistance mechanisms. As EMB resistance remains constant over four generations without continued selection (Espedal *et al.* 2013), it is likely that baseline changes would accumulate and remain in the absence of EMB.

In both sexes of the resistant population, enrichment and high fold change was identified in overexpression of degradative transcripts (e.g., *trypsin-1*, *carboxypeptidase b*). Additionally, overexpressed genes in the resistant males were enriched for collagen degradative transcripts (males are thought to be more resistant than females in general; Igboeli *et al.* 2013b). Other transcriptomic studies have found basal overexpression of peptidase transcripts associated with resistance, for example in pyrethroid resistant *Anopheles gambaie* (Vontas *et al.* 2005) and in both field- and laboratory-selected DDT-resistant *Drosophila melanogaster* (Pedra *et al.* 2004). Functional enzymatic studies indicate increased proteolytic activity in insecticide resistant house fly *Musca domestica* (Ahmed *et al.* 1998). Additionally, *trypsin* and *chymotrypsin* were overexpressed in a deltamethrin-resistant population of the mosquito *Culex pipiens pallens*, and these genes were demonstrated in cell culture to confer protection against deltamethrin when stably expressed (Gong *et al.* 2005). It has been proposed that these enzymes are generating energy to alleviate costs of metabolic detoxification of DDT (Pedra *et al.* 2004). However, in Atlantic *L. salmonis*, no fitness costs were associated with EMB resistance (Espedal *et al.* 2013). Resistance is not always associated with costs, for example common green lacewing *Chrysoperla*

carnea with 300-fold increase in EMB resistance had shorter development time, increased fecundity, and other indicators of increased fitness relative to the sensitive control (Mansoor *et al.* 2013). Since no costs have been identified yet in EMB resistance in *L. salmonis*, the reason behind the basal increase in peptidase transcripts needs to be explored further.

It is important to note that both Atlantic populations used in this study may have some resistance to EMB, only to differing extents (Igboeli *et al.* 2013b), and therefore neither would be expected to be as sensitive as Pacific *L. salmonis*. Pre-resistant Atlantic *L. salmonis* incurred 74-100% mortality from bioassay concentrations of 30-100 ppb EMB (Tribble *et al.* 2007), whereas even the sensitive population used in the present study was more resistant (Igboeli *et al.* 2013b); Table 17). In the present study, large variance in EC50 is probably due to the concentrations used with large differences between doses. However, these doses were selected to be comparable to the Pacific exposures as well as to cause phenotypic effects in Atlantic lice. Assuming some resistance in both populations, genes responding to EMB similarly in all Atlantic lice may be reliable response genes to EMB, and potentially could contain resistance-related mechanisms. For example, *calcium activated channel slowpoke* increased expression from EMB; this potassium channel is involved in membrane repolarization (Atkinson *et al.* 1991; Miller 2000), of interest considering the function of EMB in hyperpolarizing neuromuscular junctions (Arena *et al.* 1995). Also up-regulated in response to EMB were probes annotated as *alpha-* and *beta-taxilin*. These genes are involved in calcium-dependent exocytosis of neuroendocrine cells (Nogami *et al.* 2003) and may be involved in the promotion of motor nerve regeneration (Itoh *et al.* 2004). Finally, a probe annotated as *kynurenine 3-monooxygenase* was down-regulated; inhibition of this protein increases kynurenic acid and decreases glutamate (Zwilling *et al.* 2011), of interest considering glutamate is the ligand for the target site of EMB (Arena *et al.* 1995). These genes and others

responding in general to EMB exposure will be interesting to investigate further in their relation to EMB polygenic resistance.

Different levels of resistance occur in Atlantic lice from different areas, suggesting multiple resistance mechanisms (and possibly polygenic resistance). Polygenic resistance can be favoured when the drug exposure is within the range of tolerance of a proportion of the controlled population (ffrench-Constant *et al.* 2004). In contrast, monogenic resistance or *knock-down resistance* (*kdr*) occurs through a mutation in a target site and will usually result in complete resistance to the compound, and can be favored when the treatment is outside of the tolerance range of the controlled animals. Due to issues such as differential medicated feed ingestion in farm salmon (Igboeli *et al.* 2013a), it is possible that some animals will not receive enough EMB to kill all lice in a managed area, and hence the accumulation of multiple minor resistance mechanisms may be favored in this situation.

As evident from the PCA (Figure 19), a response to the gradient of EMB dilutions was noted in the female resistant lice only (e.g., Figure 20). No dose-dependent response of this manner was noted specific to males or to sensitive females. Interestingly, these clusters indicate a return to the level of the other conditions, for example the female resistant population reducing to the level of all other conditions (cluster *iii*), or starting similar to the resistant males and increasing to the level of the sensitive populations (cluster *i* and *iv*). The nature of these responses is not clear, and genes found within are not typical resistance candidates. It is possible that this response is reflective of the increased sensitivity viewed for females relative to males within the resistant population. Clusters appear to change between 0 and 0.1, and it is not known if a concentration this low would actually affect anything significantly. However, the largest difference is between 0 and the 300 or 1000 ppb, and the genes do continue increasing or

decreasing between the 0.1 ppb and the higher doses. Also interesting was the observation that genes responding to EMB in all Atlantic lice had highest expression in sensitive lice at the highest dose (1000 ppb). More work will be needed to understand the nature of these induced response genes.

The effect of sex surpassed that of population (Table 18). Furthermore, the basal expression of many genes was dependent on both sex and population. Even though differences between the responses of males and females are to be expected (Igboeli *et al.* 2013a; Saksida *et al.* 2013), the number of genes in these comparisons may indicate other effects not directly related to resistance.

In contrast to the Atlantic lice, the Pacific lice can all be considered sensitive to EMB (Saksida *et al.* 2013). In the Pacific *L. salmonis*, the only response to EMB was a down-regulation of genes from the control; no up-regulation over the doses of 10-50 ppb was identified. The down-regulated genes were enriched for peptidase activity (13 genes; p = 8E-11), and this suppression was specific to the 50 ppb condition. The Atlantic lice also decreased degradative enzymes and peptidases over the concentrations of EMB. In EMB resistant *C. rogercresseyi* from Chile there was no up-regulation of *heat shock protein* in response to the EMB exposure (sHSP; Cárcamo *et al.* 2011). Similarly, in spot prawn *Pandalus platyceros*, the stress response was not indicated in response to EMB, although other biological functions were induced, including genes related to transcription and translation control (Veldhoen *et al.* 2012). In comparison, salmon lice exposed to reduced salinity induced cellular stress responses specific to the level of the stressor, involving many protein folding or apoptotis-related transcripts (Sutherland *et al.* 2012). In the present experiment, several probes annotated as *heat shock protein beta-1* were up-regulated at only the highest dose of EMB in the Atlantic lice, but apoptosis or other cellular stress response

genes were not up-regulated. It may be that the nature of the xenobiotic stressor does not induce the same sort of cellular damage as could be expected from an environmental perturbation, or that these types of coping strategies would not be useful in response to this stressor. It is possible that other parasiticidal drugs used in aquaculture may have different induced responses, including stress responses. For example, stress response genes were induced by permethrin in the pyrethroid-resistant *A. gambiae* (Vontas *et al.* 2005). These authors also found the strongest response at 6-10 hours of permethrin exposure, and less effect at 2 or 24 hours, so it is also possible that our timing may have missed a stress response occurring at 10 hours post exposure.

The presence of many suppressed digestive enzymes in the response of Pacific lice may be due to the EMB exposure interrupting signals for production of these enzymes. Collagenase genes were down-regulated in response to higher doses of EMB in the Atlantic lice as well. If calcium signaling, or a related mechanism affected by EMB presence, is involved in regulating these enzymes, it may explain the suppression at higher doses of EMB. For example, calcium (Ca^{2+}) influx in a rat exocrine pancreatic cell line affects the transcriptional regulation of *trypsin* (increased), *chymotrypsin*, *amylase*, and *carboxypeptidase-a1* (decreased; Stratowa & Rutter 1986). Another possibility behind the suppression may include time prior to molt, which can reduce *trypsin* and *chymotrypsin* expression (Vanwormhoudt *et al.* 1995; Klein *et al.* 1996; Sanchez-Paz *et al.* 2003). Although randomization was applied to the present experiment and the likelihood of one condition being staggered in development is low, it is interesting to note that EMB is implicated in inducing early molt in lobsters (Waddy *et al.* 2002). During starvation, digestive enzymes can be suppressed (e.g., white shrimp *Penaeus vannamei*; Muhlia-Almazan & Garcia-Carreno 2002), although are not always (e.g., black tiger shrimp *P. monodon*; Lehnert & Johnson 2002). Additionally, antinutritional factors such as tannins can reduce protease and

invertase activity in the cotton pest *Heliothis zea* haemolymph (Klocke & Chan 1982). The presence of other genes such as non-digestive enzymes and transporters suggest this is likely not the reason for the suppression. The more likely explanation is that the suppression is due to regulatory issues occurring due to the EMB impact on the organism. Transcriptional control is related to enzyme activity for these enzymes (Klein *et al.* 1996), and so the effect of the down-regulation would be expected to result in physiological consequences.

Clusters of genes can change within a stage depending on time since previous molt, or time prior to egg production in females, as identified in *L. salmonis* (Eichner *et al.* 2008) among others. Response clusters were also identified in Atlantic lice responding to EMB exposure (Carmichael *et al.* 2013). It is likely that there are other clusters of genes that will change together dependent on other unexpected variables (e.g., effectiveness of feeding or of defending against host). Once these clusters can be characterized further and better understood, it will improve the interpretability of the results of *L. salmonis* transcriptomic responses. This effort may be assisted by the meta-analysis of existing and forthcoming *L. salmonis* transcriptome datasets. This will also help to assign characteristics to unannotated genes (e.g., relation to cluster or variable; Pavey *et al.* 2012). Connecting specific marker genes to a cluster will potentially allow for the incorporation of these unknown variables in transcriptome response models.

The reason for the lack of resistance to EMB in *L. salmonis* from Western Canada remains unknown. It is possible that refugia of sensitive alleles and influx of pressure from wild salmon carrying outbred lice in a panmictic population (Messmer *et al.* 2011) is inhibiting the development of resistance, as suggested by Saksida *et al.* (2013). It is necessary to be proactive about monitoring and managing resistance to insecticides (Zhao *et al.* 2006). The importance of understanding the mechanisms of resistance development has prompted use of transcriptomics to

evaluate differential responses between populations varying in sensitivity to drugs, such as the pyrethroid deltamethrin in mosquitoes *Anopheles gambiae* (Bonizzoni *et al.* 2012). Increased hydrolysis or sequestration through increased expression of esterases has been noted as leading to resistance in several insect species (ffrench-Constant *et al.* 2004). Transcriptomics may provide insight on the inputs of the different mechanisms known to be involved in insecticide metabolism: cytochrome P450s; glutathione S-transferases; and carboxyesterases; and informing the generation of molecular tools to detect resistance evolution in the field (David *et al.* 2005). Although the differences between a louse ingesting EMB from feeding and immersion (i.e. bioassay) are currently unknown, this work increases our understanding of the louse response to EMB. Continued work identifying the types of resistance induced in response to EMB selective pressure will also help in understanding the nature of the selective pressures (e.g., monogenic/polygenic resistance).

5.6 Conclusions

Together with previous studies, the present work suggests that polygenic resistance mechanisms are present to provide some protection from EMB in *L. salmonis*. In Atlantic *L. salmonis* originating from different regions and differing in sensitivity to EMB, differential expression between populations was larger in terms of number and fold change differences of genes than the induced responses. While no genes were up-regulated in Pacific lice in response to EMB, several interesting induced responses were noted in Atlantic *L. salmonis*, however more work would be needed to characterize these genes. At only the highest dose of EMB in the Atlantic lice (1000 ppb) *heat shock protein beta-1* was induced, but evidence for response to stress was minimal, even though lice were being immobilized by the dose, suggesting that cellular stress responses may not be important in EMB resistance. Interestingly, in the sensitive Pacific lice, and both

populations and sexes of Atlantic lice, degradative enzymes were down-regulated over the doses of EMB, potentially indicating disruption of signaling for expression due to EMB. Resistant populations of both sexes were associated with increased basal expression of degradative enzymes, and of collagen degradative enzymes in resistant males only. Additional work on single nucleotide polymorphism (SNP) differences among populations should continue to increase our understanding of EMB resistance in *L. salmonis*, as well as continued profiling of some of the markers identified here and in previous work. Transcriptome response interpretations will be improved with improved understanding of co-expressed gene modules in *L. salmonis* through meta-analysis of existing and forthcoming transcriptome studies.

5.7 Chapter acknowledgements

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Chapter 6: General Discussion

6.1 Salmon defences against sea lice in a complex environment

In the present work, it is apparent that a salmon response to *L. salmonis* infection requires the expenditure of a certain amount of energy that is likely specific to the type and scale of the mounted responses. If that energy is not available to expend, detrimental effects can occur for the host. Certain stages of salmon life history would be particularly sensitive to this type of energy expenditure and infection, for example, while in a development stage not typically encountering a large infection with the agent (Krkošek *et al.* 2007) or shortly after smoltification while the immune response is less able to mount a defense (Maule *et al.* 1987). As in an ecological immunology framework, energy allocated to immune-related responses may be drawn from reserves stored for other purposes (Sadd & Schmid-Hempel 2009; Martin *et al.* 2011).

Response to lice infection may in fact result in the formation of a stage that is sensitive to other agents, for example a viral infection. In Chapter 3, the response of multiple salmon species, including the least susceptible included in the experiment (pink salmon; Jones *et al.* 2007) indicated a suppression of antiviral response-related transcripts. This may indicate that cells expressing these genes are in circulation in a normal state for these fish as an antiviral surveillance defense. It is possible that the signal indicating a form of immunosuppression observed here could be due to immunosuppressive compounds secreted by lice (e.g., prostaglandin E_2; Fast *et al.* 2007a). However, the immune suppression coincident with up-regulation of other more relevant innate immunity components suggests that this is not directly louse-controlled. This signal could also be suppressed due to the inverse regulation of the Th1 and Th2 subsets of the immune system (Allen & Maizels 1997; Romagnani 1997). Regardless of the mechanism behind the suppression, it may indicate the induction of a more susceptible state

of salmon to viral infections. This is of particular interest considering the potential for salmon lice to act as a mechanical vector for bacteria and viruses (Barker *et al.* 2009; Jakob *et al.* 2011). However, it may be that the presence of a virus would rapidly re-induce an antiviral response, and that this suppression is ephemeral and easily reversed. This is an important avenue of future research that will improve our understanding of the complex impacts of parasites and pathogens in the salmon environment.

The transcriptomic approach identified interesting response mechanisms not typically considered in the response of salmon to lice infections, for example up-regulation of *acidic mammalian chitinase* (Chapters 2 and 3), acute phase proteins and lectins, as well as a suite of iron protective mechanisms (Chapter 3). Similar to anemia of inflammation, which can occur to reduce the amount of iron available to pathogenic bacteria (Nemeth & Ganz 2006), it is possible that reducing circulating iron or hemoglobin (also via ferritin; Easy & Ross 2009) could serve in a preventative role (reduced attraction) or a protective role against the lice or other opportunistic infections. Responses of the refractory pink salmon were consistent with the earlier described importance of innate immunity for louse defense (see Jones 2001). Furthermore, as discussed in previous work regarding teleost inflammation and wound repair (Chadzinska *et al.* 2008; Skugor *et al.* 2008; Tadiso *et al.* 2011) the increased expression of matrix metalloproteases was also identified here in the response to the lice infection (all species but chum salmon). In the sensitive juvenile pink salmon, tissue remodelling through matrix metalloproteinase activity may incur a cost detrimental to development at this stage, as has been described as a chronic infection in Atlantic salmon (Skugor *et al.* 2008). These mechanisms will be important to consider in continuing research into characterizing a successful response to the louse infection.

6.2 Transcriptome (co)regulation in salmon lice

Sequencing technology advances are reducing boundaries for non-model organism genomic or transcriptomic studies (Wang *et al.* 2009), in addition to custom microarray designs based on large scale EST projects, such as that described in Chapter 4. Furthermore, sequence similarity and (assumed) functional similarity in important genes and gene families allows the use of response characterization methods, such as through Gene Ontology enrichment analysis (Ashburner *et al.* 2000), which has been highly useful for non-model organisms (Primmer *et al.* 2013) through tools like DAVID bioinformatics (Huang *et al.* 2009) and others. However, with copepods, in addition to other highly divergent organisms, the closest relative is still a large evolutionary distance away (Bron *et al.* 2011; Colbourne *et al.* 2011), and this can generate many unknown genes even with sequencing tools enabling discovery. Without predicted function, these are often are overlooked in analyses (for example in Chapter 4 and 5).

In addition to the challenge of unknown genes in salmon lice, another interesting aspect is the co-regulated gene modules that occur without apparent explanation given the biological and technical explanatory variables included in the study (*personal observation*). These unexplained differences could be due to differences in feeding behaviours, individual louse health or other possibilities. Improved characterization and annotation of clusters as well as genes central to the cluster may improve our utilization of these gene lists, as may be performed by a clustering method such as the Weighted Gene Co-Expression Network Analysis (Langfelder & Horvath 2008; Filteau *et al.* 2013). Genes identified as central to a pathway or function could even be included as markers for characterizing aspects of the louse transcriptome that are not identifiable from external phenotypes. Coupled with methods proposed for annotation based on ecological associations (Pavey *et al.* 2012), these types of characteristics could also be used to annotate

unknowns, if they are typically found in specific clusters, especially if the function of the cluster becomes known.

Several datasets of *L. salmonis* transcriptomes have been collected including several that have used expression clustering for characterization of responses (Eichner *et al.* 2008; Carmichael *et al.* 2013; Chapter 4 and 5). It may be particularly relevant to profile individual lice for interpretation of clusters, even though this can reduce biological replication (e.g., within a pooled sample). Based on the present work and previously published work mentioned here, it would appear that expression modules are present in salmon lice, likely under the same transcriptional regulators. Some interesting questions will be posed with improved characterization. For example, how do these clusters of responses differ between Atlantic and Pacific lice? Or further, what are the transcriptional regulators involved in controlling these genes? Or even, do some gene clusters respond in association with the presence of specific microbiota within the louse? This is certainly an area open for more advancement that could be improved through meta-analysis.

An additional aspect of this work that has great potential to advance the field is the determination of the variance in the microbiome of lice, and the correlation of this variation in important aspects of lice biology, such as pathogenicity, rapid development, or even resistance development.

6.3 Emamectin benzoate resistance – polygenic mechanisms?

Based on the variable levels of emamectin benzoate (EMB) resistance in different populations of lice in the East Coast of Canada (Igboeli *et al.* 2013b) or on the moderate sensitivity of hybrids of pure resistant or sensitive forms of lice in Norway (Espedal *et al.* 2013), and the accumulation of transcriptional changes between populations with differing EMB sensitivity (Chapter 5), it would

appear that lice resistance to EMB involves polygenic mechanisms, not a single mutation. With this being said, a single nucleotide polymorphism (SNP) or genome resequencing analysis of resistant and sensitive populations will also improve our understanding of genes with a role here, and this would be important to pursue. Although the importance of identifying markers for easy identification of resistance in the field has been discussed (Igboeli *et al.* 2013a), this still is a more challenging assay than a diagnostic SNP marker for resistance development. However, since resistance can emerge in numerous and varied ways, we may be left to continue global profiling rather than targeted. Conducted properly, with control of other variables (something difficult to do in nature), it is possible that we could identify expression markers that could be useful at least in part for diagnosing resistance development, for example, in something with such a large expression difference as *peroxidasin* in Chapter 5 (>100 fold higher expressed in resistant lice). It will also be interesting to continue the work in profiling the responses to xenobiotics in *L. salmonis*, both for these practical monitoring applications (Igboeli *et al.* 2013a), as well as to investigate cross-resistance, to continue understanding stressor context of genes and what indicates a stress response in a salmon louse. The relatively few genes similar in response between the EMB-exposed lice (characterized by down-regulation of peptidase transcripts) and the low salinity-exposed lice (characterized by protein folding transcripts, apoptosis-related transcripts) indicates stressor specific responses. It was interesting that *heat shock protein beta-1* was identified in response to the highest level of stressor in both the high resolution salinity work (at 25 ‰) and the general response of both Atlantic populations and sexes to EMB (1000 ppb), indicating this may be a general louse stress response gene. Other identified genes could be annotated with stressor-specific annotations, and together with additional work could be used to evaluate relative levels of stress.

6.4 Transcriptomics as a diagnostic tool

One of the most useful aspects of the transcriptomic approach is the ease of profiling a large number of transcripts that can each carry information about the response without being previously linked to the response. This large amount of data must be handled appropriately and can be used in a number of sophisticated techniques to discriminate between samples (e.g., principal components analysis), as well as perform an exploratory function to identify new components of a response not previously considered (as discussed above). Principal components analysis or other hierarchical clustering methods can allowing for the separation of groups, and linking genes contributing to this separation (Ringner 2008). This approach has been applied in many diverse studies ranging from classification of cancers based on machine learning and discriminant analyses (Khan *et al.* 2001) to predicting behaviour of honey bees based on brain gene expression profiles (Whitfield *et al.* 2003). In fish biology, the separation of sockeye salmon based on gene expression profiles was correlated with the probability of their survival to reach spawning grounds, opening some exciting new avenues for research on viral signatures that were associated to the separating signals (Miller *et al.* 2011). These multidimensional reducing techniques take these analyses beyond what can be separated out by the researcher, and allows a systematic method of determining groups.

Another highly useful approach is the clustering of genes based on expression level (Eisen *et al.* 1998). This approach was particularly useful in determining the correlated expression of the lice studies in the present dissertation (i.e. Chapters 4 and 5). Whether the co-regulated genes are associated in function requires the assessment of functional annotation, but similar expression profiles across a range of samples provides an easy way to reduce the interpretation to a few key response types.

As these approaches continue to be refined, and as technical artifacts are reduced (e.g., reduced noise in oligonucleotide arrays, and beyond), these methods will continue to be useful diagnostics of different signals. The more information about phenotypes that can be obtained and used as anchors for the interpretation of gene expression analysis can improve the attribution of contributing factors to separations determined by discriminant analyses, and the proper recording and access to these factors will be important to have accessible for meta-analyses of multiple transcriptome datasets.

Determination of cellular stress based on the transcriptome response has to be considered carefully; for example in Chapter 4, temperature was found to not have as large of an effect as salinity over the short term. However, over the long term it is highly likely that elevated temperatures can reduce the timing of molts of lice, and potentially contribute to outbreaks and epidemiology of the species (Boxaspen 2006; Groner *et al.* 2014). However, over the short term that was evaluated here, it was clear that the salinity stress induced rapid changes in coping mechanisms indicating a cellular stress, as could be expected from phenotypic studies of free-swimming lice at 25 ‰ (Bricknell *et al.* 2006). It will also be important to continue the connecting of genes associated to stress to the specific stressor. For example, in the emamectin benzoate work (Chapter 5), many of the stress-associated genes identified in the salinity work (Chapter 4) were not found to be induced by this xenobiotic stress, with the exception of *heat shock protein beta-1*. If stress-associated genes from the salinity work were the only genes to be evaluated in the chemical response work without accompanying bioassays and phenotypic responses, it would be less obvious that these lice were being negatively impacted by the chemical stress. In conclusion, it is important to remember that while transcriptome profiling is a useful tool for assessing the response of an organism, it is only a view at a single time point in the

organism's continued response. An example of this related to the current work is the emamectin benzoate response in *C. rogercresseyi* was found to be strongest at 10 hrs, not at 24 hrs (Cárcamo *et al.* 2011). Time-course studies, such as that conducted in Chapter 3 are also highly useful to look for consistent responses over the course of a stress or perturbation, and high replication also improves reliability of results. Additionally, phenotypic responses are always important to carefully record and use to interpret responses, some of which may not be attributable to the factors directly being tested. For example, in an additional study to those presented here, using a lower emamectin benzoate concentration exposure of Pacific lice, clusters of genes were expressed in an on-or-off manner not explainable by any of the recorded variables (*personal observation*).

The increased sensitivity of transcriptomic methods may change several areas of study, for example in determining ecotoxicological impacts of different waste products, or in studying local adaptation by looking for changes in the transcriptome that may indicate the adaptation to a particular location (Tymchuk *et al.* 2010; Larsen *et al.* 2011). Although barcoding and genomics have been used for the identification and resolution of species and populations of fish, it is possible that higher resolution methods, such as transcriptomics, will eventually be used to identify sub-populations of individuals with novel and highly beneficial adaptive gene regulatory networks and responses to environment (Nielsen & Pavey 2010). Predictive potential of these studies will continue to aid conservation efforts, for example transcriptomics was recently applied to predict the migratory potential of steelhead salmon *O. mykiss* while all fish were still in a common hatchery environment, generating markers that can be used to evaluate new techniques of increasing the potential for more fish to migrate rather than reside in freshwater streams (Sutherland *et al.* 2014a). Studies will likely continue to identify signatures that precede

phenotypic manifestations, and introduce new testable hypotheses (i.e. exploratory science informs questions which are then tested). The potential for these tools to be used in ecological work is exciting, and will continue to bring new knowledge advances that will continue to be incorporated in policy determination or conservation biology. The sensitivity of these methods may change the way we view health of populations, biodiversity, and how stress is defined.

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Appendix A
Primers used in qPCR experiments

Collected here for completeness, the following pages contain the primers used in the qPCR components of the preceding chapters. In each table, if primers have been published previously it is noted below the table.

Table 19. *O. gorbuscha* mixed tissue qPCR primers with amplicon size and efficiency values.

Gene	Forward primer	Reverse primer	Size	Eff. (%)
Actin cytoplasmic 1 (*actb*)	CCTCCTTCCTCGGTATGG	ATGTCCACGTCACACTTC	76	87
Eukaryotic translation initiation factor 4H (*eif4h*)	CACAGTACAAGGAGACATAG	AGGAGAGCACCATCATAC	162	92
Plastin-1 (*pls1*)	ATAATCAACTGGGTCAAC	TGTCTAACAAGTCTATCAC	112	99
Ubiquitin (*ubiq*)	TTCTGTTCTGGCAAATGTTC	TTCCTATACTTCATAAGTTCCATTG	147	97
Transducer of erbB-2 1 (*tob1*)	CCTCAACTTTATTATTTCC	CCTTATATGGCTTATCAG	131	104
Cyclin-dependent kinase 4 inhibitor B (*cdkn2b*)	ATCCTTGTCCAATTCCTC	ACTTTCTATTGCCAAATCG	78	96
Sestrin-1 (*sesn1*)	TTCCAGCACTCCGAGAAG	TCAGAGCGTAGAGCAGTTC	79	103
B-cell translocation gene 1 protein (*btg1*)	GCTGTCTACTCTACCTTG	TACAACTTTCGCACAATC	130	97
Transcription factor DP-1 (*tfdp1*)	CTTCAGACAGAGCCTTAC	ACCTTCAGTTCTCCATTG	82	96
Superoxide dismutase-1 (*sod1*)	TACCGGGACCGTATTCTTTG	TCTCCATAAGCATGGACGTG	114	100
TGF-beta receptor type-1 (*tgfbr1*)	AAGGAGTTTGCTTCGTGTCC	ATGGGGTAGATGCCAGTGTC	148	92
Peptidyl-prolyl cis-trans isomerase (*fkbp7*)	ACATTCTACTTCAAGGTG	TTATCAATCCATCTCTATCG	121	96
Nucleolysin (*tia1*)	ATGGGTAAGGAGGTTAAAG	ATGGAAATGATTGCTTGTG	75	102
Gamma-interferon-inducible lysosomal thiol reductase (*ifi30*)	CTTGGATTGTCATAAATGG	TAACTTCTGTGTCGTTTC	142	100
Matrix metalloproteinase-9 (*mmp9*)	TTTCTCGGGGAGACATATGAAG	TCAGAGTTTCCACCAATCACAG	146	95
Collagenase-3 (*mmp13*)	GCTTCACCACCTTCGACAAT	ATGGAGTTGTCCACCTCAGC	103	99
Metalloproteinase inhibitor 2 (*timp2*)	TATCGACGCCATCTTCACTG	ATACTCCTTGTTGCCGTTGG	76	95
Titin (*ttn1*)	ATGGAAGTTGAAGGCCACAG	TCTCCAGGCACATCCTCTTC	149	101
Ras GTPase-activating-like protein (*iqgap1*)	TAAGGCACATAATGAATAC	GAACAGAACATCTAACAG	100	103
SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily E member 1 (*smarce1*)	ACTACAGGCTGGGAGGGAAT	TTGGAGGCTTTGACTTGGTC	145	97
Protein disulfide isomerase A3 (*pdia3*)	CCAGTGATCCCAACATTG	GGCTCATTTTCTGTCCAG	120	100
Phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger 1 (*prex1*)	ATCAAGAGGGTCTGCTTC	CTGTTACTGTTACATTCACTG	101	90
Ribonuclease P protein subunit p21 (*rpp21*)	ACAACCTATTAGCATTACATC	TCGTTCCTGAACTTACTG	147	95
Dual specificity protein phosphatase 26 (*dusp26*)	TGGTGGCTATTAAAGAAC	TCCGATGTAGAGATTAGG	92	105
Suppressor of G2 allele of SKP1 homolog (*sugt1*)	GCTCCGATAACACTTTCC	TCTCCTTTCCATTCTGATTG	78	97

All primers are from (Sutherland *et al.* 2011).

Table 20. Salmon anterior kidney and skin qPCR primers with amplicon sizes and efficiency values for each tissue and species tested. AK = anterior kidney; S = skin

				Efficiency (%)					
				O. gorbuscha		*S. salar*		*O. keta*	
Gene	Forward primer	Reverse primer	Size	AK	S	AK	S	AK	S
15-hydroxyprostaglandin dehydrogenase [NAD+] (*hpgd*)	AGGGAAGGATTTGAAGGCTG	ACCGTTTTCTGAAAGGCATC	114	105	-	92	-	93	-
Collagenase-3* (*mmp13*)	GCTTCACCACCTTCGACAAT	ATGGAGTTGTCCACCTCAGC	103	99	106	102	103	103	101
Hepcidin-1 (*hamp1*)	TTCAGGTTCAAGCGTCAGAG	AGGTCCTCAGAATTTGCAGC	99	88	98	99	112	88	112
Interferon regulatory factor 7 (*irf7*)	CCTGGTTCAGCTCCACTACC	AGGATGCGGTTGGTGTATTC	117	-	105	-	114	-	98
Complement component 7 (*c7*)	AGTGCTTTGACACCTCCGTC	TGACCACATCAAACTGCTCC	120	-	110	-	109	-	104
Galectin-3-binding protein (*lgals3bp*)	ACCAACGAGAATGTTCCAGG	CACATGTTAGGAGCAGTCGG	120	-	103	-	103	-	97
Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	AGCTACGCTGAGATCAAGGC	AGGAGTGGGTGTCTCCAATG	118	-	105	-	109	-	96
Thioredoxin (*txn*)	TGGCCAAACACTGTGACATC	ACCTTCTCCTCCAGTTTGGC	109	-	103	-	112	-	108
Interleukin-1 beta** (*il-1b*)	CGTCACATTGCCAACCTCAT	ACTGTGATGTACTGCTGAAC	200	-	104	-	103	-	109
Interleukin-8** (*il-8*)	GAATGTCAGCCAGCCTTGTC	TCCAGACAAATCTCCTGACCG	226	-	106	-	102	-	101
Prostaglandin D synthase*** (*pgds*)	CCTACACCAACCTGAACGCTGATG	ACGCTGGCTGGTGAAGGTGAAG	98	-	98	-	83	-	94
Tumour necrosis factor alpha** (*tnf-a*)	GGCGAGCATACCACTCCTCT	TCGGACTCAGCATCACCGTA	124	-	84	-	88	-	100
Eukaryotic translation initiation factor 4H (*eif4h*)	AGAACCCTCTGACGAGGAGAG	ATATGGCAGAGTTGGGGTTG	104	105	91	101	104	105	95
U6 snRNA-associated Sm-like protein (*lsm8*)	TTGACCAGACCATCAACCTG	CAGCAACGTTGTCTCCTCTG	117	107	104	100	106	103	96
mRNA turnover 4 homolog (*mrto4*)	GGGAGACACACTAACCCCTG	GTCGCTCGTTTCAGAGTTCC	109	107	98	101	107	101	95
Dynein light chain 1, cytoplasmic (*dynll1*)	ACATCGAGAAAGACATCGCC	TCTCATGGGTCACGTAGCTG	111	106	101	102	108	103	104

All primers are from (Sutherland *et al.* 2014b), except those noted **(Fast *et al.* 2007b) and ***(Braden *et al.* 2012).

Table 21. *Lepeophtheirus salmonis* mixed tissue qPCR primers with amplicon sizes and efficiency values.

Gene	Forward primer	Reverse primer	Size	Eff. (%)
Chromobox protein homolog 1 (*cbx1*)	TCATTGGAGCCACAGATTCC	TCACTGTTTGAGGACATCGC	117	99
Chromobox protein homolog 2 (*cbx2*)	CAAATGCCACCAATCTCTCC	CATCGTGATCAAATTCACCG	118	111
Histone-binding protein (*rbbp4*)	GAGAAGTGAATCGTGCTCGG	CACGAGAACATCAGAGCTGG	80	97
Heat shock protein HSP 90-alpha (*hsp90aa1*)	CGGGATAACTCAACTGTCGG	CATTCTTGTCAGCATTTGCC	109	93
T-complex protein 1 subunit zeta (*cct6*)	CATGAAGGCTGCCAATAAGC	ACTTCAAAAGCTCCAGCACC	123	97
Protein disulphide isomerase A3 (*pdia3*)	CCCATCTACGAGGAACTTGG	GGAACATCATTTGCCGTAGC	83	101
Calreticulin (*crt*)	CGACCCTGAAGCATCTAAGC	CATTTACCCTTGTATGCGGG	138	103
Apoptosis-stimulating of p53 protein 2 (*tp53bp2*)	GGACTCCTCTTCATTGTGCC	AACCATGAAAGCCTTCCTCC	150	116
Programmed cell death protein 4 (*pdcd4*)	TCAATCGTAAGATGCCGTCC	CCAGTATTCCTTGAATCGGC	77	105
Growth arrest-specific protein 1 (*gas1*)	GTGAGGAACAGGAAACAAATCC	ACAACATCCGTTTCACCTCC	106	105
Adenine phosphoribosyltransferase (*aprt*)	GTTGAGGAAAAAGCATTGCC	TTGGAACAAAAGGAACTCCG	118	111
GTP-binding protein (*sar1b*)	GTCCAGTTCTCATTTTGGGC	CCTTTCCCGGTAGTTTGACC	103	102
FK506-binding protein 4 (*fkbp4*)	ATGGTTCCCAAAGAAGAGGC	ATCGCTCTTTGGAGTGTTCC	145	95
Myosin heavy chain, muscle (*mhc*)	GGAACTCACTTATGCCACGG	TTTGCTTCTTGTAGGAGCGG	101	90
High-affinity copper uptake protein 1 (*slc31a1*)	CTACAAATCCCACTGAATGCC	AATTGAAGGACGTGCAGAGC	106	102
Structural ribosomal protein S20 (*rps20*)**	GTCACCTCAACCTCCACTCC	TGACTTGCCTCAAAGTGAGC	274	94
Glutathione S-transferase 1, isoform D (*gstd1*)	GGAGCTCCAACAACTTCAGC	AAGGAAGCTCTCTCGCACC	115	101
Tubulin beta chain (*tubb*)	TGCGGCTATATTTAGAGGGC	AGGTGGAATGTCACAAACGG	136	110
Vinculin (*vcl*)	AGATTCCAACACTGGGAACG	CAGAGTCCATTTTTGCTCCC	78	105
Aquaporin-9 (*aqp9*)*	ATTGGATTGGGACTCACTGC	AAGGGACCACTTCCATACCC	125	93
Matrix metalloproteinase-9 (*mmp9*)*	TAATGGCGTGTTTTGGTGTG	GGGGGAGCATGAGTTTCTTC	105	101
Acidic mammalian chitinase (*chia*)*	CTCTCCCCTTTATGGCCTTC	TTTTGGAGCACCATTTTGAAC	109	92
Trypsin-1 (*tryp1*)*	TGTGCATCCGGTAAAGGTAAG	TACCCCATGAAACAATTCCAA	94	94
Carboxypeptidase b (*cpb*)*	AGGAGGAATGTTTGGCTTTG	TCGAACACAACTTCCATTGC	87	96

All primers from (Sutherland *et al.* 2012), except those denoted with * are from Chapter 5 (submission in prep) and ** are from (Frost & Nilsen 2003)