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GENETIC ARCHITECTURE OF PHENOTYPIC AND TRANSCRIPTIONAL VARIATION IN SALMON

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GENETIC ARCHITECTURE OF PHENOTYPIC AND TRANSCRIPTIONAL VARIATION IN SALMON

By

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Genetic Architecture of Phenotypic and Transcriptional Variation in Salmon

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:
This thesis also incorporates the outcome of a joint research undertaken under the supervision of professor DD Heath. In all cases, the contribution of co-authors was primarily in an advisory capacity. The key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author with the exception of Chapter 2 where FP Thrower set up the crosses and reared the fish and of Chapter 4 where part of the data was obtained from CA Bryden.

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Thesis Chapter	Publication title/full citation	Publication status
Chapter 2	Aykanat T, Thrower FP, Heath DD (2011) Rapid evolution of osmoregulatory function by Modification of Gene Transcription in Steelhead Trout. *Genetica*, **139**, 233-242.	*Published in Genetica*
Chapter 3	Aykanat T, Heath JW, Heath DD. Additive, non-additive and maternal effects of cytokine transcription in response to immunostimulation with *Vibrio* vaccine in Chinook salmon *(Oncorhynchus tshawytscha).*	*Submitted to Evolutionary Applications*
Chapter 4	Aykanat T, Bryden CA, Heath DD. Sex-biased genetic component distribution among populations: Additive genetic and maternal contributions to phenotypic differences among populations of Chinook salmon	*Submitted to Journal of Evolutionary Biology*

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ABSTRACT

Understanding the genetic determinants of phenotypic variation is crucial for a predictive evolutionary theory. Although Fisher's fundamental theorem provides a simple quantitative framework for evolutionary processes, the underlying assumptions regarding the heritability and variability of traits and population structure can diverge from real systems drastically. Therefore, the genetic architecture of traits associated with fitness should be explored to verify the theory's relevance to evolutionary changes and its universality, but this isn't practiced much in natural systems.

Pacific salmon provide an excellent model system to examine genetic architecture and variance structure in and among populations. Here, I analyzed trait inheritance in salmon, and characterized the underlying adaptive significance under different ecological scenarios. Using transcriptional traits, I examined the relationship between plasticity and genetic differentiation shaping salmon populations. I employed common garden rearing and factorial mating designs to evaluate the genetic architecture of traits under physiological stress (i.e. saltwater, temperature and immune) to explore phenotypic variance under different environments.

In Chapter 2, I showed osmoregulation gene transcription diverged after anadromous steelhead trout (*Oncorhynchus mykiss*) were introduced to a landlocked lake, and non-additive inheritance of traits was common among diverging populations. In Chapter 3, the variation in innate immune response gene transcription was shown to be mediated by non-additive effects in farmed Chinook salmon (*O. tshawytscha*), and the effect was elevated after the immune stimulation with *Vibrio* vaccine. In Chapter 4, significant maternal components in traits closely related to fitness confounded the

differences observed among populations. Finally, in Chapter 5, I characterized the among-population variance structure associated with individual response to immune stimulation using a multigene microarray approach. Overall, my research suggests that transcription and phenotypic plasticity is different among salmon populations, can rapidly evolve, and that non-additive genetic effects in transcriptional and phenotypic variation is common in salmon.

In general these results are important to question applicability of fundamental theorem for salmon populations, hence conservational strategies based on evolutionary concerns. Furthermore, it presents a framework of population differentiation in salmon based on modifications to physiological response. These two combined would help us to unravel how salmon populations are structured in space and time.

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Understanding the causes of phenotypic variation within and among populations is fundamental to Biology. In general, phenotypic variation provides the opportunity for differential response among individuals along an environmental gradient, therefore allowing species a wide array of high efficiency outcomes. Furthermore, if phenotypic differences have an underlying genetic basis, those differences would provide building material by which evolutionary forces can shape populations which would ultimately result in evolutionary processes such as adaptation and speciation. Indeed, variation in phenotype within a species could have a genetic and environmental basis or a complex combination including higher order interactions. The magnitude of each factor and its relative contribution to the overall variation is crucial to predict the viability of populations under changing environmental conditions.

It was not until 1940s, within the framework of modern synthesis, that phenotypic variation was merged into Mendelian "particulate" inheritance which formally explains evolutionary processes in populations with population genetic principles and continuous variation observed in most of the phenotypic traits. In particular, Ronald Fisher and Sewall Wright outlined the main principles in the first half of 20^{th} century, and provided the necessary mathematical framework that enables us to express genotypic values (of phenotype) as a function of allelic frequencies, and furthermore provided the statistical background that enables us to partition phenotypic variance into genetic and

environmental components (Lynch and Walsh 1998). This advancement resulted in the formation of the current paradigm in Biology that places populations as the unit of evolution, and population genetic principles as the basis for evolution. Quantitative genetics has advanced as a major branch of evolutionary biology by conceptualizing the problems of the relationship between phenotype and genotype with a population genetic perspective to explain the evolutionary process (Lande 1979; Lynch and Walsh 1998).

Breeding programs have experienced the most rapid advancement through the integration of the modern synthesis and quantitative genetics to practical problems in agriculture. For example, since the 1940s, great improvements in agriculture productivity have been achieved by systematic selection programs using quantitative genetic principles (Gjedrem 2004; Hill and Kirkpatrick 2010). On the other hand, relatively few studies make use of quantitative genetics in natural systems, partially because of the laborious and time consuming nature of experimental designs needed to adequately estimate components of phenotypic variation. However, quantitative genetics can be employed to address several fundamental and practical questions of evolution in natural populations (Frankham 1999; Hill and Kirkpatrick 2010). For example quantitative genetics is extremely valuable in contemporary evolution studies, as well as for local adaptation and adaptive radiation studies, which are increasingly acknowledged in recent years owing to the growing awareness of climate change and human anthropogenic impacts on natural systems (*e.g.* Bernatchez 2004). Similarly, understanding the mechanisms that maintain genetic variation in the face of natural selection is of fundamental importance in evolutionary biology (Kruuk 2008). Also, from a conservation point of view, quantitative genetics provides a framework on which to understand population viability under low

effective population size and with high inbreeding pressures (Frankham 1999; Moran 2002).

SALMON AS A MODEL ORGANISM AND TRANSCRIPTION AS A PHENOTYPIC TRAIT TO STUDY EVOLUTIONARY QUANTITATIVE GENETICS

Salmon are a collection of vertebrate species for which ecological and evolutionary questions conveniently overlap with what quantitative genetics can offer. More than 30 years of systematic aquaculture efforts on salmonids provides strong background knowledge and practical experience for quantitative geneticists and theorists to test ecological and evolutionary models. This thesis examines the dynamics of phenotypic variation and genetic architecture in salmon at different spatial scales and ecological settings. For the most part (Chapters 2, 3 and 5) I employ transcriptional traits as the phenotypes of interest. Transcription is a well-suited proxy for molecular biochemistry, and the combination of molecular biochemistry and quantitative genetics are noted for being a candidate for the next synthesis in population genetics (Moran 2002; Larsen 2011). In the following two sections, I briefly introduce and justify salmon as the model species and transcription as a unique and valuable phenotypic trait to be quantified and analyzed.

SALMON

Salmonidae belongs to the Salmoniformes order of the teleost subclass of Actinopterygii fishes. The family includes three subfamilies which includes Coregoninae (white fishes), Thymallinae (graylings) and Salmoninae, the last of which consists of five genera: *Brachymystax* (Asiatic trouts), *Hucho, Salmo* (*i.e.* Atlantic salmon and brown trout),

Salvelinus (*i.e.* Char, Brook Trout and Lake Trout) and *Oncorhynchus* (*i.e.* Pacific salmon and trout; Kinnison and Hendry 2004). All salmonids spawn in fresh water, but in many cases, the fish spend most of their life at sea, returning to the rivers to reproduce after which they may die or live to spawn again: these types of life cycles are described as anadromous and semelparous/iteroparous. They are predators, feeding on small crustaceans, aquatic insects, and smaller fish.

Pacific salmon (genus *Oncorhynchus*) spawns in the fresh waters around the northern Pacific Ocean basin. The genus has expanded its range and established populations north as the ice sheet retreated about 10000 years ago. *Oncorhynchus* is a heritage genus and highly respected by native tribes of the Pacific coasts, who quite understand its significance in the river ecosystems, indeed, salmon are a frequently used icon in totems. Pacific salmon, being semelparous (with the exception *Oncorhynchus mykiss*) and anadromous, transport large amounts of biomass, and hence energy and essential minerals, from the oceans to inland riparian ecosystems, which is very important for those ecosystem (*e.g.* supporting diversity: Schindler et al. 2003; Janetski et al. 2003).

In general, salmon are very well researched fishes. In addition to their importance in aquaculture, and as the result of conservation concerns associated with human mediated habitat loss, evolutionary and ecological properties of salmonids make them excellent natural model for adaptation studies (Carlson et al 2011). Genetic differentiation among populations, or stocks, (as a result of strong homing behavior) and considerable habitat heterogeneity across their geographic range suggests salmonids are likely locally adapted, and some empirical examples have demonstrated local adaptation in salmon populations (Taylor 1991; Adkison 1995; Garcia de Leaniz et al. 2007). Furthermore, salmonids can maintain high levels of genetic variation within populations and rapidly

adapt to environmental changes, which makes them resilient to unstable environmental conditions and successful in colonizing new habitats (such as their range expansion after the retreat of the glaciers and their successful introduction into the southern hemisphere; Quinn 2005). In contrast, salmonid populations tend to have low effective population sizes and undergo frequent bottlenecks which would effectively reduce genetic diversity, and their potential to respond to selection (*e.g.* Heath et al. 2002; Koskinen et al. 2002; Shrimpton and Heath 2003). Such population dynamics suggests salmon populations lack an evolutionary "equilibrium". Quantitative genetics framework is useful in understanding the underlying genentic basis that mediate adaptive potential in salmonids under such ecological setting (Roff 1997, Carlson and Seamons 2010).

More speculatively, salmon may provide a "testable" or at least a very useful indirect model for the shifting balance theory of evolution which posits the importance of non-additive genetic variation as the basis of "adaptive novelties" in subdivided populations where effective population size is small (Wade and Goodnight 1998; Merila and Sheldon 1999). Non-additivity in transcriptional traits has been documented in salmonids, among diverging steelhead salmon populations (Chapter 2) and between wild-farmed Atlantic salmon hybrids (Normandeau et al. 2009; Roberge et al. 2008), therefore evolutionary predictions that assume only additivity may be unrealistic for salmon (Kawecki and Ebert 2004). Furthermore, several properties of salmon populations (outbreeding or inbreeding depression, small effective population size) are not compatible with the Fisherian paradigm of evolutionary process (Wade and Goodnight 1998). Such a

paradigm shift[*] in explaining the evolutionary process can predict salmon population dynamics better and also improve conservation efforts.

TRANSCRIPTION

Transcription is the first step in the information flux by which genetic information stored on the DNA molecule translates into the secondary molecules by which form and function of an organism is realized. RNA is the molecule which is synthesized from and complements DNA as the result of the transcription process. Every protein expressed starts with transcription and has an RNA intermediate. RNA can also affect organisms' function and phenotype independent of translation in several ways (e.g. regulatory non coding RNAs such as siRNAs, miRNAs), which makes transcription and its evolutionary ontology indispensable for understanding the complexity of life. In general transcription variation is thought to be in good concordance with protein level, therefore it correlates functional differences (Yates 1998), however many post-transcriptional and post translational regulations can alter the signal created by the production of the mRNA, thus potentially decouple transcription from function (Wilkins et al. 1999; Eddy 2001; Bartel 2004; Lee et al. 2007).

Transcription Evolution: Since King and Wilson (1975) transcriptional evolution has been accepted as playing a major role in the diversification of species; however, only recently has variation within and among populations been shown at the transcription level (*e.g.* Oleksiak et al 2002; Whitehead and Crawford 2005; Schadt et al. 2003; Morley et al.

[*] Many evolutionary biologists state that the shifting balance theory of evolution is not testable, hence cannot be an alternative for the Fisherian mode of evolution (Coyne et al. 1997). However, I believe the controversy is on philosophical grounds, by which the shifting balance theory of evolution can be viewed as a 'paradigm shift" rather than a matter of fulfilling Popper's falsifiable criterion (Kuhn 1962).

2004), although studies showing the significance of transcriptional variation are expanding greatly over time.

In its simplest sense, a transcriptional trait is very similar to any other phenotypic trait such that its expression is a combination of genotypic and environmental factors (Cheung and Spielman 2002). However, the ontology of transcription complements the statistical properties of quantitative genetics by providing biological mechanisms for complex interactions predicted by the theory (*i.e.* gene-gene, gene-environment interactions) and can provide biochemical explanations to complex evolutionary processes such as rapid adaptive response, and short term tolerance responses (Gibson and Weir 2005). Within a population, genetic variation can be maintained at higher levels in traits linked to transcription relative to protein polymorphism. First, gene transcription is highly modular and likely to be regulated by many loci. Secondly, intrinsic stochastic changes in gene transcription levels elevate the "noise" in this phenotype, thus reducing the efficiency of selective pressures upon genetic variance (Raser and O'Shea 2005). Thirdly, mechanisms inclusive to gene expression such as phenotype canalization (Rutherford 2000) and phenotypic plasticity are likely to buffer transcriptional genetic variance further.

Furthermore, many of the previous practical disadvantages of transcription research are rapidly diminishing. Next generation sequencing, ever-improving gene sequence and function databases and increasing numbers of annotated genomes are making functional transcription variation analysis very feasible and affordable. Additionally, higher computational power is leading to a growing interest in the computationally intense areas of physiological epitasis and gene networks, from which evolutionary quantitative genetic studies will soon benefit, especially due to the growing

interest in non-additive genetic interactions (Gibson and Weir 2005). Non-additivity is indeed remarkable in transcription as evidenced in the few studies targeting that question (*i.e.* examples in Gibson and Weir 2005, and Maverez et al. 2009). Therefore transcription research is indispensable to understand the true nature of evolutionary process.

THESIS OBJECTIVES

This thesis explores the genetic architecture of salmon populations with an emphasis on transcriptional and non-additive genetic variation. The genetic architecture of a trait can be defined as the underlying genetic properties of a phenotypic trait. Several different genetic effects make up genetic architecture, such as additive effects (each alleles' effect on phenotype is fixed and additive), dominance effects (joint effect of two or more alleles in one locus differs from their additive effects), episatsis (joint effect of two or more gene loci differs from their additive effects), maternal effects (only maternal genes affect phenotype). Throughout this thesis non-additivity is used to refer all genetic effects except additive genetic effects. Both non-additivity and transcription are important for salmon evolution and life history, yet the empirical evidence as well as theoretical considerations are scarce. Therefore I explore those areas at different spatial and temporal scales in the next four chapters of this thesis.

In Chapter 2, the evolution and genetic architecture of transcription at four candidate genes was explored in recently diverged Steelhead trout (*Oncorhynchus mykiss*) populations. The chapter provides an example of rapid evolution of transcription in osmoregulation upon introduction to a freshwater habitat. The evolutionary response was rapid and complex, as evidenced by deviation from the mid-parent mean values of the

reciprocal crosses in a common garden environment. The response seemed to be associated with reduced energy expenditure in the lake system. These results highlight unpredictable phenotypic outcomes of hybridization among locally adapted populations and the need for caution when interbreeding populations for conservation purposes.

In chapter 3, within-population variance components of transcription for six genes before and 24 hours after immune stimulation with *Vibrio* vaccine was estimated using juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in a replicated factorial breeding experiment. Four of six genes explored were early response cytokine genes (*IL1*, *TNF*-α, *IL-8, IL8-R*) and two were control genes (*IgM* and *RPS-11*). Additive genetic effects were small and not significant but I found significant maternal effects in pre-challenge and non-additive genetic (interaction) effects in post-challenge cytokine transcription. Gene expression was correlated among cytokine genes, but not between pre- and post-challenge states. The lack of additive genetic effects in cytokine transcription showed these traits are not likely to be good candidates for selection programs to improve immune function in Chinook salmon. On the other hand, my results support the general finding that non-additive effects in salmonids are prevalent, and cannot be overlooked when exploring evolutionary ecology and adaptive responses in salmonids.

In chapter 4, I explored the underlying genetic architecture of differentiation among local populations, which is important to understand the dynamics of local adaptation. I analyzed two factorial breeding experiments which include fish from different stocks kept in a common garden environment for a generation. Overall, 17 traits were evaluated for differentiation among populations and the relative contribution of maternal and additive effects to population differentiation was estimated. Although among-family variance was mostly dominated by additive effects, among-stock

differences were explained mostly through dam effects. These results signify that maternal effects are a primary component of Chinook salmon population differences. Also, I concluded that a single generation in a common environment is not sufficient to negate maternal effects among populations, thus common garden and translocation experiments designed to measure the additive genetic contribution to local adaptation would not be conclusive unless the translocation involved individuals from a controlled breeding program or reciprocal crosses to correct for the possible non-additive effects.

In chapter 5, I performed a large scale transcriptome analysis using a 652 gene element Chinook salmon microarray among four populations of Chinook salmon. The design included three environmental conditions: Control (resting), 24 hours after a 4° C water temperature increase and 24 hours after immune stimulation with *Vibrio* vaccine. The experiment demonstrated the dynamics of differentiation among populations among three different environments. My results suggest acclimation response (to immune stimulation) mediates differences among salmon populations.

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CHAPTER 2

RAPID EVOLUTION OF OSMOREGULATORY FUNCTION BY MODIFICATION OF GENE TRANSCRIPTION IN STEELHEAD TROUT*

INTRODUCTION

Increasing awareness of global ecological degradation and human anthropogenic impacts, combined with the need for better natural resource management, has directed more attention to conservation biology, and in particular to studies concerning population viability in rapidly changing conditions. Indeed, a growing body of evidence documents examples of rapid evolution in a variety of taxa and ecosystems (*e.g*. Hendry and Kinnison 1999). Rapid trait divergence is thought to be correlated with changing environmental factors, for example: precipitation and Galapagos finches (Grant and Grant 2002), host/food networks and soapberry bugs (Carroll et al. 2001), spawning habitat and salmon (Hendry et al. 2000), predator-prey interactions in guppies (Reznick et al.1997), and invasion of a novel environment in sticklebacks (Barrett et al. 2008). The conceptual link among all of those studies is rapid adaptive phenotypic change in natural populations.

Empirical examples of rapid evolutionary change refute the once commonly accepted idea that fitness-related traits are expected to have low additive genetic variance (Mousseau and Roff 1987), and hence be incapable of rapid evolutionary change. Fitness-related traits can, in fact, evolve as fast as neutral traits (Houle 1992; Kinnison and Hendry 2001; Merila and Sheldon 2000). This apparent contradiction of quantitative

*Aykanat T, Thrower FP, Heath DD (2011) Rapid evolution of osmoregulatory function by modification of gene transcription in Steelhead trout. *Genetica*, **139**, 233-242.

genetics theory and experiment is resolved through non-additive genetic models which provide a basis for preserving genetic variation and ongoing capacity for rapid evolution in traits associated with fitness (Cheverud and Routman 1996; Goodnight 1988; Merila and Sheldon 1999). The molecular genetic mechanisms behind rapid evolution and non-additive genetic contributions to evolutionary change are generally poorly understood; however, one exception is gene transcription. Transcription is a polygenic trait and harbors substantial genetic variation that can contribute to phenotypic evolution (Gilad 2006; Oleksiak et al. 2002; Roberge et al. 2007; Roelofs et al. 2006). The phenotypic plasticity (Ghalambor et al. 2007), stochasticity (Raser and O'Shea 2004) and significant non-additive genetic components of transcription (Gibson et al. 2004; Hedgecock et al. 2007) provides further buffering of genetic variation against loss by selection. Given the expectation for a role of transcription modification in the evolutionary response to environmental perturbation and the reduced costs and technical difficulty of transcription quantification, transcription has become the focus of a number of evolutionary population studies. Such studies have been designed to test for local adaptation in natural populations (Giger et al. 2008; Jeukens et al. 2009; Larsen et al. 2007; Larsen et al. 2008; Nilsen et al. 2007), rapid adaptive changes in captive populations (Normandeau et al. 2009; Roberge et al. 2006; Roberge 2008), and ecotypic divergence in the wild (Roberge et al. 2007).

An important, but little studied, genetic outcome of local adaptation and rapid divergence is the change in the non-additive genetic variance component in populations experiencing strong selection pressures (Carroll et al. 2001; 2003). For example, hybridization between wild and farmed Atlantic salmon (*Salmo salar*) results in remarkable non-additive variation in gene transcription, where farmed escapees that interbreed with wild fish would produce offspring with unpredictable phenotypes that

would likely reduce their viability (Normandeau et al. 2009; Roberge 2008). However, the genetic architecture of transcription upon hybridization of naturally diverging wild populations has not yet been explored.

The ecological and demographic properties of salmonids provide an excellent natural system to test for rapid evolution of gene transcription and the genetic architecture of transcriptional divergence. Salmonid populations, naturally or as a result of human impact, tend to have low effective population size and undergo frequent bottlenecks (*e.g*. Heath et al. 2002; Koskinen et al. 2002; Shrimpton and Heath 2003; Thrower et al. 2004a) hence, they are expected to have relatively low additive genetic variation. Yet, they exhibit considerable genetic variation in transcriptional traits within and among populations (*e.g*., Derome and Bernatchez 2006; Roberge et al. 2006) and have a high capacity for rapid evolution (*e.g*., Heath et al. 2003; Hendry et al. 1998; Hendry et al. 2000; Kinnison et al. 1998; Koskinen et al. 2002).

Here I document evolutionary change at four osmoregulatory genes in steelhead trout (*Oncorhynchus mykiss*) introduced into a freshwater lake 80 years (14 generations) ago and the genetic architecture of these genes upon hybridization with the ancestral population. I targeted genes that play central roles in the osmoregulatory changes associated with the parr-smolt transformation (in preparation for saltwater migration) in salmonids (i.e., *CFTR I, NaK ATPase1αa, NaK ATPase1αb* and *GHRII*; see Materials and Methods*).* All are known to respond to short-term salt water challenge, and three of them are known to require high energetic input for expression (*NaK ATPase1αa, NaK ATPase1αb* and *GHRII*). Here I test two predictions; 1) the transplanted freshwater fish will exhibit a reduced transcriptional response to the saltwater challenge due to evolutionary loss of function, and 2) the freshwater expression of the osmoregulatory

genes will be reduced in the landlocked fish due to selection favoring lower energetic costs in the lake habitat. My results support the second prediction, and I propose that energy constraints may play a role in the transcriptional evolution of osmoregulatory genes in the landlocked population. Also, since I found that gene transcription had a substantial non-additive component, the potential for hybridization among salmon populations to result in unpredictable and possibly maladaptive transcriptional profiles is high, and it should be considered in the planning of future conservation and management action.

MATERIALS AND METHODS

The study system: In 1926, wild steelhead trout (anadromous form of *O. mykiss*) from Sashin Creek (Alaska, USA) were introduced into fishless Sashin Lake, upstream of Sashin Creek (Figure 2.1). The lake is isolated from the lower stream by impassable waterfalls that prevent upstream migration (Figure 2.1) A large resident population of rainbow trout was established in the lake, with a low number of founding individuals (3-8 founding females; Thrower et al. 2004a). Some gene flow from lake population to downstream creek population was revealed and up to 25 % of individuals in Creek population are estimated to have been lake origin (Pella and Masuda 2001). However, substantial phenotypic differentiation with high heritability was documented between the two populations for life history traits such as size, growth and smoltification, after approximately 14 generations (80 years; Thrower et al. 2004b). High heritability in morphological and developmental traits also suggested the populations were capable of responding to selection, despite the small founding population (Thrower et al 2004b).

Breeding and Rearing: Fish from both the anadromous and introduced (resident) lake populations (wild-caught fish in 1996; Thrower et al. 2004b) were bred and reared in a common hatchery environment for two generations. In May 2004, sexually mature fish (Table 2.1) from two pure lines were bred to generate four cross-types: pure resident (RxR; 8 families), pure anadromous (AxA; 10 families), female resident by male anadromous (RxA; 8 families), and female anadromous by male resident (AxR; 10 families). The RxR dams were significantly smaller than AxA and AxR dams, while no other groups differed (Table 2.1). The difference in the size of the females used could lead to maternal effects affecting both offspring size and possibly gene transcription; however, the effects associated with maternal size generally become indistinguishable by the time of my sampling, at the age of 2 years (Thrower et al. 2004b; Heath and Blouw 1998). Offspring from the various families within each cross-type were mixed and reared in a common hatchery environment in four identical tanks, thus minimizing the likelihood of tank or family effects. The two generations of common rearing environment likely minimized or eliminated source-related environmental and maternal effects (Roff 1997).

Saltwater challenge and sampling: The experiment included 2-yr-old fish at the parr-smolt transformation stage. Parr-smolt transformation is the process by which the morphology, physiology, and behavior of salmonids change for saltwater acclimation prior to ocean migration (McCormick and Saunders 1987). Smolting fish were identified by their characteristic silver coloration and loss of parr marks and non-smolt fish were identified by retained parr marks, light colored fins and the normal, cryptic stream coloration (Thrower et al. 2004b). Both smolt and non-smolt offspring from all cross types were randomly selected from pooled families. The fish were sampled prior to, and after, exposure to 30 ppt salt water for 24 hours. A 24 hour saltwater challenge (at 30 ppt

salt) is a standard protocol for the physiological measurement of saltwater tolerance in anadromous salmonids (*e.g.*, Blackburn and Clarke 1987). Fish were humanely euthanized by a blow to the head, and gill tissue was immediately removed and preserved in RNA preservation medium (3.5 M Ammonium Sulfate; 15 mM EDTA; 15 mM Sodium Citrate; pH: 5.2,) at -20°C for later RNA extraction. All fish were individually measured for wet body mass (g). Eight fish of the smolt phenotype and seven of the non-smolts from each cross and treatment were assayed in this study.

Genes assayed: I targeted four osmoregulatory genes (*CFTR I, NaK ATPase1αa*, *NaK ATPase1αb* and *GHRII*) whose functions are relatively well characterized and are known to play key roles in saltwater acclimation. Other assayed genes included; Elongation factor 1a (*EF1a*) as the reference for normalization of quantification, *β-actin* and immunoglobulin M heavy chain (*IgM*) as "control" genes to assess neutral expectations of change between the two populations (since neither gene is expected to be under strong directional selection in either environment). Both *β-actin* and *IgM* have been shown to exhibit variable transcription under stress in Pacific salmonids (Ching et al. 2009).

Cystic fibrosis transmembrane receptor (CFTR I) is a chloride channel, located apically in the gills in Atlantic salmon (*S. salar*), and is important for saltwater adaptation (Singer et al. 2002). Transcription of *CFTR I* gene is upregulated during saltwater exposure and expression varies among strains (Singer et al. 2002). Landlocked Atlantic salmon have been shown to have reduced levels of *CFTR I* expression (Nilsen et al. 2007).

NaK ATPase1αa and NaK ATPase1αb are the two isoforms of the active subunit (*1α*) of the sodium potassium ATPase pump (Blanco et al. 1998). Saltwater exposure

downregulates *NaK ATPase1αa* expression, while upregulating *NaK ATPase1αb* in *O. mykiss,* 24 hours after exposure (Richards et al. 2003). Protein and immunohistochemistry studies further supports these two subunits having different functions in fresh and salt water (McCormick et al. 2009). The protein is expressed in gills and kidney, is highly ATP-dependent, and the protein activity is correlated with smoltification and saltwater tolerance in Atlantic salmon (Kiilerich et al. 2007). Nilsen et al. (2007) showed seasonal expression in *NaK ATPase1α* subunits (a and b) is elevated in anadromous Atlantic salmon (*S. salar*) compared to landlocked populations.

Growth hormone receptor II (GHRII): Growth hormone (GH) has wide range of functions in teleost fish, and it known to influence somatic growth, lipid metabolism and saltwater acclimation (Bjornsson 1997; McCormick 2001; Deane and Woo 2009; Kiilerich et al. 2007). GHRII acts to modulate tissue-specific activity of GH (Norbeck et al. 2007). The role of GHRII as the receptor of growth hormone has been verified by protein-protein interaction experiments (Reindl et al. 2009). GHRII is upregulated during saltwater exposure (Poppinga et al. 2007), differentially expressed among anadromous and landlocked strains of Atlantic salmon (*S. salar*), and exhibits seasonal elevations that are associated with smolting and growth in Atlantic salmon (Nilsen et al. 2008).

RNA extraction and cDNA synthesis: Gill tissue was homogenized in 1.0 mL with a glass mortar and pestle. Total RNA was isolated by acid guanidium thiocyanate, phenol chloroform extraction using TRIZOL reagent (Invitrogen) following Chomczynski and Sacchi (1987). A subset of the total RNA extracts was evaluated for quality and quantity using a bioanalyzer (Agilent Technologies). RNA concentrations and RNA integrity number (RIN) values ranged from 0.2 μg/μL to 1.2 μg/μL and 5.7 to 9.4, respectively (mean RIN = 7.7 ± 1.2 SD). For cDNA synthesis, 0.5 μL total RNA was reverse

transcribed using reverse transcriptase (Invitrogen SuperScript II), 0.5 µg Oligo (dT), 50 ng random hexamers, 10 mM dNTP with total RNA, incubated for 5 minutes at 65°C and chilled on ice. Subsequently, 5X RT buffer (Invitrogen), 40 units of RNaseOUT (Invitrogen) and 0.1 mM dithiothreitol (DTT) was added to the reaction and incubated 2 minutes at 42ºC. Finally, 100 units of reverse transcriptase was added and the reaction was incubated at; 42ºC for 10 min., 25ºC for 10 min. and 42ºC for 20 min. The enzyme was inactivated at 70ºC for 15 minutes. The resulting cDNA was washed with 70% ethanol twice and resuspended in 10 mM TRIS (pH 8.0) prior to quantitative real time PCR.

Quantitative real-time PCR (qRT-PCR): Assayed genes were quantified in eight smolt and seven non-smolt offspring from each cross-type in fresh water and after 24 hours in salt water (except for *IgM* which was assayed only for freshwater transcription). Salmon have a tetraploid ancestry, and many of their genes have two isoforms with similar DNA sequences. I therefore designed my probes and primers in regions where the isoform sequences are most dissimilar, and that lie across intron-exon boundaries (Table 2.2). All assays were developed for this study, except *β-actin* and *IgM* which are described in Ching et al. (2009). The *CFTR I* gene of *O. mykiss* had not been characterized, and thus I amplified and sequenced it using degenerate primers designed from *Salmo salar CFTR I* & *II*. Sequence information for the other genes for *O. mykiss* was obtained from GenBank cDNA sequences (Table 2.2). Quantitative real-time PCR analyses were performed in triplicates for low expression genes (*CFTRI* and *GHRII*) and in duplicates for the others (*EF1a, NaK ATPase 1αa, NaK ATPase 1αb,* β-*actin,* and *IgM*). qRT-PCR critical threshold (Ct) values were obtained using ABI's 7500 System SDS software and assayed genes were quantified using the efficiency-corrected method

(Pfaffl 2001) and were normalized to the Elongation Factor 1a (*EF1a*). qRT-PCR efficiencies are presented in Table 2.2.

Quantifying response to saltwater challenge: I report plasticity of gene transcription as the response of the various cross-types to a 24 hour saltwater challenge (30 ppt). The same number of fish that were sampled in fresh water were subjected to a 24-hr saltwater challenge and RNA was extracted post-challenge. Here I report transcriptional response (saltwater minus freshwater gene transcription) to the 24 hour saltwater challenge, rather than gene expression in saltwater, since transcription response is a more functional measure of evolutionary "loss of function" in the landlocked population. I calculated response by subtracting the average transcription value in fresh water from the individual fish transcription values after the 24 hour saltwater challenge.

Statistical analysis: Pure-type cross analysis: First, I used t-tests to test for significant differences in freshwater transcription and response to salt water (relative to EF1a) for each gene between pure-type crosses (i.e., RxR and AxA). Since I performed multiple tests, I calculated global p-values and false discovery rates (FDR) by permutating the data 1000 times. I calculated the global p-value as the ratio of the number of permutations with greater significance than the actual t-test divided by the total number of permutations, and FDR as the random expectation of the number of significant comparisons divided by the observed number of comparisons. For the random expectation of the number of significant comparisons I used the average number of significant comparisons per permutation. For t-tests and permutations, I used R software version 2.10.1 (R Development Core Team, 2009). I also tested whether the observed transcriptional response to the 24 hr saltwater challenge was significantly different from zero for each gene using t-tests (SYSTAT v7.0.1, SPSS Inc., Evanston, Illinois). Unless

otherwise noted, all other statistical analyses were performed using SYSTAT v7.0.1 (SPSS Inc., Evanston, Illinois).

Reciprocal cross analysis: I compared freshwater transcription and saltwater challenge response in each reciprocal cross with the pure-type crosses using two-sample t tests. Non-additive genetic effects are identified as significant deviations of reciprocal cross trait values from the midpoint of the pure-type cross trait values. I did not include *IgM* and *β-actin* in the reciprocal cross analysis, since those genes were solely included to characterize transcriptional evolution (drift) associated with pure-type crosses at genes not under osmoregulatory selection pressure.

Body size effects: Body size can influence the transcription of genes involved in osmoregulation, since smolting is sensitive to body size variation (McCormick and Saunders 1987, McCormick 2001). Since AxR non-smolts were significantly smaller than other cross-types (Table 2.1), I tested for an effect of individual body mass on variation in transcriptional traits among the four cross-types using an analysis of covariance (ANCOVA), with body mass as the covariate.

Q_{ST} calculation: I estimated phenotypic divergence (Q_{ST}) of transcriptional traits for smolts and non-smolts using the formula: $Q_{ST} = \sigma^2_{GB} / (\sigma^2_{GB} + 2\ \sigma^2_{GW})$, where σ^2_{GB} and σ^2_{GW} are among-population and average within-population components of genetic variance respectively (Whitlock 2008). Variance components (σ^2_{GB} and σ^2_{GW}) were estimated using ANOVA, and 95% confidence intervals (CI) were estimated by bootstrapping the data 30 times. The average within-population genetic variance (σ^2_{GW}) also includes environmental variance and thus may be overestimated, which may lead to an under-estimation of the true Q_{ST} (Whitlock 2008). No differential selection is expected

in the two populations at *β-actin* or *IgM*, thus transcriptional differentiation at those genes should reflect primarily neutral (drift) divergence.

RESULTS

Pure-type cross analysis: CFTR I and *NaK ATPase1αa* showed significant up-regulation and down-regulation respectively in AxA smolts in response to salt water (results not shown), consistent with previously published results (Richards et al. 2003; Singer et al. 2002). Up-regulation of *GHRII* and down-regulation of *NaK ATPase1αa* in response to salt water was significant in RxR non-smolts. All other comparisons of pre- and post-challenge transcription levels were not significant. Contrary to Richards et al (2003), *NaK ATPase1αb* transcriptional response to the saltwater challenge was not significant, although I did observe a non-significant up-regulation trend.

Differences between pure lines (AxA versus RxR) were significant in five comparisons (Figures 2.2 and 2.3), while neither *IgM n*or *β-actin* expression were significantly different between pure-type crosses ($p > 0.20$, results not shown). Multiple test analyses showed that my significance estimates are highly meaningful with FDR = 0.183 and a global *p*-value of 0.005. All significant diffrences between the two pure cross-types was either *GHRII* or *NaK ATPase1αa* transcription. Comparisons among pure cross-types were not significant for *CFTR I* and *NaK ATPase1αb* in either the smolt or non-smolt trials, both in fresh water and in response to the saltwater challenge (Figures 2.2 and 2.3).

The transcription of genes which are associated with high energy demand (i.e. *GHRII*, *NaK ATPase1αa* and *NaK ATPase1αb*) were consistently lower in the RxR fish in the fresh water (t=0, Figure 2.2). However, differences in the transcriptional response to

salt water were not as consistent: in some cases the RxR crosses showed a greater change in response to the saltwater challenge, in others, a lower change (Figure 2.3).

Reciprocal cross analysis: I measured gene transcription in reciprocal crosses (AxR and RxA) to assess additive versus non-additive genetic variance contribution to the expression of the selected genes. Dominance and epistatic effects would be evident by reciprocal cross transcription values that depart equally from the midpoint between the two pure-type crosses, while reciprocal cross transcription at the midpoint would indicate primarily additive genetic variance. Two sample t-tests identified significant departures from additive genetic variance expectation in reciprocal crosses in 5 of 16 cases (Figures 2.2 and 2.3). In all cases, the departures from additivity were characterized by a single reciprocal cross exhibiting overdominance, while the other did not deviate from additivity (Figures 2.2 and 2.3). *NaK ATPase1αa* and *NaK ATPase1αb* did not significantly deviate from additivity. Interestingly, *CFTR I* transcription shows significant non-additivity (non-smolts in fresh water and in response to salt water; Figures 2.2 and 2.3), despite no significant difference in transcription between the pure lines.

Body size effects: Individual body mass was not a significant factor for most transcriptional traits among cross-types (ANCOVA). Mass was marginally significant ($p = 0.04$) for freshwater transcription expression of *NaK ATPase1αb* in non-smolts. Out of 22 comparisons, this one significant effect may be due to chance alone, and post-hoc Bonferonni correction renders it non-significant.

Q_{ST} calculation: Q_{ST} estimates for transcription varied considerably. The Q_{ST} values for β-*actin* and *IgM,* which are expected to be under little or no selection in this system, averaged 0.33 (95% CI = ± 0.07, Figure 2.4). *CFTR I* showed generally low Q_{ST} values, indicative of no strong selection. Overall, most Q_{ST} estimates ranged between 0.2

and 0.4, whereas traits with elevated Q_{ST} estimates were generally in agreement with significant pure-type cross differences as determined by t-test (Figure 2.4).

DISCUSSION

In Pacific salmon (*Oncorhynchus sp*.), both genetic and environmental mechanisms have been proposed as contributing to the observed diversity in life history (*e.g.*, Heath et al. 2008). In the Alaskan steelhead trout, I demonstrate both rapid evolution in transcriptional traits as well as plasticity of transcription as a response to a saltwater challenge (i.e., differences in transcription between the freshwater and saltwater environments). Thus, gene transcription provides a single mechanism for both the rapid evolution of adaptive life history characters as well as the well known physiological plasticity associated with gene expression.

Ecological dissimilarities between the two habitats (ionic and energetic) are ideally suited to promote rapid transcriptional evolution at genes associated with osmoregulation and seaward migration during the parr-smolt transformation (Barrett et al. 2008; Leonard and McCormick 2001). Generally my results in the freshwater environment are in accordance with my prediction of reduced energetic expenditure for osmoregulation in the resident freshwater population. For example, the two ATP dependent isoforms of the sodium-potassium pump (*NaK ATPase1αa*, *NaK ATPase1αb*), which are highly energy dependent and expressed at high levels in fish (Tseng and Hwang 2008), were expressed at lower levels in the landlocked population relative to the ancestral population. On the other hand, *GHRII* is not ATP dependent, but is associated with high energy demanding physiological processes such as smoltification, osmoregulation and growth (Kiilerich et al. 2007; Nilsen et al. 2008; Norbeck et al 2007).

GHRII shows downregulation in the resident smolts and non-smolts in the fresh water, consistent with my first prediction.

Overall, the landlocked population shows an evolutionary shift towards a lower energy consumption state, which is consistent with the low productivity of northern lakes and the high energetic costs of osmoregulation (Tseng and Hwang 2008). Similarly, juvenile anadromous Arctic charr exhibit lower rates of growth than their resident freshwater counterparts, despite having higher feeding rates, and the authors suggested that the anadromous fish had higher metabolic costs associated with their saltwater environment (Morinville and Rasmussen 2003). Thus, I suggest that gene transcription at selected loci adaptively evolved in the landlocked population due to energetic constraints. However, the energetic cost of osmoregulation is still under debate (Boeuf and Payan 2001; Tseng and Hwang 2009), and the direct measurement of O_2 consumption, or a microarray based approach to investigate energy related metabolic pathways could be implemented to confirm my conclusions.

Several previous studies have confirmed that the expression of the osmoregulatory genes used in this study do respond to abrupt salinity changes (*e.g.*, Singer et al. 2002; Richards et al. 2003; Poppinga et al. 2007). These genes are also known to change during the seawater preparatory period of anadromous salmon (Nilsen et al. 2007). Despite the documented association between gene expression and saltwater acclimation, experiments demonstrating the direct role of variation in gene transcription in osmoregulation have yet to be done.

The resident freshwater population is known to have experienced hard selection for traits that are correlated with seaward migration (i.e., over the waterfalls; Thrower et al. 2004a, b). In osmoregulatory gene expression, I predicted the resident fish would

exhibit a loss of response to saltwater exposure if selection favored a saltwater intolerant state. However, I did not observe a pattern of transcriptional response that was consistent with the hypothesis of an evolutionary loss of saltwater response in the resident fish. Two examples of the predicted loss of response to the saltwater challenge in the resident fish was for the *NaK ATPase1αa* and the *GHRII* genes in smolts, which showed virtually no change in expression for the RxR, while the AxA showed a negative and positive response, respectively, to the saltwater challenge. Curiously, I observed significant transcriptional differences in the saltwater response in non-smolts for both genes, but the direction of the difference was contrary to my predictions; the RxR non-smolts exhibited a greater transcription response than the AxA non-smolts. However, the transcriptional response to short-term saltwater stress is not well characterized in non-smolt salmonids, hence it is difficult to interpret the functional significance of my non-smolt results. A more exploratory approach (such as microarray analyses) would perhaps identify additional genes that have responded to the environmentally-based selection between the semi-isolated populations in this study.

The inheritance of transcription is more complex than simple additive genetic variance models can account for since transcription includes substantial non-additive genetic effects (Gibson et al. 2004; Hedgecock et al. 2007; Roberge et al. 2008). The non-additive genetic component of variance in *CFTR I* and *GHRII* transcription reported here is likely an important factor in the maintenance of genetic variation and evolutionary potential in small and isolated salmon populations. On the other hand, non-additive genetic variance results in the disruption of co-adapted genotypes and can lead to extreme phenotypes and generally reduced fitness (Tymchuk et al. 2007). The non-additive effects I identified at *CFTR I* and *GHRII* are curious, since the reciprocal crosses differ

substantially. Classically, reciprocal cross divergence is explained by sex-linkage or extra-nuclear inheritance, although sex-linked epistatic effects or maternal imprinting are also possible explanations (Falconer and Mackay 1996; Tuiskula-Haavisto and Vilkki 2007). There is no evidence for sex-linkage or extra-nuclear inheritance of the genes assayed here, thus sex-linked epistatic effects or (origin of parent specific) genetic imprinting are more likely explanations. However, genetic imprinting has not yet been reported in lower vertebrates (Xie B et al. 2009).

The non-additive response in *CFTR I* expression is particularly notable, since there was no significant difference in transcription between the pure-type crosses. This suggests that stabilizing selection for transcription may be acting at *CFTR I*, but the disrupted genomic background generated in reciprocal crosses affected the transcription control, likely resulting in the observed anomalous gene expression response. Such unexpected gene expression patterns in hybrid offspring highlight the need for caution when crossing individuals from putatively locally adapted populations for conservation or management purposes (Roberge 2008; Tymchuk et al. 2007).

This study presents empirical evidence of rapid transcriptional evolution in a recently colonized population of steelhead trout. Transcriptional variation can not only mediate the evolution of physiological traits (such as osmoregulatory function), but it is also recognized as a primary mechanism for phenotypic plasticity associated with physiological acclimation. Transcriptional modification thus plays a role in the rapid adaptation and acclimation processes necessary for local adaptation in a changing environment. My results also show that interbreeding locally adapted populations may increase the overall phenotypic variation but, in a cautionary conservation note, it can

give rise to anomalous gene transcription responses in genes closely related to survival and performance (Tymchuk et al. 2007).

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Table 2.1: Mean body mass (g) with one standard error in parentheses, for parental and offspring experimental fish by cross–type. Parental fish weight is given for dam and sire separately. Freshwater and saltwater challenged fish are pooled for each cross–type in the offspring. Significant differences among parental crosses are indicated with different letters for Dams (ANOVA, Tukey multiple comparison test, $p<0.05$). Differences were not significant among sire groups.

		Dam	Sire		Smolt		Non smolt
Cross-type	**N**	**Mass**	**Mass**	**N**	**Mass**	**N**	**Mass**
AxA	10	3250[a] (862)	2810 (660)	16	104 (21.7)	14	64.1 (22.0)
AxR	10	3380[a] (798)	2490 (449)	16	101 (14.5)	14	46.8 (16.0)
RxA	8	2640[ab] (774)	2840 (714)	16	105 (21.7)	14	68.4 (14.0)
RxR	8	2130[b] (791)	2820 (693)	16	110 (24.9)	14	56.5 (22.4)

Table 2.2: Quantitative real time PCR details for selected genes in steelhead trout. PCR efficiency, final product length, and primer-probe sequence information (with concentration in parentheses) is provided. Intron-exon junctions are underlined. *EF1a* was used as endogenous control.

Gene	PCR Efficiency %	Product length (bp)	Species (GenBank accession) used for assay development	TAQMAN MGB Probe, forward and reverse primer (nM)
CFTR I	92	112	*S. salar* (AF161070, AF155237)	TAA AAC TGG CGG TGC TC (150) CGA TAG GAC ACA GGT GCA GTG A (350) TGG AGA TGT CCA CCA GAA TAC ATA TT (350)
GHRII	83	85	*O. mykiss* (AY861675, AY751531)	CTG GGC GAC CAC CCT (250) ACC CTG AGC TCT TCA AGA AAG GTA (900) CAG TAC AGC TCT GGC CTC AGG T (900)
NaK ATPase 1αb	88	69	*O. mykiss* (AY319390)	CCT ACT ACT GAC AAA AAG A (200) CAG GAG GTT GGG TGG AAC AG (900) GAC ATT GAG TGA TCC TGG GGA TA (900)
NaK ATPase 1αa	93	99	*O. mykiss* (AY319391)	TAT TGA GAC GAA GAG GCC (200) CCC AGG AGG TTG GGT GTA CC (450) TGC ATT ACA AGG CAA TAC TGC A (450)
β-actin	90	64	See reference: Ching *et al.* (2009)	CAC AGC TTC TCC TTG ATG T (250) ACG GCC GAG AGG GAA ATC (900) CAA AGT CCA GCG CCA CGT A (900)
IgM heavy chain	93	69	See reference: Ching *et al.* (2009)	ACCTTGGTAAAGAAAGC (250) CGCTGTAGATCACTTGGAAAACC (900) TCTCCTCCAGTCTCCCTCTTGT (900)
EF1a	84	80	*O. mykiss* (AF498320)	TGC GTG ACA TGA GGC (100) AAT ACC CTC CTC TTG GTC GTT TC (450) CTT GTC GAC GGC CTT GAT G (450)

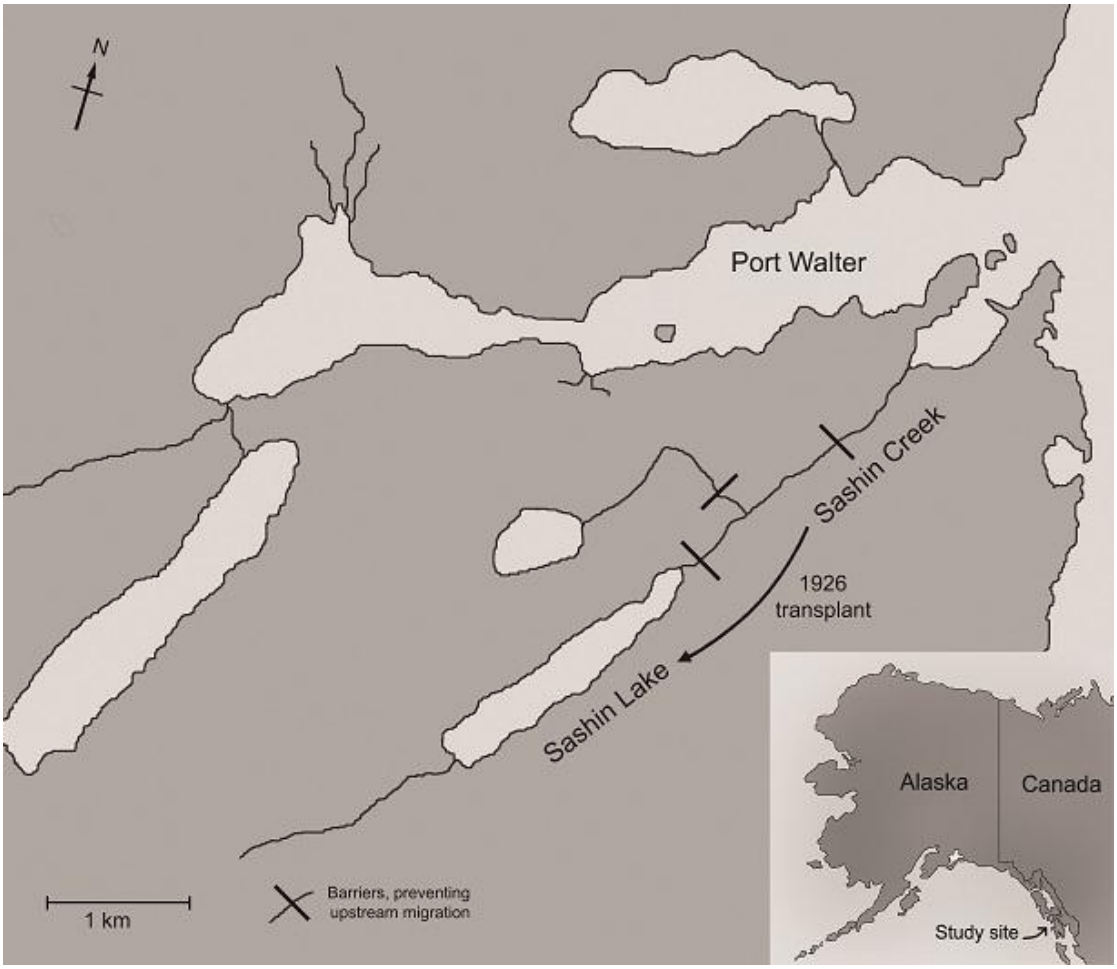

Figure 2.1: Map of Alaska (USA) showing the source of anadromous steelhead trout (Sashin Creek) and the site of introduction of the resident population in Sashin Lake in 1926 (adapted from Thrower et al. 2004a). Impassable barriers to upstream migration are marked with black bars across rivers.

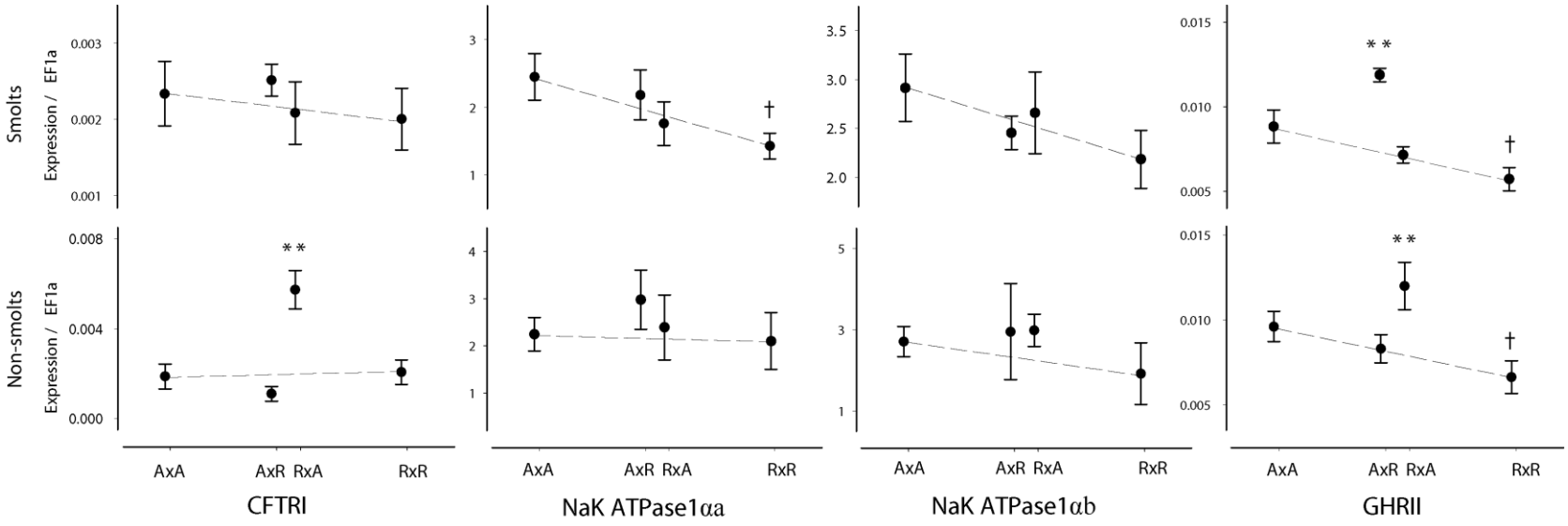

Figure 2.2: Mean gene transcription (± 1 standard error of the mean, SEM) normalized to *EF1a* for four osmoregulatory genes in steelhead trout (*Oncorhynchus mykiss*) from two divergent populations (anadromous – "A"; and land-locked, or resident – "R") and their reciprocal crosses in fresh water. Relative transcription is shown as the comparison between pure (AxA and RxR) and reciprocal crosses (RxA and AxR). Significant differences between pure types are indicated with † (t-test, $p < 0.05$). Deviation of reciprocal crosses from additive expectation are estimated using t-test and indicated with * ($p < 0.05$) and ** ($p < 0.001$). Smolt and non-smolt phenotypes are presented in upper and lower panels respectively.

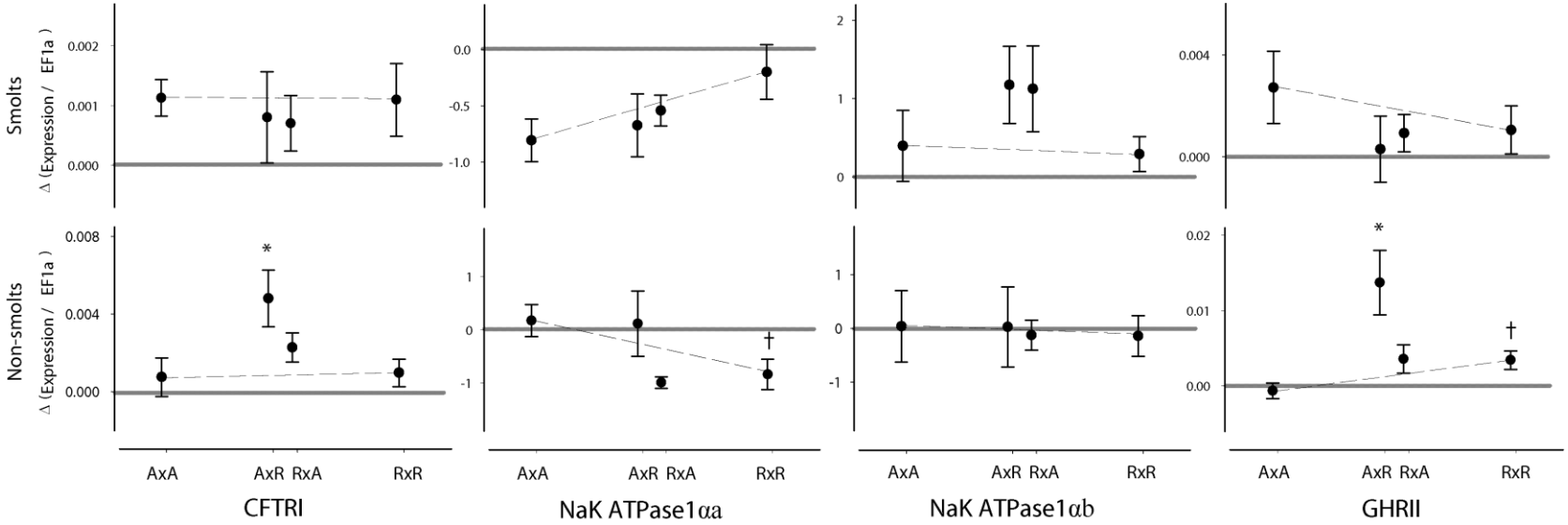

Figure 2.3: Mean gene transcription response (± 1 SEM) normalized to *EF1a* at four osmoregulatory genes in steelhead trout (*Oncorhynchus mykiss*) from two divergent populations (anadromous – "A"; and land-locked, or resident – "R") and their reciprocal crosses as a response to 24-hour saltwater challenge. The difference between relative transcription (t=24 – t=0) is shown as the comparison between pure (AxA and RxR) and reciprocal crosses (RxA and AxR). Significant differences between pure types are indicated with † (t-test, $p < 0.05$). Deviation of reciprocal crosses from additive expectation are estimated using t-test and indicated with * ($p < 0.05$) and ** ($p < 0.001$). The "no response" line is indicated with a thick gray bar. Smolt and non-smolt phenotypes are presented in upper and lower panels respectively.

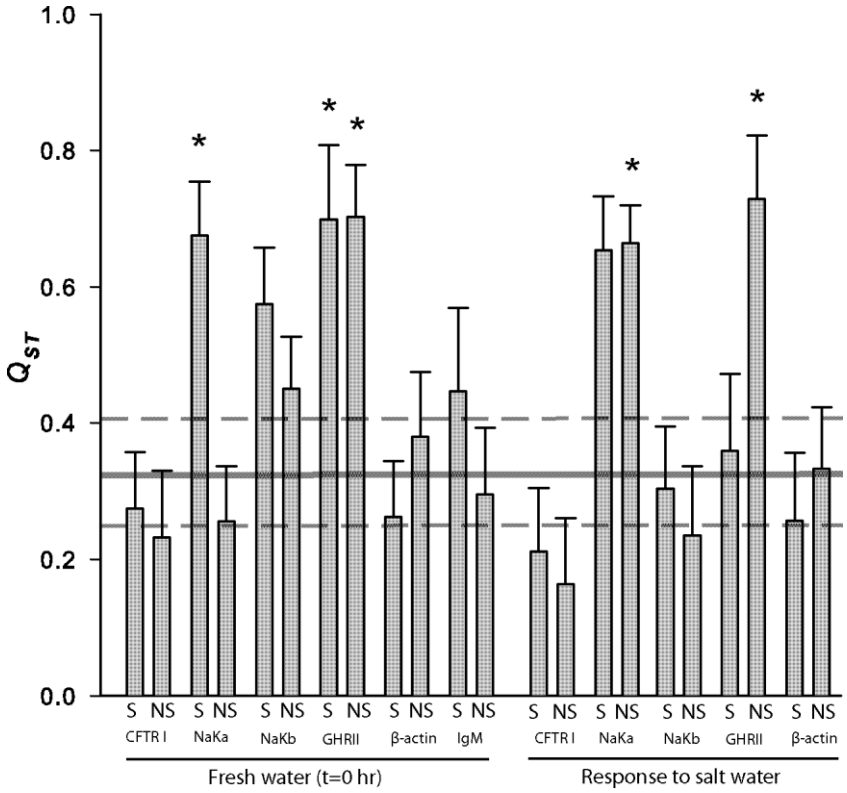

Figure 2.4: Q_{ST} estimations and 95 % CI of investigated traits. Significant differences between pure cross types are also included in the figure and denoted with * ($p < 0.05$). Abbreviations, S: smolt, NS: non-smolt, NaKa: *NaK ATPase 1αa*, NaKb: *NaK ATPase 1αb*. Mean and 95 % CI for putatively neutral response (*IgM* and *β-actin*) are marked with a line and dashed lines, respectively.

CHAPTER 3

ADDITIVE, NON-ADDITIVE AND MATERNAL EFFECTS OF CYTOKINE TRANSCRIPTION IN RESPONSE TO IMMUNOSTIMULATION WITH *VIBRIO* VACCINE IN CHINOOK SALMON *(ONCORHYNCHUS TSHAWYTSCHA)*[*]

INTRODUCTION

Improving disease resistance and overcoming disease associated losses are crucial for cost-effective aquaculture (Cnaani 2008). Healthier and disease resistant fish also encourage environmentally friendly aquaculture practices by reducing the likelihood of transmitting disease to wild fish, and by minimizing the need for antibiotics. In contrast to many other economically important traits (i.e., growth rate, feed conversion efficiency, and flesh quality) genetic improvement of disease resistance appears to be problematic and relatively little has been accomplished through selection programs (Tave 1995; Gjedrem 2005; Sahoo et al. 2008). Part of the problem is that "disease resistance" is difficult to define and direct measurements are not straightforward (Tave 1995; Wiegertjes et al. 1996; Gjedrem 2005). Commonly used indirect health indicators, such as plasma lysozyme or cortisol levels (Fevolden et al. 1994; Balfry et al. 1997; Fevolden et al. 1999; Johnson et al. 2003), and survival rates after disease induction (Amend and Nelson 1977; Johnson et al. 2003), are heavily influenced by environmental factors (Saurabh and Sahoo 2008) and by the immune status of the fish (Roed et al. 2002), and may not accurately reflect disease resistance (Tave 1995; Wiegertjes et al. 1996;

[*] Aykanat T, Heath JW, Heath DD. Additive, non-additive and maternal effects of cytokine transcription in response to immunostimulation with *Vibrio* vaccine in Chinook salmon *(Oncorhynchus tshawytscha)*. Submitted to *Evolutionary Applications*.

Gjedrem 2005). Clearly, new markers that accurately reflect disease resistance and that have a significant genetic component are needed.

One approach to developing such markers would be to characterize the initial stages of the immune response. The rate and timing of the response should be closely related to functional disease resistance, and could thus be used as traits for selection to improve immune function. One set of candidate markers (used in this study) are pro-inflammatory cytokines which are known to facilitate immune function at the very early stages of infection (Secombes 1996). The cytokine-mediated immune network provides communication among immune cells and between body and immune cells by affecting cell motility, chemotaxis, phagocytosis and cytotoxicity (Frankenstein et al. 2006). The fundamental role of cytokines in the modulation of the fish immune system has recently been explored in functional studies which monitored cytokine gene expression in response to immune stimulation (Purcell et al. 2004; Fast et al. 2007; Mulder et al. 2007; Raida and Buchmann 2008; Ching et al. 2010), and physiological effects following recombinant cytokine injection (Hong 2003; Martin et al. 2008; Zhang et al. 2009). Thus, cytokines represent candidate markers for selective breeding programs to improve disease resistance; however, the genetic architecture of cytokine expression is unknown in salmonids, or in any other teleost.

A highly sensitive method to detect early changes in disease resistance is to monitor transcriptional variation in immune related genes. Functional genomics and transcription studies are becoming more common and recognized as valuable in the study of genetic variation and adaptation in fish (Goetz and MacKenzie 2008; Naish and Hard 2008). In contrast, the quantitative genetics of transcription has not been well characterized (Goetz and MacKenzie 2008), and only a few studies have partitioned

genetic variation and estimated heritability for transcription (Roberge et al. 2007; Normandeau et al. 2009). However, selection-based broodstock development in aquaculture would benefit from quantitative genetic analysis of transcriptional traits if sufficient additive genetic variation were to be found in transcriptional traits. Virtually every gene involved in the immune response could be screened for its potential as a marker for disease resistance. Furthermore, transcription-based markers would provide high sensitivity for genes that are expressed at low levels (such as cytokines) which make protein-based detection problematic. Despite these advantages, no studies as yet provide estimates of the genetic variance components for the transcription of immune function genes.

Salmon are intensively farmed and have been of interest to breeders since the beginning of systematic salmon farming activities in the mid 1970s (Gjedrem 2005). Chinook salmon (*Oncorhynchus tshawytscha*) is a Pacific salmon species native to British Columbia, Canada, where it remains the most important native salmon commercially farmed (Kim et al. 2004). Here, I describe a quantitative genetic experiment using a North Carolina II breeding design to partition genetic variance of cytokine transcription in Chinook salmon, before and after immunostimulation with *Vibrio* vaccine. In contrast to traditional nested breeding designs, the North Carolina II factorial scheme can partition additive and non-additive genetic components as well as provide an estimate of maternal effects (Lynch and Walsh, 1998). Non-additive effects have been reported in a number of salmonids (Gharrett et al. 1999; Gink et al. 2004; Pitcher and Neff 2007; Roberge et al. 2008; Evans et al. 2009; Normandeau et al. 2009; Aykanat et al. 2011), thus such effects should not be overlooked in any comprehensive quantitative genetic analysis of complex

traits in salmon, since non-additive effects can fundamentally affect response to selection and evolutionary pathways.

In this study, I assayed three pro-inflammatory cytokine genes (*Interleukin 1, IL1; Interleukin-8, IL8;* and *Tumor necroisis factor-α, TNF-α*), *Interleukin-8 receptor* (*IL8-R*) and two control genes (*IgM* and *RPS-11)*. My results showed cytokine transcription in Chinook salmon possesses little additive genetic variation, but substantial maternal and non-additive variation.

MATERIALS AND METHODS

Breeding Design: In November 2005, 24 3-yr-old male and 24 3-yr-old female domestic Chinook salmon (*O. tshawytscha*) were randomly selected and mated in a North Carolina II design at a commercial salmon farm (Yellow Island Aquaculture Limited; YIAL, Quadra Island BC, Canada). In addition to additive genetic effects, the factorial North Carolina II design allows the estimation of maternal and non-additive effects. Two sires and two dams were mated to produce four families in a 2 x 2 factorial design. The cross was replicated 12 times generating a total of 48 families. Eggs were fertilized and families were incubated separately in vertical-stack incubation trays divided into 16 compartments. In February 2006, all families were transferred to individual but identical rearing tanks (150L), and fed to satiation four times daily until sampling.

Immune stimulation and sampling: Gill tissue from four fish per family was sampled in June 2006, at seven months post-fertilization, before and 24 hours after the immune challenge. The immune challenge consisted of immersion in a 20 L bath of 10% vaccine solution (inactivated strains of *Vibrio anguillarum* serotypes 01 & 02; MICROVIB, Microtek International Inc.). The fish were challenged in batches of

approximately 40-50 fish (average individual mass = 5.46 g ± 1.16). After three minutes of immersion, the fish were transferred to holding tanks (identical to the original) and sampled 24 hours later. The challenge was performed after noon (14:00 to 18:30) over four days, with a randomized family order. Pre-challenge fish were sampled in the morning (8:00 to 13:00) of the same days.

For sampling, the fish were anesthetized in a clove oil solution (0.5 mL of clove oil diluted 1:10 in EtOH and added to 2 L of water). The fish were then immediately weighed, and three gill arches were sampled and stored in RNA preservative solution (3.5 M Ammonium Sulfate; 15 mM EDTA; 15 mM Sodium Citrate; pH: 5.2). The samples were stored at 4° C overnight and subsequently transferred to -20° C until they were shipped to the laboratory in Windsor, Ontario for subsequent lab work.

RNA extraction and cDNA synthesis: RNA was extracted from four fish per family, per treatment. Gill tissue was homogenized in a pre-chilled 0.8 mL TRIZOL solution in a 2 mL tube with glass beads using a bead-based homogenizer. Despite the fact that gills are not a primary tissue for the immune response (such as spleen and head kidney and thymus), their role is vital for an effective defense against pathogens, since they are exposed to the environment and are known to provide a primary defensive barrier in response to immunostimulants (Press and Evensen 1999). Furthermore, the gill mucosal layer not only provides a physical barrier to pathogen entry, mucosa-associated lymphoid tissue contains leucocytes for active local immune responses (Press and Evensen 1999). Therefore gills represent an excellent tissue to monitor important and relevant early immune responses to pathogens. In salmonids, gene expression of pro-inflammatory cytokines is known to be upregulated in gill after immunostimulant exposure (Hong et al. 2003).

Total RNA was isolated by acid guanidium thiocyanite, phenol-chloroform extraction using TRIZOL reagent (Invitrogen), following Chomczynski and Sacchi (1987). A subset of the total RNA extracts was evaluated for integrity using agarose gel electrophoresis and quantified with a small sample volume spectrophometer (Nanovue, General Electric). All RNA samples were normalized to 250 ng/μL using DEPC treated water, and subsequently were treated with DNAase following the manufacturers' instructions (Fermentas #EN0521). For cDNA synthesis, 10 μL total RNA, 1μL Oligo (dT_{12-18}; 500μg/mL) and 1 μL dNTP (10 mM each) were mixed and incubated at 65°C for 10 min and chilled on ice. Subsequently, 5X RT buffer (Invitrogen), 40 units RNaseOUT (Invitrogen) and 0.1 mM dithiothreitol (DTT) were added to the reaction and incubated for 2 min at 42ºC. Finally, 100 units of reverse transcriptase (Invitrogen, SuperScript II) were added, the reaction incubated at 42ºC for 2 h, and the enzyme was inactivated at 70ºC for 15 min.

Quantitative real-time PCR: I assayed three pro-inflammatory cytokine genes (*Interleukin 1β*; *IL1*, *Tumor necrosis factor-α*; *TNF-α*, and *Interleukin-8*; *IL-8*), and one cytokine receptor (*interleukin-8 receptor*, *IL8-R*) which are elements of innate immune system and have roles in mediating the non-specific immune response (Secombes et al. 1996). Innate immunity offers protection against a wide array of pathogens and provides primary protection and the first line of defense against infections (Magnadottir 2007). Furthermore, the innate immune response primes the adaptive immunity, which takes place weeks after the initial infection to occur (Magnadottir 2007). Therefore a properly functioning innate immune system is vital for fish. I also assayed one non-innate immune gene *immunoglobin M* (*IgM*), and a ribosomal protein gene, *RPS-11*, to contrast and

verify innate immune genes expression patterns (e.g. between pre- and post-challenge gene expression) and Elongation Factor *1a* (*EF1a*) was used as the endogenous control gene. Primer and probe sequence information for all markers except *RPS-11* are described in Ching et al. (2009). *RPS-11* primers and probe designed for Chinook salmon are: forward primer: *CCCTCAGCAAGACAGTCAGGTT*, reverse primer: *TGGCTCCAGCAGCCTTTG* and Taqman MGB probe: *AACGTCCTCAAGGTC*. Transcription of the selected genes was quantified for four individuals per family per sampling time (t = 0 h and t = 24 h post-challenge).

Quantitative real-time PCR analyses were performed in triplicate for the endogenous control gene Elongation Factor 1a (*EF1a*) and in duplicate for others. PCR conditions were: 2 min at 50°C and 10 min at 95 °C, followed by 40 cycles of incubation at 95°C (15 s) and 60 °C (1 min). Quantitative real-time-PCR critical threshold (C_T) values were obtained using ABI's 7500 System SDS software. Assayed genes were quantified using efficiency corrected ΔC_T method (Pfaffl 2001), and normalized to *EF1a* expression. I calculated PCR efficiency for each marker by averaging individual efficiency scores of each sample run estimated by the window of linearity method in LinRegPCR (Ramakers et al. 2003). Individual marker efficiency estimates are: 110% for *IL1*; 100% for *TNF*-α; 105% for *IL-8*; 100% for *IL8-R*; 110 % for *IgM*, 100% for *RPS-11* and 80% for *EF1a*.

Statistical analysis: Unless otherwise stated, statistical analyses were performed using R software (R Development Core Team, 2009). Transcription values relative to endogenous control (*EF1a*) were log-transformed to achieve normal distributions. Normality was evaluated in each family and at each time point for each gene using

Shapiro-Wilk normality tests. The relationships between individual fish mass and transcription were evaluated using least square regression with fish mass as the independent variable.

a) Response to disease challenge: A two-way ANOVA in which families were nested in time (pre- and 24 hours post-challenge) was performed to test for significant changes in transcription of each gene resulting from the disease challenge.

b) Estimates of genetic components: Under the North Carolina II design, phenotypic variance was partitioned using the following model:

$$z_{ijk} = \mu + s_i + d_j + I_{ij} + e_{ijk}$$

Where z_{ijk} is the phenotypic value of *k*th offspring of *i*th sire and *j*th dam, μ is the mean phenotype of the sample, s_i and d_j are the effects of *i*th sire and *j*th dam, I_{ij} is the family (non-additive) effect due to the interaction of *i*th sire and *j*th dam, and e_{ijk} is the deviation of the *k*th individual of *i*th sire and *j*th dam (Lynch and Walsh, 1998). Total phenotypic (transcription and mass-at-age) variance (σ^2_P) was then partitioned into paternal half sib (σ^2_s), maternal half sib (σ^2_d), interaction (σ^2_I) and residual error (σ^2_e) variance components with restricted maximum likelihood (REML) using lme4 package in R (Bates and Maechler 2009). I assume environmental effects do not vary among families. Within a Bayesian framework, the REML fitted parameters were taken as priors to calculate the parameters' posterior highest probability densities (HPD) with Markov Chain Monte Carlo method (MCMC; 1000 runs) using the languageR package in R (Baayen 2010). Next, the median HPD and 95% confidence intervals (CI) were estimated for all variance components in the model. The significant effects were identified as when the HPD 95% CI did not include zero. To test for significant maternal effects; I subtract 1000 posterior σ^2_s estimate from corresponding σ^2_d estimate (σ^2_d - σ^2_s) and if the number

of negative estimates are not significant ($p< 0.05$; less than 50 times out 1000 cases) I conclude σ^2_d is significantly higher than σ^2_s indicative of significant maternal effects.

I also assessed the potential effects of within-family sample size on my variance analysis. I randomly withdrew one individual from every family, recalculated the REML fitted variance parameters 1000 times, estimated the median HPD for each run and plotted these estimates (with standard deviation; SD) against the variance estimates generated with the original data set. If within-family sample size plays a significant role in my analysis, the plot of N= 3 versus N= 4 will differ from the expected 1:1 line. I also evaluated the effect of within-family sample size using the data for mass-at-age estimates; since I could combine before and after challenge mass-at-age data, I was able to compare the variance component estimates for mass-at-age for pre-treatment fish (four fish total; M_{t0}), post-treatment fish (four fish total; M_{t24}), and fish from both treatments combined (eight fish total; $M_{n=8}$) to test for the effect of increased sample size on my variance component estimates. Furthermore I could also compare the variance estimates based on a large sample size from the same population by using fish that were weighed, but not sampled for transcription ($M_{n\sim40}$; 30-45 fish per family, median=42).

Additive genetic variance (V_A) was calculated as four times the sire component of variance (σ^2_s; assuming no paternal effect; Lynch and Walsh 1998), and narrow sense heritability (h^2) was estimated as the additive genetic variance divided by the total phenotypic variance (i.e., V_A/V_P). The HPD median value was used in h^2 estimation and parameter estimates from each MCMC run were used to calculate the h^2 error (SD).

c) Phenotypic / genetic correlations: Estimating (additive) genetic correlations is technically difficult (Lynch and Walsh 1998); however, family mean phenotypic correlations can be used to predict genetic correlations (Lynch and Walsh 1998).

Theoretically, the relationships are equivalent when the two traits have h^2 equal to one, but gradually becomes weaker, and genetic correlations are overestimated, with lower h^2 (Lynch and Walsh 1998). On the other hand, similarities between phenotypic and genetic estimates of correlations are widespread, and striking (Roff 1996; Lynch and Walsh 1998). Therefore, I used mean family phenotypic correlations as a proxy for estimating genetic correlations (Roff 1996; Lynch and Walsh 1998). Pairwise Pearson correlation among family means was used to estimate correlations among transcription values. For visual simplicity and conceptualization of associations among cytokine transcription, principal components analysis (PCA) was used to generate orthogonal principal components of the variation among family mean transcription values (SYSTAT 11, SAS Institute Inc., Cary, USA). Furthermore, significant principal components (PC; eigenvalue>1) are partitioned into their genetic variation components as surrogate estimates of the genetic architecture of complex gene associations. For example, PCA analysis is useful to eliminate (individual) residual variation within traits and reflects shared variation concisely yet more precise among correlated traits.

RESULTS

There were no substantial or consistent departures from normality in the log-transformed transcription data. Only 33 out of 480 (p= 0.069) cases depart from normality, which is slightly higher than what would be expected by chance alone (p< 0.05). One exception was *RPS-11* of which the transcription was not normal and indeed had a bimodal distribution. Individual fish mass was not significantly correlated with transcription for any marker except TNF-α post-challenge expression (p < 0.001, adjusted R^2 = 0.057). When I included fish mass as a fixed effect into the model for TNF-α, I did not find any

substantial changes in the variance component estimates.

a) Response to disease challenge: All cytokine genes (*IL1*, *TNF*-α, *IL8-R* and *IL8*) were highly significantly up-regulated ($p < 0.0001$) after the immune challenge, while *RPS-11* and *IgM* genes showed no significant difference between pre- and post-challenge gene expression (Figure 3.1).

b) Estimates of genetic components and heritability: Post-challenge *IL1* and *TNF*-α gene expression showed significant sire x dam interaction effects (σ^2_I), indicative of non-additive genetic components (Table 3.1). Dam component variance (σ^2_d) was substantial in pre-challenge cytokine gene transcription; three out of four cytokines (*IL1*, *IL8-R* and *TNF*-α) had significant dam variance component (σ^2_d) while none had significant sire variance components (σ^2_s; Table 3.1), indicative of maternal effects on those transcriptional values. Post- challenge *IL8-R* was the only cytokine with significant sire variance component (σ^2_s). *RPS-11* gene expression displayed substantial σ^2_d and σ^2_s in both pre- and post-challenge fish and *IgM* gene expression showed no significant genetic variance components (Table 3.1).

None of my maternal effects estimates ($\sigma^2_d - \sigma^2_s$) were significant for gene transcription. Maternal effects were significant only for PC2 and for mass-at-age ($M_{n=8}$) estimates ($p < 0.05$). Despite the lack of significance at the individual gene level, maternal effects appear to be substantial for pre-challenge cytokines, for which σ^2_d estimates were always higher than σ^2_s estimates, and more importantly was indicated by the significant maternal effect in PC2, which correlates with pre-challenge cytokines (Figure 3.2). Maternal effects were significant for mass-at-age ($M_{n=8}$), as expected for sub-yearling fish (Heath et al. 1999).

The narrow sense heritability (h^2) estimates were moderate to low for cytokine transcription, but with high standard deviation and were thus not significantly different from zero (Table 3.1). Heritabilities of post-challenge cytokine gene expression were lower than pre-challenge. Although *IL8-R* showed slightly higher h^2 than for the cytokine transcription, the only h^2 estimates significantly greater than zero were for *RPS-11* (Table 3.1).

Despite a low within-family sample size, I had robust variance component and h^2 estimates. First, there were no substantial difference in the median or in the SD of h^2 estimates for mass-at-age obtained using different sample sizes ($M_{n\sim40}$, $M_{n=8}$, M_{t0}, and M_{t24}, respectively; Table 3.1). Furthermore, there was strong agreement between variance component estimates obtained from my original data and the reduced data sets that were generated by random subtraction of a single individual from each family (Figure 3.3). However, for higher variance estimates, the values I estimated with four individuals per family (N= 3) tended to be marginally higher than the median of the bootstrapped four individuals per family (N= 3) variance estimates, possibly as a result of inflated error variance in N= 3 estimates (Figure 3.3).

c) *Phenotypic/ genetic correlations:* Mean family cytokine transcription was strongly correlated within treatments, but not between pre- and post–challenge cytokine genes (Table 3.2). One exception was *IL8-R*, in which pre- and post-challenge transcriptions were significantly correlated (Table 3.2). As expected, pre- and post-challenge transcriptions were strongly correlated in *IgM* and *RPS-11*, and transcription of these genes showed no significant correlations to others' (Table 3.2).

The first three PCs resulting from the PCA were significant, and they explain 71% of the observed variation (32%, 25% and 14%, respectively) among cytokine genes.

Three clear clusters emerged when the eigenvectors of the response variables were plotted (Figure 3.4). Cytokine ligands (*IL1*, *TNF-α, IL8*) were clustered together within the same treatment but were separated between treatments (i.e., pre- and post-challenge). In contrast, post- and pre- challenge *IL8-R* transcriptions clustered, but were apart from other cytokines (Figure 3.4). Orthogonal variables generated by PCA plotted against cytokine transcription demonstrated that PC1, PC2 and PC3 are associated with pre- and post-challenge cytokines and *IL8-R* transcription, respectively (Figure 3.2). Interestingly, *IL8-R* post-challenge expression is correlated with both PCA1 and PCA3 but in different directions, suggesting independent antagonistic regulation of *IL8-R* during immune stimulation (Figure 3.2). Finally, the variance components for the principal components were in concordance with the PC-associating gene transcription, yet with tighter confidence limits; i.e., PC1 and PC2 show highly significant non-additive genetic and maternal effects respectively, while PC3 exhibits significant h^2 (Table 3.1)

DISCUSSION

Improvement of immunocompetence in farmed fish is crucial for sustainable production without high antibiotic use. Selection on end-point health indicator traits (such as lysozyme concentration or outbreak survival) may not serve to improve functional immune response to disease. I suggest that health indicators that reflect the early response to an immune challenge may be used as effective markers to select for improved disease resistance. Here, I focus on cytokine genes, since they are critical for activating the first line of defense against pathogens, as well as for activating the specific immune system for longer-term and more specific lines of defense. Furthermore, I chose to quantify transcriptional variation as a potentially reliable genetically-based marker for use in

selective breeding. Although transcriptional markers reflect mRNA concentration, they should be closely related to the actual protein level for cytokines, since transcription of cytokines is known to be transient, and their mRNA is short-lived (Secombes et al. 1996). My study is not designed to address whether pro-inflammatory cytokine expression is a good predictor for disease outcomes (although it is a reasonable expectation since cytokines are mediators of the immune system), but rather to estimate the genetic components contributing to cytokine transcriptional variation, with the ultimate aim of applications in selective breeding for salmon aquaculture and improved conservation-based breeding in government hatcheries.

Immunostimulation by means of vaccine immersion was sufficient to initiate a regulatory pathway alteration of transcription of cytokines 24 hours post-treatment, which indicates post-challenge transcription can be reasonably extrapolated as a proxy for early cytokine response. This effect was mainly inherited non-additively, as evidenced by significant interaction effects in post-challenged cytokine transcription (*IL1*, *TNF-α*) and in the associated PC (PC1). I also observed a significant non-additive genetic component for mass-at-age. The presence of non-additive effects with little or no additive genetic variation makes the phenotype of the progeny unpredictable based on parental trait values. Indeed, non–additive genetic effects are interpreted as an undesirable mode of genetic inheritance for breeding purposes (Rye and Mao 1998; Pante et al. 2002), and their implications both in theory and practice are often ignored in quantitative genetic studies (i.e. Carlson and Seamons, 2008). However, the prevalence of non-additive genetic variance components in salmon transcription is recurrent; remarkable departures from additive genetic variation in gene expression have been reported upon hybridization between wild and farmed Atlantic salmon (*Salmo salar*; Roberge et al. 2008;

Normandeau et al. 2009), and in reciprocal crosses of diverging populations of steelhead trout (*Oncorhynchus mykiss*; Aykanat et al. 2011). Non-additive genetic effects have also been reported for early life traits in Chinook salmon (Pitcher and Neff 2007; Evans et al. 2009) and between populations of pink salmon (*Oncorhynchus gorbuscha*; Gharrett et al. 1999; Gilk et al. 2004). Thus, non-additive genotypic variation may provide a genetic framework to explain the complex life history and biological diversity of salmon, that is, non-additive genetic variance may allow the preservation of genetic variation despite genetic bottlenecks and directional selection (Cheverud and Routman 1995, 1996; Carroll et al. 2001, 2003; Aykanat et al. 2011). Perhaps non-additive genetic contributions to phenotype are common in salmon due to their natural history of small and fluctuating population size, and that variation may provide the capacity for rapid adaptation potential in heterogeneous and changing environments.

Pre-challenge cytokine gene transcription appears to primarily reflect maternal effects. In fish, epigenetic maternally-inherited immunity is of critical importance for survival in the early stages of life (Oshima et al. 1996; Swain and Nayak 2009). Maternally-inherited immunity is the prime protection against pathogens until immune function and immunocompetence are developed later in the fry stage. Furthermore, maternally-inherited immunity can affect offspring immune function later in life; Tyndale et al. (2008) showed that disease resistance in fry seven months post-fertilization (with their complete immune repertoire present) was significantly affected by their maternal egg provisioning. Such delayed maternal effects are not expected (Heath et al. 1999). The maternal effects I observed here may be a result of immunological priming associated with earlier epigenetic maternally-inherited immunity (Tyndale et al. 2008).

Additive genetic effects and narrow sense heritabilities (h^2) were not significant in pro-inflammatory cytokines (IL1, TNF-α, IL8-R, IL8). Prior to this study, the genetic variance components of cytokine transcription were only characterized in humans, and were found to be highly additive (Craen et al. 2005). I found a mean value of h^2 estimates for cytokine transcription of ~0.25, which agrees with the median of compiled h^2 estimates from salmonids (Carlson and Seamons 2008). My cytokine h^2 estimates are likely robust estimates of the true h^2, however, are not significant due to high associated error (Table 3.1). My mean h^2 estimate for mass-at-age, which is strongly selected for under farm practices, was also low (~ 0.15; Table 3.1), and it was substantially smaller than the compiled average mass-at-age h^2 value for salmonids (~ 0.32; Carlson and Seamons 2008). Likewise, post-challenge cytokines, which are more likely to be associated with disease resistance response, and hence under selection, had lower mean h^2 estimates than pre-challenge cytokines (Table 3.1). Yellow Island Aquaculture Ltd. (YIAL; my salmon farm partner) follows organic farming practices, and has not treated their salmon stocks with antibiotics for over four generations, despite repeated disease outbreaks. Therefore, the population has been under strong selection for disease resistance and this may have already depleted additive variation in immune components in YIAL stocks. A comparison of farmed stocks with wild population fish could provide a valuable test of this possibility. Overall, I conclude that there is not much room for mean-trait selection of cytokine transcription in YIAL Chinook salmon, but cytokine transcription should be further explored for their value as a predictor of disease outcomes.

Family mean correlations between pre- and post-challenge cytokine transcription were not significant, which suggests immune-stimulated (post-challenge; t= 24 hrs) cytokine transcription is regulated independent from resting (t= 0 hr) transcription. On the

other hand, significant correlations among cytokine transcription within the same sampling time (i.e. either pre- or post-challenge) suggest a coordinated immune response in the pro-inflammatory immune response. *IL8-R* transcription appears to be under two different genetic regulatory systems, as it is correlated with both PC1 and PC3; one seems unique to *IL8-R* with a substantial h^2, (as evident in PC3), while the other is associated with post-challenge cytokine expression (as evident in PC1). Furthermore, the *IL8-R* transcription correlation with PC1 and PC3 were antagonistic (opposite sign slopes), suggesting a balancing action of the two regulatory mechanisms on *IL8-R* transcription level. Similar to our observations, in rainbow trout (*O. mykiss*), *IL8-R* transcription is suppressed in response to a viral hemorrhagic septicemia virus (VHSV) infection, while IL8 protein induces the receptor's transcription level. However the functional importance of that regulation pattern is not known.

I observed significant additive effects in *RPS-11*. However, *RPS-11* gene expression does not fit a normal distribution, but rather exhibited a bimodal transcription pattern in both pre- and post-challenge fish (Figure 3.5). The bimodal distribution of *RPS-11* transcription is curious. In humans, *RPS-11* is a constitutively expressed ribosomal protein, indeed it is used as an endogenous control for qRT-PCR in human cell lines (Zuidervaart et al. 2003; Filali et al. 2008). Recently, downregulation of *RPS-11* during development was found to be correlated with longevity in *Caenorhabditis elegans* (Curran and Ravkun 2007). Yet, in Chinook salmon, this gene appears to have a primary on/off transcriptional control mechanism. This is not consistent with the putative function of this gene, and I conclude that more work needs to be done to identify the function of this gene in salmon.

This study describes one of the first reports of genetic variance partitioning for immune-related gene transcription with an emphasis on non-additive genetic effects in fish. The lack of substantial additive genetic contribution to cytokine gene transcription will limit the effectiveness of artificial group selection approaches. However, the non-additive genetic variation does provide the potential for adaptive responses in natural populations (e.g. Goodnight 1988; Cheverud and Routman 1996). My analyses point to complex and coordinated expression control for the cytokine genes in Chinook salmon. It is clear that transcription cannot be assumed to be a simple additive quantitative trait, nor can it be assumed to evolve in a manner predicted for more traditional and better-studied traits. My work serves to highlight the need for additional exploration of the inheritance of transcriptional control of key fitness-related traits in captive and wild populations of fish.

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Table 3.1: Variance components for transcription at six genes, body mass (calculated using different sample sizes), and the first three principal components from my principal component analysis (PCA) of all cytokine gene transcription data. Significant variance components are indicated in bold-face type. Narrow sense heritability (h^2) estimates and standard deviation (SD) are also presented. The response variable grand mean is denoted by μ.

		μ	σ^2_d	(%)	σ^2_s	(%)	σ^2_I	(%)	σ^2_e	(%)	h^2	(SD)
Pre-challenge (t=0 hr)	***IL1***	-5.086	**0.0031**	**(21)**	0.0016	(11)	0.0001	(1)	0.0098	(67)	0.44	(0.28)
	TNF-α	-4.420	**0.0020**	**(15)**	0.0012	(9)	0.0003	(2)	0.0096	(73)	0.36	(0.23)
	IL8-R	-5.012	**0.0192**	**(12)**	0.0124	(8)	0.0048	(3)	0.122	(77)	0.31	(0.26)
	IL8	-3.410	0.0006	(8)	0.0001	(1)	0.0003	(4)	0.0065	(87)	0.05	(0.15)
	IgM	-4.679	0.0005	(1)	0.0041	(4)	0.0002	(0)	0.0871	(95)	0.18	(0.18)
	RPS-11	-1.756	**0.0197**	**(11)**	**0.0402**	**(22)**	0.0029	(2)	0.1166	(65)	0.90	(0.28)
Post-challenge (t=24 hrs)	***IL1***	-4.658	0.0009	(4)	0.0015	(6)	**0.0043**	**(18)**	0.0176	(72)	0.25	(0.20)
	TNF-α	-4.183	**0.0023**	**(13)**	0.0002	(1)	**0.0021**	**(12)**	0.0133	(74)	0.04	(0.16)
	IL8-R	-4.272	0.0015	(2)	0.0081	(10)	0.0016	(2)	0.0678	(86)	0.41	(0.25)
	IL8	-3.094	0.0013	(11)	0.0006	(5)	0.001	(8)	0.0094	(76)	0.19	(0.22)
	IgM	-4.715	0.0004	(0)	0.0004	(0)	0.0004	(0)	0.0928	(99)	0.02	(0.10)
	RPS-11	-1.829	0.0234	(13)	**0.0317**	**(17)**	0.0016	(1)	0.1245	(69)	0.70	(0.28)
Mass , N / family	**N~ 40**	4.81	**0.068**	**(5)**	0.03	(4)	**0.068**	**(5)**	1.232	(87)	0.15	(0.10)
	N=8	5.46	**0.144**	**(11)**	0.032	(2)	0.036	(3)	1.124	(84)	0.10	(0.14)
	N=4,t0	5.45	0.063	(5)	0.044	(4)	0.020	(2)	1.061	(89)	0.14	(0.19)
	N=4,t24	5.47	0.152	(11)	0.084	(6)	0.072	(5)	1.081	(78)	0.24	(0.23)
PCA	**PC1**	0	**0.252**	**(11)**	0.071	(3)	**0.324**	**(14)**	1.633	(72)	0.12	(0.21)
	PC2	0	**0.349**	**(19)**	0.089	(5)	0.023	(1)	1.384	(75)	0.19	(0.21)
	PC3	0	0.061	(6)	**0.161**	**(16)**	0.044	(4)	0.748	(74)	0.64	(0.28)

Table 3.2: Pairwise mean family correlations among six genes' transcription under two treatments in juvenile Chinook salmon. Significant correlations after Bonferroni correction are highlighted in bold ($p < 0.05$).

	IL1 (t=0)	*TNF-α* (t=0)	*IL8-R* (t=0)	*IL8* (t=0)	*IgM* (t=0)	*RPS-11* (t=0)	*IL1* (t=24)	*TNF* (t=24)	*IL8-R* (t=24)	*IL8* (t=24)	*IgM* (t=24)	*RPS-11* (t=24)
IL1 (t=0)	**1**											
TNF-α (t=0)	**0.64**	**1**										
IL8-R (t=0)	0.16	0.13	**1**									
IL8 (t=0)	0.44	**0.49**	0.01	**1**								
IgM (t=0)	-0.13	0.06	0.13	0.03	**1**							
RPS-11 (t=0)	0.12	-0.11	0.01	-0.03	-0.16	**1**						
IL1 (t=24)	0.09	0.18	0.16	-0.05	0.13	0.10	**1**					
TNF (t=24)	0.10	0.20	0.09	-0.06	0.00	0.26	**0.68**	**1**				
IL8-R (t=24)	0.10	-0.01	**0.52**	-0.16	-0.08	0.34	**0.47**	0.39	**1**			
IL8 (t=24)	-0.15	0.01	0.04	0.03	0.00	0.06	**0.61**	**0.77**	0.40	**1**		
IgM (t=24)	-0.14	-0.12	-0.03	0.11	**0.54**	-0.07	0.08	0.15	-0.09	0.19	**1**	
RPS-11 (t=24)	-0.16	-0.22	-0.01	-0.15	-0.13	**0.73**	-0.02	0.15	0.28	0.08	-0.08	**1**

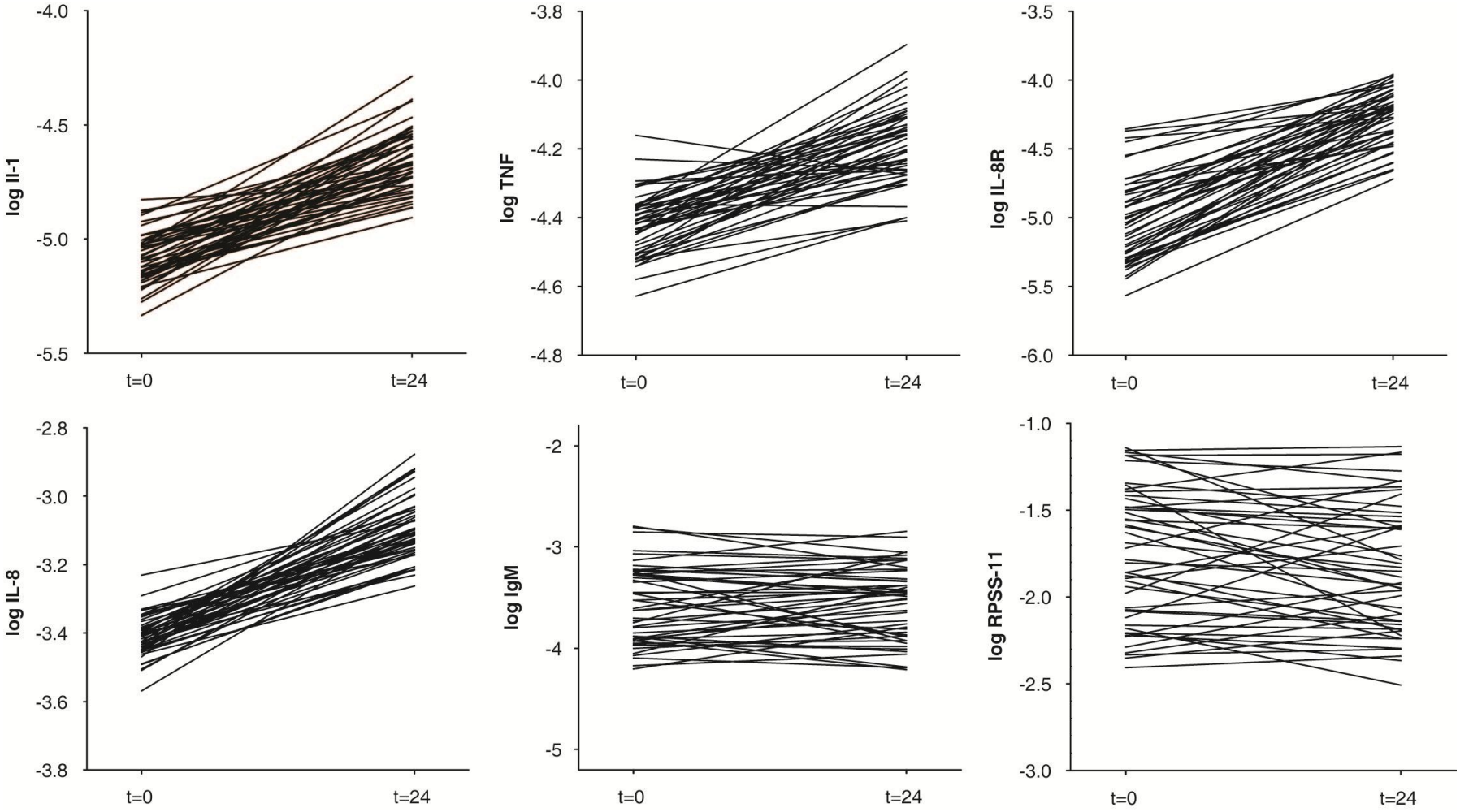

Figure 3.1: The logarithm of relative transcription for 6 genes in juvenile Chinook salmon, before and 24 hours after a *Vibrio* vaccine challenge. Each line represents individual full-sib families (N = 48).

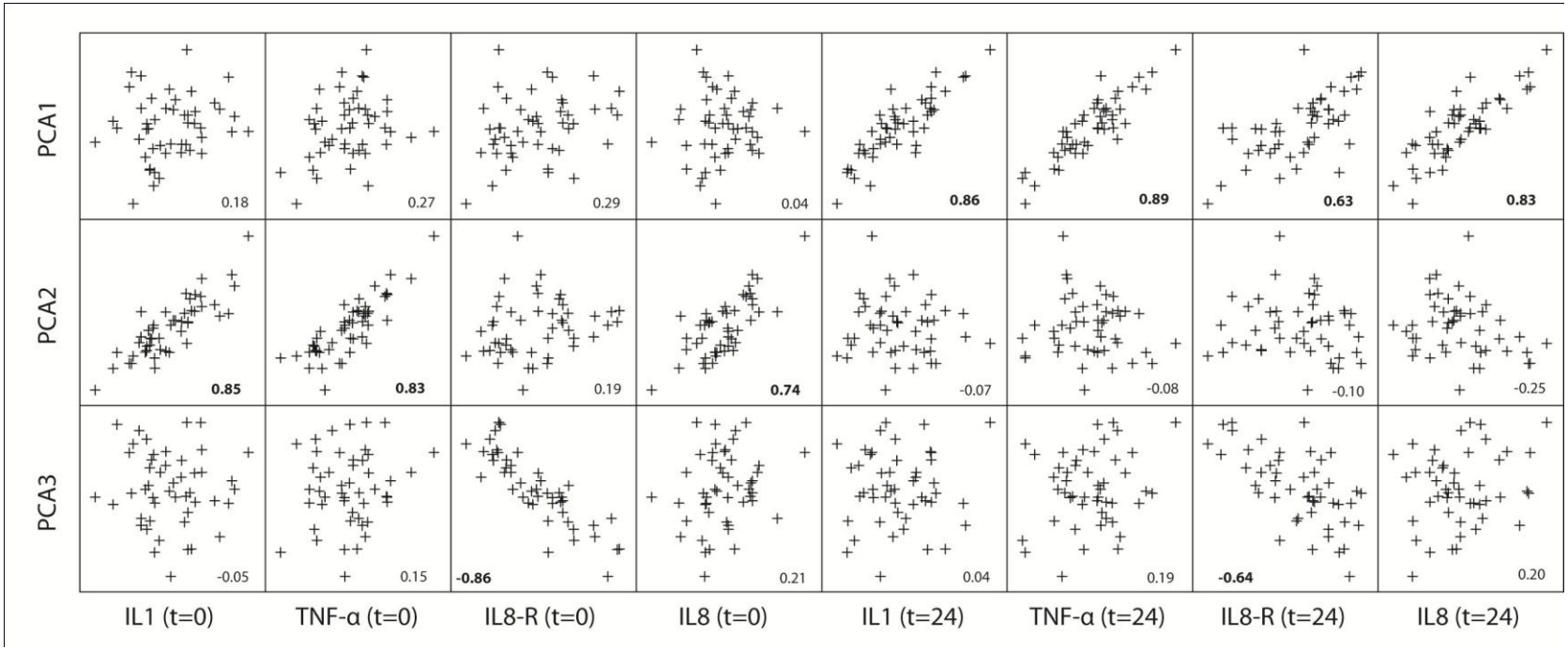

Figure 3.2: Scatter plots of mean family cytokine transcription (*IL1*, *TNF-α, IL8-R*, *IL8*) before (time = 0) and 24 hours after a challenge versus the first three principal components derived from all the data combined. Correlation coefficients are given at the bottom of each panel: significant correlations are in bold-face type ($p < 0.05$).

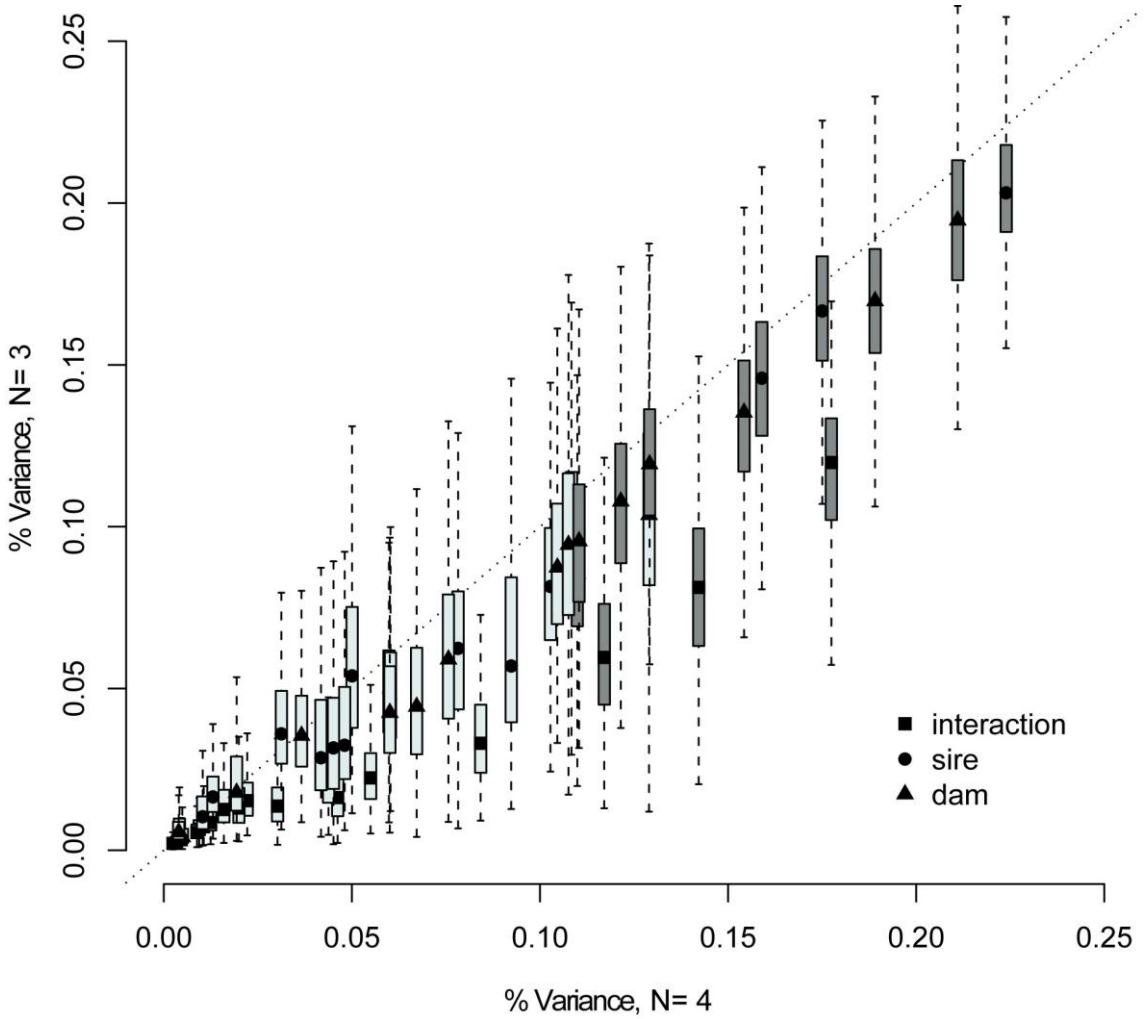

Figure 3.3: Comparison of percent (%) variance components from my analysis (N= 4/ family) and the distribution of variance components generated over 1000 bootstrap simulations with a reduced sample size (N= 3/ family). Box plots indicate the 25-75% interval, and the dotted whiskers are the 95% confidence interval respectively. The dotted line shows the slope of one (x=y) line. Variance components that are significantly greater than zero are indicated with darker grey box plot.

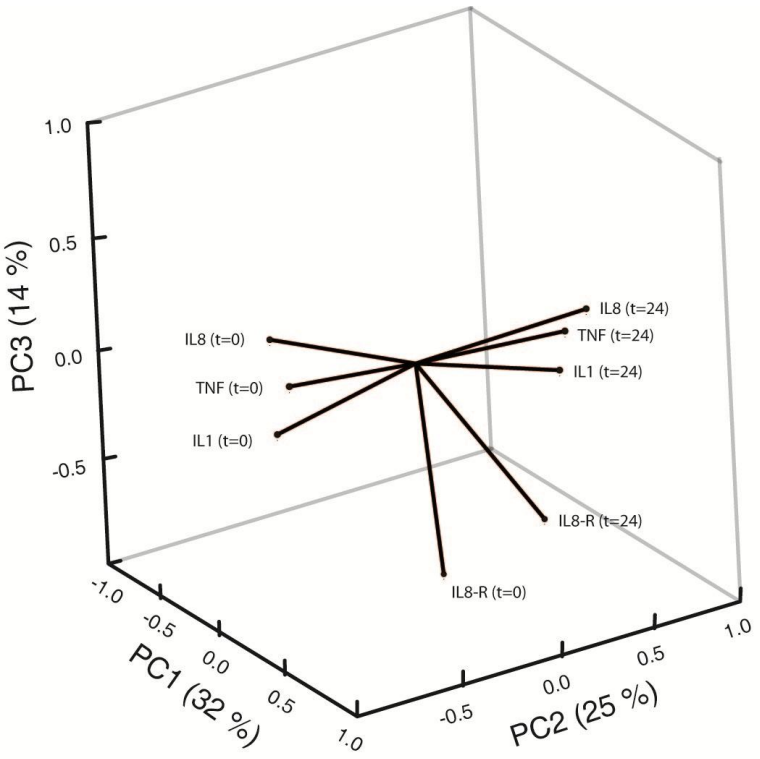

Figure 3.4: Decomposed eigenvectors of cytokine transcription before and 24 hours after a *Vibrio* vaccine challenge plotted against the first three principal components (PC) in juvenile Chinook salmon. The percent variation explained by each PC is given in parenthesis on each axis. PC1 and PC2 separate pre- and post-challenge cytokine transcription, while PC3 separates IL8-R from the other transcriptional values.

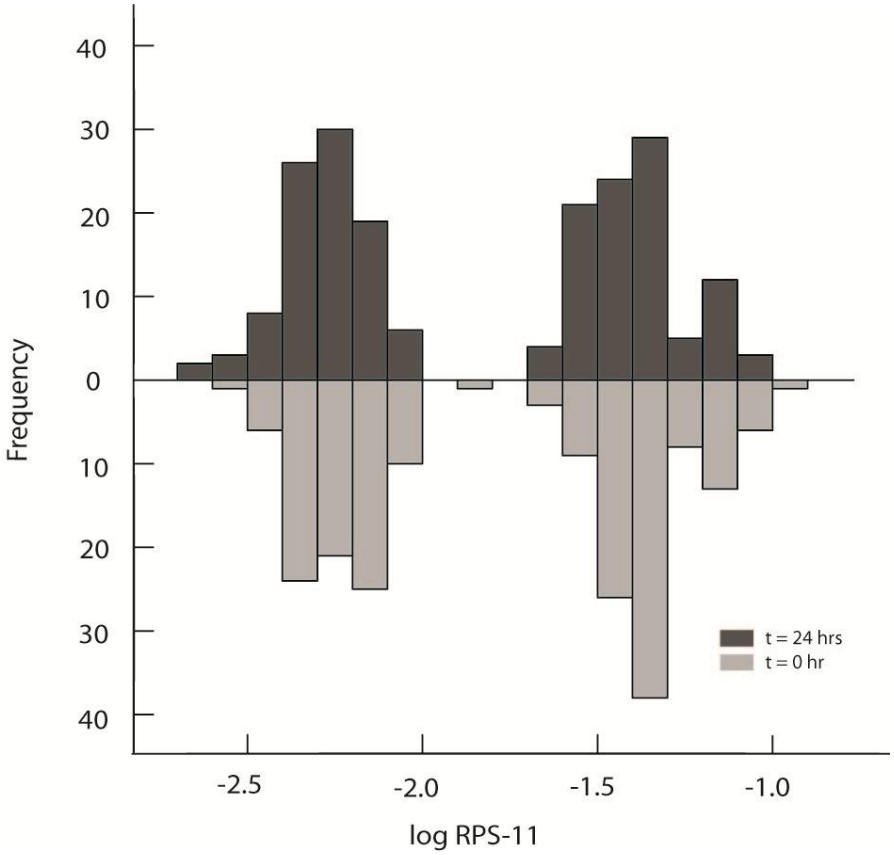

Figure 3.5: Frequency histograms showing the bimodal distribution of log-transformed *RPS-11* gene expression in Chinook salmon before (t=0 hr, histogram down) and after (t = 24 hrs, histogram up) challenge. Note the similar transcriptional distributions at the before and after challenge time points.

CHAPTER 4

SEX-BIASED GENETIC COMPONENT DISTRIBUTION AMONG POPULATIONS: ADDITIVE GENETIC AND MATERNAL CONTRIBUTIONS TO PHENOTYPIC DIFFERENCES AMONG POPULATIONS OF CHINOOK SALMON*

INTRODUCTION

Many phenotypic differences among salmon populations are hypothesized to be as a result of adaptation to the local environment, genetic drift, or as a result of physiological acclimation to the environment, with no genetic effects involved (Taylor 1991; Adkison 1995; Fraser et al. 2011). Generally it is very difficult to distinguish among those possibilities, and it may be that in most cases population differences are due to a combination of those processes. Yet local adaptation is given special attention in salmon since the presence of local adaptation has serious consequences for conservation and management strategies. Specifically, if differences among populations are as a result of local adaptation, reintroduction or enhancement efforts would have limited success when the introduced fishes' genomes do not match the local environment (Garcia de Leaniz et al. 2007). Furthermore, if an introduction is successful, and the introduced fish hybridize with the locally adapted fish, the average fitness of the population is expected to decline as a result of outbreeding depression (Gilk et al. 2004).

Demonstrating local adaptation is not straightforward (Kawechki and Ebert, 2004; Fraser et al. 2011; Garcia de Leaniz et al. 2007). In salmon, only a handful of studies have

* Aykanat T, Bryden CA, Heath DD. Sex-biased genetic component distribution among populations: Additive genetic and maternal contributions to phenotypic differences among populations of Chinook salmon. Submitted to *Journal of Biology*

effectively shown local adaptation (*e.g.*, Unwin et al. 1997; Riddell et al. 1981). One precondition for demonstrating local adaptation, which often is not evaluated, is to show that the divergence of fitness trait among populations has an additive genetic basis. Indeed, studies that do attempt to estimate additive genetic variation are usually flawed in that non-additive sources (*i.e.* maternal, dominance, and epistatic effects) of variation are often confounded within the estimate, under the assumption that non-additivity contributed negligibly to the variance structure (reviewed in Heath and Blouw 1999 for maternal effects, see also; Pante et al. 2002; Gallardo et al. 2010). In fact, non-additive genetic effects may constitute a substantial component of phenotypic variation within and among populations (*i.e.* Gilk et al. 2004; Pitcher and Neff 2006; Roberge et al. 2008; Aykanat et al. 2011). Therefore, the underlying genetic architecture (relative contribution of additive and the various non-additive genetic variance components) of trait divergence among populations should be carefully investigated when exploring potential local adaptation.

In salmonids, one non-additive source of phenotypic variation common in early life history traits is maternal effects (Heath and Blouw 1998; Heath et al. 1999). Many common garden experiments designed to evaluate local adaptation employ only one generation of common rearing (*e.g.*, Valdimarsson et al. 2000; Jonsson et al. 2001; Stewart et al. 2002; Jensen et al. 2008; Kavanagh et al. 2010). Common garden designs are assumed to minimize environmental effects, including maternal effects, and that differences in mean trait values reflect primarily additive genetic effects. However, maternal effects and other non-genetic effects may persist for more than one generation of common garden rearing (Roff 1997; Richards 2006), and therefore estimates of the additive genetic variance component for such traits would be inflated.

Here I perform two quantitative genetic experiments to partition phenotypic variance into additive (sire component of genetic variance; σ^2_{sire}) genetic and maternal (dam component of genetic variance; σ^2_{dam} minus σ^2_{sire}) components of variance for 17 traits in Chinook salmon (*Oncorhynchus tshawytscha*) which were held in a common environment from fertilization. I use a modified North Carolina II factorial breeding design with which I explored population-specific maternal (dam) and paternal (sire) contributions to phenotypic differentiation among populations by analyzing among-population genetic variance structure. My results showed maternal effects can account for most of the among-population differences observed in experiments which employ only a single generation of common garden rearing. My results highlight the essential role maternal effects may play in among-population trait divergence and that they must be taken into account when designing experiments to test for local adaptation. Furthermore, given their role in salmon population trait divergence, maternal effects should be investigated in more detail for their impact on salmon evolution and population viability.

MATERIALS AND METHODS

Data from two different breeding experiments are used in this study. Hereafter the two experiments are referred as the YIAL and QRRC experiments after the hatchery where the rearing took place.

The YIAL experiment involved breeding wild and domestic stocks of Chinook salmon *(Oncorhynchus tshawytscha)* to characterize the genetic architecture underlying phenotypic differences between a natural and a domestic population. Mature wild Chinook salmon from the Big Qualicum River and mature domestic Chinook salmon from Yellow Island Aquaculture Limited (YIAL; Quardra Island, BC) were mated to

create 104 families. Breeding design details are described in Bryden et al. (2004), but briefly, one male and one female from each of the two stocks were mated to produce four families in a 2x2 factorial design. The cross was replicated 26 times with different parent fish to generate 104 families. Husbandry conditions are described in Bryden et al. (2004). Due to losses during the experiment, the numbers of families used in my analyses ranged between 80 and 94 (Table 4.1).

Fourteen traits were measured in the YIAL experiment (Table 4.1): six were body size traits (wet weight and fork length measures at age 420 and 615 days post fertilization, female offspring wet weight excluding gonads at 3 and 4 years post fertilization), two were osmotic stress response traits after saltwater challenge (Hematocrit count and plasma chloride ion concentration), four were survival measures (Egg, eyed egg, fry and natural vibriosis outbreak survival), an adult reproductive trait (relative fecundity at age 3) and the final trait was a fluctuating asymmetry index (FA). Survival measures were coded with each fish represented as an independent binomial data point such that a "0" was assigned for a mortality event and a "1" for the survivors. FA index was calculated using eight bilaterally measured traits, as described in Bryden and Heath (2000). Hematocrit (percent packed red blood cells x 100) and plasma chloride ion concentration (meq/L) in response to a 24-hour saltwater exposure challenge were calculated as described in Bryden et al. (2004). Conventional salmon aquaculture rearing practices were followed for all fish.

Many of the traits studied here are likely to be important components of fitness and hence good candidates for possible locally adaptive traits. While survival measures are clearly direct measures of fitness, traits such as body size at age and the fluctuating asymmetry index are considered potential proxies for performance or fitness (Clark 1995;

Garcia de Leaniz et al. 2007). Saltwater tolerance is a vital physiological process for anadromous salmon, and variation associated with it is important for survival (*i.e.* Kreeger 1995; Leonard and McCormick 2001).

The QRRC experiment involved cross-breeding four wild stocks of Chinook salmon (*Oncorhynchus tshawytscha*) and was designed to partition the genetic variance components underlying phenotypic differences among natural populations. I included this second breeding experiment to provide a comparison to the results of the YIAL experiment. Fish from Harrison River (HR), Quinsam River Hatchery (QN), Big Qualicum (BQ), and Robertson Creek (RC) were crossed to generate pure-type and reciprocal families. On 17th Oct 2005, eggs and milt were obtained from parental fish at the river of origin, and were immediately shipped to the Quesnel River Research Center (QRRC) on ice. Eggs and milt were received on the same day from all 4 populations (within 24 hours of collection), and fertilizations were performed on that day. Milt from one male, and eggs from one female from each of four stocks were crossed in a 4X4 factorial design to generate 16 families. This breeding design was replicated five times with different individual fish to generate 80 families in total. Fin clip tissues from the parental fish were sampled for later microsatellite genotyping.

Fertilized eggs were incubated in vertical stack incubation trays, each family separated by dividers. At the eyed egg stage (18th November 2005), 200 eggs from 16 families from each 4X4 cross were pooled and reared together to minimize tank effects (total 3200 individuals per 4X4). Three families had less than 200 eggs survive to the eyed stage: BQxBQ, BQxCH and BQxQN crosses (dam-first notation) had 153, 65 and 182 eggs respectively. These reduced numbers had little effect on the final rearing density since each group consisted of ≈3200 fish. In Jan 2006, each of the pooled 4X4 crosses

had reached the first-feeding stage (mean mass= 0.41 g) and were transferred to five outdoor 6 m^3 freshwater troughs (flow rate = app. 8 L/sec). In May 2006, a sub-sample of fish from all five 4X4 crosses (troughs) were sampled and weighed, and fin–clips were taken for subsequent DNA analysis for parentage assignment.

Egg survival (to eyed egg stage), fry survival and total length at 210 days data were used as the fitness-related traits for the phenotypic variance partitioning in the QRRC experiment (Table 4.1). Similar to survival data from YIAL, early egg survival was calculated by binning the total number of fish per family, where each egg provides an independent binomial data point; "0" for a dead egg, and "1" for a surviving egg. However, for the fry, I was not able to genotype and assign all fry in each mixed-family group to family of origin to calculate actual family survival, instead I genotyped and assigned a sub-sample of the fish from each trough. Thus, fry survival is estimated as the occurrence of each family in each trough relative to the total number of fish sampled in that trough. Since each trough is a replicate, I have one survival estimate for each family, replicated four times. I sampled 406, 280, 437 and 339 fish from each 4X4 cross. (I have excluded one of the 4X4 crosses for fry survival because of poor resolution in the parentage assignment.) Using two or three microsatellite loci, individual fish were assigned to their parental cross (Supplementary Table 4.1). There were a few ambiguous genotype combinations that failed to provide positive assignment of individuals to their parental crosses (Supplementary Table 4.A1), hence my relative occurrences data for the affected crosses were weighted accordingly.

Statistical analysis: Unless otherwise stated, statistical analyses were performed using R software (R Development Core Team, 2009). In the YIAL experiment, I tested for differences in the mean values for the various traits between the wild and domestic

population pure-type crosses using t-tests and non-parametric Kruskal-Wallis test for normally distributed and for non-normally distributed traits, respectively. In the QRRC experiment, pure-type cross differences among the four wild populations were tested using ANOVA and Tukey's HSD post-hoc multiple comparison test, and Kruskal-Wallis test with multiple comparison test for normally distributed and for non-normally distributed traits, respectively.

Phenotypic variance was partitioned using the following model (Lynch and Walsh 1998), which I refer to as the "basic model":

$$\text{Basic model:} \qquad z_{klm} = \mu + d_k + s_l + e_{klm}$$

Where z_{klm} is the phenotypic value of m^{th} offspring of k^{th} dam and l^{th} sire, μ is the mean phenotype of the sample, d_k and s_l are the effects of k^{th} dam and l^{th} sire and e_{klm} is the error term of m^{th} individual (Lynch and Walsh 1998). To evaluate the relative contribution of population-specific sire and dam effects, I included population sire and population dam as fixed effects to the basic model as follows:

$$\text{Full model:} \qquad z_{ijklm} = \mu + Pd_i + Ps_j + d_k + s_l + e_{ijklm}$$

$$\text{Sire model:} \qquad z_{jklm} = \mu + Ps_j + d_k + s_l + e_{jklm}$$

$$\text{Dam model:} \qquad z_{iklm} = \mu + Pd_i + d_k + s_l + e_{iklm}$$

Where Pd_i and Ps_j are the fixed effects of i^{th} and j^{th} population dam and sire effects respectively. The notation of the individual phenotypic value and the error term is adjusted according to inclusion of the population fixed effects. I fit all model parameters

using restricted maximum likelihood (REML) in the lme4 package in R (Bates and Maechler 2009). I then compared the basic model to "forward step-wise" models using a log-likelihood test where the log-likelihood ratio statistic is chi square (χ^2) distributed with degrees of freedom equal to the number of factors omitted. Significant improvement ($p < 0.05$) in the fit of the model with the inclusion of population fixed effects would indicate the significance of the added effect and be detected by an increase in the log-likelihood of the model. If the basic model is improved by the inclusion of both population-dam and population-sire effects similarly, than I conclude the differentiation among populations has an additive genetic basis. If, on the other hand, the model is improved by the inclusion of the population-dam model only, I ruled out an additive genetic explanation and conclude maternal effects are the basis of the observed divergence among the study populations. Similarly, an improvement by the inclusion of the population-sire model would indicate that paternal effect are basis of phenotypic divergence.

Phenotypic variance (σ^2_P) was partitioned to paternal sib (σ^2_s), maternal sib (σ^2_d), and residual error (σ^2_e) components within a Bayesian framework, where the REML fitted parameters were simulated 10000 times using Monte Carlo simulation using the languageR package in R (Baayen 2010). Posterior distribution of the parameters of fitted model is then used to calculate the parameters' highest probability densities (HPD). Random effects were identified as significant when the HPD 95% confidence interval (CI) did not include zero. Maternal effects are estimated by subtracting the σ^2_s estimate from the σ^2_d estimate (*i.e.*, $\sigma^2_d - \sigma^2_s$), while additive genetic variance (V_A) was estimated as $4\sigma^2_s$

RESULTS

In the YIAL experiment, 9 of 15 traits show a difference between pure-type crosses of wild Big Qualicum and domestic YIAL fish (Table 4.2). All four measures of survival showed differences among pure-type crosses. The wild population has higher early survival, but the disease outbreak data shows that the domestic fish have significantly higher survival rates later in life (Table 4.2). Body size traits (weight and length at 420 days and 615 days post-fertilization) had significant population differences, but the differences gradually reduced with age and become non-significant by the age of four years post-fertilization (Table 4.2). Fluctuating asymmetry was also significantly different between the populations, with the domestic line displaying higher FA than the wild population (Table 4.2). Relative fecundity and saltwater challenge response traits (hematocrit and plasma chloride ion concentration) were not significantly different between the populations (Table 4.2).

In the YIAL experiment, when population-dam effects are included (dam model), the model likelihood significantly increased for 11 traits, compared to 5 traits where the likelihood increased when population specific sire effects are included (sire model; Figure 4.1, Supplementary Table 4.2). In all cases, the population-dam model improved the likelihood more than the sire model. Egg survival, eyed egg survival, fry survival and FA were improved by only the dam and full model (Figure 4.1, Supplementary Table 4.2).

In the QRRC experiment, egg survival and fork length at 210 days were significantly different among four populations (Table 4.3). For length at 210 days, all models significantly improved the likelihood of the model over the basic model (Figure 4.1, Supplementary Table 4.2). For fry survival, only the full model was significantly better than the basic model, although the dam model was marginally significant ($p =$

0.06). For egg survival only the dam model gave a significantly better fit than the basic model (Figure 4.1, Supplementary Table 4.2).

The total variance explained by random effects ($\sigma^2_{P=} \sigma^2_{d+} \sigma^2_{s+} \sigma^2_R$) for traits measured in the QRRC and YIAL experiments was reduced by a maximum of 15 % (to 0.85 σ^2_P ratio) in the full model relative to the basic model (Table 4.4, last column). For most traits, the decrease in total variance was explained by a decrease in the dam component of variation (σ^2_d; Table 4.4, Figure 4.2).

DISCUSSION

I found significant phenotypic differences among the study populations held in common environments for a number of traits that are expected to contribute to fitness and performance in natural populations of salmon. Curiously, my results show that additive genetic variance has relatively little impact on population differentiation for several of those traits, despite sizeable population differences. This is especially notable for YIAL body size at age traits and for early survival traits. Instead, differentiation between the wild and domestic populations is driven by population-dam (maternal) effects. On the other hand, body size variation among the populations in the QRRC experiment has more of an additive genetic basis, suggesting that additive genetic effects do play a role in population differences for some traits and some population comparisons, or that the nature of the genetic architecture differs between domestic-wild versus wild-wild comparisons.

The substantial role played by maternal effects in this study, especially for body size at older ages (*i.e.* weight and length at 420 days and 615 days post-fertilization) is surprising, since maternal effects in salmon are expected to erode one year post-

fertilization (Heath and Blouw 1998; Heath et al. 1999). It may be that the among-population maternal effects I demonstrated are not simply maternal environment effects, but rather include more profound, longer acting maternal influences on offspring. Although I cannot rule out genetic maternal effects (*i.e.*, genetic imprinting, sex-linkage, mitochondrial inheritance) as a contributor to the variation among populations observed here, such effects have been rarely reported in salmon (Perry et al. 2005; Houde et al. 2011). Although environmental maternal effects are not inherited across generations, theoretical and empirical studies indicate that such effects can have dramatic effects on short-term evolutionary responses to selection (Cheverud and Moore 1994; Riska 1991; Kawecki and Ebert 2004)

One important implication of my study is that common garden experiments designed to test for genetic contributions to population differences which employ less than one generation of common environment rearing overestimates the additive genetic basis of differentiation among populations. This also holds true for translocation studies, where first generation differences in the traits of interest may not reflect longer-term differences or additive genetic variance. Unfortunately, common garden experiments lasting less than two generations are common in local adaptation research (Garcia de Leaniz et al. 2007; Fraser et al. 2011) and such studies of the genetic basis for local adaptation are biased towards finding an additive genetic basis for trait differentiation among populations. Either multiple generations of common garden rearing, or half-sib breeding designs (or ideally, both) should be used to show that differences do reflect the additive genetic variance necessary for local adaptation arguments. Studies such as ours that use more sophisticated quantitative genetic breeding designs (compared to full-sib

breeding) allow the partitioning of the variance components contributing to traits that show potential for local adaptation.

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Table 4.1: A list of the traits measured in this study for both the YIAL (wild vs domestic) and QRRC (four wild populations) experiments. The number of families and individuals within each family are also indicated.

	Trait	Short description	# of families	Median per family (range)
YIAL	FA	Fluctuating asymmetry index 278 days post fertilization	80	3 (1-3)
	FL-615	Fork Length at 615 days post fertilization	82	10 (2-18)
	W-615	Weight at 615 days post fertilization	82	10 (2-18)
	FL-420	Fork Length at 420 days post fertilization	82	7 (1-28)
	W-420	Weight at 420 days post fertilization	82	7 (1-28)
	Hct	Hematocrit count after saltwater challenge, 230-234 days post fertilization	91	8 (5-11)
	[Cl]	Plasma Cl^- concentration (meq/l) after saltwater challenge. 230-234 days post fertilization	91	8 (5-11)
	Egg survival	Egg survival	94	200
	Eyed egg survival	Eyed egg survival	94	159 (65-195)
	Fry survival	Fry survival	91	155 (63-195)
	Outbreak survival	Natural vibriosis outbreak survival 520-610 days post fertilization	82	143 (61-192)
	Relative fecundity	Relative fecundity 3 years post fertilization	49	2 (1-10)
	W at 3 yrs	W at 3 years (Female only, no gonads)	49	2 (1-10)
	W at 4 yrs	W at 4 years (Female only, no gonads)	53	2 (1-9)
QRRC	Egg survival	Egg survival	80	470 (284-1110)
	Fry survival	Fry survival	64	1
	FL-210	Fork length at 210 days	64	24 (3-41)

Table 4.2: Comparisons of mean pure-type cross traits in the YIAL experiment (domestic versus wild populations). The standard error of the mean is given in parenthesis. Significant *p* values ($p < 0.05$) are marked with bold face type. Comparisons made using the non-parametric Kruskal-Wallis test are marked with "†".

Trait (unit)	Pure wild cross	Pure domestic cross	*p* value
FA	2.07 (0.18)	2.99 (0.32)	**0.019**
FL-615 (cm)	31.8 (0.3)	29.3 (0.4)	< **0.001**
W-615 (g)	395 (13)	319 (10)	< **0.001**
FL-420 (cm)	22.4 (0.2)	20.2 (0.3)	< **0.001**
W-420 (g)	145 (3.9)	111 (4.6)	< **0.001**
Hct (100 x % RBC content)	44.1 (0.6)	44.2 (0.5)	0.573
[Cl] (meq/L)	155 (2)	161 (2)	0.069
Egg survival (%)	0.84 (0.03)	0.64 (0.03)	< **0.001**†
Eyed egg survival (%)	0.99 (0)	0.93 (0.02)	< **0.001**†
Fry survival (%)	0.99 (0)	0.97(0.01)	**0.021**†
Outbreak survival (%)	0.83 (0.01)	0.92 (0.01)	< **0.001**†
Relative fecundity (%)	1224 (70)	1059 (57)	0.34
W at 3 yrs (kg)	1.95 (0.14)	1.62 (0.06)	0.056
W at 4 yrs (kg)	1.88 (0.10)	1.82 (0.07)	0.514

Table 4.3: The effect of population on the mean trait values in the QRRC experiment pure-type crosses. The standard error of the mean is given in parenthesis. Significant post-hoc comparison differences are marked with different letters ($p < 0.05$). Egg survival data distribution was non-normal and comparison made with non-parametric Kruskal-Wallis test.

	Population				
Trait	HR	QN	BQ	RC	*p* value
Egg survival	0.81(0.06)[b]	0.98(0.01)[a]	0.78(0.12)[b]	0.96(0.01)[a]	< 0.001
Fry survival	7.20(0.36)	7.16 (0.86)	6.36 (0.21)	5.28 (0.98)	0.22
FL-210	7.34 (0.05)[a]	7.15 (0.15)[ab]	6.95 (0.04)[c]	6.98(0.22)[bc]	< 0.002

Table 4.4: Variance structure for 18 traits measured in both the YIAL and QRRC breeding experiments for the full and basic models, where the full model includes population specific sire and dam effects as fixed factor. Significant variance components (σ^2_s and σ^2_d) are indicated in bold-face type. Genetic variance components (V_A and V_M) are given as percentages only. σ^2_P is the total phenotypic variation explained by random variation ($\sigma^2_s + \sigma^2_d + \sigma^2_e$). "$\sigma^2_P$ ratio" is the ratio of variances explained by random effects between full and basic models (*i.e.*, $\sigma^2_{P\text{-Full}} / \sigma^2_{P\text{-Basic}}$).

		Full Model					Basic Model					σ^2_P ratio
	Trait	σ^2_s (%)	σ^2_d (%)	σ^2_P	% V_A	% V_M	σ^2_s (%)	σ^2_d (%)	σ^2_P	% V_A	% V_M	
YIAL	FA	0.023 (0.6)	0.006 (0.1)	3.74	2.4	-0.5	0.074 (1.9)	0.004 (0.1)	3.89	7.6	-1.8	0.96
	FL-615	**0.44 (5.7)**	**0.83 (10.7)**	7.76	22.6	5.0	**0.33 (4.0)**	**1.47 (17.5)**	8.41	15.9	13.5	0.92
	W-615	**462 (4.5)**	**1063 (10.4)**	10177	18.2	5.9	**436 (4.1)**	**1524 (14.3)**	10692	16.3	10.2	0.95
	FL-420	**0.20 (10.3)**	**0.21 (11.0)**	1.91	41.1	0.7	**0.23 (10.1)**	**0.46 (20.7)**	2.24	40.5	10.6	0.85
	W-420	**52 (7.7)**	**76 (11.1)**	679	30.9	3.4	**60 (7.8)**	**144 (18.8)**	767	31.1	11.1	0.89
	Hct	**1.2 (6.4)**	**1.7 (8.9)**	18.9	25.4	2.5	**1.1 (5.8)**	**1.80 (9.5)**	18.9	23.4	3.7	1
	[Cl]	5.9 (2.0**)**	0.9 (0.3)	300	7.9	-1.7	5.4 (1.8)	0.9 (0.3)	300	7.2	-1.5	1
	Egg survival	**0.012 (7.0)**	**0.011 (6.2)**	0.174	28.0	-0.7	**0.012 (7.0)**	**0.015 (8.8)**	0.18	27.8	1.8	0.97
	Eyed egg survival	**1 x 10^{-4} (0.2)**	**0.0026 (6.8)**	0.0377	0.7	6.6	1 x 10^{-4} (0.1)	**0.0032 (8.4)**	0.038	0.6	8.2	0.98
	Fry survival	1 x 10^{-4} (0.6)	**6 x 10^{-4} (3.2)**	0.0194	2.4	2.6	**1 x 10^{-4} (0.5)**	**7 x 10^{-4} (3.8)**	0.02	2.0	3.3	1
	Outbreak	**0.001 (1.2)**	**0.001 (1.0)**	0.113	4.8	-0.2	**0.002 (1.6)**	**0.002 (1.6)**	0.114	6.3	0.1	0.99
	Relative fecundity	188 (0.4)	1328 (2.8)	48133	1.6	2.4	188 (0.4)	1166 (2.5)	47386	1.6	2.1	1.01
	W at 3 yrs	0.002 (1.9)	0.002 (1.9)	0.103	7.5	0.1	0.003 (2.8)	0.002 (1.5)	0.116	11.2	-1.3	0.89
	W at 4 yrs	0.009 (0.7)	0.012 (1.0)	1.177	2.9	0.3	0.004 (0.4)	0.016 (1.4)	1.219	1.6	1.0	0.97
QRRC	Egg survival	**0.003 (2.7)**	**0.019 (16.4)**	0.113	10.8	13.7	**0.002 (2.2)**	**0.022 (19.2)**	0.117	8.9	17.0	0.97
	Fry survival	0.06 (2.3)	0.10 (3.9)	2.63	9.3	1.6	0.10 (3.1)	0.22 (7.0)	3.08	12.4	3.9	0.86
	FL-210	**0.022 (4.8)**	**0.022 (4.8)**	0.454	19.2	0.0	**0.048 (9.5)**	**0.046 (9.0)**	0.506	38.1	-0.5	0.9

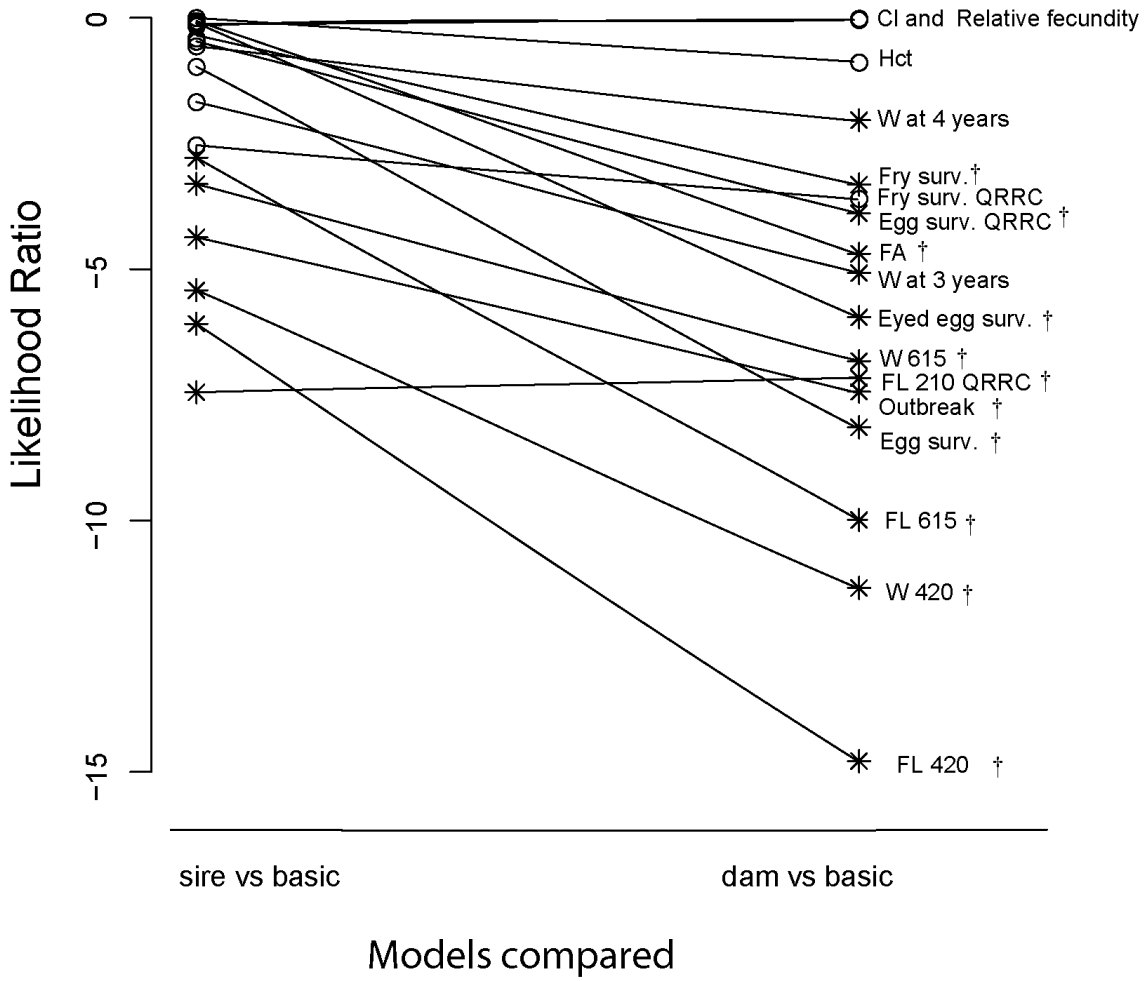

Figure 4.1: Likelihood ratios for population specific sire and dam models relative to the basic model (which does not incorporate population effects). Crosses (†) next to the trait description indicates that the trait is significantly different among pure-type crosses. Stars (*) on the data points denotes models that represent a significant improvement over the basic model ($p < 0.05$), such that the population-dam and/or population-sire effects are contributing significantly to the phenotypic differences among populations.

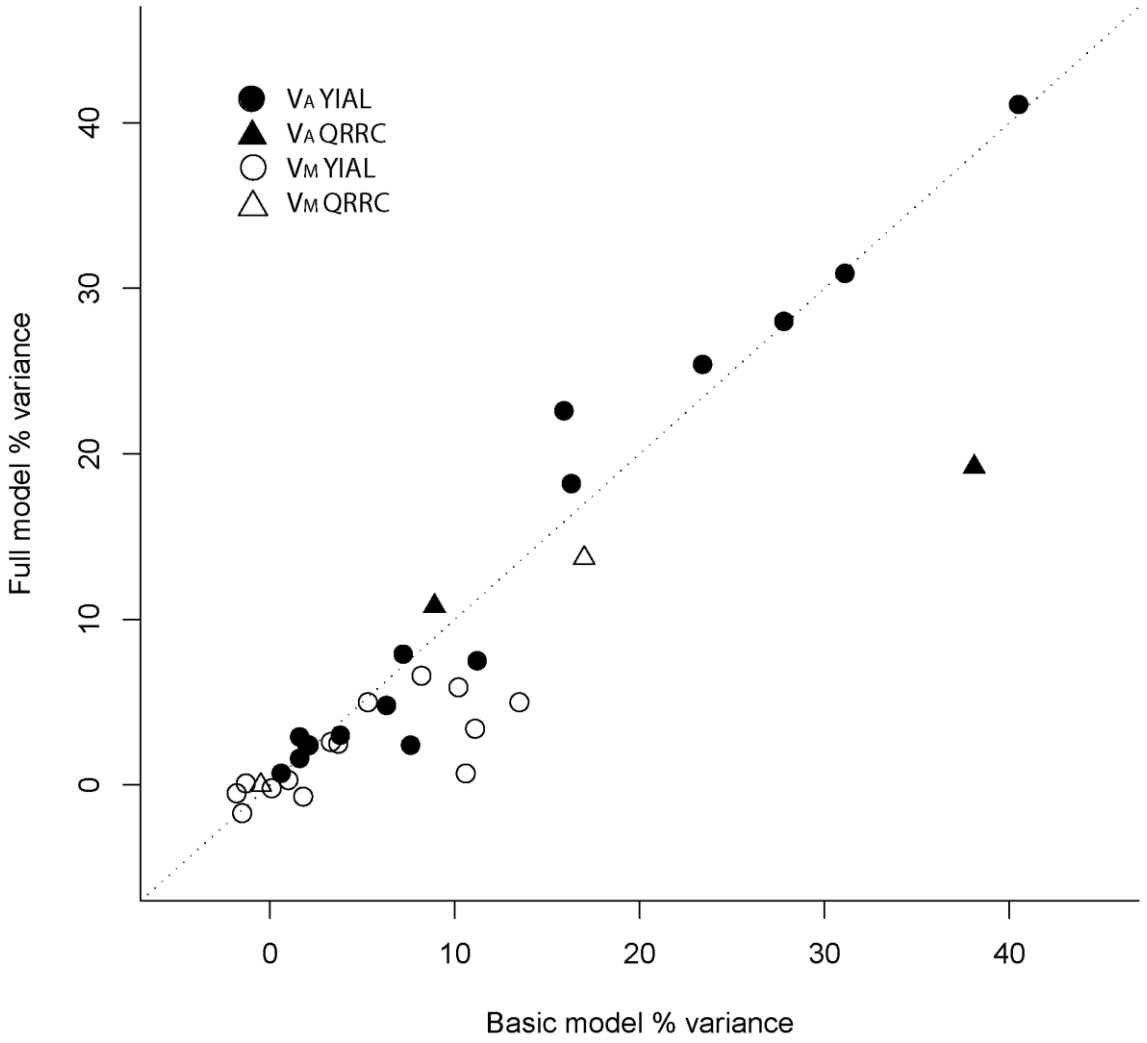

Figure 4.2: A visual representation of differences in the genetic components of variation (%) between the basic and full models. Additive genetic variation (V_A) and maternal effects (V_M) are denoted by filled and empty figures, respectively. YIAL and QRRC traits are denoted by triangles and circles, respectively. The dotted line has slope = 1.0 and signifies no difference between the two components.

Supplementary Table 4.1: Microsatellite genotypes of parental fish in the QRRC crosses that were used to assign mixed-family offspring to parental cross. Probable null alleles are indicated with "N".

		Dam; population of origin				Sire; population of origin			
Trough	Marker	HR	QN	BQ	RC	HR	QN	BQ	RC
1	*Omy325*	85/101	91/99	91/101	87/99	85/101	85/85	85/99	85/85
	Otsg432	123/167	115/131	131/131	159/159	107/N	107/115	127/131	107/137
	Otsg68	215/231	183/243	231/251	175/271	199/271	183/227	238/243	219/251
2	*Otsg78b*	286/N	290/342	254/266	254/N	318/N	246/298	258/N	266/282
	Otsg68	275/299	243/259	175/239	235/263	239/271	223/239	251/255	175/183
3	*Otsg432*	123/167	123/127	111/127	127/131	111/131	143/251	155/163	127/171
	Otsg68	191/207	211/211	235/267	183/183	187/207	211/239	207/267	187/303
4	*Otsg78b*	278/286	324/328	258/274	262/N	270/N	294/306	270/278	262/N
	Otsg68	187/223	199/219	227/239	179/299	223/299	207/211	199/255	231/235

All PCRs were performed at 12 µl and for 30 cycles. Annealing temperature are 54 C for otsg68 and otsg432 and 56-54 C (10 and 20 cycles) for omy325 and otsf78b.
QNxQN and QNxBQ genotypes in trough 1 overlap at one out of 32 possible combinations.
BQxCH & BQxRC genotypes in trough 3 overlap at one out of 8 possible combinations.
RCxCH and RCxRC genotypes in trough 3 overlap at one out of 8 possible combinations.
Null alleles did not affect our ability to assign offspring to their parental crosses

Supplementary Table 4.2: Log-likelihood of the four mixed REML models used for the 18 fitness-related traits measured in this study. Log likelihood ratios for three comparisons (basic model vs. sire model, basic model vs. dam model and basic model vs. full model) and *p* values for the likelihood ratio test statistics are also given. Significant *p* values ($p < 0.05$) are in bold-face type. Degrees of freedom for the test distributions are; 2 for Basic vs. full model comparison and 1 for basic vs. dam and basic vs. sire model comparisons.

	Traits	**Model log likelihood**				**log likelihood ratio of models comparison**			***p* value**		
		Full	Sire	Dam	Basic	basic-sire	basic-dam	basic-full	basic-sire	basic-dam	basic-full
YIAL Traits	FA	-444	-449	-444	-449	-0.1	**-4.7**	**-4.8**	0.718	**0.002**	**0.009**
	FL-615	-1940	-1949	-1942	-1952	**-2.8**	**-10.0**	**-12.0**	**0.018**	**<0.000**	**<0.000**
	W-615	-4835	-4841	-4837	-4844	**-3.3**	**-7.0**	**-9.6**	**0.010**	**<0.000**	**<0.000**
	FL-420	-1154	-1168	-1159	-1174	**-6.1**	**-14.8**	**-19.8**	**<0.000**	**<0.000**	**<0.000**
	W-420	-3208	-3219	-3213	-3224	**-5.4**	**-11.3**	**-16.0**	**0.001**	**<0.000**	**<0.000**
	Hct	-2160	-2161	-2160	-2161	0.0	-0.9	-0.9	0.940	0.183	0.410
	Cl	-3236	-3236	-3236	-3236	-0.2	0.0	-0.2	0.565	0.856	0.834
	Egg survival	-4568	-4576	-4569	-4577	-1.0	**-8.1**	**-9.1**	0.163	**<0.000**	**<0.000**
	Eyed egg survival	2344	2338	2343	2337	-0.1	**-6.0**	**-6.1**	0.647	**0.001**	**0.002**
	Fry survival	5314	5311	5314	5310	-0.4	**-3.4**	**-3.8**	0.367	**0.009**	**0.022**
	Outbreak	-2655	-2662	-2659	-2666	**-4.4**	**-7.4**	**-11.4**	**0.003**	**<0.000**	**<0.000**
	Relative fecundity	-740	-741	-741	-741	-0.1	0.0	-0.2	0.619	0.776	0.833
	W at 3 yrs	-29	-34	-31	-36	-1.7	**-5.1**	**-6.9**	0.067	**0.001**	**0.001**
	W at 4 yrs	-166	-168	-166	-168	-0.6	**-2.0**	-2.5	0.288	**0.043**	0.085
QRRC Traits	Egg survival	-9900	-9903	-9900	-9904	-0.4	-3.7	-4.0	0.870	**0.043**	0.236
	Fry survival	-118	-122	-121	-125	-2.5	-3.5	-6.2	0.167	0.069	**0.037**
	FL-210	-991	-998	-998	-1005	**-7.2**	**-6.9**	**-14.3**	**0.002**	**0.003**	**<0.000**

CHAPTER 5

DIFFERENTIATION AMONG CHINOOK SALMON (*ONCORHYNNCHUS TSHAWYTSCHA*) POPULATIONS: GENE EXPRESSION VARIATION UNDER THREE DIFFERENT ENVIRONMENTAL REGIMES

INTRODUCTION

Acclimation and adaptation represent the two mechanisms by which the individual and population respectively respond to environmental change through phenotypic modification towards an optimum performance (and hence to higher fitness). Furthermore, these two processes are both mediated by gene transcription, which has been shown to be instrumental in acclimation (Schulte 2001; Schulte 2004; Roelofs et al. 2010) and adaptive evolutionary responses among populations (*e.g.* St-Cyr et al. 2010; Jeukens et al. 2010; Aykanat et al. 2011). Acclimation is an individual organisms' response to adjust to a change in its environment, and does not result in evolutionary change. This property of acclimation has driven the long-established idea which posits that acclimation is an opposing force to adaptive evolution through its buffering of individual survival across an environmental gradient, thus constraining selection pressures (Wright 1931; Adkison 1995; deJong 2005).

On the other hand, phenotypically plastic traits can facilitate adaptive evolution (*e.g.*, West-Eberhard 2003; Prize 2003; Ghalambor 2007). Although the two models for the role of acclimation, or phenotypic plasticity, in evolution are difficult to reconcile, there are good lines of physiological evidence for the association of phenotypic plasticity and adaptive evolution (*e.g.* Schulte 2001). The parallelism between acclamatory

response to environmental change and the evolution of gene expression among populations experiencing similar environmental gradients is well established (Feder et al. 2000; Schulte 2004). For example, the constitutive expression of the *Ldh* gene among killifish *(Fundulus heteroclitus*) populations is elevated in colder northern populations than the warmer southern populations, which parallels the gene's physiological response to temperature (Schulte et al. 2000). Although such studies clearly show associations between plastic traits and adaptive evolution, they provide little insight on the actual mechanism of evolution, nor the divergence of plastic response among populations.

Examples of evolutionary changes in plasticity are rare (*e.g.* Nussey et al. 2005; McCairns and Bernatchez 2009; Scoville and Pfrender 2010). However, one notable recent example identified a reduction in pigmentation associated with the response of the melanin gene (*Ddc*) to UV stimulation in *Daphnia melanica* populations (Scoville and Pfrender 2010). *Daphnia melanica* adopted lower expression of the *Ddc* gene following UV exposure following the introduction of visual predators to the system, the associated reduced pigmentation and visibility, presumably led to lower predation. Such exceptional studies provide empirical support that phenotypic plasticity is subject to adaptive evolution, yet more examples and broader ecological scales are needed to better characterize the ecological and genetic basis for the evolution of plasticity in natural populations.

The heterogeneous nature (both temporally and spatially) of salmonid environments can promote both local adaptation and, potentially, the evolution of plasticity (Via et al. 1995). Salmonids therefore provide an ideal system to test the evolution of plasticity within the context of local adaptation. Local adaptation is the process whereby populations evolve through increased fitness under local environmental

conditions and promotes adaptive differentiation among salmon populations (Garcia de Leaniz et al. 2007; Fraser et al. 2011). In many cases, local adaptation is thought to have been driven by biotic and abiotic environmental factors, which also cause physiological acclimation responses among individuals at smaller spatial and temporal scales. Provided physiological acclimation responses could be manifested as "reversible" plastic traits (*e.g.* McCairns and Bernatchez 2009), they are candidates for testing the basis of the evolution of plasticity. Therefore, quantifying population differentiation for plastic traits in salmon will provide an opportunity to test for the adaptive role of plasticity in nature. For example, temperature and pathogen susceptibilities are known to vary among salmonid populations (reviewed in Garcia de Leaniz et al. 2007) and are therefore ideally suited to test for the evolution of plasticity. Here, in a common garden experiment, I test whether phenotypic plasticity of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) gene transcription is differentiated among populations, using a custom cDNA microarray. Gene transcription provides an ideal medium to test for the eco-evolutionary dynamics of plasticity, and a microarray platform provides a powerful means to address such questions (Goetz and Mackenzie 2008; Shiu et al. 2008). I measured the gene transcription response of individuals within full-sib families across two environmental challenges (temperature and immune), and test for differences among four populations of Chinook salmon, which were collected from Vancouver Island and mainland British Columbia, Canada (Figure 5.1). An immune challenge was included to explore differential pathogen response among populations while a temperature challenge was used as a proxy for tolerance to river water temperature fluctuations. My multi-gene microarray design indicated significant genetic differentiation among populations as well as plasticity among populations. The data also provide comparative evidence that observed population

differences are likely adaptive in nature. Specifically, I identified a set of immune-response related candidate genes that were differentially expressed among populations in which the observed changes are consistent with adaptation to local conditions.

MATERIALS AND METHODS

Breeding and rearing: On 17 Oct 2005, eggs and milt were obtained from Chinook salmon *(Oncorhynchus tshawytscha)* individuals from four populations; Harrison River (HR), Quinsam River (QN), Big Qualicum (BQ), and Robertson Creek (RC), and were immediately shipped to the Quesnel River Research Center (QRRC) on ice (Figure 5.1). Eggs and milt from four male and four female Chinook salmon were received on the same day from all four populations, and fertilizations were performed on the same day by crossing four pairs of fish (one-to-one breeding) from the same origin to generate four families of fish from each wild stock (Table 5.1). Fin clip tissue from the parental fish was collected and stored in -20° C for later genetic analyses.

Fertilized eggs were incubated in vertical stack incubation trays, each family separated by dividers. At the eyed egg stage (18th November 2005), 200 eggs from one family from each pure-type cross were pooled and reared together to minimize among-population tank effects. In Jan 2006, each of the pooled crosses had reached the first-feeding stage (mean mass = 0.41 g) and were transferred to four outdoor 4 m^3 freshwater "Capilano" troughs (flow rate = app. 8 L/sec, Table 5.1). Water was provided from an artesian well with a stable temperature of approximately 4.8° C.

Disease challenge, temperature challenge and sampling: Gill tissue from approximately 300 fish from each of the four replicate troughs was sampled in May 2006 at seven months post-fertilization. Gill tissue was also sampled 24 hours after an immune

challenge and 24 hours after the start of a temperature challenge (N = 300 per trough per treatment). I used gill tissue for my analysis because the gills are exposed to the environment and gene expression in gill tissue is known to be responsive to environmental perturbations (Bonga 1997; Evans et al. 2005). The immune challenge consisted of immersion in a 20 L bath of 10% vaccine solution (inactivated strains of *Vibrio anguillarum* serotypes 01 & 02; MICROVIB, Microtek International Inc.). The fish were challenged in batches of approximately 40-50 fish (average individual mass = 3.9 ± 0.9 SD). After two minutes of immersion, the fish were transferred to holding tanks (identical to the original) and sampled 24 hours later. For the temperature challenge, fish were transferred from their rearing troughs to identical troughs in which warmer water was supplied from a river source (app. 8.2° C, flow rate ~2 L/sec). The river water temperature was inconsistent throughout the day and was warmer in the afternoons (up to 11° C). However transfers and sampling for temperature challenge was performed in the mornings for all batches, hence this variation is uniform for all temperature challenges.

To sample gill tissue, the fish were humanely euthanized in a clove oil solution (0.5 mL of clove oil diluted 1:10 in EtOH and added to 2 L of water). The fish were then immediately weighed, and three gill arches were collected and stored in an RNA preservative solution (3.5 M Ammonium Sulfate; 15 mM EDTA; 15 mM Sodium Citrate; pH: 5.2). The samples were stored at 4° C overnight and subsequently transferred to -20° C until they were shipped to the laboratory in Windsor, Ontario for subsequent lab work.

Genetic analyses: Individual offspring ranging from 90 to 190 per trough per treatment were assigned to their population of origin by parentage assignment based on two or three diagnostic microsatellite loci (Supplementary Table 5.1). I selected four

individuals from each of the four populations in each trough for each of the three treatments. There were a total of five individuals missing from the described design due to not sampling enough fish from three families; two individuals were absent in one BQ family and one individual from a single HR family in the immune challenged fish. Additionally, two individuals were absent from a single RC family in the temperature challenged group. In total there were 187 individuals sampled for gill tissue across all replicate troughs and all treatments.

Microarray slide preparation and specifications: A Chinook salmon cDNA microarray chip was used in this study to assess gene transcription at 693 genes; however, the number of elements was reduced to 652 before hybridization, due to malfunction of one of 16 pins during printing.

The cDNA library to be spotted on the glass slides was amplified by PCR, ethanol precipitated and resuspended in 30% (vol/vol) DMSO at approximately 200 ng/μL. The glass slides (Gold Seal #3010) used in this study were coated with poly-L-lysine, using a standard poly-L-lysine coating procedure (DeRisi Lab, 2002). Spotting was performed on slides aged at least two weeks using a SpotArray 24 Micro Array Spotting System (Perkin Elmer) with 16 pins. DNA denaturation and slide blocking was performed using the succinic anhydrate blocking protocol (Massimi et al. 2002) and DNA spots were cross-linked to the slides by UV irradiation. The final printed array consisted of 2608 spots distributed in three blocks (top, center and bottom) where each block consisted of four replicates spots of each gene side by side, totaling 12 replicates overall (three blocks X four replicates per block; Figure 5.2).

RNA extraction, cDNA synthesis and dye incorporation: RNA was extracted from four fish per family, per treatment (except three families X treatment that had less individuals, see above). Gill tissue was homogenized in a pre-chilled 0.8 mL TRIZOL solution in a 2 mL tube with glass beads using a bead-based homogenizer. RNA was resuspended in 40 μL of high quality de-ionized water (18 Ω), and subsequently treated with DNase following the manufacturers' instructions (Fermentas #EN0521). RNA was quantified using a NanoVue Plus spectrophotometer (General Electric, UK), while RNA quality was assessed by gel electrophoresis where two clear rRNA bands indicated high quality RNA product.

To prepare cDNA for subsequent microarray hybridizations, SuperScript Indirect cDNA Labelling System (Invitrogen) was used according to the manufacturers directions with slight modifications. For cDNA synthesis, 10 μg total RNA and 1μL Oligo (dT_{20}; 2.5 μg/μL) were mixed and incubated at 70°C for 5 minutes, then chilled on ice. Subsequently, 5X RT buffer (Invitrogen), 40 units RNaseOUT (Invitrogen), 0.1 mM dithiothreitol (DTT), 400 units of reverse transcriptase (Invitrogen, SuperScript II) and and 0.8 μL dNTP (including amino modified nucleotides for dye incorporation) were added, and the reaction incubated at 42ºC for 4 h. The enzyme was then inactivated at 70ºC for 15 min. cDNA was purified by column clean up and ethanol precipitated, according to the manufacturer's instructions (Invitrogen). Purified cDNA was fluorescently labeled with Alexa Fluor 555 (Invitrogen) according to the manufacturers instructions, except for freshly prepared coupling buffer (0.1M $NaHCO_3$ in PBS buffer; Massimi et al. 2002) using half the recommended amount of Alexa Fluor 555 dye. Labeled cDNA was purified to remove unincorporated dye.

Microarray slide hybridization and scanning: I used a one color microarray design in my experiment due to its flexibility and simplicity of analysis without any sacrifice in sensitivity (Patterson et al. 2006). All hybridizations were performed in Corning™ slide hybridization chambers using 7 μL of the labeled cDNA sample and 43 uL of hybridization solution. The hybridization solution consisted of 25% Hi-Di formamide (Applied Biosystems, Foster City, USA), 0.1% sodium dodecyl sulfate (SDS), 5X SSPE buffer (3.0 M sodium chloride, 0.2 M sodium hydrogen phosphate, 0.02 M EDTA, pH 7.4), 10% dextran sulfate, 1 μL polyadenylic acid potassium salt (polyA; Sigma-Aldrich, St. Louis, USA) and 3 μL Human *cot-1* DNA (Invitrogen). After denaturing for 3 min at 95° C, the mixture was gently dispensed onto the microarray slides, followed by positioning the cover slip, taking care not to leave any air bubbles. Wells in the hybridization chambers were filled with 10 uL of 1X TE to achieve uniform humidity. The incubations took place in a 42°C water bath for 16 h in the dark. Hybridized arrays were consecutively washed for two minutes in each of the washing buffers: 1X SSC (with 0.2 % SDS), 0.2X SSC (with 0.2 % SDS), 0.1X SSC. Arrays were dried in slide boxes by centrifugation at 1000 rpm for one minute.

Fluorescence on each slide was quantified using a ScanArray Express microarray scanner (PerkinElmer) and ScanArray Express Microarray Analysis System software v. 4.0 (PerkinElmer) was used to analyze the data. Briefly, quantification was performed using an adaptive circle segmentation method which compensates for morphological variation in the array spots. Further quality control of spot configuration was performed by manual visual analysis and poor quality spots (*i.e.* smudges) were removed from the

data set prior to data analyses. The three replicate blocks on each slide were quantified and digitalized separately.

Data analysis: Unless otherwise stated, all statistical analyses were performed using R software (R Development Core Team, 2009). The digitalized array scans were processed using limma package in R. Out of 187 hybridized arrays, 172 (92 %) were accepted for further processing. In other words I analyzed 172 individuals for the experiment, in total (Table 5.1); the 15 excluded arrays showed essentially no measurable fluorescence (mean array intensity < 100). I filtered the data prior to the analysis by removing spots with low signal to noise ratio (Signal to noise ratio < 2) and eliminated genes from the analysis if they had less than 10 % of its repertoire remaining after the initial spot quality screening: that is, with 172 slides x 12 spot replicates/slide (= 2064 total spots per gene), if there were less than 206 spots per gene remaining for the analysis, the gene was removed from all subsequent analyses. In total, 562 genes were included for further analysis. I used BLAST2GO software, which allows multiple query BLAST searches and gene ontology to identify array gene homologues in the GenBank with BLASTn and BLASTx algorithms (Conesa et al. 2005). Overall, 356 genes were identified in GenBank. The complete gene list is deposited on the Heath Lab website (http://www.uwindsor.ca/glier/daniel-heath).

I performed a series of standard microarray corrections and normalizations to finalize pre-processing of the data prior to analysis. I first performed background correction using a simple subtraction of background intensity from individual spot intensities. As a consequence of the one color microarray design used here, gene intensities are correlated with the average intensity of whole array, therefore I used the residuals of the regression of individual gene element intensity versus whole-array

intensity as my gene transcription variable for further analysis (Supplementary Figure 5.1, Bolstad et al. 2003). Finally, I used quantile normalization to stabilize among-array variance (Bolstad et al. 2003; Smyth and Speed 2003).

I used a stratified statistical analysis to provide maximum flexibility for the analysis of one color microarray data. I used a mixed effects model (see below) as the general framework for the analysis, and changed the model specifications to address the contribution of specific effects of interest, for example, the effects of the treatments and population of origin. For each gene, the observed variance was partitioned using a mixed model where random factors are nested as follows:

$$z_{abijklm} = \mu + T_a + R_b + P_i + F_{j(i)} + I_{k(ij)} + B_{l(ijk)} + e_{abijklm}$$

Where, $z_{abijklm}$ is the normalized average intensity value over the replicate spots in the l^{th} block which is nested within k^{th} slide, which is nested within j^{th} family, which is nested within i^{th} population. The model includes treatment (T_a) and replicate trough effects (R_b) as fixed effects. The interpretation of the trough and family effects, and their interaction, warrant some caution since the two effects are partially confounded (Table 5.1). However, by including replicate trough effects (R_b) as a fixed effect, my estimate of family variance components within each population will be unbiased by potential trough ("tank") effects, although the value may be underestimated.

I first estimated the effect of treatments (immune and temperature) on gene expression by comparing the likelihood change in the model when the fixed treatment effect (T) was excluded from the model. The analysis was performed for the immune challenge vs. control (N=115 slides) and the temperature challenge vs. control (N=116 slides) separately to produce independent estimates of significance at each treatment level. For this analysis, the parameters are estimated with maximum likelihood (ML) and

the two contrasting models (with and without the treatment fixed effect) were compared with ANOVA. The statistically significant contrasts were identified as "responsive genes" using two α levels (α = 0.05 and, α= 0.01). For this particular model comparison, I used maximum likelihood (ML) instead of restricted maximum likelihood (REML), since change in likelihood estimation in REML does not depend on fixed effect variance structure; hence, it cannot be used to contrast fixed effects.

Next, for each treatment group, I partitioned the observed variance by estimating the model parameters using REML. REML fitted parameters were then taken as priors to calculate the parameters' highest probability densities (HPD) with the Markov Chain Monte Carlo method using languageR package in R (MCMC; 1000 runs; Baayen et al. 2010). Median HPD values were used as estimates for variance components in the model. Subsequently, I measured genetic differentiation among populations using Q_{ST} values, which is an analogue of F_{ST} in neutral markers and here is used to measure the degree of differentiation based on transcription profiles from the microarrays. I calculated Q_{ST} using the formula; $Q_{ST} = \sigma^2_{GB} / (\sigma^2_{GB} + 2\ \sigma^2_{GW})$, where σ^2_{GB} and σ^2_{GW} are the among-population and within-population components of the observed variance, respectively (Whitlock 2008). Q_{ST} was estimated for transcription at each gene within treatment, using family and population variances resulting from the MCMC analysis as parameters.

I performed a meta-analysis to characterize the specific role of the "responsive" genes in population differentiation. For a particular treatment (*i.e.*, immune and temperature), responsive genes are those that exhibit significant transcriptional response to the challenge, hence signifying the set of genes in the array that are functionally related to the environmental factors associated with the treatment. If the function has a role in local adaptation (*i.e.* biotic factors such as pathogen diversity, abiotic factors such as

temperature fluctuations, Garcia de Leaniz et al. 2007) I would expect responsive genes to differ more among populations than the non-responsive gene set. To test this, I compared the magnitude of the among population variance and phenotypic differentiation (*i.e.* Q_{ST}) between two groups of genes (responsive and non-responsive) using the non-parametric Wilcoxon rank sum test.

The ontology and patterns of population-specific expressed genes among the treatment groups signify the possible role of those genes in local adaptation. I identified population-specific expressed genes by contrasting models with and without the random population effects within each treatment (*i.e.* normal, immune, temperature). The model parameters were estimated with restricted maximum likelihood (REML), and the resulting two contrasting models were compared with ANOVA.

RESULTS

Overall, the challenges had significant effects on gene expression. The model comparison indicated that 46 and 161 genes (out of 562) showed significant ($p < 0.05$) temperature and immune treatments effects, respectively. The number of significantly responding genes dropped to 12 for the temperature and 92 for the immune treatment when the alpha level was decreased to $\alpha = 0.01$ (Supplementary Table 5.2). The results suggest the immune challenge by vaccine exposure had more profound effects on the expression of the gene repertoire in the array than the temperature challenge did.

Variance analysis showed that, within each treatment, among-block and among-slide variance components were similar and comprised around 18 % and 20 % of the total variance respectively (Figure 5.3, Table 5.2). Among-slide variance did not differ between treatments, but among-block variance was significantly different in the

temperature treatment relative to the other two (Table 5.2). Among-block variance was composed entirely of experimental error and similarly, among-slide variance was composed of largely experimental error, although some within-family variation is confounded in the among-slide variance component; however, it likely contributes a small proportion, since the magnitude of the within-slide (*i.e.*, among-block) variance is comparable, as expected if error contributed primarily to the among-slide variance component. These two "non-biological" variance components showed a normal distribution, as expected for error variation (Figure 5.3).

For each treatment group, family and population variance components each explained around 5 % of the total variance (Table 5.2). However their distributions were skewed towards zero, in contrast to the normal distribution of variances for the error-related observed components of variation (Figure 5.3). This suggests that the majority of the genes on my microarray displayed little transcriptional variation attributable to family- or population level effects in response to my three different treatments (control, immune and temperature). There were no differences among the three treatments for among-family variance (Table 5.2). On the other hand, among-population variance was higher in the immune challenge group relative to control or temperature treatments. In addition, this difference was explained by genes that respond to immune challenge (Table 5.2). When genes responding to immune challenge were clustered together, they showed significantly higher among-population variation than observed across all genes in the immune challenged samples (Table 5.2). Similarly, when responding genes were excluded, among population variance in immune group genes was similar to other groups (Table 5.2).

Using the estimates of within- and among-population variances, I calculated *Qst* among the four populations for all of the genes on the array. Overall, average *Qst* values were similar across all three treatments, however the mean Q_{ST} for immune challenge samples was marginally higher than the control samples after Tukey's HSD multiple comparison test (p=0.055; Table 5.2). Similar to the pattern that I observed in among-population variance comparison, Q_{ST} values for immune responsive genes were significantly higher than those other genes in immune treatment group (Table 5.2). The distribution of Q_{ST} was skewed, indicating most gene transcription do not differ among populations (Figure 5.4). On the other hand, immune responsive genes were less at lower Q_{ST} values (Figure 5.4).

There were a total of 23 genes that showed population-specific transcriptional differences among populations using model contrasts ($p< 0.05$, Figure 5.5, Table 5.3). Overall, these genes had very high Q_{ST} estimates (0.78 ± 0.11 SD). Of the 23 genes, 14 showed differences in the immune treated group, and six and three of them were from the control group and temperature group, respectively (Figure 5.5, Table 5.3). The notable genes that appear to have relevant function are "complement component c9", "complement factor h1 protein", "interferon inducible protein", "c1 inhibitor", and "complement c3-like"which encode for immune-related functions, more specifically, elements of the innate immune system (*i.e.* complement system, Table 5.3). Additionally the "warm temperature acclimation-related protein", which is mostly associated with general stress response also showed a relevant function as well as differential expression among populations.

Genes associated with immune response (based on GeneBank homology and Gene ontology) was consistently expressed at higher levels in the Robertson Creek population

and at an intermediate level in the Quinsam River population (Figure 5.5a). These genes were “warm temperature acclimation-related protein”, “complement component c9”, “interferon inducible protein”, “complement factor h1”, “c1 inhibitor” and “complement c3-like”, of which three also displayed significant treatment effects to immune challenge (“warm temperature acclimation-related protein”, “complement component c9”, “interferon inducible protein”; Figure 5.5a).

DISCUSSION

In this study I have reliably utilized microarray technology to address evolutionary problems in natural populations, specifically the differentiation of acclimation among salmon populations, as well as to detect genes that are expressed differentially among populations. The powerful nested design of the experiment allowed me to characterize biological variation by which a large portion of error variation was partitioned (i.e., among-block and among-slide replicates). Furthermore, by partitioning the biologically-relevent variation into among-family and among-population variances, I was able to account for family variance and estimate population differentiation (*i.e.* Q_{ST}). This suggests it is importnat to have replicate block and slide designs and include family structure in the design to detect population specific effects in ecological/evolutionary application of microarrays.

In this study, I showed that genes that showed an acclimation response to immune stimulation (a “reversible” type of plasticity), are more differentiated than the non-responsive group of genes among Chinook salmon populations. This provides evidence that genetic differentiation among populations is mediated by plastic genes. Salmon populations differ in their response to environmental change. Most probably, mechanisms

(environmental variation) that induce a plastic response (*i.e.* immune response) in individuals also contribute to population differentiation at higher spatial and temporal scales.

The observed gene transcription divergence among my four study Chinook salmon populations likely has an adaptive component, perhaps as part of locally adapted suites of traits. I suggest this for 3 reasons: 1) the transcriptional variation I measured has a genetic basis since I employed a common garden design by which environmental variation was minimized. 2) I see more differentiation in the genes that showed response to immune stimulation. This indicates that the differences are "non-random", or directional, suggesting a selective constraint. 3) I used a functional challenge experimental design which will highlight gene transcription related to temperature tolerance and immune function, thus identifying traits likely to be fitness- related. A significant portion of the genes that showed population-specific expression patterns are related to innate immune function, which is closely associated with fitness (Magnadottir 2006; Whyte 2007). Although I do not have a direct association between fitness and the transcriptional differentiation described here, the indirect evidence listed above strongly suggests that the transcriptional differences are adaptive in nature.

Population specific gene expression differences indicated that the Robertson Creek population expressed innate immune related genes at higher levels than other populations. On the other hand, it is difficult to interpret associations between population specific patterns and functional relevance. I believe the higher response to immune stimulation in the Robertson Creek population indicates the population is more sensitive to infections, and therefore allocates more energy to the innate immune system (Cotter et al. 2004). Similarly, Harrison River and Big Qualicum, which showed lower immune

responses, might be more resistant to immune stimulation. I have two weak but supportive lines of evidences for this: First, fry survival at QRRC to be lowest for Robertson Creek families (Chapter 4, Table 4.4). The survival figures in Table 4.4 of Chapter 4 are not significant; however, the fifth trough was excluded from the analyses due to poor parentage resolution at parentage assignment (see Materials Methods Chapter 4). However the fifth trough also showed similar pattern of survival, and when included to ANOVA model, Harrison River and Robertson Creek survival comparison became significant ($p < 0.05$ after Tukey HSD). This finding suggests that Robertson Creek population is actually less resistant to pathogens. Secondly, Harrison River and Big Qualicum populations are in closer proximity to human impact compared to Robertson Creek and Quinsam populations (Figure 5.5), therefore Harrison River and Big Qualicum populations may experience human mediated immune stimulation more often. Therefore, those two populations could have evolved to a lower innate immune response state, in order to reduce the energetic costs associated with it (*i.e.* Cotter et al. 2004).

Both of the challenge treatments I performed were relatively mild, since neither were lethal or semi-lethal (*i.e.* extreme temperature stress or injection of live pathogen). It was my intent in this experiment to mimic more natural ranges of stress, which salmon populations may experience frequently throughout their life cycle. Lethal or semi-lethal challenges are not representative of the type of environmental challenges wild populations of salmon are likely to encounter, and perhaps more importantly, such events would be expected to be rare, and not the basis for long-term local adaptation. Indeed, immunostimulation by vaccine bath exposure provided a good basis to test for the relationship between genetic differentiation and transcription plasticity, and represents the core of this paper. Very intriguingly if I hadn't included an immune challenge, I would

not have detected the same level of transcription-based population differentiation, such that the number of population-specific expressed genes would have beenreduce substantially. My results suggest that stress challenge response may play a larger role in the functional population differentiation of salmonid populations than simple resting gene expression patterns. On the other hand, the type of stress challenge is important for the magnitude of the population signal; for example, the observed response to a mild temperature challenge was not as remarkable as for the immune challenge. The temperature stress in this study consisted of an approximately 3.5° C increase, which was possibly not enough to evoke population differences. It is also possible that the duration of the temperature challenge (24 hours) was not optimal to detect population-level responses. I know that temperature is indeed an important environmental factor that likely shapes population differentiation (Schulte et al. 2000; Garcia de Leaniz et al. 2007), and further explorations of the effect of temperature challenges on the transcriptome of salmon populations would provide important insights into thermal tolerance.

My results are promising for the application of microarrays to ecologically and evolutionarily relevant questions; however substantial replication is necessary to overcome array error issues. The cost of microarray experiments can be alleviated by lower-cost custom microarrays, thus allowing larger sample sizes (*i.e.* Giger 2008). In my experiment I used 192 custom printed microarray slides which made a multi-factor ecological experiment feasible. Despite the fact that many of the population-specific gene expression differences are marginally significant (at $\alpha = 0.05$), the meta-analysis methodology presented coupled with relevant gene ontology makes microarray applications powerful and compelling.

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Table 5.1: Trough design and allocation of populations in the troughs. Each trough contains one family from each population. The final numbers of individuals per family and per treatment are indicated in each box. HR: Harrison River, QN: Quinsam River, BQ: Big Qualicum, and RC: Robertson Creek.

Treatment	Trough 1				Trough 2				Trough 3				Trough 4			
	BQ	QN	HR	RC	BQ	QN	HR	RC	BQ	QN	HR	RC	BQ	QN	HR	RC
Control	3	4	4	4	4	4	4	4	3	4	3	4	3	4	4	3
Temperature	4	4	4	4	2	4	4	2	3	4	4	4	4	3	3	3
Immune	4	4	4	3	3	4	4	3	4	4	2	4	3	4	4	3

Table 5.2: Comparison of percent variance components and Q_{ST} values at different clusters of genes. The first three rows consist of mean values of all genes in the array, while the letter rows consists of genes grouped filtered according to the given criteria. Two types of comparison involved are: a) comparisons among the three treatment class; significant differences are labeled with different letters (p<0.05 after Tukey's HSD test). b) Comparison between filtered clusters and the original set of genes within the same treatment class. Significant difference in filtered clusters are labeled with † for $p < 0.1$, * for $p < 0.05$, and ** for $p < 0.001$. To attain normality, family and population variance components, and Q_{ST} values are log normalized before analysis. "Immune responding genes at $p < 0.001$" group of genes included in the table to highlight the contrast further displayed by immune responsive group of gene.

			% Variance components				
Treatment Class	**Selected genes**	**# of genes**	**Block**	**Slide**	**Family**	**Pop.**	**Q_{ST} (SD)**
Control	All genes	562	0.181[a]	0.203	0.045	0.044[a]	0.34 (0.26)
Immune Challenge	All genes	562	0.180[a]	0.200	0.046	0.051[b]	0.38 (0.26)
Temperature Challenge	All genes	562	0.171[b]	0.205	0.042	0.038[a]	0.35 (0.25)
Immune Challenge	Immune responding genes at $p < 0.001$.	39	0.174	0.199	0.060†	0.078**	0.42 (0.26)*
Immune Challenge	Immune responding genes at $p < 0.05$.	161	0.179	0.200	0.047	0.063*	0.43 (0.23)**
Control	Immune responding genes at $p < 0.05$.	161	0.185†	0.194	0.048	0.043	0.33 (0.26)
Immune Challenge	Indifferent to immune response at $p > 0.05$.	401	0.180	0.201	0.046	0.047	0.36 (0.25)
Temperature Challenge	Temperature responding genes at $p < 0.05$.	46	0.171	0.194	0.037	0.042	0.39 (0.27)
Control	Temperature responding genes at $p < 0.05$.	46	0.187†	0.192	0.039	0.041	0.38 (0.27)
Temperature Challenge	Indifferent to temperature response at $p > 0.05$.	516	0.171	0.206	0.042	0.038	0.35 (0.25)

Table 5.3: 23 genes that showed significant population specific expression within three treatment groups and the associated gene ontologies. Highly significant differences ($p < 0.01$) are marked with asterisk (*) next to unique Heath Lab code. The responsive genes are also indicated. % sim. indicates mean % similarity to first three BLAST hits. General gene ontology (GO) categories include biological processes (P), molecular functions (F) and cellular compartments (C).

Response	Treatment Group	Unique Heath Lab code	BLAST Sequence description	% sim.	Gene ontology, (GO) terms
	Immune	MT_EE3b12	Unknown		-
	Immune	MT_EE5e1	*Salmo salar* clone ssal-rgf-524-172 glutathione mitochondrial precursor complete cds	89.33%	P:glutathione metabolic process; F:glutathione-disulfide reductase activity; P:oxidation reduction; P:cell redox homeostasis; F:oxidoreductase activity; C:cytoplasm; F:NADP or NADPH binding; F:FAD binding
	Normal	GRASP_5038	*Salmo salar* clone ssal-rgb2-568-196 40s ribosomal protein s2 complete cds	98.00%	F:structural constituent of ribosome; F:RNA binding; P:translation; C:small ribosomal subunit; C:ribonucleoprotein complex; C:ribosome; C:intracellular
YES	Immune	MS_RD1_470*	*Oncorhynchus mykiss*, omu34341 28s ribosomal RNA partial sequence	87.33%	-
	Normal	MS_RD1_164	*Salmo salar* aldolase fructose-bisphosphate 1 mRNA	86.00%	-
	Temperature	BR_TS1_743	*Danio rerio* ubiquitin specific peptidase 32 mRNA	68.67%	-
	Normal	BR_TS1_1386	*Salmo salar* fam100a mRNA	90.00%	-
	Normal	BR_TS1_1270	*Salmo salar* clone ssal-rgf-508-055 pentraxin fusion protein precursor pseudogene cds	93.00%	P:glycine catabolic process; F:methyltransferase activity; C:cytoplasm; F:aminomethyltransferase activity; F:transferase activity; F:transaminase activity
YES	Immune	BR_TS1_179*	*Salmo salar* DNA-directed RNA polymerases I and III subunit rpac1 mRNA	82.33%	F:DNA-directed RNA polymerase activity; P:transcription; F:protein dimerization activity; F:DNA binding
	Immune	LV_TS1_744	*Salmo salar* lipolysis stimulated lipoprotein receptor mRNA	80.33%	-
	Immune	LV_TS1_408	*Oncorhynchus mykiss* c1 inhibitor mRNA	93.00%	F:serine-type endopeptidase inhibitor activity
YES	Immune	LV_TS1_360	*Oncorhynchus mykiss* complement component c9 mRNA	95.67%	C:integral to membrane; C:membrane; C:membrane attack complex; P:cytolysis; C:extracellular region; P:innate immune response; P:complement activation, classical pathway; P:complement

					activation, alternative pathway; C:plasma membrane
	Immune	LV_TS1_354	*Oncorhynchus mykiss* complement factor h1 protein mRNA	93.67%	-
YES	Immune	LV_TS1_352	*Plecoglossus altivelis* mRNAwarm temperature acclimation-related protein (wap65 gene)	71.67%	-
	Immune	LV_TS1_348	*Salmo salar* cj011 protein mRNA	81.67%	F:protein binding
	Immune	LV_TS1_337	*Salmo salar* keratinocytes-associated protein 2 mRNA	94.00%	P:biological_process; C:cellular_component
	Normal	LV_TS1_677	*Caligus clemensi* clone ccle-evs-509-283 cathepsin l1 precursor complete cds	97.00%	P:proteolysis; F:hydrolase activity; F:cysteine-type peptidase activity; F:peptidase activity; F:cysteine-type endopeptidase activity
	Immune	LV_TS1_652	complement c3-like		P:inflammatory response; C:extracellular region; F:protein binding; P:innate immune response; P:complement activation, classical pathway; F:endopeptidase inhibitor activity; P:complement activation, alternative pathway; P:complement activation; C:extracellular space; P:positive regulation of angiogenesis; P:positive regulation of phagocytosis; P:positive regulation of type IIa hypersensitivity; P:blood coagulation; F:cofactor binding; P:positive regulation of activation of membrane attack complex
	Normal	LV_TS1_615	*Hypophthalmichthys molitrix* fibrinogen gamma polypeptide complete cds	69.67%	P:protein polymerization; F:receptor binding; C:extracellular space; F:protein binding, bridging; C:fibrinogen complex; P:platelet activation
	Temperature	LV_TS1_522	*Rattus norvegicus* zinc dhhc-type containing 14 mRNA	85.67%	F:metal ion binding
YES	Immune	LV_TS1_215	Unknown		-
YES	Immune	LV_TS1_186*	*Oncorhynchus mykiss* interferon inducible protein mRNA	91.33%	P:response to biotic stimulus; C:integral to membrane
	Temperature	LV_TS1_153	*Oncorhynchus mykiss* clone omyk-evo-510-135 histone complete cds	93.00%	-

Table 5.3 (*Continued*)

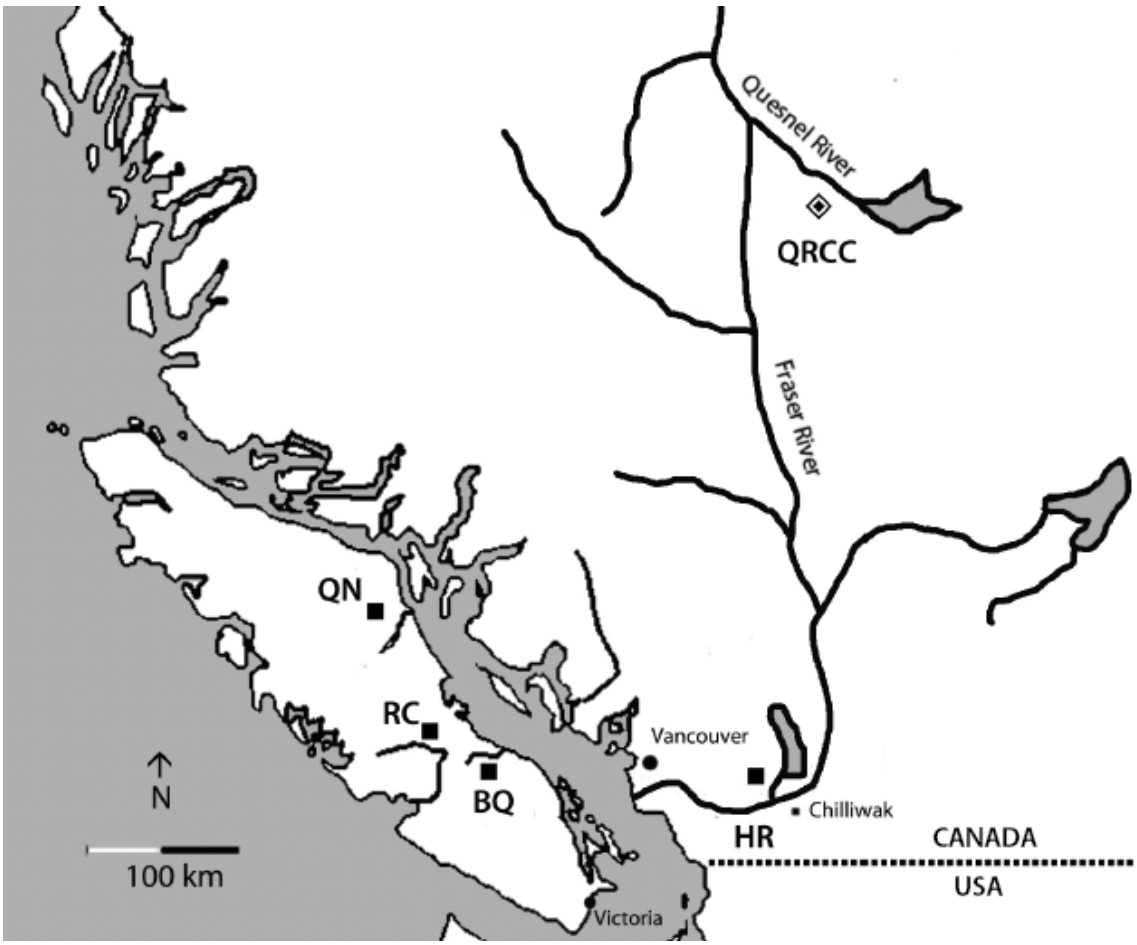


Figure 5.1: Map showing the location of the four Chinook salmon populations used in this study, as well as the Quesnel River Research Center (QRRC) where the fish were reared, challenged and sampled. HR: Harrison River, QN: Quinsam River, BQ: Big Qualicum, and RC: Robertson Creek.

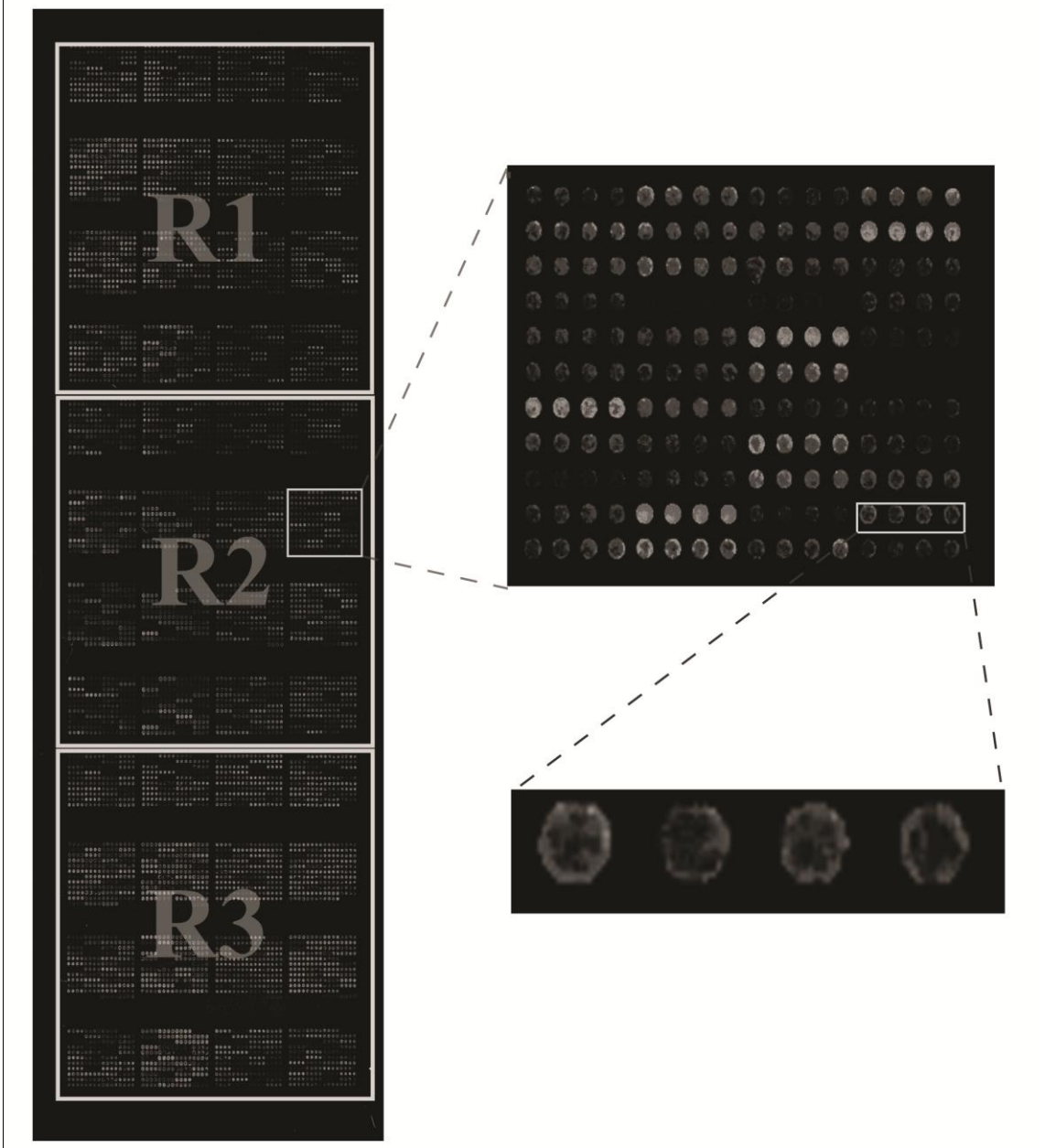


Figure 5.2: Block and spot replicate design in the microarray slides. There are there block replicates within a slide and each gene is replicated four times in each replicate block. The middle figure shows one out of 16 spot panels in the middle block replicate.

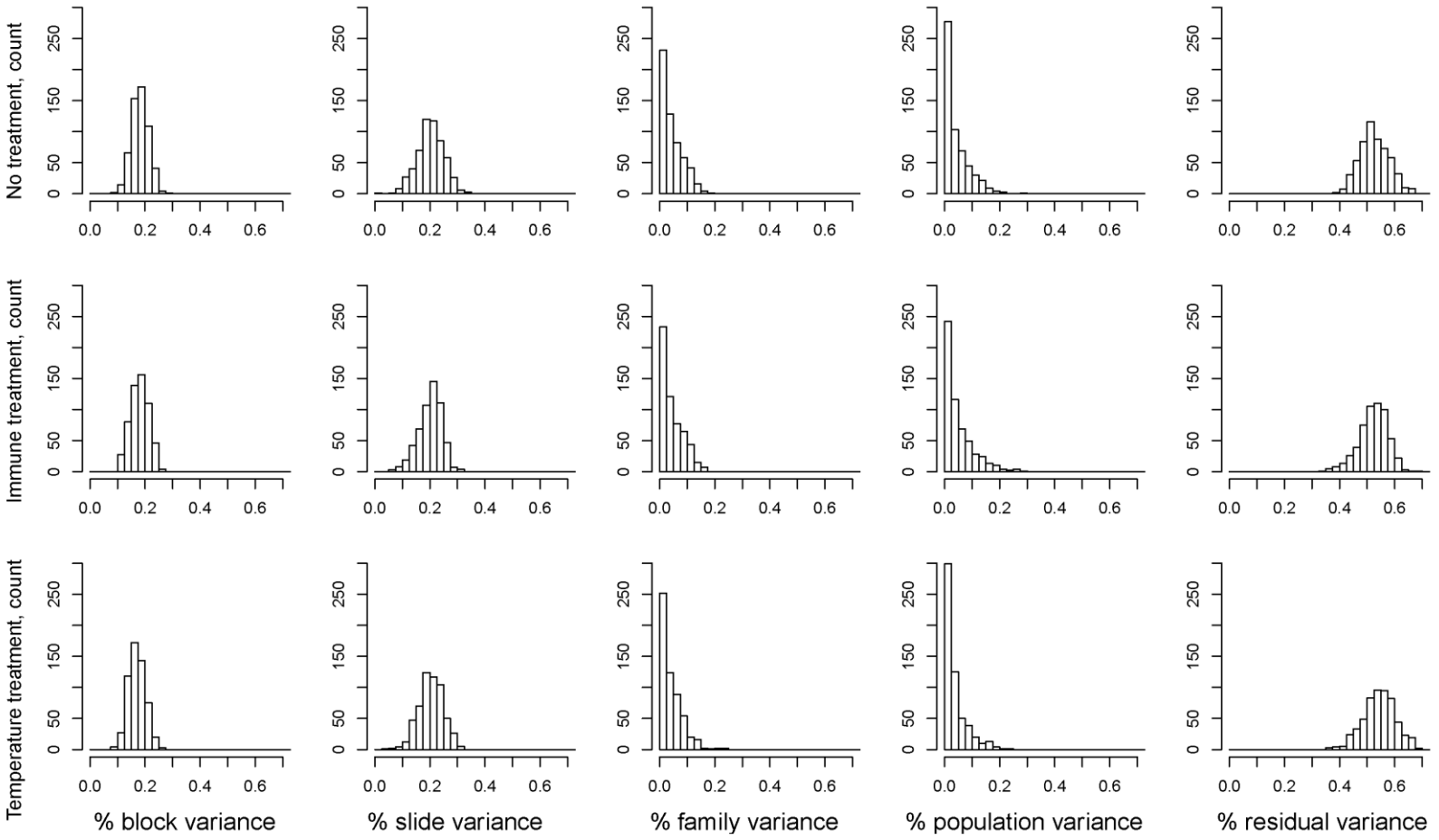


Figure 5.3: Frequency histograms (numbers of genes) of observed variance components for transcription of all genes, categorized by treatment group. Variance components are indicated by percentage and labeled at the bottom of the panel. Treatment groups are indicated on the left side of each row of panels.

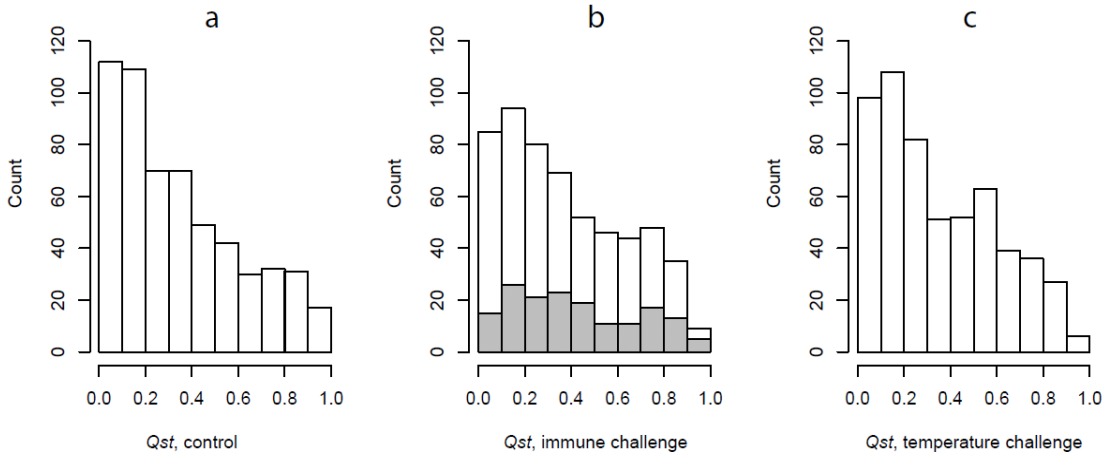


Figure 5.4: Frequency histograms (numbers of genes) for Q_{ST} estimates for all genes on the microarray categorized by control (a), immune (b) and temperature (c) treatment groups respectively. The shaded bars in panel (b) show the distribution of immune responsive genes (*i.e.* those that were significantly different in the challenge relative to the control treatments).

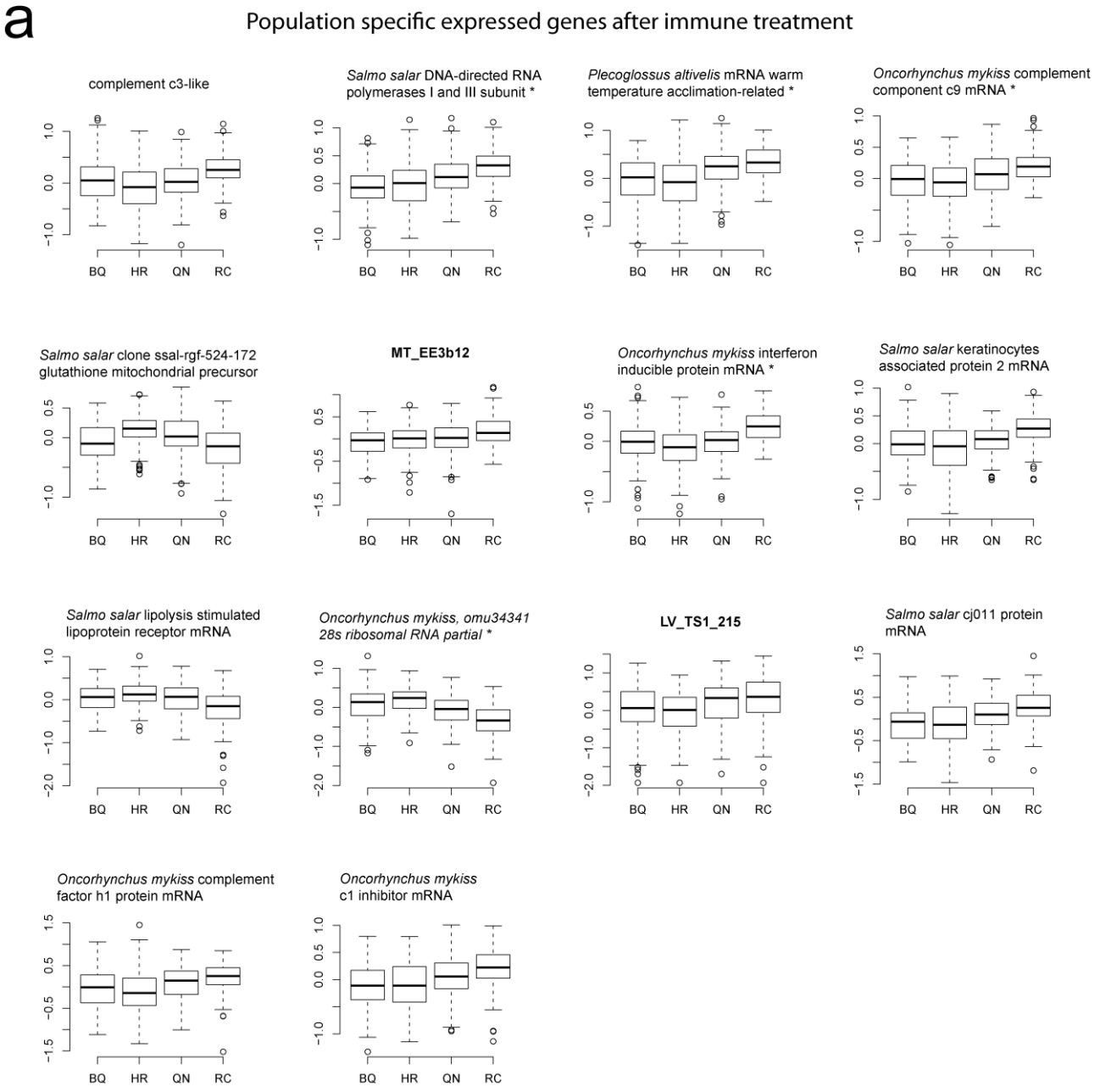


Figure 5.5 (*Continued*)

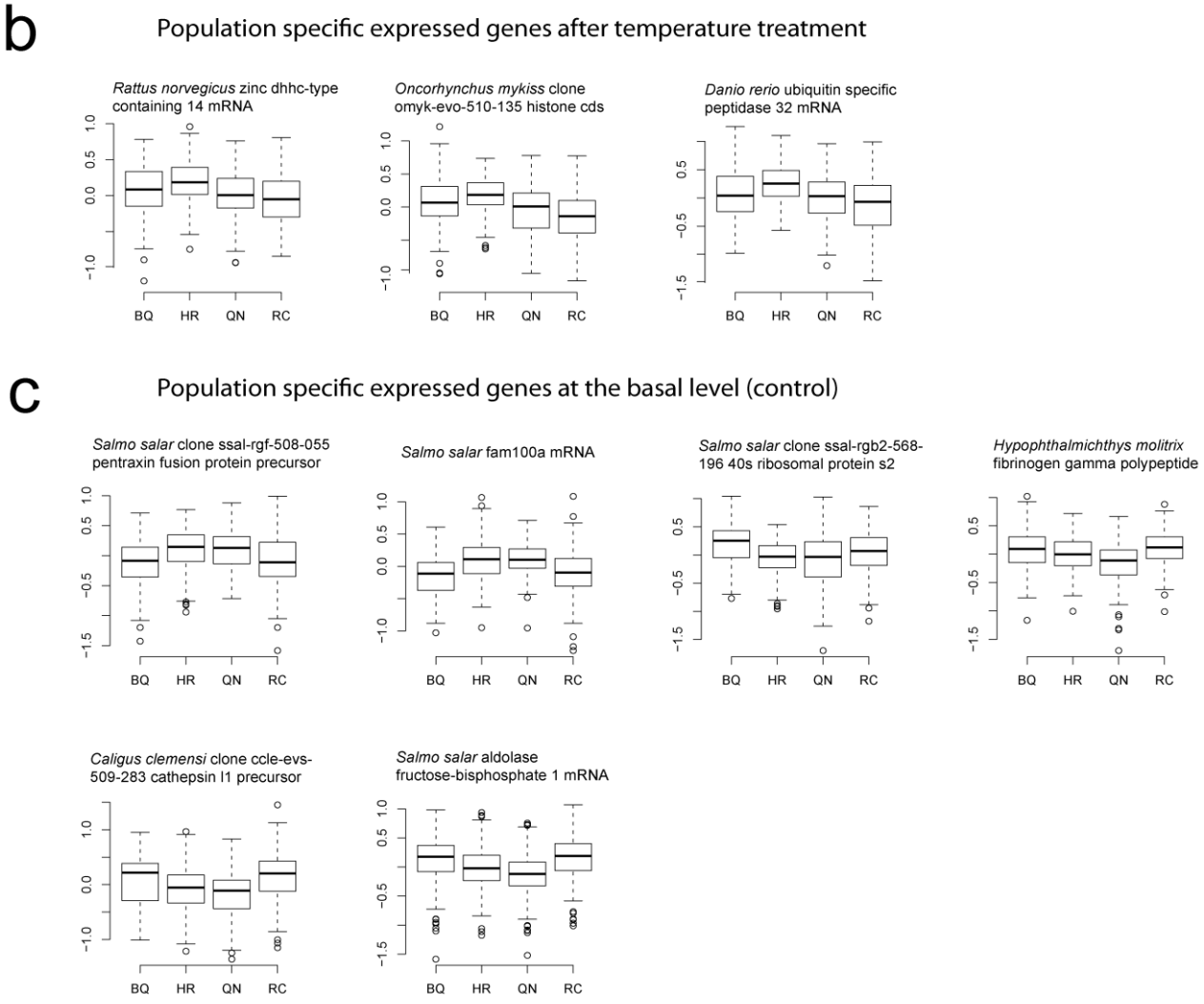


Figure 5.5: Population specific random effect of 23 genes that shows significant differences among populations ($p< 0.05$). Treatment group at which the gene was significant among populations are displayed at panel a for immune treatment, panel b temperature treatment and panel c for control. Genes are named after the most similar gene in the GenBank or after unique Heath Lab code if gene is unknown. Genes that showed significant response to treatment is marked with asterisk after its name. HR: Harrison River, QN: Quinsam River, BQ: Big Qualicum, and RC: Robertson Creek.

Supplementary Table 5.1: Microsatellite genotypes of parental Chinook salmon from four populations used to identify the parentage of the offspring use in this study. Null alleles are indicated with an "N". HR: Harrison River, QN: Quinsam River, BQ: Big Qualicum, and RC: Robertson Creek.

		Dam; population of origin				Sire; population of origin			
Trough	Marker	HR	QN	BQ	RC	HR	QN	BQ	RC
1	*Omy325*	85/101	91/99	91/101	87/99	85/101	85/85	85/99	85/85
	Otsg432	123/167	115/131	131/131	159/159	107/N	107/115	127/131	107/137
	Otsg68	215/231	183/243	231/251	175/271	199/271	183/227	238/243	219/251
2	*Otsg78b*	286/N	290/342	254/266	254/N	318/N	246/298	258/N	266/282
	Otsg68	275/299	243/259	175/239	235/263	239/271	223/239	251/255	175/183
3	*Otsg432*	123/167	123/127	111/127	127/131	111/131	143/251	155/163	127/171
	Otsg68	191/207	211/211	235/267	183/183	187/207	211/239	207/267	187/303
4	*Otsg78b*	278/286	324/328	258/274	262/N	270/N	294/306	270/278	262/N
	Otsg68	187/223	199/219	227/239	179/299	223/299	207/211	199/255	231/235

All PCRs were performed at 12 ul and for 30 cycles. Annealing temperature are 54 C for otsg68 and otsg432 and 56-54 C (10 and 20 cycles) for omy325 and otsf78b.
Null alleles did not affect my ability to assign offspring to their parental crosses

Supplementary Table 5.2: 104 genes with significant response to treatment ($p < 0.01$).

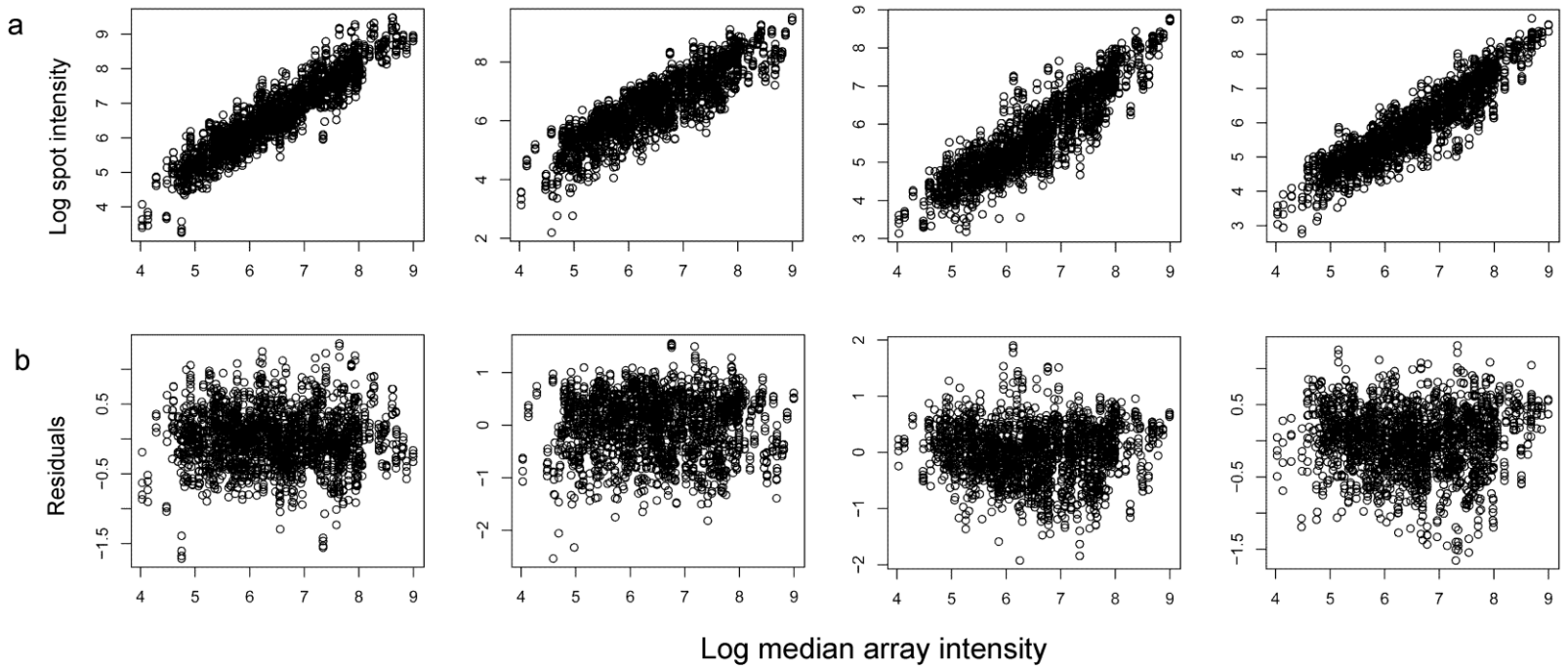
Treatment	Unique Heath Lab code	BLAST top hit sequence description	Mean % similarity to first three BLAST hits
Immune	MT_EE1c4	*Gasterosteus aculeatus* clone ch213- complete sequence	94.33%
Immune	MT_EE2b9	Unknown	
Immune	MT_EE2b10	Unknown	
Immune	MT_EE2g8	*Oncorhynchus mykiss* partial mRNA for ribosomal protein s8	93.00%
Immune	MT_EE3b8	*Oncorhynchus mykiss* c-type mbl-2 protein mRNA	96.67%
Immune	MT_EE3g8	Unknown	
Immune	MT_EE4c7	*Salmo salar* clone ssal-rgf-502-004 insulin-like growth factor-binding protein 7 precursor complete cds	92.33%
Immune	MT_EE4d1	*Oncorhynchus mykiss* heavy subunit mRNA	97.67%
Immune	MT_EE4d2	*Oncorhynchus mykiss* small inducible cytokine a13 mRNA	91.00%
Immune	MT_EE4d5	*Oncorhynchus mykiss* complement factor h1 protein mRNA	93.00%
Immune	MT_EE4d9	*Salmo salar* clone ssal-rgf-002-282 rho gdp-dissociation inhibitor 1 complete cds	95.00%
Immune	MT_EE4d11	*Samia cynthia* ricini pgrp-d mRNA for peptidoglycan recognition protein- complete cds	99.00%
Immune	MT_EE4e6	*Oncorhynchus mykiss* niemann-pick type c2 mRNA	96.67%
Immune	MT_EE5e4	*Salmo salar* clone ssal-plnb-025-229 guanylin precursor complete cds	96.00%
Immune	MT_EE6a8	*Samia cynthia ricini* pgrp-d mRNA for peptidoglycan recognition protein- complete cds	98.00%
Immune	MT_EE6c2	*Oncorhynchus mykiss* serum albumin partial cds	92.67%
Immune	MT_EE6c3	*Salmo salar* clone ssal-rgf-502-338 aspartate mitochondrial precursor complete cds	96.00%
Immune	MT_EE6d1	*Salmo salar* clone ssal-rgg-506-243 ependymin precursor complete cds	98.00%
Immune	MT_EE7a9	*esox lucius* clone eluc-evq-519-379 elastase-1 complete cds	77.00%
Immune	MT_EE7b6	*samia cynthia ricini* pgrp-d mRNA for peptidoglycan recognition protein- complete cds	99.00%

Immune	MT_EE7b7	*anoplopoma fimbria* clone afim-evh-505-213 cathepsin l precursor complete cds	87.67%
Immune	MT_EE7c1	*Oncorhynchus mykiss* complement factor h1 protein mRNA	93.00%
Immune	MT_EE7g4	*Salmo salar* clone ssal-rgf-536-260 tropomyosin-1 alpha chain complete cds	94.00%
Immune	MT_EE7g7	*Oncorhynchus masou* formosanus RNAse 2 complete cds	98.33%
Immune	MT_EE7g10	*Oncorhynchus mykiss* nadh dehydrogenase 1 alpha subcomplex subunit 1 mRNA	95.67%
Immune	GRASP_5142	*Salmo salar* clone ssal-rgh-511-049 cytochrome c oxidase polypeptide mitochondrial precursor complete cds	94.00%
Immune	GRASP_5225	*Salmo salar* elongation factor 1-gamma mRNA	89.00%
Immune	GRASP_5279	*Salmo salar* major facilitator superfamily domain-containing protein 4 mRNA	94.00%
Immune	GRASP_5367	*Salmo salar* ribulose-5-phosphate-3-epimerase mRNA	96.00%
Immune	GRASP_5445	*ornithorhynchus anatinus* plastin 3 (t-isoform) mRNA	73.00%
Immune	GRASP_5514	*Oncorhynchus mykiss* clone omyk-evo-507-248 lysozyme g complete cds	97.33%
Immune	GRASP_5533	*Salmo salar* partner of nob1 homolog (cerevisiae) mRNA	89.33%
Immune	GRASP_5717	*Salmo salar* clone ssal-evf-579-342 transgelin complete cds	97.00%
Immune	MS_RD1_279	*Salmo salar* clone hm5_1684 enolase 3-2 complete cds	87.33%
Immune	MS_RD1_644	*tetraodon nigroviridis* full-length cDNA	81.67%
Immune	MS_RD1_618	*Oncorhynchus mykiss* clone omyk-evn-509-061 60s ribosomal protein l5 complete cds	94.00%
Immune	MS_RD1_347	*Oncorhynchus keta* mRNA for myosin heavy complete cds	86.67%
Immune	MS_RD1_267	*bos taurus* gametogenetin binding protein 2 mRNA	78.33%
Immune	MS_RD1_131	*Oncorhynchus masou* formosanus ribosomal protein l8 complete cds	94.00%
Immune	MS_RD1_89	*Salmo salar* clone ssal-evf-542-261 40s ribosomal protein s4 complete cds	91.00%
Immune	MS_RD1_41	*Salmo salar* creatine mitochondrial 2 nuclear gene encoding mitochondrial mRNA	81.67%
Immune	BR_TS1_528	Unknown	
Immune	BR_TS1_514	Unknown	
Immune	BR_TS1_509	Unknown	

Immune	BR_TS1_1179	*Oncorhynchus mykiss* mhc class i a complete and partial cds	96.00%
Immune	BR_TS1_1149	Unknown	
Immune	BR_TS1_761	*Dicentrarchus labrax* chromosome sequence corresponding to linkage group top complete sequence	76.00%
Immune	BR_TS1_743	*Danio rerio* ubiquitin specific peptidase 32 mRNA	68.67%
Immune	BR_TS1_505	*Salmo salar* clone ssal-rgf-518-260 fam134a partial cds	85.33%
Immune	BR_TS1_500	Unknown	
Immune	BR_TS1_279	*Danio rerio* novel protein vertebrate taf4 RNA polymerase tata box binding protein -associated 135kda mRNA	70.33%
Immune	BR_TS1_21	*Mus musculus* bac clone rp23-404j7 from complete sequence	74.00%
Immune	BR_TS1_48	*Danio* rerio transmembrane 9 superfamily member 1 mRNA	76.00%
Immune	BR_TS1_40	*Callorhinchus milii* BAC clone IMCB_Eshark-91H12 from chromosome unknown, complete sequence	80.00%
Immune	BR_TS1_16	*Oncorhynchus mykiss* sypg1 phf1 and rgl2 complete cds d complete sequence lgn- pbx2 notch-tap1 and brd2 complete cds and mhcii-alpha and raftlin-like complete sequence	92.33%
Immune	BR_TS1_1476	*Salvelinus alpinus* pituitary-specific transcription factor pit-1 intron 1	79.67%
Immune	BR_TS1_1366	*Oncorhynchus* tshawytscha clone 1 interferon alpha 1-like complete sequence growth hormone 1 complete cds and skeletal muscle sodium channel alpha subunit-like and myosin alkali light chain-like complete sequence	81.67%
Immune	BR_TS1_1350	*Salmo salar* clone ssal-evd-552-121 cyclin-g1 complete cds	86.33%
Immune	BR_TS1_1348	*Oncorhynchus mykiss* clone omyk-evo-506-263 nicotinamide riboside kinase 2 complete cds	93.67%
Immune	BR_TS1_1227	Unknown	
Immune	BR_TS1_1150	*Danio* rerio si:dkeyp- (si:dkeyp-) mRNA	77.33%
Immune	BR_TS1_1145	*Salmo salar* surfeit locus protein 2 mRNA	90.00%
Immune	BR_TS1_1136	*Salmo salar* clone ssal-rgb2-549-134 vacuolar atp synthase 16 kda proteolipid subunit complete	87.33%

		cds	
Immune	BR_TS1_824	*Salmo salar* locus a genomic sequence	77.33%
Immune	BR_TS1_742	*Salmo salar* clone ssal-rgh-508-110 zinc finger protein 271 complete cds	77.33%
Immune	BR_TS1_545	Unknown	
Immune	BR_TS1_527	*Salmo salar* clone ssal-evf-530-292 lipocalin precursor complete cds	85.67%
Immune	BR_TS1_491	*Oncorhynchus* masou formosanus ribosomal protein l6 complete cds	83.33%
Immune	BR_TS1_482	*Salmo salar* stathmin-like 4 mRNA	79.33%
Immune	BR_TS1_344	*Oncorhynchus mykiss* mitogen-activated protein-binding protein-interacting protein mRNA	90.67%
Immune	BR_TS1_290	*Salmo salar* clone 272p16 chaperonin complete cds myosin 1 partial cds and tcr-gamma partial sequence	78.33%
Immune	BR_TS1_280	Unknown	
Immune	BR_TS1_194	*Oncorhynchus mykiss* 14-3-3g1 protein mRNA	92.00%
Immune	BR_TS1_179	*Salmo salar* DNA-directed RNA polymerases i and iii subunit rpac1 mRNA	82.33%
Immune	LV_TS1_499	omu61753*Oncorhynchus mykiss* complement component c3-3 partial cds	87.00%
Immune	LV_TS1_398	*Oncorhynchus mykiss* complement protein component c7-1 (c7-1) mRNA	79.00%
Immune	LV_TS1_385	*Salmo salar* phospholipase a-2-activating protein mRNA	79.00%
Immune	LV_TS1_352	plecoglossus altivelis mRNA for warm temperature acclimation-related protein (wap65 gene)	71.67%
Immune	LV_TS1_1	Unknown	
Immune	LV_TS1_508	*Salmo salar* clone ssal-rgf-519-210 scavenger receptor cysteine-rich type 1 protein m130 precursor pseudogene cds	88.33%
Immune	LV_TS1_243	*Salmo salar* 40s ribosomal protein s2 mRNA	92.00%
Immune	LV_TS1_222	Unknown	
Immune	LV_TS1_215	Unknown	
Immune	LV_TS1_186	*Oncorhynchus mykiss* interferon inducible protein mRNA	91.33%
Immune	LV_TS1_157	*Carassius auratus gibelio* fetuin-b complete cds	84.00%

Immune	LV_TS1_153	*Oncorhynchus mykiss* clone omyk-evo-510-135 histone complete cds	93.00%
Immune	LV_TS1_133	Unknown	
Immune	LV_TS1_82	*Salmo salar* clone ssal-rgf-522-184 cold-inducible RNA-binding protein complete cds	82.00%
Immune	LV_TS1_424	*Salmo salar* docking protein 5 mRNA	90.00%
Immune	BR_TS1_6	Unknown	
Immune	BR_TS1_272	Unknown	
Immune	BR_TS1_1328	Unknown	
Temperature	MT_EE4b10	integral membrane protein 2b	98.67%
Temperature	MT_EE5c3	heavy subunit	98.67%
Temperature	GRASP_4868	heat shock cognate 70 kda protein	97.00%
Temperature	MS_RD1_644	pleckstrin homology domain-containing family f member 2-like	93.67%
Temperature	MS_RD1_618	ribosomal protein l5	87.00%
Temperature	BR_TS1_1059	Unknown	
Temperature	BR_TS1_1183	Unknown	
Temperature	BR_TS1_1091	Unknown	
Temperature	LV_TS1_745	Unknown	
Temperature	LV_TS1_83	middle subunit	78.00%
Temperature	LV_TS1_186	interferon-induced transmembrane protein 3	98.00%
Temperature	BR_TS1_637	Unknown	



Supplementary Figure 5.1: Scatterplots that shows relationship between median array intensity and specific spot intensity a) before and b) after correcting for the relation. Four columns are arbitrarily selected spots from the array. In each figure, each dot represents an array.

CHAPTER 6

CONCLUSION

INTRODUCTION

Conservation and management madates to reverse population declines and to improve productivity are perhaps the main applied motivations for research on the evolution and ecology of salmon (Waples and Hendry 2008). For such applications it is important to understand the genetic basis of differentiation, and factors driving the differentiation, among populations. It is equally vital to understand the basis of populations' response to anthropogenic changes. Therefore, evolutionary genetics provides a framework to address important demographic aspects of salmon biology relevant to their conservation and management. Additionally, the outstanding life history and genetic properties of salmon offer evolutionary biologists intriguing puzzles to unravel and makes salmon ideal study species (Groot and Margolis 1991; Waples 2001).

The scope of this thesis, which is includes by both applied and basic science, is the quantitative genetic foundations and ecological rationale for evolution by transcriptional modification in salmon populations. As outlined in Chapter 1, the observations that salmon populations are highly genetically structured (*e.g.*, Waples 2001; Heath et al. 2006) with generally low effective populations sizes (*e.g.*, Heath et al. 2002; Koskinen et al. 2002), yet retain a high capacity to evolve and adapt (*e.g.*, Hendry et al. 1998; Hendry et al. 2000; Koskinen et al. 2002; Heath et al. 2003) cannot be explained by simple additive genetic effects or as a result of single gene action, but are consistent with non-additive models of evolution (Wade and Goodnight 1998; Lee 2002; Feinberg and

Irizarry 2010). Furthermore, transcriptional machinery provides a biochemical basis that makes complex genetic mechanisms feasible (*e.g.* Wray et al. 2003), thus providing a framework for the evolution of adaptation and phenotypic plasticity in salmon (and other species) populations.

Throughout this thesis, I explored the genetics of transcription by investigating underlying genetic architecture within and among salmon populations (Chapters 2, 3 and 4) and of transcription itself (Chapters 2 and 3). Furthermore, I paid special attention to phenotypic plasticity, or acclimation since environmentally responsive gene regulation is an indispensable property of transcription, and is a very important component in the fitness and survival of all living organisms. Therefore, I characterized the genetics of, and variation in, the acclimation response (*i.e.*, salinity tolerance, immune response, temperature tolerance) within and among populations (Chapters 2, 3 and 5). My findings show that transcription is important in salmon population structure and can rapidly evolve, but does not necessarily follow an additive genetic mode of inheritance. This suggests predictions based on Fishers' fundamental theorem (which is based on gene action additivity) do not provide adequate explanations for transcriptional evolution in salmon.

CONTRIBUTIONS

Genetic architecture within and among salmon populations with emphasis on transcription: In Chapters 2, 3 & 4, I explored the genetic architecture of phenotypic variation within and among salmon populations, with special emphasis on transcriptional traits in Chapters 2 &3. In Chapter 2, I showed that genetic differences in transcription can rapidly evolve in diverging populations by both additive and non-additive genetic

mechanisms. That chapter is especially important in that it identifies non–additive genetic effects as a potential basis for rapid evolutionary change when wild salmon populations are exposed to changing environmental conditions. In Chapter 3, I showed transcription is heritable (and can therefore respond to selection), but also possesses substantial non-additive genetic variance components. Furthermore, this is the first time that transcriptional variation has been partitioned into additive, non-additive and maternal components in salmon with highly sensitive quantitative real time PCR. With a more technical perspective, I found in Chapter 4 that maternal effects comprise a significant portion of phenotypic variation among populations reared in a common garden environment, especially at younger stages of life. This result is important because maternal effects (along with other non–additive genetic effects) are confounded with additive genetic variance estimates (under the assumption that non-additive effects are negligible) in many studies which screen genetic differences among populations. This leads to an upward bias in the resulting published additive genetic variance estimate.

Genetics of acclimation: I explored the genetics of the acclimation response within and among salmonid populations (Chapters 2, 3 and 5). An effective acclimation response is essential for individual performance and survival in changing environments, and it acts by modifying the phenotype towards an optimum state. Salmonids have a very developed suite of acclimation responses by which they can function well in a variety of different (and variable) environmental conditions (Groot and Margolis 1991). Therefore, the acclimation response is important for salmon survival; however, the genetic basis for acclimation is poorly understood. Therefore, I explored gene transcription for adaptively important acclimation responses (*i.e.*, salinity tolerance, immune response) at different organizational scales (i.e. among families, among populations). I analyzed acclimation

with a reaction norm perspective in Chapter 2, and employed a character state approach for Chapters 3 & 5 (reaction norms assume the response function to the environmental change is the trait of interest, while the character state approach considers the traits as separate entities in different environments, albeit with some genetic correlation; Roff 1997).

In Chapter 2, I showed the response to salinity in a landlocked steelhead trout population had diverged in its response to osmotic stress from the ancestral population, suggesting acclimation has the capacity to rapidly evolve. Furthermore, the non-additive component to the differences was also pronounced. In Chapter 3, I explored the structure of the genetic variance underlying cytokine transcription, before and after immune stimulation. The results were interesting, showing elevated non-additive genetic effects in transcriptional traits upon immunostimulation. Those results suggest non-additivity is more prevalent in traits that are more important for survival, (*i.e.* innate immune transcription after immune stimulation was more non-additive than before immune stimulation). Finally, in Chapter 5, I presented a large scale microarray experiment designed to investigate transcriptional differentiation among wild Chinook salmon populations, and compare the differentiation among populations before and after immune and temperature challenges. The results showed gene expression is more differentiated in immune responsive genes than in the non-responsive genes. Furthermore, the direction of differentiation is consistent among populations suggesting the differences are adaptive. This study provides a novel contribution to salmon biology and evolution, providing empirical evidence that salmon populations have a differentiated response to an environmentally important cue (*i.e.* immune stimulation) which has a genetic basis and is likely adaptive.

FUTURE DIRECTIONS

Transcription is becoming more important for studies aimed at understanding the basis of differences in form and function that underlie salmonid variation (*i.e.* Giger et al. 2006; Giger et al. 2008; St Cyr et al. 2008; Roberge et al. 2007; Seear et al. 2010). However, non-additive effects are often disregarded in some reviews, where quantitative genetic structure of salmon population (Carlson and Seamons 2011) and population structure with emphasis on local adaptation and conservation (*i.e.* Fraser et al. 2011, Garcia de Leaniz et al. 2007) was investigated in detail. In general, non-additive genetic effects in salmon are mostly investigated at a conceptual level, where it emerges as potential product of hybridization (*e.g.* Normandeau et al., 2009, Mavarez et al. 2009, Roberge et al. 2008). This may be partly due to the fact that models which adopt non-additive effects as a mechanism structuring populations are difficult to reconcile with empirical data, and perhaps do not stand on firm theoretical grounds (see: Cheverud and Routman 1995; Wade and Goodnight 1998, Coyne et al. 1997).

Non-additive genetic variation can be substantial and has a role in shaping populations. Several empirical studies demonstrate non-additive traits divergence in a variety of species including Pacific salmon (*Oncorhynchus*; *e.g.* Pitcher and Neff 2006; Evans and Neff 2009; Aykanat et al 2011), fruit flies (*Drosophila melanogaster* Gibson et al. 2004), yeast (Jasnos and Corona 2004; Dettman et al. 2007), pitcher plant mosquitos (*Wyeomyia smithii*; Armbruster et al. 1997), Soapberry bugs, (*Jadera haematoloma;* Carroll et al. 2003), Pacific oysters (*Crassostrea gigas*; Hedgecock et al. 2007), guppies (*Poecilia reticulate*; Lindholm et al. 2005), and corn (Zea mays; Tabanao and Bernardo 2005). The ecological and evolutionary context of trait divergence includes population

establishment and bottleneck events (Armbruster et al. 1997, Aykanat et al. 2011; Carroll et al. 2011; Lindholm et al. 2005; Jasnos and Corona 2004), rapid divergent evolution (Aykanat et al. 2011; Carroll et al. 2011; Dettman et al. 2007), and inbreeding effects (Gibson et al. 2004; Tabanao and Bernardo 2005; Hedgecock et al. 2007), where the evolutionary response is only predictable with non-additive models (Wade and Goodnight 1998; Feinberg and Irizarry 2010). Such non-additive models also predict that populations with (naturally) high inbreeding coupled with environmental variation still have a high capacity to evolve and invade (as a result of high non-additive genetic variance in fitness), but such a prediction has not been tested globally at the multi species level. Salmon population demography provides an ideal opportunity to test ecological and evolutionary hypotheses with a non–additive genetic approach, and my results show that salmon genetic architecture is consistent with that framework. Non-additivity should not be overlooked by salmon biologists, and more effort on this subject should be promoted, both to improve our theoretical understanding and expand the relevant empirical data available for future researchers.

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“The fish doesn't think because the fish knows everything”

Goran Bregović

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