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Epigenetic contributions to early life history variation in Chinook salmon

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EPIGENETIC CONTRIBUTIONS TO EARLY LIFE HISTORY VARIATION IN CHINOOK SALMON

By

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

In all cases, the key ideas, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by the author under the supervision of Daniel Heath. Mattias Johansson co-authored Chapter 2 and assisted with data analysis, interpretation, and writing of the manuscript. Chapter 3 was co-authored by Oliver Love, who assisted with the writing of the manuscript, and Jane Drown, who assisted with experimental design and setup of the project. Kyle Wellband co-authored Chapter 4 and assisted with experimental design, setup, parentage analyses, and writing the manuscript. Chapter 5 was co-authored by Ben Sutherland and Terry Beacham, who provided microsatellite and SNP data and contributed to the writing of the manuscript.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

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II. Previous Publication

This thesis includes four original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Chapter 2	Venney CJ, Johansson ML, Heath DD. 2016. Inbreeding effects on gene-specific DNA methylation among tissues of Chinook salmon. Mol Ecol. 25(18):4521–4533.	Published
Chapter 3	Venney CJ, Love OP, Drown EJ, Heath DD. 2020. DNA methylation profiles suggest intergenerational transfer of maternal effects. Mol Biol Evol. 37(2):540-548.	Published
Chapter 4	Venney CJ, Wellband KW, Heath DD. Rearing environment affects the genetic architecture and plasticity of DNA methylation in Chinook salmon. Heredity.	Accepted for publication
Chapter 5	Venney CJ, Sutherland BJG, Beacham TD, Heath DD. Population differences in Chinook salmon (*Oncorhynchus tshawytscha*) DNA methylation: genetic drift and environmental factors. Mol Ecol.	Submitted

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ABSTRACT

DNA methylation has been proposed as an epigenetic, evolutionary mechanism for acclimation, transgenerational plasticity, and local adaptation without changes in DNA sequence. In this thesis, I assess the highly targeted evolutionary nature of DNA methylation in Chinook salmon from the tissue to the population level, with important implications for organism survival and evolution.

First, I developed a PCR-based bisulfite assay for Next-Generation sequencing for genes involved in growth, development, immune function, stress response, and metabolism (Chapter 2). Locus- and tissue-specific methylation was assessed in inbred and outbred Chinook salmon at two developmental stages (fry and yearling). This chapter established DNA methylation as a mechanism targeted to specific loci, tissues, levels of inbreeding, and developmental stages/environmental contexts.

I assessed the role of DNA methylation in the propagation of maternal effects at three early developmental stages (egg, alevin, and fry; Chapter 3). Two 6x6 fully factorial Chinook salmon breeding crosses were used to estimate maternal effects. DNA methylation was assessed using bisulfite sequencing and both locus-specific and CpG-specific maternal effects were identified. This chapter established DNA methylation as a potential mechanism for the transmission of maternal effects, which can have important influences on offspring development and fitness.

I quantified the effects of early environment on the genetic architecture of DNA methylation using 6x6 factorial crosses reared in two environments: a hatchery and a semi-natural channel (Chapter 4). Additive, non-additive, and maternal variance components, combined with environmental and GxE effects for DNA methylation were calculated. Rearing environment caused gene-specific plasticity in methylation, as well as differences in the genetic architecture of methylation. This chapter identified the importance of both genetic and environmental variation in controlling methylation, with important implications for methylation as an acclimation or adaptive mechanism.

Finally, I characterized differences in locus-specific methylation among eight populations of Chinook salmon (Chapter 5). The significant population differences in locus-specific methylation were tested for correlation with environmental variables from natal streams, and pairwise F_{ST} estimates (microsatellite and SNP data). I identified no effects of rearing environment, but a weak among-population correlation between methylation and microsatellite F_{ST} indicating that genetic drift is influencing methylation. Population-level differences in DNA methylation suggest methylation may contribute to local adaptation and is certainly an important additional source of phenotypic variation.

In conclusion, my doctoral research evaluated the role of DNA methylation from the tissue to the population level. My results support DNA methylation as a novel, potentially adaptive mechanism, contributing to normal organism function, transgenerational plasticity through maternal effects, plasticity, and population-level acclimation or adaptation.

DEDICATION

To Zach, Marvin, and Winston,
who are always there when I need them.

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CHAPTER 1 - GENERAL INTRODUCTION

The goal of quantitative genetics is to relate genetic variation (or traditionally, genetic relatedness) to specific phenotypic variation (Barton 1989; Kruuk et al. 2008; Milocco and Salazar-Ciudad 2020). Quantitative genetics thus has important implications for the evolution and fitness of organisms (Houle et al. 2010; Shaw and Etterson 2012; Gienapp et al. 2017; Milocco and Salazar-Ciudad 2020), particularly in times of rapid evolutionary change (Shaw and Etterson 2012). Traditionally, the study of natural populations has focused on measures of genetic divergence (Balloux and Lugon-Moulin 2002; Selkoe and Toonen 2006) such as differences in mitochondrial gene sequence (Selkoe and Toonen 2006) and microsatellite allele variation (Balloux and Lugon-Moulin 2002; Selkoe and Toonen 2006). New approaches involving functional genetic variation, such as single nucleotide polymorphism (SNP) assays (Wellenreuther et al. 2019) and quantitative trait loci (QTL; Kruuk et al. 2008; Gienapp et al. 2017) seek to characterize the genetic basis of phenotypic variation in natural populations at the genomic sequence level. However, the study of evolutionary genetics has expanded from solely considering sequence variant allele frequencies to considering the influences of gene expression (Mori et al. 2005; Derome et al. 2006), gene regulation (Duncan et al. 2014), chromosomal structure and inversions (Wellenreuther et al. 2019), epigenetics (Johannes et al. 2008; Banta and Richards 2018), and other sources and forms of genomic and epigenomic variation on organismal phenotype. Phenotype is the culmination of the effects of genetic variation, gene expression, and various contributing molecular mechanisms. For example, organisms with little or no genetic variation based on allelic differences can express their genes in different ways (Larsen et al. 2007), resulting in phenotypic and physiological differences in the absence of genetic variation. Thus, while

differences in DNA sequence are important for characterizing and describing populations, gene expression differences are perhaps of greater relevance when studying evolutionary and phenotypic adaptation (Whitehead and Crawford 2006a). Gene expression is one of the main traits upon which natural selection acts, thus the regulatory mechanisms behind differences in gene expression are expected to be an important basis for evolutionary change and adaptation, in contrast to the traditional focus on allelic variants (King and Wilson 1975; Whitehead and Crawford 2006a,b; López-Maury et al. 2008). However, the study of genetic and genomic (gene expression/regulation, epigenetic, etc.) sources of variation are not sufficient on their own in the study of quantitative genetics. The environment in which an organism resides is an important consideration in quantitative genetic studies, as the environment often influences gene expression (Gibson 2008; Hodgins-Davis and Townsend 2009; Grishkevich and Yanai 2013) and phenotype (Barton 1989; Kruuk et al. 2008; Houle et al. 2010; Banta and Richards 2018), resulting in phenotypic plasticity.

Phenotypic plasticity refers to the ability of a single genotype to produce different phenotypes under different environmental conditions (Mori et al. 2005; Pfennig et al. 2010; Richards et al. 2010; Torres-Dowdall et al. 2012). Plasticity has been observed across environmental clines (Bentz et al. 2011) and in response to environmental stress (Scheiner and Lyman 1989; Mori et al. 2005; Bentz et al. 2011; Torres-Dowdall et al. 2012; Wellband et al. 2018), and can result in diversification of organism phenotypes due to environmental pressures (Pfennig et al. 2010). Since plasticity can occur rapidly in response to changing environmental conditions (Pfennig et al. 2010) and may be genetically encoded (Scheiner and Lyman 1989; Pfennig et al. 2010; Richards et al. 2010;

Bentz et al. 2011), it can respond to natural selection for the capacity for an organism to exhibit plasticity (Scheiner and Lyman 1989; Pfennig et al. 2010), and lead to increased resilience in stressful and variable environments (Crispo 2008; Bentz et al. 2011; Torres-Dowdall et al. 2012). Environmental effects on the genome (Scheiner and Lyman 1989), sometimes leading to environmentally-induced non-additive interactions producing novel phenotypes (Bentz et al. 2011), are the primary sources of plasticity in organisms. Differences in the capacity for plasticity can occur among organisms; cyclical or predictably variable environments favour plasticity despite the cost of maintaining the capacity for plasticity, while stable environments favour reduced plasticity (Angers et al. 2010; Sultan and Spencer 2013). Under certain conditions, phenotypic plasticity can be passed on to offspring through intergenerational inheritance.

Intergenerational inheritance refers to the passage of non-DNA sequence-based information through the germline to progeny (Jablonka and Raz 2009; Miska and Ferguson-Smith 2016; Perez and Lehner 2019) that is unidirectional from parent to offspring. While intergenerational inheritance may or may not be adaptive, adaptive intergenerational plasticity involves the plastic response of an offspring arising from parental transmission of environmental cues in order to improve offspring fitness dependent on successful prediction of offspring environment by the parent (Galloway and Etterson 2007; Marshall 2008; Sheriff and Love 2013; Jensen et al. 2014; Donelan et al. 2020). Intergenerational plasticity includes both maternal and paternal effects (Uller 2008; Jensen et al. 2014; Shama et al. 2014; Donelan et al. 2020), which were thought to be troublesome complications in evolutionary biology until their adaptive potential recently became apparent (Mousseau and Fox 1998). Maternal effects generally have

stronger influence on offspring phenotype than paternal effects (Shama et al. 2014) due to greater female gamete size and investment into reproduction (Guillaume et al. 2016) in most (but not all) vertebrate species. On the other hand, paternal effects occur primarily through epigenetic mechanisms due to low paternal investment into offspring (Crean and Bonduriansky 2014). For adaptive intergenerational plasticity to occur, (i) environments must be heterogeneous, (ii) environments must provide parents accurate cues for the environment their offspring will experience, and (iii) the cost of transmitting plasticity to offspring must be low (Marshall and Uller 2007; Uller 2008). When parents successfully predict their offspring's environment, they can improve offspring fitness through intergenerational plasticity, which can aid in the resilience of populations to environmental stress (Jensen et al. 2014; Shama et al. 2014), though there can be complex trade-offs where intergenerational plasticity results in improved offspring performance in some aspects but decreased performance in others (Marshall 2008). However, if parents are unsuccessful at predicting offspring environment, it can result in reduced offspring fitness and survival (Galloway and Etterson 2007; Sheriff and Love 2013; Jensen et al. 2014; Shama et al. 2014). The signals underlying intergenerational plasticity can persist across multiple generations – that is, they are not limited to parent-offspring transmission, but can persist into grand-offspring generations and beyond (Donohue 2014; Donelan et al. 2020) - which is unlikely to be adaptive unless environments remain predictable or cyclical across several generations (Donohue 2014). Thus, a thorough understanding of the mechanisms underlying parental effects is necessary to understand the fitness consequences of intergenerational plasticity and its effects on the evolutionary trajectory of organisms.

Epigenetic processes have frequently been proposed as important mechanisms underlying intergenerational plasticity, non-genetic inheritance, and evolutionary response to environmental stress. Epigenetic mechanisms modify organism function and phenotype without changes in the DNA sequence (Angers et al. 2010; Richards et al. 2010), and include mechanisms such as DNA methylation, RNA interference, histone modifications, and other effects on chromosome structure (Richards et al. 2010; Miska and Ferguson-Smith 2016). Epigenetic mechanisms add another layer of complexity to our understanding of the genetic basis of phenotype, and the mechanisms underlying phenotypic plasticity and evolution (Crews et al. 2007; Angers et al. 2010; Richards et al. 2010). Modern quantitative genetic studies often focus on DNA sequence variation, though both genetic and epigenetic variation likely contribute to phenotypes, and the heritability of phenotypes (Kruuk et al. 2008; Banta and Richards 2018). Since epigenetic mechanisms are sensitive to environmental context (Angers et al. 2010) and can be passed on to offspring (Angers et al. 2010; Donohue 2014), they represent a novel short-term evolutionary mechanism to cope with environmental stimuli and increase the resilience (the ability of an organism to respond to and recover from stress) and evolutionary potential of organisms facing environmental stress (Bossdorf et al. 2008; Duncan et al. 2014; Varriale 2014; Bernatchez 2016). Genetic change is slow and generally requires generations of selection to occur, but epigenetic changes can occur rapidly in response to environmental context, and can persist in stable environments (Angers et al. 2010; Richards et al. 2010). Thus, epigenetic mechanisms present an interesting evolutionary phenomenon that can influence organismal fitness (Crews et al. 2007; Crews 2008; Angers et al. 2010) when organisms are under selection, yet can

persist within populations until genetic change can catch up (Angers et al. 2010; Richards et al. 2010). While epigenetic mechanisms have clear implications for evolutionary biology and ecology, their role in phenotypic variation and evolutionary change has not been extensively studied.

The most studied epigenetic mechanism is DNA methylation, the addition of a methyl group to the 5' carbon of a CpG cytosine base, which controls gene expression by suppressing the transcription of the methylated gene (Jaenisch and Bird 2003). Methylation is a common mechanism for gene expression regulation and can act through an organism's lifespan: it plays a role in controlling transcription during early development (Jaenisch and Bird 2003), sexual development (Morán and Pérez-Figueroa 2011), and senescence (Richardson 2003). Patterns of methylation differ among developmental phases (Mhanni and McGowan 2004; Morán and Pérez-Figueroa 2011; Fang et al. 2013) and cell types (Strömqvist et al. 2010) leading to transcriptional differences and altered physiology (Strömqvist et al. 2010; Morán and Pérez-Figueroa 2011). DNA methylation is sensitive to environmental signals which can result in phenotypic and physiological changes that align with an organism exhibiting a stress response (Jaenisch and Bird 2003; Schrey et al. 2012; Herman et al. 2014). Previous studies have shown that a wide array of stressors, including contaminants (Reamon-Buettner et al. 2008; Baccarelli and Bollati 2009; Koturbash et al. 2011), salinity change (Morán et al. 2013), changes in ambient temperature (Pecinka et al. 2010; Anastasiadi et al. 2017; Metzger and Schulte 2017), and artificial rearing environments (Le Luyer et al. 2017; Rodriguez Barreto et al. 2019) affect DNA methylation. The resultant phenotypic changes can be selected for if the environmental stressors remain and the altered

phenotype confers a selective advantage over other methylation states (Crews et al. 2007; Crews 2008; Angers et al. 2010). If environmental conditions persist, these selective pressures can result in a methylation state becoming fixed within a population (Verhoeven et al. 2010), leading to population-level epigenetic differences. Alternatively, it has been suggested that differences in DNA methylation are a form of bet-hedging via increased inter-individual variance in offspring and in response to environmental stress, providing a greater range of phenotypes for selection to act upon (Angers et al. 2010; Herman et al. 2014). Since epigenetic states can be passed down through generations (Jaenisch and Bird 2003; Uller 2008), epigenetic mechanisms likely contribute to the propagation of parental effects, either through the transmission of a beneficial epigenetically-derived phenotype (Herman et al. 2014), or through multi-generational adaptive plasticity (Angers et al. 2010; Herman et al. 2014) with implications for offspring fitness. At present, epigenetic mechanisms have been suggested as likely mechanisms for multigenerational adaptive effects, though the mechanism and genetic basis of intergenerational transfer of methylation states remain unclear (Perez and Lehner 2019). It is also unclear if DNA methylation dynamically changes in response to environmental stressors in a stochastic way, or if methylation responses are targeted to specific genes to elicit an adaptive response (Angers et al. 2010). While different mechanisms may hold true among species or among populations of a given species (Angers et al. 2010), achieving an understanding of the epigenetic basis of acclimation and population-level differentiation will revolutionize our understanding of the evolutionary mechanisms conferring fitness benefits unexplained by genetic differences.

The study of epigenetics is particularly important in light of anthropogenic climate change and habitat degradation, which place additional stress upon natural populations.

The goal of this thesis is to characterize the role of DNA methylation in the propagation of maternal effects, in response to environmental variation, and in population-level variation, all in Chinook salmon (*Oncorhynchus tshawytscha*). Chinook salmon exhibit substantial maternal effects during early life and fine-scale local adaptation (higher fitness in native vs. non-native environments) due to their large native range across habitats with considerable environmental differences. Thus, Chinook salmon are an ideal study species for this thesis, and for the study of the evolutionary significance of DNA methylation. Methylation has been hypothesized to serve as an adaptive mechanism filling the temporal gap between short-term acclimation and long-term genetic adaptation. Since DNA methylation is heritable, changes through development, and is altered by the environment, it is a potential mechanism for the transient parental effects observed in Chinook salmon, preparing offspring for a predicted early environment, responding to environmental cues as a mechanism for phenotypic plasticity, and responding to the chronic stressors populations endure.

For DNA methylation to serve as an adaptive mechanism, it must be targeted to specific genes, rather than occurring stochastically across the genome. **Chapter 2** addresses this question by developing and testing a gene-targeted bisulfite sequencing DNA methylation assay for Next-Generation sequencing in Chinook salmon. Bisulfite sequencing allows for the identification of methylated cytosine bases by leaving methylated cytosines unaffected and converting unmethylated cytosines to uracil (Figure 1.1). DNA methylation was assayed at individual genes in four tissues of freshwater fry

and ocean-dwelling yearling salmon sampled from both inbred and outbred populations. This chapter highlights the highly targeted nature of DNA methylation, and the importance of gene-specific analyses for analyzing individual variation in DNA methylation.

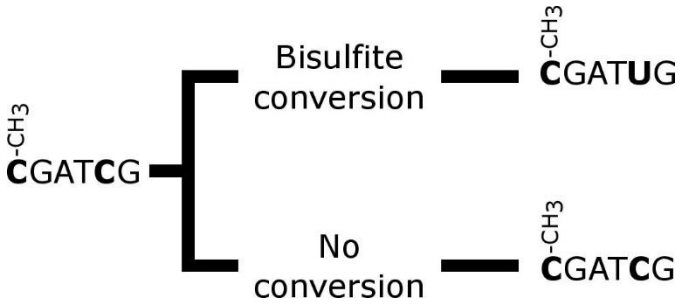

Figure 1.1: Visualization of methylated cytosines through bisulfite conversion and DNA sequencing.

To further assess the role of DNA methylation in interindividual variation, it is important to consider the role of DNA methylation in non-genetic mechanisms of inheritance such as intergenerational plasticity. Since maternal effects contribute to among-family variation and offspring fitness in juvenile Chinook salmon, **Chapter 3** assesses the role of DNA methylation in the transmission of maternal effects by combining a factorial breeding design with the gene-targeted methylation assay to measure maternal effects on locus-specific and CpG site-specific transmission of DNA methylation states in three early developmental stages. This chapter reinforces the targeted nature and strict developmental control of DNA methylation and solidifies DNA methylation as a mechanism for the propagation of maternal effects with important implications for offspring function and fitness.

While characterizing the role of DNA methylation in the propagation of maternal effects is important, it is also necessary to consider how environmental context influences the genetic architecture of traits. Many studies have reported plasticity in DNA methylation in response to a change in environmental context, yet the effects of environmental context on the genetic architecture of DNA methylation remain unclear. The genetic effects of hatchery versus natural rearing are of particular interest in salmonid conservation and supplementation efforts due to decreases in fitness following a single generation of hatchery rearing. **Chapter 4** uses a factorial breeding design and gene-targeted methylation assay to compare the genetic architecture of DNA methylation in hatchery and semi-natural reared Chinook salmon at two developmental stages. The plasticity of DNA methylation and importance of genotype x environment effects on DNA methylation is also assessed. Since DNA methylation can influence organism phenotype and fitness, understanding the genetic architecture and phenotypic plasticity of DNA methylation is an important potential mechanism underlying trait variation within and among populations.

Local adaptation and phenotypic differences are evident among natural populations, though most studies focus on genetic differences among populations. The role of DNA methylation in population-level differentiation and divergence remains unclear. **Chapter 5** measures population-level differences in gene-specific DNA methylation. Population-level methylation differences are compared to environmental variables from the populations' spawning rivers to determine whether environmental factors influence gene-specific methylation in offspring. Methylation data is also compared to neutral genetic differentiation based on microsatellite and single nucleotide

polymorphism (SNP) data to determine whether population-level differences in methylation align with divergence due to genetic drift. This study provides insight into the presence and causes of gene-specific population differences in DNA methylation, with important implications for our understanding of the role of DNA methylation in natural populations.

The work detailed in this thesis advances our knowledge of the role played by DNA methylation in ecological, evolutionary, and environmental contexts. My research advances our understanding of the role of DNA methylation in organism function, phenotypic and intergenerational plasticity, and standing non-genetic variation in populations. These studies are important early steps in understanding the genetic basis of DNA methylation and its role in evolutionary biology and response to environmental stress.

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CHAPTER 2 - INBREEDING EFFECTS ON GENE-SPECIFIC DNA METHYLATION AMONG TISSUES OF CHINOOK SALMON

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Summary

Inbreeding depression is the loss of fitness resulting from the mating of genetically-related individuals. Traditionally, the study of inbreeding depression focused on genetic effects, though recent research has identified DNA methylation as also having a role in inbreeding effects. Since inbreeding depression and DNA methylation change with age and environmental stress, DNA methylation is a likely candidate for the regulation of genes associated with inbreeding depression. Here we use a targeted, multi-gene approach to assess methylation at 22 growth, metabolic, immune, and stress-related genes. We developed PCR-based DNA methylation assays to test the effects of intense inbreeding on intragenic gene-specific methylation in inbred and outbred Chinook salmon. Inbred fish had altered methylation at three genes, CK1, GTIIBS, and hsp70, suggesting that methylation changes associated with inbreeding depression are targeted to specific genes and are not whole-genome effects. While we did not find a significant inbreeding by age interaction, we found that DNA methylation generally increases with age, though methylation decreased with age in five genes, CK1, IFNɣ, hnrL, hsc71, and FSHb, potentially due to environmental context and sexual maturation. As expected, we found methylation patterns differed among tissue types, highlighting the need for careful selection of target tissue for methylation studies. This study provides insight into the role of epigenetic effects on aging, environment and tissue function in Chinook salmon and shows that methylation is a targeted and regulated cellular process. We provide the first evidence of epigenetically-based inbreeding depression in vertebrates.

Introduction

Variation in gene expression has been documented at all biological organization levels, including among cells, tissues, individuals, populations, and species. Although all cells within an organism share the same genome, most cells are specialized for specific functions and different cell types can have drastically different phenotypes (Sul et al. 2009). This specialization is the result of differences in gene expression patterns at loci responsible for cellular function and communication, and is thus highly regulated to ensure the normal function of tissues and organs (Linney et al. 2004). Through development, gene expression can change drastically over short periods (Hashimoto and Heinrich 1997; Von Schalburg and Sherwood 1999; Lam et al. 2004; Linney et al. 2004; Helterline et al. 2007), resulting in tissues with different functions (Linney et al. 2004; Helterline et al. 2007; Sul et al. 2009). Despite strong developmental and functional constraints, variation in gene expression is observed among individuals (Linney et al. 2004; Whitehead and Crawford 2006) and among genetically similar populations (Larsen et al. 2007). Considering the breadth of variation in gene expression, little is known about the inherited, organismal, and environmental cues driving variation in gene expression.

When an organism experiences environmental stress, it responds via rapid changes in physiology and gene expression that comprise the acclimation response (Scott et al. 2004). Acclimation occurs through behavioural, physiological, or gene expression changes that work to alleviate the impacts of environmentally or experimentally induced strain (Bowler 2005). Changes in gene expression vary among individuals, resulting in a range of responses to the same stressor (Larsen et al. 2007; López-Maury et al. 2008). This variation is observed even in the absence of genetic variation, making epigenetic

effects a likely contributor (Larsen et al. 2007). Gene expression and regulation thus have evolutionary consequences (Whitehead and Crawford 2006) since heritable variation in gene expression can be selected upon when it affects the biological function and fitness of an organism, whether driven by inherited genetic or epigenetic factors (Fangue et al. 2006; Whitehead and Crawford 2006; Fisher and Oleksiak 2007). Independent of the mechanism driving the variation in gene expression, that variation can be adaptive or maladaptive.

Inbreeding depression refers to decreased offspring fitness, a maladaptive result of the mating of genetically related individuals (Su et al. 1996; Pante et al. 2001; Keller and Waller 2002; Ayroles et al. 2009; Fessehaye et al. 2009; Zajitschek et al. 2009; Biémont 2010; Kristensen et al. 2010). The two main hypotheses for the genetic basis of inbreeding depression are the dominance hypothesis, which states that increased homozygosity in inbred organisms results in the unmasking of recessive deleterious alleles, and overdominance, which suggests that heterozygotes are generally superior to homozygotes across the genome (Kristensen et al. 2010). Inbreeding depression is critical for conservation efforts as inbreeding is common in small, declining, or fragmented populations (Keller and Waller 2002). Inbreeding depression occurs across taxa (Keller and Waller 2002), and is observed phenotypically through reduced growth and body weight (Su et al. 1996; Pante et al. 2001), gamete quality (Zajitschek et al. 2009), fecundity (Su et al. 1996; Fessehaye et al. 2009), and immune function (Sarder et al. 2001; Arkush et al. 2002). The severity of inbreeding depression generally increases with age (Keller and Waller 2002) and environmental stress (Auld and Relyea 2010; Kristensen et al. 2010). Differences in expression of metabolic, stress resistance, and

protein folding/degradation genes have been reported between inbred and outbred individuals, though the molecular basis of these differences has not been determined (Kristensen et al. 2010). Thus, while dominance and overdominance effects contribute to inbreeding depression, differences in gene expression are likely an additional mechanism driving inbreeding depression (Biémont 2010; Vergeer et al. 2012) with genetic and epigenetic factors cumulatively resulting in the decreased fitness of inbred organisms. While many studies have analyzed the role of genetics in inbreeding depression, the study of epigenetic inbreeding depression is in its infancy.

One of the most studied epigenetic mechanisms known to affect gene expression is DNA methylation, which involves the addition of a methyl group to the 5' carbon of CpG cytosines (Jaenisch and Bird 2003; Morán and Pérez-Figueroa 2011). DNA methylation can occur throughout the genome with varying effects on transcription (Lorincz et al. 2004; Ball et al. 2009; Heyn et al. 2013; Kulis et al. 2013; Shenker et al. 2015). Intragenic methylation is an understudied, important regulator of gene expression (Heyn et al. 2013) resulting in the suppression of the majority of genes assayed (Heyn et al. 2013) though it has also been associated with increased gene expression (Ball et al. 2009; Heyn et al. 2013; Kulis et al. 2013) when genes are hyper- or hypomethylated (highly methylated or exhibiting low levels of methylation, respectively; Ball et al. 2009). Methylation is a common mechanism for regulating gene expression during development (Jaenisch and Bird 2003) and cell specialization (Strömqvist et al. 2010; Massicotte et al. 2011), and in tissue function (Strömqvist et al. 2010; Massicotte et al. 2011). DNA methylation also changes in response to DNA damage by silencing the damaged gene (Cuozzo et al. 2007; O'Hagan et al. 2008), and to various environmental stressors,

resulting in altered gene expression (Morán et al. 2013; Farmen et al. 2014). Since changes in DNA methylation can be transmitted through generations and result in phenotypic variation, it is an important response to changing environments (Angers et al. 2010; Donohue 2014). While recent research in plants suggests that epigenetic effects have a role in inbreeding depression, the mechanisms are not well understood (Biémont 2010; Nakamura and Hosaka 2010; Vergeer et al. 2012). Two studies, one in the perennial plant *Scabiosa columbaria* and one in the potato *Solanum chacoense*, determined that genome-wide methylation is higher in inbred relative to outbred individuals (Nakamura and Hosaka 2010; Vergeer et al. 2012). To our knowledge, no studies on epigenetically-based inbreeding depression have been reported using vertebrate models, or using methods which provide the identity of differentially methylated genes or genomic regions.

At present, studies on the role of DNA methylation either target a single gene or functional group of genes, or use methylation sensitive amplified polymorphism (MSAP), a reduced-representation whole-genome technique that quantifies differences in methylation among individuals. At best, these studies provide information on a single gene or functional group (Blouin et al. 2010). However, new methods that capitalize on massively parallel ("Next-Generation") sequencing technology provide gene-specific methylation data that can improve our understanding of how an organism's genome interacts with environmental, ontogenetic, and heritable cues to elicit an epigenetic response at specific gene loci. This approach is particularly useful for the study of non-model organisms with limited genomic data where the results of MSAP followed by sequencing of bands showing differential methylation would be difficult to interpret. The

Next-Generation sequencing approach ensures that the same CpG sites are compared among all individuals and allows the analysis of multiple CpG sites within a given gene, and can be scaled up to a large number of genes. Sequencing-based methods have high sensitivity to allow the detection of differentially methylated sites, while MSAP has been shown to have extremely low sensitivity at sites of intermediate levels of methylation (Blouin et al. 2010).

The aim of this project is to use a multi-gene bisulfite Next-Generation sequencing approach to determine the gene-specific effects of inbreeding on intragenic DNA methylation in four tissues in Chinook salmon. The inbred salmon were the highly inbred product of self-fertilization of a hormonally-induced functional hermaphrodite followed by full-sibling mating, the offspring of which showed reduced survival and growth (D. Heath, unpublished data). Since inbreeding depression effects generally increase with age (Charlesworth and Hughes 1996), we studied how inbreeding and age interact to affect DNA methylation at the single gene level. We postulate two possible scenarios for differential methylation between inbred and outbred organisms: (1) If DNA methylation is a maladaptive response to increased genetic load in inbred individuals, we expect methylation to be elevated genome-wide in inbred individuals, serving as a mechanism for the loss of fitness in inbred individuals via loss of appropriate gene transcription regulation; (2) if methylation in inbred organisms is a potentially adaptive response that silences damaged or deleterious alleles (Cuozzo et al. 2007; O'Hagan et al. 2008) or compensates for increased genetic load by activating or repressing genes, methylation or demethylation should be targeted to specific genes to reduce the severity of inbreeding depression. Since DNA methylation is energetically costly (Chiang et al.

1996), and whole-genome hypermethylation (high levels of methylation) would result in the dysregulation of genes and metabolic pathways necessary for normal cellular function, a whole-genome increase in methylation with inbreeding would be detrimental to an individual. Targeted methylation of single genes would be less energetically costly and serve as a mechanism for counteracting inbreeding depression without interfering with necessary cellular processes. We expected that differences in DNA methylation between inbred and outbred individuals would be targeted to specific genes or functional classes of genes, rather than occurring at a whole-genome level. Since inbreeding depression is known to increase with age, and age effects are associated with a locus-specific loss of function, we predicted that intragenic DNA methylation would increase with age (Richardson 2003) and with level of inbreeding (Nakamura and Hosaka 2010; Vergeer et al. 2012) in a targeted tissue- and gene-specific manner. Specifically, we predicted that the divergence of DNA methylation levels between inbred and outbred individuals would increase with age. By understanding the role DNA methylation plays in inbreeding depression, the study of inbreeding will shift from focusing on genetic effects to incorporating genetic and epigenetic effects, which will broaden our understanding of the phenotypic and physiological effects of inbreeding. Such a paradigm shift in our view of inbreeding effects will alter how conservation biologists address inbreeding in small, fragmented or captive populations of species at risk.

Methods

Study species and sampling

Saltwater yearling (age 16 months since fertilization) and freshwater fry (age eight months since fertilization) Chinook salmon were sampled in February and June of

2015, respectively, at Yellow Island Aquaculture Ltd. (YIAL). YIAL is a low-density, pesticide- and antibiotic-free commercial salmon farm on Quadra Island, BC, Canada. At each sampling, we sampled 10 fish from the outbred production stock (with a low level of inbreeding) and 10 fish of the same age from a highly inbred stock of fish (estimated inbreeding coefficient=0.63). The inbred fish were generated by hormonally treating female fish to create a functional hermaphrodite which was self-crossed to create the F1 generation (estimated inbreeding coefficient=0.5). F1 fish were subsequently crossed using full-sibling mating to produce the sampled F2 generation. Full-sib mating creates an estimated inbreeding coefficient of 0.25, thus self-fertilization followed by full-sib mating results in an inbreeding coefficient=(0.50)+(0.50x0.25)=0.63. The estimated inbreeding coefficient is likely an underestimate as our calculation assumes an initial inbreeding coefficient of 0 for the fish used to create the F1 generation. Additionally, an F_{IS} of 0.18 (ranging from -0.04 to 0.47 across eight loci) was calculated using microsatellite data for the F2 generation (J. Drown, unpublished data) using GenePop 4.2 (Raymond and Rousset 1995; Rousset 2008).

The inbred and outbred fish were incubated in freshwater vertical stack incubation trays following standard hatchery protocols. Hatched embryos were transferred to 2,500 L freshwater tanks where they were reared until nine months old, when they were transferred to saltwater net cages where they were raised following standard YIAL protocols. Fry were collected by dip-netting from the freshwater tanks and yearlings were captured by cast net in the saltwater net cages; all fish were immediately humanely euthanized. Fin, gill, liver, and spleen tissues were sampled; fin tissue is primarily skin, and is thus expected to be metabolically inactive, while the other three tissues play

important roles in metabolism and immune function. The tissues were preserved within five minutes of euthanasia in a high salt buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) for later DNA extraction.

Sample processing and bisulfite conversion

DNA was extracted from the samples using the Wizard® Genomic DNA Purification kit following manufacturer instructions. Extracted DNA was quantified using a Quant-IT PicoGreen® dsDNA Assay kit on a Victor V3 plate reader. Using this data, 500 ng of the DNA underwent bisulfite conversion using an EZ-96 DNA Methylation-Lightning kit following the provided protocol.

DNA methylation assay and Next-Generation sequencing

To quantify the proportion of methylated CpG sites, a bisulfite-sequencing PCR assay was designed for Chinook salmon. Primers specific to the bisulfite-converted DNA sequence were designed for highly conserved intragenic regions of five growth, seven immune and stress, and 10 metabolic genes using publicly available genomic DNA and mRNA GenBank sequences (22 genes total, Appendix 1). Growth- and immune-related genes were chosen since development and immune function change with level of inbreeding and age. Additionally, many genes were included that are not expressed in the assayed tissues: for these genes, we would not expect to see significant changes in methylation if methylation is targeted since the genes should not be expressed. The region between primers ranged from 25 bp to 199 bp, with a total of 2,700 bp amplified across all genes. Next-Generation sequencing libraries were developed using a two-stage PCR approach. The first PCR amplified the target gene region using gene-specific PCR

primers with overhanging adaptor sequences at the 5' end of the forward primer sequence (5'-ACCTGCCTGCCG-3') as well as the 5' end of the reverse primer (5'-ACGCCACCGAGC-3'). The first stage PCR reactions (12.5 µL) contained 1.25 µL 10x *Taq* buffer, 0.9 µL $MgCl_2$, 0.9 µL 10 mM dNTPs, 0.5 µL of each primer (10mM), 0.1 µL bovine serum albumin, 0.05 µL GenScript *Taq* polymerase, and 0.5 µL (approximately 8 ng) bisulfite-converted template DNA. PCR thermocycler conditions consisted of (i) an initial denaturation step at 95ºC for 2 min, (ii) 30 cycles of 95ºC for 30 s, 30 s at various melting temperatures, and 72ºC for 1 min, followed by (iii) a final elongation at 72ºC for 10 min. Sample amplification was verified on a 2% agarose gel run for 30 minutes at 105 V.

PCR amplicons from the first-stage PCR were pooled for each tissue in each individual and subsequently cleaned with Agencourt AMPure XP to remove primers, primer-dimer and truncated sequences. Each sample was assigned one of 384 unique IonX barcodes for multiplexing on the Ion Torrent; IonX barcodes are short, 10-12 bp oligonucleotides that allow the differentiation of samples through parallel sequencing. The IonX barcode/sequencing adaptor construct was ligated to the first stage PCR product via short-cycle PCR amplification. The second stage PCR primer construct included the complementary sequence to the overhanging sequence on the first stage PCR primer at the 3' end. The second stage PCR (ligation reaction) contained 2.5µL of 10x Taq Buffer, 1.0µL $MgCl_2$, 0.5µL 10mM dNTPs, 0.5µL of both the second stage primers (10mM), 0.1µL of *Taq* polymerase, and 10µL of cleaned PCR product. Short-cycle PCR thermocycler conditions consisted of (i) an initial denaturation at 94ºC for 2 min, (ii) six cycles at 94ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for one minute, and (iii)

one cycle at 72ºC for five minutes. The ligated products were cleaned using Agencourt AMPure XP. All samples were combined and concentrated via isopropanol precipitation and subsequently gel extracted using an EZ-10 Spin Column DNA Gel Extraction kit to ensure maximal purity. After purification, the extract was run on an Agilent 2100 Bioanalyzer using a High Sensitivity DNA kit to quantify the DNA and to ensure the sample contained no primer-dimer or short strand DNA. The concentrated, pooled DNA was diluted and sequenced on the Ion Personal Genome Machine® (PGM™) system using an Ion 318™ Chip kit with an Ion PGM™ Sequencing 400 kit; our 160 samples were sequenced with 192 other samples, thus we expected 2.3 million total reads, with an average of 180 reads per gene in a given sample and a maximum read length of 400 bp.

Data processing

The bioinformatics software mothur (Schloss et al. 2009) was used to remove low quality sequences, separate sequence data by unique IonX barcode, and remove adapter and barcode sequences. Trimmed sequences were aligned to existing sequence data for the locus of interest using the bwa-meth add-on (Pedersen et al. 2014) for the Burrows-Wheeler Aligner (Li and Durbin 2009). Multiple GenBank sequences were used, and the sequencing accuracy was verified for each gene using existing Next-Generation sequencing or pyrosequencing data generated prior to assay design. A maximum of two alignment mismatches were allowed to ensure that the aligned sequence was consistent with the locus of interest, and not a product of non-specific binding and genome simplification typical of bisulfite sequencing PCR. The use of conserved gene regions ensured that individuals were homozygous for the genes assayed. Bwa-meth generated a

data table for each sample with a summary of the number of methylated and unmethylated cytosines at each CpG site detected.

Statistical analysis

All statistical analyses were conducted using R software (R Development Core Team 2016). Data tables generated by bwa-meth were imported into R for statistical analysis. Initially, we analyzed the combined CpG site sequence data across all individuals, tissues and treatments to ensure even distribution of sample sizes. CpG sites that were represented in less than 80% of individuals (with a minimum of five sequences for each individual) were excluded from the analysis for all individuals to ensure that the same number and position of CpG sites were compared across all individuals and tissues. This removed CpG sites with low coverage across individuals, tissues and treatments due to truncated sequencing. Additionally, CpG sites detected by bwa-meth that occurred at low abundances in a single individual were excluded as they were likely sequencing artefacts. Quality control also ensured that multiple bisulfite-converted sequences were used in the analysis for each individual; using more sequences gives a more accurate estimate of the proportion of methylation at each CpG in each individual. The average percent methylation at each gene was determined by averaging the percent methylation estimates for individual CpG sites within a gene. The Shapiro-Wilk test for normality was performed on the average percent methylation data for each gene by treatment and by tissue in addition to checking normality visually using q-q plots. Outliers were excluded using Rosner's test for outliers; significance levels for the tests were adjusted with the Bonferroni correction for multiple comparisons to ensure only extreme outliers were identified. Outliers were likely due to low sequence coverage in a given sample at a

particular locus (i.e. the site was sequenced in at least 80% of individuals, but the outlier likely had low sequencing depth resulting in an inaccurate estimate of percent methylation at that locus) rather than a biological effect. We tested for the fixed effects of age and inbreeding, in addition to two-way interaction effects, on DNA methylation for each tissue separately using a two-way analysis of variance (ANOVA) for each gene. P-values were adjusted using the Benjamini-Hochberg procedure for false discovery rates, a powerful method which is less conservative than the Bonferroni correction and allows for the detection of biologically relevant population effects (Narum 2006).

Results

Our analysis included data for 100 out of an expected 153 CpG sites (65% based on consensus sequence data) across the 22 gene regions after quality control. Twenty-two outlier values representing the percent methylation for specific tissue, individual, and gene region were excluded out of a total of 3,520 measurements (0.63%). We found considerable variation in methylation levels with age and inbreeding status (Figure 2.1), but no significant difference in methylation by age (Figure 2.1A) or inbreeding status (Figure 2.1B) or their interactions were observed for 10 of the 22 gene regions included in the study. The methylation status of 11 genes was significantly affected by age, while three genes showed a significant inbreeding effect after FDR correction. No significant age by inbreeding interaction effects were detected.

Age effects

For the 11 genes that showed an age effect on methylation levels, DNA methylation increased with age in six of the genes in at least one tissue (Figure 2.2). Four

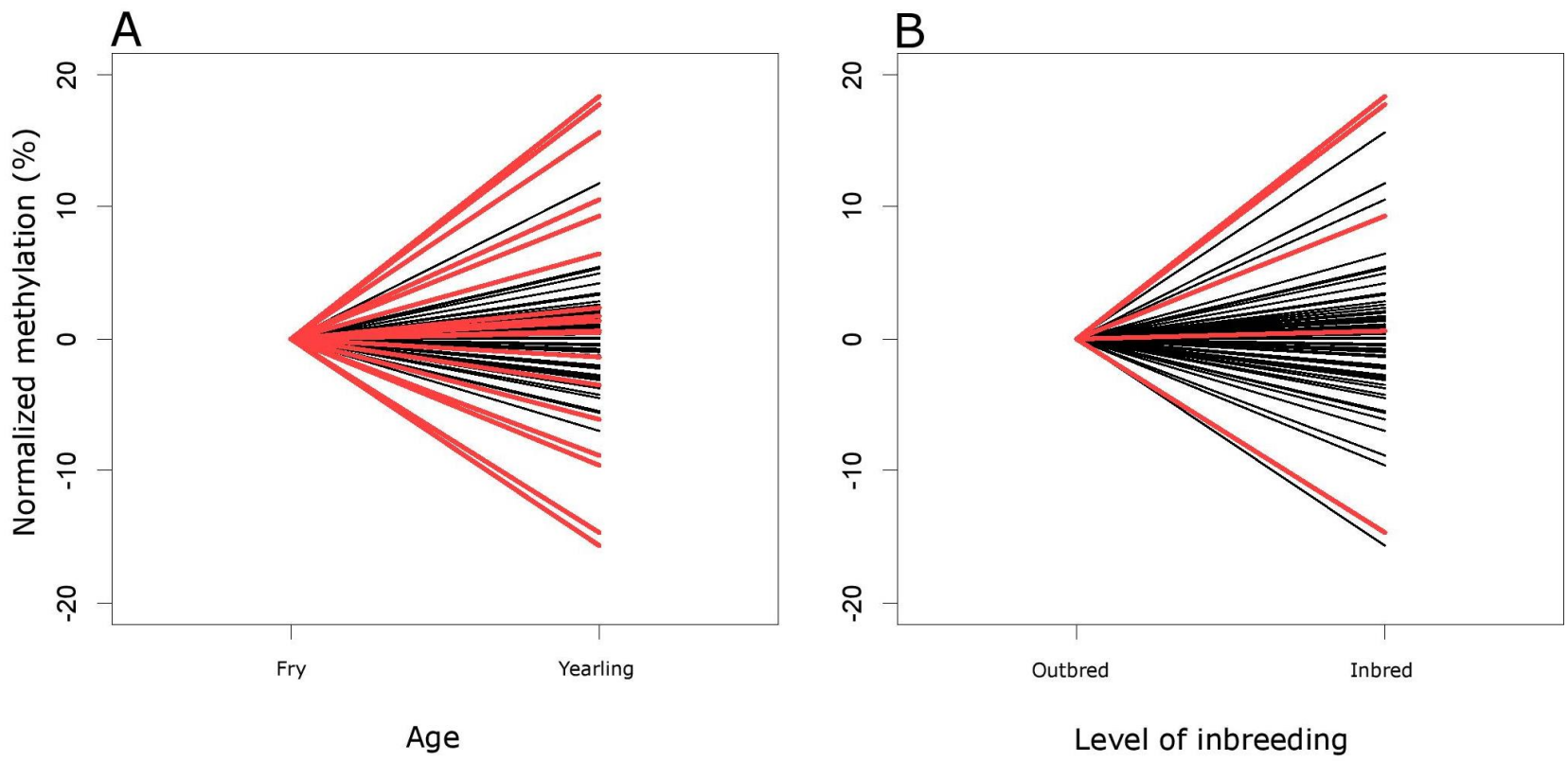

Figure 2.1: Line graphs showing the change in percent methylation with age (A) and level of inbreeding (B) for 22 genes across four tissues. Estimates of change in methylation were normalized by subtracting the mean methylation values for the fry stage and the outbred population fish, thus the normalized methylation for the fry and outbred individuals are zero. A positive slope indicates increased methylation with age or level of inbreeding, while a negative slope indicates decreased methylation with age or level of inbreeding. Statistically significant differences are denoted by red lines, while non-significant results are displayed in black.

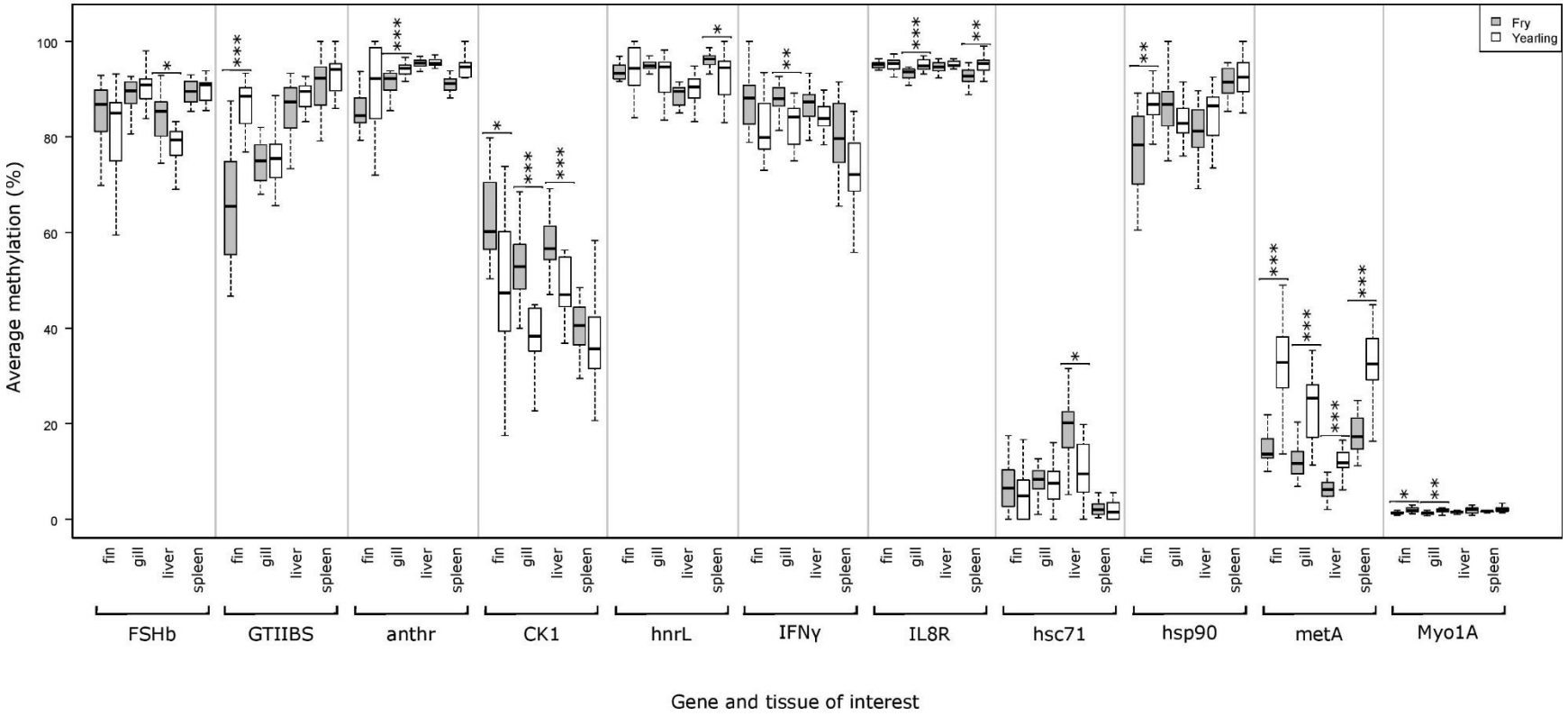

Figure 2.2: Box plots showing mean DNA methylation within the eleven genes that showed significant age effects (fry versus yearling) on DNA methylation in at least one tissue (all tissue data shown across all CpG sites). *p<0.05; **p<0.01, ***p<0.001

genes showed significant effects in more than one tissue: metA in all four tissues, CK1 and IL8R in three tissues, and Myo1A in two tissues (Figure 2.2). Gill showed the highest number of significant effects, while spleen showed the least. Five genes showed decreased methylation with age ($p<0.05$ after FDR): CK1 (fin, gill, and liver), IFNɣ (gill), hnrL (spleen), FSHb (liver), and hsc71 (liver).

Inbreeding effects

Three genes were differentially methylated between inbred and outbred fish (Figure 2.3): CK1 in gill and spleen, GTIIBS in liver and spleen, and hsp70 in fin ($p<0.05$ after FDR correction). Fish from the inbred stock had higher DNA methylation than outbred fish for CK1 and GTIIBS in all tissues (but statistically significant in only two tissues; Figure 2.3). Fin tissue showed differential methylation between inbred and outbred fish for hsp70, with outbred fish showing higher methylation than inbred fish (Figure 2.3).

Age by inbreeding interaction

Before Benjamini-Hochberg false discovery rate (FDR) correction, significant age by inbreeding interaction effects were found in FSHb, GTIIBS, hsp70, metA, IL8R, hsp47, hsp70a, CK1, hnrL, and anthr in at least one tissue ($0.046 \geq p \geq 0.005$ before FDR correction). Some genes showed an increased difference in methylation between inbred and outbred organisms with age, while other genes showed that inbred organisms increased methylation with age while outbred fish decreased methylation (or vice versa). However, the two-way ANOVA revealed no significant effect of inbreeding by age interaction after FDR correction.

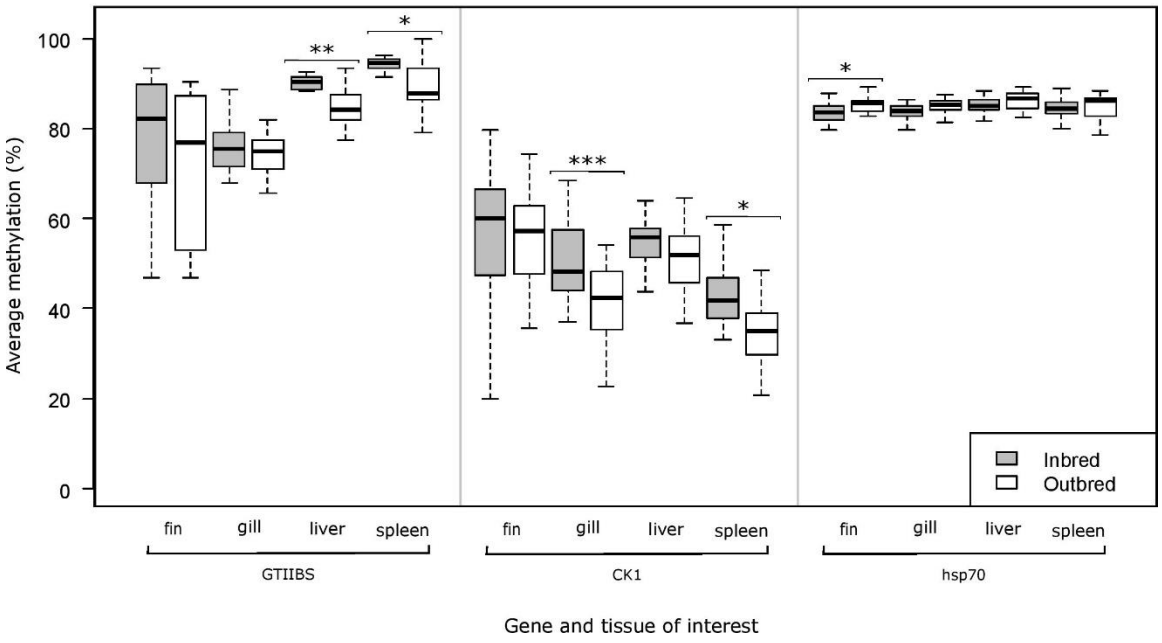

Figure 2.3: Box plots showing mean DNA methylation for the three genes with significant inbreeding effects (inbred versus outbred) on DNA methylation in at least one tissue. *P<0.05; **P<0.01

Discussion

The study of the genetics of inbreeding depression traditionally focuses on the role of recessive deleterious alleles and the loss of heterotic effects; however, there is increasing interest in determining the role of epigenetics in inbreeding depression (Biémont 2010; Vergeer et al. 2012). Epigenetic changes in the regulation of genes could result in increased or reduced inbreeding depression, independent of genetic differences between inbred and outbred organisms (Nakamura and Hosaka 2010; Vergeer et al. 2012), thus epigenetic gene regulation could modify the more traditionally accepted paradigm of inbreeding depression. We found higher methylation in the inbred stock relative to the outbred fish in two genes: chemokine-1 (CK1) in gill and spleen, and gonadotropin-II beta subunit (GTIIBS) in liver and spleen. Heat shock protein 70 (hsp70)

showed decreased methylation in fin tissue of inbred fish. CK1 localizes white blood cells to sites of infection and is an important regulator of the immune system (Lally et al. 2003). GTIIBS stimulates gametogenesis by regulating the amount of gonadotropins produced within a cell, and is expressed throughout gametogenesis in both mature and immature fish (Gomez et al. 1999). Previous studies determined that inbred fish have increased infection rates and severity of infection (Sarder et al. 2001; Arkush et al. 2002), decreased growth (Su et al. 1996; Pante et al. 2001), decreased egg production (Su et al. 1996), and reduced sperm quality relative to outbred populations (Fessehaye et al. 2009; Zajitschek et al. 2009). Since the observed increase in methylation of CK1 and GTIIBS is likely to result in their suppression, our results support our hypothesis that DNA methylation plays a mechanistic role in the physiological changes associated with inbreeding depression. With inbreeding, offspring are more likely to inherit genetic and epigenetic factors that are identical by descent which can have downstream effects on gene expression. Reduced expression or dysregulation of certain genes would prevent normal cellular function and organismal development in the early stages of life. Thus, dysregulation of both alleles of genes critical for normal cellular function and development should be purged due to high embryonic and early life mortality (Keller and Waller 2002; Pedersen et al. 2005). However, the effects of dysregulation in immune and reproductive genes, such as CK1 and GTIIBS respectively, would not interfere with normal cellular function, though it could result in the decreased immune resistance and reproductive success characteristic of inbreeding depression. Inbreeding depression is also associated with the expression of damaged or deleterious alleles, which can result in protein instability (Kristensen et al. 2002; Pedersen et al. 2005; Cheng et al. 2006). Hsp70

acts as a chaperone to ensure the proper folding of proteins (Pedersen et al. 2005; Cheng et al. 2006; Kristensen et al. 2010). Increased hsp70 expression has been reported in inbred *Drosophila buzzatii* (Kristensen et al. 2002), *D. melanogaster* (Kristensen et al. 2002; Pedersen et al. 2005; Ayroles et al. 2009), and Pacific abalone *Haliotis discus hannai* Ino (Cheng et al. 2006) in the absence of heat stress, which is consistent with the lower hsp70 methylation we observed in inbred fish. The increased expression of hsp70 in inbred organisms is hypothesized to be an adaptive response to the genetic stress associated with the unmasking of deleterious alleles (Pedersen et al. 2005). Thus, our study shows differences of methylation between inbred and outbred fish that are targeted to specific genes associated with inbreeding. Our results lead to the exciting possibility that epigenetic factors may alter dominance effects in inbreeding depression; however further research is necessary to determine the inheritance and mechanisms of putative epigenetic dominance effects.

To our knowledge, there are only two other studies on epigenetically-based inbreeding depression and both used plant models. Both studies used methylation sensitive amplified polymorphism (MSAP) and showed that DNA methylation was higher in inbred lines (Nakamura and Hosaka 2010; Vergeer et al. 2012). Our study is the first to employ targeted, qualitative analysis of sites of DNA methylation among inbred and outbred individuals, and the first to provide evidence for an epigenetic role in inbreeding depression in vertebrates. Most DNA methylation studies use MSAP due to its speed and simplicity, though it does not provide qualitative data (i.e. the identity of differentially methylated genes) and can be insensitive at intermediate or low levels of methylation (Blouin et al. 2010). Bisulfite sequencing-based methylation studies often

target a single gene or functional gene cluster, and while that approach is suitable for medical studies or studies focusing on a single known stressor under controlled conditions, the study of evolutionary and interactive epigenetics requires broader, gene-specific data. We re-analyzed our methylation data by combining the data across all 22 genes and used a Bonferroni-corrected two-way ANOVA for the fixed effects of inbreeding and age and their interaction for each tissue and found no significant effect of inbreeding or age on DNA methylation across the 100 CpG sites in our study. While our combined analysis only represents 22 functional genes out of the whole genome, it demonstrates that a targeted, gene-by-gene sequencing approach is more sensitive to differences in DNA methylation than a broad genome-wide approach. Large-scale targeted sequencing methods provide high-resolution quantitative and qualitative data on the synergistic effects of treatments, and allow a greater understanding of how an individual's epigenome responds to complex mixtures of environmental, developmental, and inherited signals.

We sampled fish at different ages to test for the effects of the interaction between aging and inbreeding on gene-specific DNA methylation as the severity of inbreeding depression has been shown to increase with age (Charlesworth and Hughes 1996; Keller and Waller 2002). We did not detect significant age by inbreeding interaction effects; however, it is important to note that fry-to-yearling development is confounded by the accompanying freshwater to saltwater transition. Since the severity of inbreeding depression is dependent on environmental context (Keller and Waller 2002) and age is confounded with environmental change in our study, it is possible that the magnitude of the age effect on inbreeding could be masked by the epigenetic response to the seawater

environment taking precedent. Alternatively, it is possible that our temporal sampling of the fish was too close together (eight months difference in age) to detect a strong aging effect on inbreeding-related DNA methylation.

We found strong age effects on DNA methylation in Chinook salmon that were independent of inbreeding effects. Previous studies have shown that whole-genome methylation decreases with age in a tissue- and gene-specific manner in vertebrates (Richardson 2003; Christensen et al. 2009). Our results indicate that DNA methylation increases in a tissue-specific manner with age (encompassing the fry-to-yearling transition) in CpG intragenic regions of six out of 22 genes. Our observation of increased methylation between fry and yearlings is not likely a result of the stress associated with moving to seawater, as DNA methylation changes associated with the transfer to seawater in salmonids are often transient (Morán et al. 2013) and the yearling fish had been in the salt water for over 10 months. It is more likely that the pattern of change in the methylation of specific genes in Chinook salmon is a result of the aging process, more specifically, ontogenetic developmental changes. Five genes, CK1, interferon gamma (IFNɣ), heterogeneous nuclear ribonucleoprotein L (hnrL), heat shock cognate protein 71 (hsc71), and follicle stimulating hormone beta (FSHb) showed decreased methylation with age. Chemokines and interferons are involved in the localization of leukocytes to sites of infection (Alejo and Tafalla 2011). HnrL is required for alternative splicing of cell membrane proteins during lymphocyte activation (Oberdoerffer et al. 2008; de la Grange et al. 2010). Hsc71 is a constitutively expressed heat shock protein (Basu et al. 2002; Deane and Woo 2004) involved in osmoregulation (Deane and Woo 2004) and immune response (Basu et al. 2002; Srivastava 2002). Thus our results suggest an

epigenetic component to previously-demonstrated elevated immune capacity associated with saltwater acclimation in fish (Boutet et al. 2006; Taylor et al. 2007). Expression of IFNγ is not associated with preparation for smoltification in salmonids (Das et al. 2007), suggesting that the decrease in methylation we observed is due to an environmental effect (i.e. transfer to seawater) rather than a developmental effect. This is consistent with previous literature reporting increased expression of immune-related genes, including cytokines, and interferon-related genes, in seawater-acclimated European seabass compared with freshwater-acclimated fish (Boutet et al. 2006). Transfer of non-smolting rainbow trout from freshwater to saltwater resulted in an immediate and sustained doubling of white blood cell concentrations (Taylor et al. 2007). HnrL is upregulated in the spleen (de la Grange et al. 2010) and in stimulated T cells during immune challenge resulting in antigen splicing and T cell activation (Oberdoerffer et al. 2008; de la Grange et al. 2010). Additionally, hsc71 expression has been shown to increase in the liver of sea bream (*Sparus sarba*) upon transfer to high salinity environments as an environmental response to osmotic stress (Deane and Woo 2004). Overall, our results suggest that fish transferred to saltwater acclimate to the marine-related immune and salinity challenge via targeted, reduced DNA methylation of immune genes in a tissue-specific manner. FSHb is the only non-immune gene which showed decreased methylation in the yearling salmon; it regulates steroidogenesis (Schulz et al. 2001; Yaron et al. 2003; Zhou et al. 2010), vitellogenesis (Yaron et al. 2003; Zhou et al. 2010), and testicular development (Schulz et al. 2001) in fish. It is primarily released from the pituitary (Yaron et al. 2003; Zhou et al. 2010) and present at high levels in the blood of sexually immature Coho salmon (Yaron et al. 2003). We found decreased methylation of FSHb in liver tissue of

Chinook salmon. FSHb causes the release of estradiol from the ovaries and subsequent release of vitellogenin from the liver (Yaron et al. 2003). Since previous studies on the tissue-specificity of FSHb gene expression used real-time PCR (Zhou et al. 2010), it is possible that FSHb is expressed at very low levels in the liver, outside the range of sensitivity (Lemmon and Gardner 2008). The observed decrease in methylation of liver FSHb may reflect the divestment of energy into sexual development during the saltwater stage of salmonids. However, it seems more likely that reduced intragenic methylation with age may indicate the suppression of FSHb in the liver since intragenic methylation is sometimes associated with gene suppression (Ball et al. 2009; Heyn et al. 2013; Kulis et al. 2013). Overall, intragenic DNA methylation increases with age in Chinook salmon; however, the genes that depart from this expectation, along with the previous research on seawater acclimation in fish, support the dynamic and potentially adaptive role of epigenetic gene regulation in Chinook salmon.

We found no differences in the methylation levels of 10 genes regardless of age or level of inbreeding. A subset of these genes are not transcribed in the tissues sampled, including brain-derived neurotrophic factor (BDNF) which is highly expressed in the central nervous system (Conner et al. 1997), recombination activating gene (RAG1) which is predominantly expressed in the thymus (Hansen and Kaattari 1995), and growth hormone 1 (GH1) and pituitary-specific transcription factor (pit1) which are primarily expressed in the pituitary gland (Su et al. 2004). These genes were included in the study to determine if DNA methylation was targeted to specific genes, or if inbreeding depression and aging effects were non-specific and distributed across the genome. We also included constitutively expressed genes in our panel of genes; these genes include

transferrin (Tf; Stafford and Belosevic 2003), heat shock proteins (Basu et al. 2002), cellular metabolism genes such as inosine triphosphatase (itpa; Burnstock 2012) and tumour antigen p53 (p53; Puzio-Kuter 2011), and natural killer enhancement factor (Nkef) which is expressed in all tissues (Zhang et al. 2001). We did not expect the genes known to be primarily expressed in tissues not sampled in this study to show inbreeding or age methylation if methylation is a targeted epigenetic response. Dysregulated methylation of the constitutively expressed genes would have incremental detrimental effects, including reduced ability to transport iron through the body (Stafford and Belosevic 2003) or maintain basic cellular structure and function (Basu et al. 2002). As we found that none of those selected gene regions showed a methylation response to either age or inbreeding, we conclude that DNA methylation is targeted to specific genes and may be part of an adaptive response to genetic and environmental stress.

The purpose of our study was to explore the role of epigenetic effects in inbreeding depression, and more specifically, the effects of inbreeding on gene-specific DNA methylation across tissues, and the interactions between inbreeding and age in Chinook salmon. Epigenetic studies have shown that intragenic DNA methylation increases through ontogeny and whole-genome methylation increases with inbreeding depression. We determined that both inbreeding and age affect DNA methylation in a locus-specific manner, which supports DNA methylation's potential role as an adaptive epigenetic response to inbreeding and environmental challenges. We provide the first evidence that DNA methylation plays a role in inbreeding depression in vertebrates, and that gene-specific methylation changes are associated with inbreeding depression. This study highlights the advantages of a targeted approach to studying DNA methylation as

our targeted multigene approach provides a mechanistic understanding of how DNA methylation may affect phenotype in response to environmental, ontogenetic, and evolutionary factors.

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CHAPTER 3 - DNA METHYLATION PROFILES SUGGEST INTERGENERATIONAL TRANSFER OF MATERNAL EFFECTS

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Summary

The view of maternal effects (non-genetic maternal environmental influence on offspring phenotype) has changed from one of distracting complications in evolutionary genetics to an important evolutionary mechanism for improving offspring fitness. Recent studies have shown that maternal effects act as an adaptive mechanism to prepare offspring for stressful environments. Although research into the magnitude of maternal effects is abundant, the molecular mechanisms of maternal influences on offspring phenotypic variation are not fully understood. Despite recent work identifying DNA methylation as a potential mechanism of non-genetic inheritance, currently proposed links between DNA methylation and parental effects are indirect and primarily involve genomic imprinting. We combined a factorial breeding design and gene-targeted sequencing methods to assess inheritance of methylation during early life stages at 14 genes involved in growth, development, metabolism, stress response and immune function of Chinook salmon (*Oncorhynchus tshawytscha*). We found little evidence for additive or non-additive genetic effects acting on methylation levels during early development; however, we detected significant maternal effects. Consistent with conventional maternal effect data, maternal effects on methylation declined through development and were replaced with non-additive effects when offspring began exogenous feeding. We mapped methylation at individual CpG sites across the selected candidate genes to test for variation in site-specific methylation profiles and found significant maternal effects at selected CpG sites that also declined with development stage. While intergenerational inheritance of methylated DNA is controversial, we show

that CpG-specific methylation may function as an underlying molecular mechanism for maternal effects, with important implications for offspring fitness.

Introduction

Maternal effects have been shown to affect offspring and maternal fitness (Galloway and Etterson 2007; Aykanat et al. 2012a; Perez et al. 2017; Fan et al. 2019) and can contribute to patterns of local adaptation (Wolf and Wade 2016). Traditionally, maternal effects were thought to be driven primarily by gamete size and maternal loading of gametes with hormones, proteins, mRNA, and energy stores (Nodine and Bartel 2012; Perez et al. 2017) although other mechanisms have been identified (Heath et al. 1999; Aykanat et al. 2012b; Nodine and Bartel 2012; Videvall et al. 2016; Falica et al. 2017). Maternal effects can affect offspring gene expression patterns (Aykanat et al. 2012b; Nodine and Bartel 2012; Videvall et al. 2016), and for these effects to be adaptive, they must be targeted to specific genes, though the mechanisms for intergenerational control of early life gene expression remain unclear. Previous research has identified maternal effects driven by epigenetic mechanisms, including transmission of small RNAs, histone modifications and parent-specific genetic imprinting to offspring (Feng et al. 2010).

Genetic imprinting, the monoallelic expression of one parent's genes in offspring, has been extensively studied in mammals and DNA methylation shown to be a contributing mechanism (Inoue et al. 2017). However, methylation is often reset at fertilization in animals such as fish, thus the mechanisms behind intergenerational inheritance of methylation are unclear (Perez and Lehner 2019). In zebrafish, DNA methylation is reset almost immediately after fertilization (Mhanni and McGowan 2004) and subsequent de novo methylation occurs (Mhanni and McGowan 2004; Mackay et al.

2007), after which sperm DNA becomes hypermethylated (highly methylated) compared to oocyte DNA in newly fertilized embryos (Mhanni and McGowan 2004; Jiang et al. 2013). Paternal methylation patterns are retained through early development, but maternal methylation patterns are lost by the midblastula stage and altered to resemble paternal methylation patterns (Jiang et al. 2013; Potok et al. 2013). While overall changes in early developmental methylation landscapes suggest that methylation may serve as a conduit for parental effects in fish (Perez and Lehner 2019), gene-specific methylation changes still occur at developmentally critical loci (Fang et al. 2013). As development progresses, fluxes in methylation levels occur (Mhanni and McGowan 2004) before stabilizing to the same levels as adult somatic tissue around the time of gastrulation (Fang et al. 2013).

Regardless of the pattern of loss of maternal methylation signatures during early development, maternal effects on offspring methylation have been reported. Since methylation landscapes differ considerably between early embryogenesis and hatching in zebrafish (McGaughey et al. 2014), DNA methylation remains a possible mechanism for the propagation of maternal effects despite genomic imprinting and resetting of methylation, reported in previous research. DNA methylation is sensitive to environmental changes, such as developmental differences (Anastasiadi et al. 2017), and inter-species variation in global methylation based on temperature (Varriale and Bernardi 2006), altered gene methylation due to seawater acclimation in brown trout (Morán et al. 2013), and hatchery-induced methylation changes in Coho salmon (Le Luyer et al. 2017). Since methylation is affected by developmental stage and environment, it is possible that maternal effects are propagated through methylation in response to the maternal

environment, with offspring gaining autonomy over methylation later in development. Previous research has shown that maternal food deprivation resulted in altered offspring gene expression and increased mortality in zebrafish (Fan et al. 2019). Exposure of female zebrafish to BPA resulted in transgenerational effects on offspring gene expression and promoter methylation up to the F3 generation (Santangeli et al. 2019). Similar effects were reported on offspring promoter DNA methylation levels up to the F3 generation when adult zebrafish were subjected to ionizing radiation (Kamstra et al. 2018). Strong family effects on DNA methylation have been reported in stickleback, which suggests a role for DNA methylation in generating inter-individual variation (Metzger and Schulte 2018). It is possible that variation in DNA methylation among families reflects intergenerational epigenetic inheritance or maternal effects (Metzger and Schulte 2018), thus epigenetic mechanisms other than imprinting are likely responsible for maternal effects on gene expression later in development. However, it is unclear whether DNA methylation is also responsible for intergenerational fine-tuning of offspring gene expression levels. For methylation to be a viable mechanism for the transmission of maternal effects, it must be targeted to specific non-canalized genes reflecting the mother's environmental experiences and genotype, but not affect genes with highly canalized expression. In contrast, random intergenerational epigenetic inheritance would align with the antiquated view of maternal effects as physiological side effects (e.g. Mousseau & Fox, 1998).

Chinook salmon (*Oncorhynchus tshawytscha)* are an ideal species for the study of maternal effects as they show high levels of individual variation in fitness-related and life history traits (Fraser et al. 2011) as well as robust maternal effects (Aykanat et al.,

2012a,b; Falica et al., 2017; Heath et al., 1999), including strong maternal effects on early life gene transcription patterns (Wellband et al. 2018). Chinook salmon have a semelparous life history where a single, terminal reproductive event (Heath et al. 1999) results in strong selection to maximize the adaptive value of maternal effects through their downstream effects on offspring fitness. Furthermore, Chinook salmon are externally fertilized and receive no parental care, allowing for sophisticated breeding designs but avoiding confounding effects of parental care or behavioural variation.

To quantify the role of DNA methylation in the propagation of maternal effects, we created replicated full-factorial (6x6 North Carolina II design) Chinook salmon crosses and estimated genetic variance components for DNA methylation levels at 14 gene loci. We selected genes involved in growth, developmental control, metabolism, stress response, and immune function (Appendix 1). We used massively parallel ("Next Generation") bisulfite sequencing in a gene-targeted DNA methylation assay for offspring from the replicated 6x6 crosses over a total of 76 CpG sites at three early developmental stages (864 offspring in total): eyed egg (embryo), alevin (larval) and fry (post-exogenous feeding). We hypothesized that if maternal effects are adaptively affecting offspring DNA methylation profiles, they would be gene-specific (Venney et al. 2016) and targeted to specific CpG sites within genes, as random-acting maternal effects during this highly regulated developmental period would be expected to be maladaptive. We further predicted that maternal influences on offspring methylation patterns should decline through development, consistent with phenotypic observations of maternal effects in salmon (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017) and in other taxa (Mousseau et al. 2009) as the offspring gains control over their genome and phenome.

Maternal effects are thought to decline during development due to a parent-offspring conflict between the mother, who predicts the offspring's environment based on her experience, and the offspring, which seeks to maximize its own fitness based on its actual environmental experience (Heath et al. 1999; Crespi and Semeniuk 2004; Falica and Higgs 2013). Maternal effects on methylation would control early-life offspring methylation based on maternal predictions of offspring environment. However, if these predictions are not accurate depictions of offspring environment, parent-offspring conflict may arise either due to (i) offspring experiencing stressful conditions and attempting to respond to their environment despite maternal influences, or (ii) mothers incorrectly predicting a stressful environment for offspring, resulting in increased offspring energetic investment into anticipating non-existent environmental stress. Despite the resetting of DNA methylation, maternal effects are successfully passed to offspring and persist until offspring gain autonomy over their own development and function. A molecular mechanism (such as DNA methylation) for maternal effects would be consistent with observed strong maternal effects across taxa, coupled with the growing realization that maternal effects likely evolved as an intergenerational signalling process that facilitates rapid adaptation to variable environments.

Methods

Breeding design and sampling

On October 31st, 2014, two North Carolina II breeding crosses were set up using Chinook salmon at Yellow Island Aquaculture, Ltd (YIAL), a commercial salmon farm on Quadra Island, BC, Canada. Sexually mature males and females (ages three to five years old) were selected for the breeding experiment, with the first cross created using

parents who had been transferred to freshwater tanks and the second cross using parents from saltwater cages. Each of the two crosses were generated by mating 6 sires with 6 dams in a factorial design, resulting in 36 families per cross (72 families total). The North Carolina II mating design allows variance to be partitioned to maternal effects by subtracting the sire (additive) component from the dam (additive + maternal) component of variance. Since Chinook salmon die after reproducing, their offspring receive no parental care, and thus any maternal effects are due to underlying egg provisioning or molecular maternal signals.

The fertilized eggs were incubated in freshwater vertical stack incubation trays following standard YIAL protocols, with two replicate cells allotted to each half-sib family. Eyed eggs were sampled from each replicate on December 19th, 2014 (~300 ATUs, 49 days since fertilization). Alevins were humanely euthanized and sampled on March 2nd, 2015 (~700 ATUs, 123 days since fertilization). The remaining alevins were transferred to 200 L freshwater tanks where they were reared until the fry stage. For the transfer, the two replicate incubation cells were pooled, and each mixed family was divided between two replicate rearing tanks. On May 6th, 2015, fry were collected by dip netting, humanely euthanized and sampled. Whole fish or eyed egg samples from all developmental stages were preserved immediately in a high salt buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) for later analysis. Fry were cut open to promote preservation. A total of four fish (two per replicate cell) per full-sibling family were used for each developmental stage.

DNA extraction and processing

Embryos from eyed eggs were dissected from the yolk and digested in 1000 µL of digestion buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS) with 10 µL of proteinase K. Alevins were cut in half and both halves digested in 6000 µL of digestion buffer with 10 µL of proteinase K (20 mg/mL). The fry (who had their livers removed for another experiment) were cut into three pieces, and the three pieces were digested together in 7000 µL of digestion buffer with 10 µL of proteinase K. While the liver is an important tissue for regulating growth and metabolism, it represented a minute portion of the total body mass of the fish, and thus the loss of DNA from the liver is not expected to affect our results. All samples were digested at 37ºC for 24 hours, and 150 µL of the digested product was used for DNA extraction using a high-throughput plate-based extraction protocol (Elphinstone et al. 2003).

Bisulfite conversion and DNA methylation assay

Extracted DNA was quantified using a Quant-IT PicoGreen® dsDNA Assay kit (ThermoFisher Scientific P11496) and 500 ng of DNA underwent bisulfite conversion with an EZ-96 DNA Methylation-Lightning kit (Zymo Research D5033) following the manufacturer's protocol. Bisulfite conversion allows for the analysis of sites of methylation: methylated cytosines are unaffected while unmethylated cytosines are converted to uracil.

Published bisulfite sequencing primers for 14 highly conserved genes involved in early development, metabolism, and stress response were used for methylation analysis (Venney et al. 2016). Primers were designed for intragenic exon gene regions with little

to no sequence variation to minimize the effects on genetic variation on methylation analysis (Venney et al. 2016). Genes were primarily chosen based on their role in early growth and differentiation, protecting the developing fish from environmental stress, and metabolic regulation. Metabolic and developmental gene expression is highly conserved; thus, these loci are unlikely targets for maternal effects on DNA methylation. Genes with less canalized expression, such as stress and immune genes, are likely candidates for maternal effects. An expected 2249 bp were amplified across the 14 genes ranging from 79 to 225 bp per gene; estimates of fragment length exclude primer sequences.

A two-stage PCR approach and sequencing protocol (Venney et al. 2016) was used to generate bisulfite sequencing libraries, which were sequenced using an Ion PGM™ Sequencing 400 kit with an Ion 318™ Chip for the Ion Torrent Personal Genome Machine® (PGM™). The samples were spread across 4 sequencing runs with an expected 500 reads per gene with a maximum length of 400 bp.

Data processing

Using the program mothur (Schloss et al. 2009), the sequencing runs were demultiplexed to create one sequence file per individual and primer sequences were trimmed. The program bwa-meth (Pedersen et al. 2014) was used to align the generated sequence data to existing sequence data for the genes of interest. The use of highly conserved genes in our methylation assay, as well as allowing a maximum of two alignment mismatches in bwa-meth, ensured that the aligned sequences represented the targeted genes. Bwa-meth generated a data table with the percent methylation for each CpG site for each gene in each individual.

Statistical analysis

Bwa-meth data tables were imported into R (R Development Core Team 2016), which was used for all statistical analyses. Data for all individuals was analyzed, and CpG sites successfully sequenced in less than 70% of individuals (with less than 5 reads per gene per individual) were excluded from the analysis to ensure the represented CpG sites were compared across all individuals.

Linear mixed models (LMM) were run in the R package lme4 (Bates et al. 2015). To determine if maternal effects were targeted to specific loci, an LMM was used to test the effects of dam, sire, dam x sire interaction, gene, and the random effects of (6x6 factorial) cross and replicate Heath tray cell on gene methylation across all loci for each developmental stage. To determine which genes were driving significant effects, an LMM was run for each gene in each developmental stage to determine whether dam, sire, dam x sire, cross, and replicate significantly affected locus-specific DNA methylation. Replicate did not significantly affect methylation and was removed from the final model. Cross was retained in the final model as it was significant for at least one gene before Bonferroni correction but non-significant after correcting for multiple comparisons. A Bonferroni correction was used to correct for multiple comparisons.

Maternal effects were calculated by subtracting dam minus sire variance components taken from the LMM. The sire component of variance represents solely additive variation, whereas the dam component represents additive + maternal variance. Significant maternal effects were identified by generating 95% confidence intervals in the fullfact (Houde and Pitcher 2016) package. Methylation data was used to generate 1000 iterations of possible datasets, which were used to calculate confidence intervals.

Maternal effects were considered significant when the confidence intervals did not overlap zero.

To test for dam and sire effects on CpG-specific methylation across all loci simultaneously, LMMs were used to test for the random effects of CpG site, dam, sire, and all two- and three-way interaction effects. Cross (freshwater or saltwater) was included initially as a fixed effect, but was non-significant in all models and excluded from the final analyses. The final model was used to test the effects of each variable on DNA methylation across all genes in each of the three developmental stages. Likelihood ratio tests were used to determine the significance of each variable in the final model and a Bonferroni correction was used to correct for multiple comparisons. To determine if specific genes were driving dam and sire effects on CpG-specific methylation or if the same effects were observed across all genes, a LMM for the effects of CpG site, dam, sire, and all interactions was tested for each gene in each developmental stage. The significance of the dam x CpG site interaction term determined whether there were differential methylation patterns within a gene based on maternal identity. A Benjamini-Hochberg false discovery rate (FDR) correction was used to correct for multiple comparisons.

Results

Average read depth across all CpG sites was 106 sequences after all quality trimming. Linear mixed models were used to test for maternal effects on DNA methylation (1) across all assayed loci combined, (2) at each locus, (3) at individual CpG sites across all loci combined, and (4) at individual CpG sites at each locus.

Overall DNA methylation

We first combined all CpG site methylation data across the 14 candidate genes to test for dam, sire, dam x sire interaction and locus effects on percent methylation levels averaged across all CpG sites for each gene. Replicate (incubation tray cell) and breeding cross were included as variables, but replicate was not significant and therefore removed from the final model. We found evidence for dam effects acting on DNA methylation across all gene loci at the eyed egg ($p < 0.05$) and the alevin ($p < 0.0001$), but not at the fry stage. Sire (additive) and dam x sire interaction (non-additive) effects on methylation were not significant, but non-additive interaction effects were significant at the fry stage ($p < 0.05$). We also found very strong locus effects ($p < 0.0001$) at all developmental stages, indicating substantial variation in methylation levels among the candidate genes, as expected.

Locus-specific methylation

Next, we tested for dam, sire, and interaction effects at each locus independently to test for gene-specific effects. At the individual gene level, we found transient gene-specific dam effects at the eyed egg and alevin stages after Bonferroni correction which subsided by the fry stage (Figure 3.1, Appendix 2). We found significant dam effects at GTIIBS and hsc71 at the eyed egg stage, and hsc71, GH1, metA, and ITPA at the alevin stage. We detected significant maternal effects (dam - sire variance) by generating 95% confidence intervals using the fullfact (Houde and Pitcher 2016) package in R (R Development Core Team 2016).

Maternal effects on methylation levels at individual gene loci were significant (i.e. confidence intervals excluding zero) for three genes (metA, hsp70a, hnrL) in the

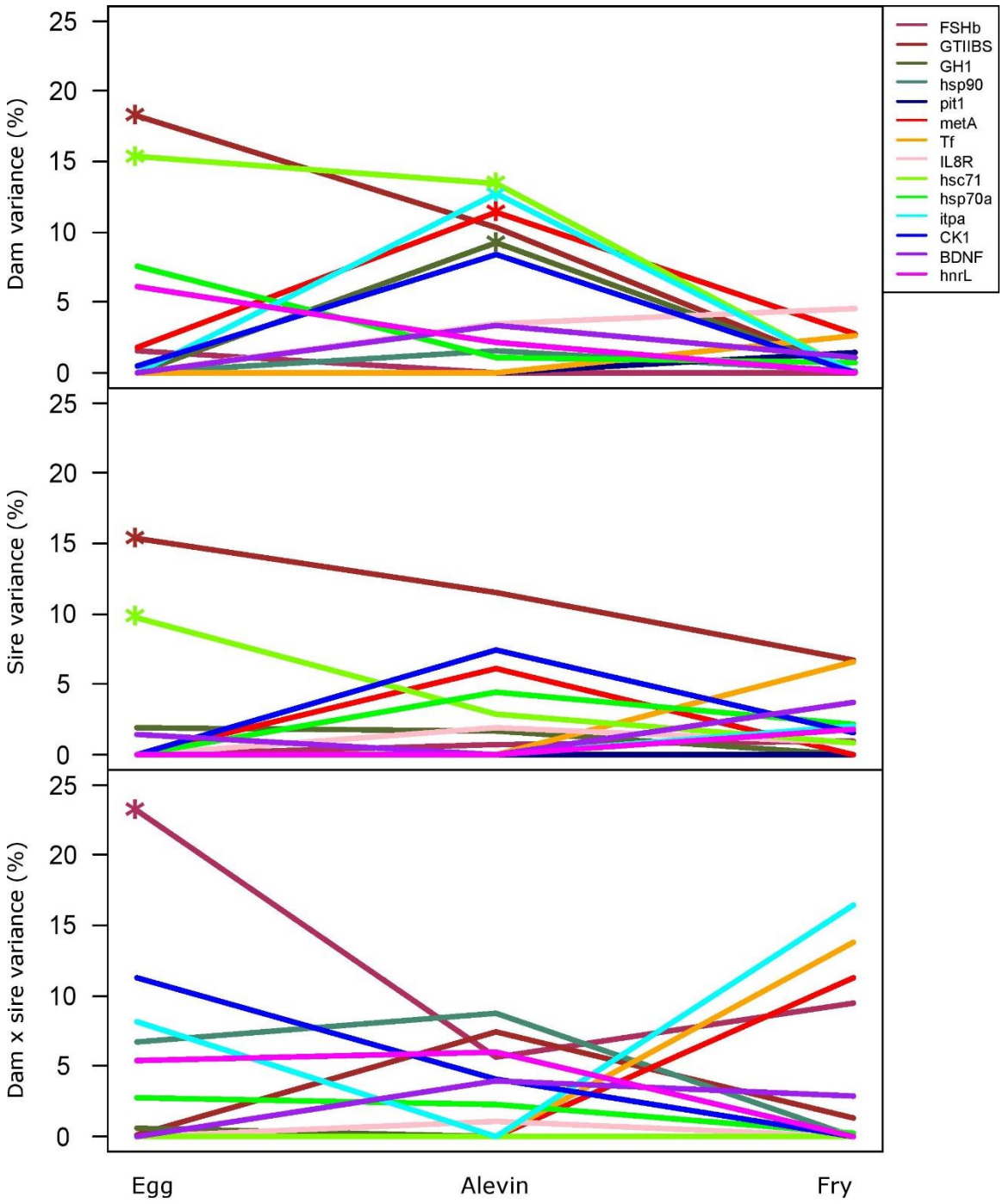

Figure 3.1: Mean dam, sire, and dam x sire effect variance component for mean gene-specific DNA methylation rates at 14 selected gene loci across three developmental stages in Chinook salmon offspring. Significant effects of the variance component on percent methylation are denoted by an asterisk.

eyed egg stage, six genes (GH1, hsp90, hsc71, itpa, BDNF, hnrL) in the alevin stage, and six genes (GTIIBS, pit1, metA, IL8R, hsc71, hsp70a) in the fry stage (Figure 3.2). At the eyed egg stage, we also found significant sire effects on two genes, GTIIBS and hsc71 ($p < 0.001$ and $p < 0.05$, respectively, after Bonferroni correction) as well as non-additive genetic effects on FSHb methylation ($p < 0.01$, after Bonferroni correction).

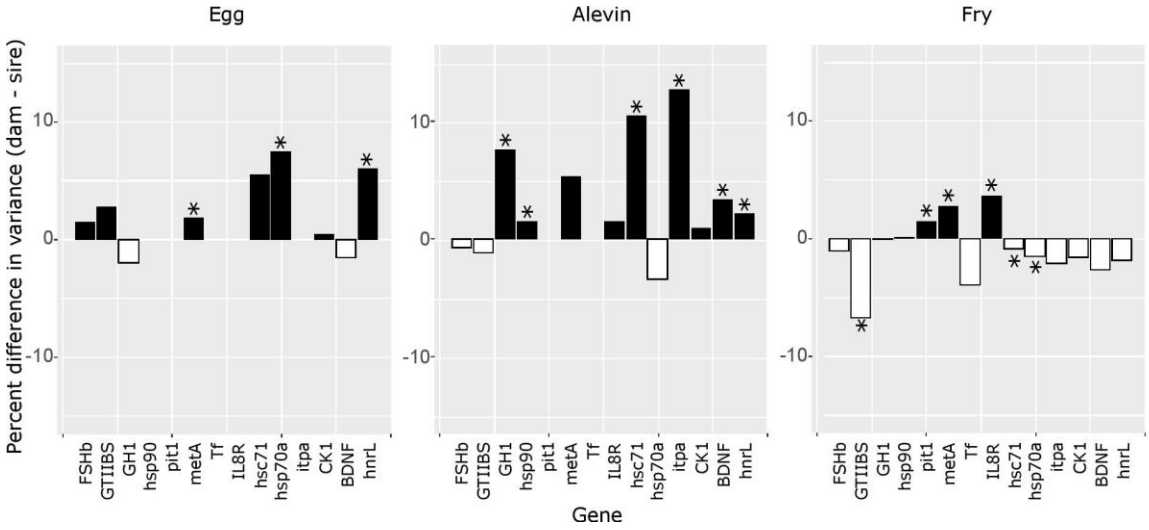

Figure 3.2: Percent difference in dam versus sire variance components (maternal effects) for mean DNA methylation at 14 gene loci across three developmental stages in Chinook salmon. Black bars indicate a greater dam component of variance and grey bars indicate a greater sire variance component. Results show that the dam component of variance is generally greater than the sire component of variance (black bars) early in development (indicative of maternal effects) but the sire component of variance is generally larger after the onset of endogenous feeding (grey bars). Significant maternal effects determined using 95% confidence intervals (see Methods) are denoted by an asterisk and gene names are provided in Appendix 1.

CpG-specific methylation

Finally, we tested for CpG-specific maternal effects. We found a strong dam x CpG site effect across all candidate loci combined ($p < 0.001$ for the eyed egg and alevin stages, but not at the fry stage) with, as expected, a strong locus effect ($p < 0.001$ for all

developmental stages). At the individual locus level, three genes showed a significant dam x CpG interaction: CK1 at the eyed egg stage, ITPA at the alevin stage, and GTIIBS at the eyed egg and alevin stage (Figure 3.3). While statistically non-significant, hsp70a methylation at the alevin stage differed based on which cross the mothers were from (Figure 3.3). No significant dam x CpG site effects were found at the fry stage.

Discussion

Maternal effects can dramatically contribute to variation in offspring phenotype, performance, and fitness at early life stages (Galloway and Etterson 2007), and result in evolutionary change at the population level (Aykanat et al. 2012a; Wolf and Wade 2016); thus, maternal effects are an important consideration in evolutionary biology. However, due to the resetting of methylation signatures across the genome during early development (Mhanni and McGowan 2004; Perez and Lehner 2019), it remains unclear whether methylation serves as a mechanism for the propagation of maternal effects across generations. Across all loci, we observed strong maternal effects on overall DNA methylation that subsided by the fry stage in Chinook salmon. Despite the reported loss of maternal methylation signatures early in development (Jiang et al. 2013; Potok et al. 2013), we found that maternal effects persist and influence DNA methylation patterns early in life. The widely reported pattern of declining maternal effects associated with offspring control over their genome matches our results, specifically, negligible maternal effects on methylation levels by the exogenous feeding fry stage (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017). We observed strong locus effects on DNA methylation at all stages. Since normal development requires strict regulation of gene

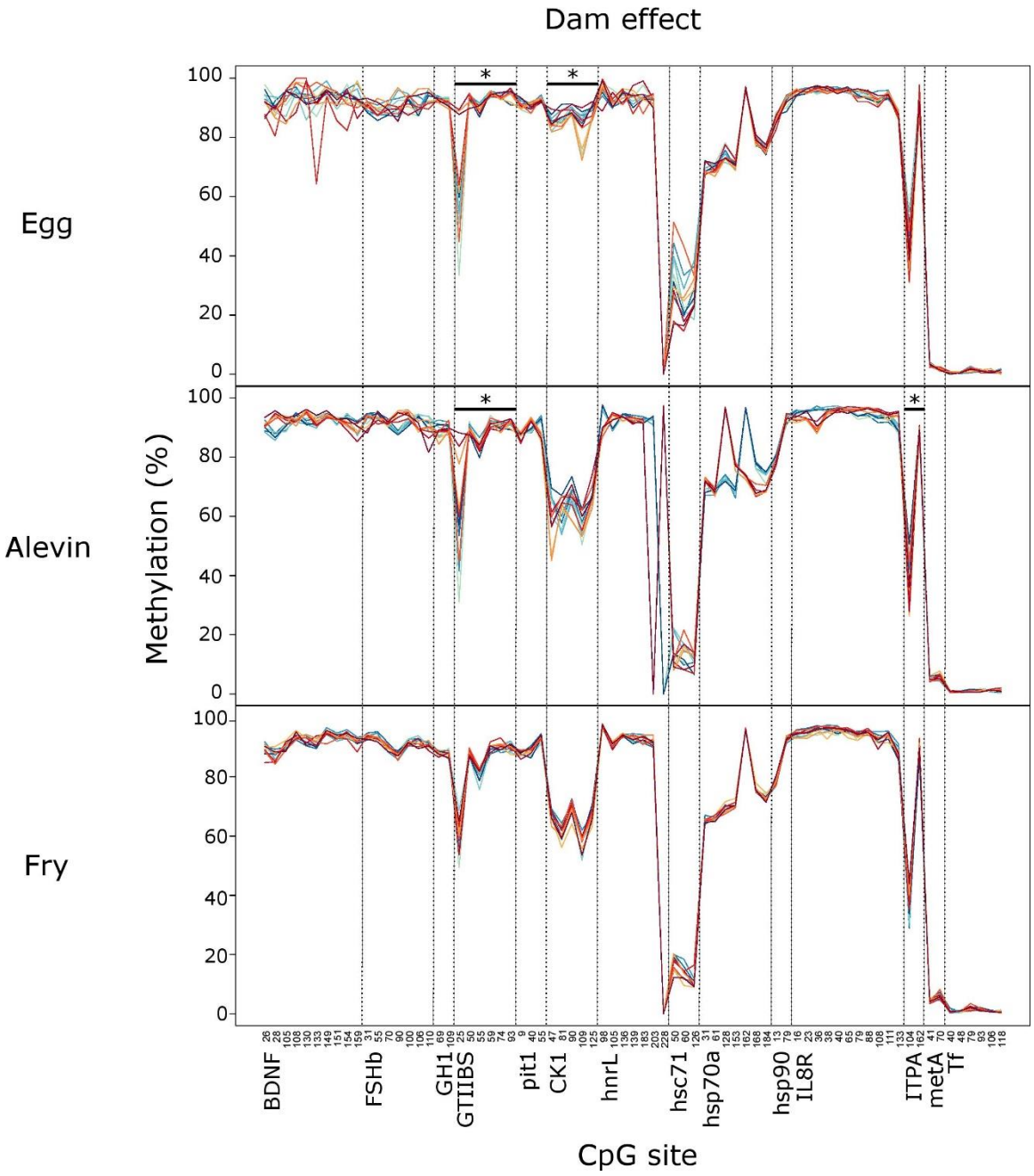

Figure 3.3: Maternal DNA methylation profiles for individual CpG methylation sites at 14 gene loci for three developmental stages in Chinook salmon offspring. Individual line graphs show dam-specific effects on CpG-specific DNA methylation rates (%) with the 12 dams used in the crosses shown in different colours. Blue lines represent dams from cross 1 and red lines represent dams from cross 2. Horizontal lines with asterisks denote significant dam x CpG effects on methylation. High levels of dam effects are present when the profiles diverge.

expression at critical developmental loci (Zeitlinger and Stark 2010), maternal effects are likely to act to "fine-tune" expression of less canalized genes and leave the expression of highly regulated and developmentally-controlled genes unaffected. While the mechanism behind the transmission of maternal effects after the loss of maternal methylation patterns during development remains unclear (Perez and Lehner 2019), our results are consistent with previous findings of intergenerational epigenetic inheritance in fish (Kamstra et al. 2018; Santangeli et al. 2019). Thus, maternal effects on DNA methylation occur in the eyed egg and alevin stages of Chinook salmon, but vary among loci, consistent with the hypothesis that maternal effects must target specific loci to be adaptive.

Our results indicate maternal effects on offspring DNA methylation in early development are gene-specific. We found transient gene-specific dam effects at the eyed egg and alevin stages after Bonferroni correction (Figure 3.1), consistent with previous research on maternal effects in Chinook salmon (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017), and a broad array of other taxa (Mousseau et al. 2009). We observed maternal effects on GTIIBS (endocrine function and sex differentiation; Patsoula et al. 2003) and hsc71 (aids in protein folding; Massicotte et al. 2006) at the eyed egg stage, and hsc71, GH1 (larval body size; Li et al. 2007), metA (influenced by maternal contaminant exposure; Wu et al. 2008) and ITPA (control of cell replication; Abolhassani et al. 2010) at the alevin stage. These genes are associated with phenotypic effects related to previously documented maternal effects, including effects on offspring size (Janssen et al. 1988; Heath et al. 1999; Falica et al. 2017) and resistance to contaminants (Wu et al. 2008). Conversely, constitutively expressed and developmentally critical genes did not show significant dam effects. Genes such as Tf, which is

constitutively expressed (Stafford and Belosevic 2003), BDNF which is involved in neural function and development (Conner et al. 1997), and pit1 which is involved in regulating growth hormone and other growth-related genes (Yamada et al. 1993) did not show significant effects on methylation at any stage, as expected for developmentally and metabolically critical genes. Previous studies have shown that parental exposure to stressful stimuli results in locus-specific methylation changes in offspring (Kamstra et al. 2018; Santangeli et al. 2019), thus our results support the occurrence of a targeted mechanism for the propagation of maternal effects, though the mechanism remains unclear. Maternal effects are associated with phenotypic and physiological variation which could prove to be adaptive (or maladaptive) depending on the correlation between maternal and offspring environments (Mousseau and Fox 1998), consistent with the theory of the evolution of adaptive maternal-offspring signalling (Sheriff and Love 2013). Our results thus strongly support the hypothesis that methylation serves as a mechanistic mediator for maternal effects (Love et al. 2013).

The discovery of maternal effects influencing offspring DNA methylation at specific loci prompted the question of whether maternal effects act on mean methylation levels across candidate gene loci, or whether maternal effects affect methylation status at specific CpG sites within genes. Since methylation can have variable effects on gene expression depending on which CpG sites are methylated (Lillycrop et al. 2008), CpG site-specific methylation provides an additional level of specificity (and complexity) to the transmission of DNA methylation-based maternal signals. Strong dam x CpG effects across the combined candidate loci at the eyed egg and alevin stage are indicative of broad-scale targeted maternal effects acting on methylation at specific CpG sites. At the

individual locus level, dam x CpG interaction effects were detected at CK1 (immune response; Lally et al. 2003) and GTIIBS at the eyed egg stage, and GTIIBS and ITPA at the alevin stage (Figure 3.3). These results support the hypothesis that mothers influence offspring DNA methylation in early development not only at specific genes, but also at specific CpG sites, consistent with a targeted mechanism for maternal effects. The individual genes with significant dam x CpG interaction terms are logical targets for adaptive maternal effects due to their non-canalized expression and role in response to environmental challenges. While the dam component of the methylation profile varies though development, it is lost at the fry (exogenous feeding) stage, as expected for transient maternal effects that are overridden by offspring methylation control as the offspring responds to its environment.

The erosion of maternal effects through early development is well documented (Heath et al. 1999; Falica and Higgs 2013; Falica et al. 2017), but the proximate mechanism of this reduction has not been explored. The loss of maternal control over offspring gene-specific methylation could be due to the degradation of maternally-derived proteins regulating DNA methylation (Inoue et al. 2017). At the fry stage, the sire component of variance generally explained more of the variance in DNA methylation than the dam component (Figure 3.2). This could be due to a delayed paternal effect, as seen in previous studies that have reported increased sire effects later in development in Chinook salmon (Falica and Higgs 2013) and paternal effects in other species (Jensen et al. 2014), or due to a negative maternal effect (Janssen et al. 1988; Heath et al. 1999). However, non-additive (dam x sire) effects on methylation became significant at the fry stage, suggesting increasing endogenous epistatic and/or dominance effects (Aykanat et

al. 2012b; Wellband et al. 2018) as the offspring genome gains control of methylation and demethylation processes. Our results suggest that intergenerational effects on DNA methylation occur at specific life stages after methylation reset, but before the offspring gains autonomy over their genome. Regardless, our results support DNA methylation as a potential novel mechanism for transient intergenerational maternal effects, which can have important consequences for offspring fitness.

Our results support the idea that CpG-specific DNA methylation has a role in mechanistically propagating maternal effects during early development, which may influence offspring growth and physiology through gene-specific methylation changes. However, further research is required to determine the mechanisms involved in transmitting maternal signals to modify methylation patterns. Our results are unexpected based on the loss of maternal methylation signals early in embryonic development in fish and subsequent adoption of methylation landscapes similar to sperm (Jiang et al. 2013; Potok et al. 2013; Perez and Lehner 2019). A process other than methylation resetting is likely responsible since we detected maternal effects on offspring DNA methylation in life stages after the expected loss of maternal methylation patterns. It is possible that our results are due to our exploration of later developmental stages. At the eyed egg stage, the earliest developmental stage we studied, the developing embryo is in the midst of organogenesis and well past gastrulation (Velsen 1980). While methylation is reset around the time of gastrulation (Mhanni and McGowan 2004), maternal effects on methylation have been reported in developmental stages undergoing organogenesis in zebrafish and are targeted to specific regions of the genome (Fan et al. 2019; Santangeli et al. 2019), consistent with our results. While Chinook salmon development is primarily

affected by temperature and time since fertilization (Beacham and Murray 1990), it is possible that some variation in observed methylation is due to differences in developmental rate. However, Chinook salmon tend to show high synchrony in developmental rate when raised in a common, controlled environment, thus we find this unlikely. While previous studies have identified phenotypic effects of intergenerational epigenetic inheritance (Fan et al. 2019), future research should relate changes in gene-specific and CpG-specific DNA methylation profiles with the well-documented phenotypic maternal effects, such as those observed in Chinook salmon (Aykanat et al., 2012a,b; Heath et al., 1999). Our data further support DNA methylation as a highly targeted mechanism in the underlying genetic architecture of intergenerational effects. Since methylation controls individual variation in gene expression, it has the potential to generate physiological and phenotypic variation upon which selection could act and, ultimately, fine-tune gene expression through maternal inputs to optimize offspring fitness. At present, it is unclear if maternal effects on DNA methylation in early life are indicative of a true mechanism for the transfer of maternal effects, or if they are a downstream consequence of changes in transcription, as reported in other studies (Pacis et al. 2019). Our results highlight the need for future studies on the effects of intergenerational DNA methylation transfer on offspring phenotype and fitness, and their timing with respect to changes in transcription.

The study of DNA methylation in an evolutionary context is in its infancy, with most published studies focused on medical or physiological applications. However, previous research has proposed DNA methylation as a novel adaptive mechanism (Aykanat et al. 2012b; Venney et al. 2016). In this study, we provide support for targeted

DNA methylation as a mechanism for intergenerational signalling in Chinook salmon. Despite loss of maternal methylation patterns shortly after fertilization, strong maternal effects on gene-specific and CpG-specific methylation, suggesting a previously unidentified mechanism allows maternal control over the offspring genome even after loss of parental methylation patterns. Parental effects can have far-reaching effects on offspring fitness, resulting in population and evolutionary change (Aykanat et al. 2012a; Wolf and Wade 2016). If parentally-induced DNA methylation profiles reflect parental environment and experiences, then epigenetic mechanisms may serve as a conduit for parents to affect early-stage offspring phenotype and physiology. Such effects could increase offspring fitness and potentially reinforce local adaptation through maternal effects, a pattern already proposed based on population-level phenotypic divergence (Aykanat et al. 2012a).

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CHAPTER 4 – REARING ENVIRONMENT AFFECTS THE GENETIC ARCHITECTURE AND PLASTICITY OF DNA METHYLATION IN CHINOOK SALMON

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Summary

Genetic architecture and phenotypic plasticity are important considerations when studying trait variation within and among populations. Since environmental change can induce shifts in the genetic architecture and plasticity of traits, it is important to consider both genetic and environmental sources of phenotypic variation. While there is overwhelming evidence for environmental effects on phenotype, the underlying mechanisms are less clear. Variation in DNA methylation is a potential mechanism mediating environmental effects on phenotype due to its sensitivity to environmental stimuli, transgenerational inheritance, and influences on transcription. To characterize the effect of environment on methylation, we created two 6x6 (North Carolina II) Chinook salmon breeding crosses and reared the offspring in two environments: uniform hatchery tanks and semi-natural stream channels. We sampled the fish twice during development, at the alevin (larval) and fry (juvenile) stages. We measured DNA methylation at 13 genes using a PCR-based bisulfite sequencing protocol. The genetic architecture of DNA methylation differed between rearing environments, with greater additive and non-additive genetic variance in hatchery fish and greater maternal effects in semi-natural channel fish, though gene-specific variation was evident. We observed plasticity in methylation across all assayed genes, as well as gene-specific effects at two genes in alevin and six genes in fry, indicating developmental stage-specific effects of rearing environment on methylation. Characterizing genetic and environmental influences on methylation is critical for future studies on DNA methylation as a potential mechanism for acclimation and adaptation.

Introduction

Phenotypic plasticity refers to the ability of a genotype to produce different phenotypes depending on environmental context (Scheiner and Lyman 1989; Uller 2008; Richards et al. 2010; Guillaume et al. 2016). Since phenotypic plasticity can occur over short time scales compared to genetic adaptation, which requires generations of selection and adequate standing genetic variation, plasticity serves as a rapid mechanism for coping with changing environmental conditions (Guillaume et al. 2016). Studies have characterized plasticity in response to a broad range of environmental cues, including plastic changes in gene expression and jaw morphology when cichlids were fed different diets (Schneider et al. 2014), plastic colouration in reef fish which resulted in increased prey capture success and fitness (Cortesi et al. 2015), changes in gill gene expression after rapid transfer to saltwater in killifish (Scott et al. 2004), changes in steelhead salmon brain growth depending on rearing environment complexity (Kihslinger and Nevitt 2006), and gene expression plasticity in response to confinement stress in Chinook salmon (Wellband et al. 2018). While plasticity is known to occur over short time periods within an organism's life, transgenerational plasticity also occurs when offspring phenotype is based on both parental and offspring environmental contexts (Galloway and Etterson 2007; Uller 2008). Transgenerational plasticity can be maladaptive if the parental environment is a poor predictor of offspring environmental conditions (Galloway and Etterson 2007; Uller 2008), or if there is the potential for parent-offspring conflict (Uller 2008). The main mechanism for the transmission of transgenerational plasticity is through maternal effects on offspring phenotype and development (Galloway and

Etterson 2007; Marshall 2008; Uller 2008), which is often an important component of the underlying genetic architecture of early life phenotypic traits.

When individual variation is at least partially genetically derived and not entirely determined by environment, components of an organismal phenotype can be explained by the genetic architecture of traits. Genetic architecture is the underlying quantitative genetic basis of variation in phenotypic traits, and includes gene effects, interaction effects among genes, and environmental factors affecting phenotype (Martínez et al. 2014). Often, genetic architecture is reported as maternal, additive, and non-additive genetic variance components (Houde et al. 2013). Maternal effects are non-genetic influences of maternal genotype and environment on offspring phenotype (Marshall and Uller 2007), often through control of gamete size and deposition of proteins, hormones, and mRNA into eggs (Nodine and Bartel 2012; Perez et al. 2017), in addition to other mechanisms (e.g. Heath et al. 1996; Aykanat et al. 2012b; Nodine and Bartel 2012; Videvall et al. 2016; Falica et al. 2017). Since maternal effects can strongly influence offspring phenotype, particularly early in life (Houde et al. 2013), they can have considerable effects on offspring development and fitness (Galloway and Etterson 2007; Marshall and Uller 2007; Perez et al. 2017; Fan et al. 2019). Additive genetic effects are heritable, predictable based on genotype, and respond to selection (Houde et al. 2013) making additive genetic variation an ideal target for selective breeding programs and predicting evolutionary trajectories of populations. Non-additive genetic effects encompass dominance effects (interactions among alleles within a locus), epistatic effects (interactions among loci) and higher-order interactions (Sheldon and Merilä 1999). While the effects of non-additive genetic variance are difficult to predict, there is abundant

evidence for non-additive genetic effects on transcription (Aykanat et al. 2012b; Wellband et al. 2018) and fitness-related traits (Aykanat et al. 2012a; Houde et al. 2013) with the potential for non-additive effects to contribute to fitness (Sheldon and Merilä 1999; Neff et al. 2011). The study of the underlying genetic architecture of traits is important to characterize the basis and breadth of phenotypic variation and the evolution of organisms, yet genetic architecture is often influenced by environment (Holloway et al. 1990; Etterson 2004; Yeaman and Whitlock 2011; Parsons et al. 2016; Wellband et al. 2018), resulting in genotype-by-environment (GxE) effects on phenotype. When GxE effects on phenotype occur, environmental variation elicits different phenotypes from the same genotype, resulting in variable fitness of a single genotype dependent on environmental context (García de Leániz et al. 2007; Sae-Lim et al. 2016). Thus, an understanding of the genetic (additive, non-additive, and maternal variance) basis of phenotypic traits, the environmental context in which organisms reside, and the interaction between genetics and the environment is critical for understanding the basis of phenotype and the evolution of organisms (Banta and Richards 2018).

Despite the importance of the role of plasticity and genetic architecture in phenotypic variation, the mechanisms behind those effects are not well characterized. Epigenetic mechanisms such as DNA methylation alter organism function without underlying changes in the DNA sequence (Bird 2007; Bossdorf et al. 2008). DNA methylation represents an exciting possible mechanism for differences in genetic architecture and phenotypic plasticity to contribute to underlying early life trait variation. Previous studies have identified plasticity in methylation levels in response to stressors, including changes in methylation in response to pollutant exposure (Fang et al. 2013; reviewed in Head

2014; Olsvik et al. 2019), temperature changes (Anastasiadi et al. 2017; Metzger and Schulte 2017; Liew et al. 2020), elevated salinity (Morán et al. 2013; Metzger and Schulte 2018; Li et al. 2020), inbreeding (Vergeer et al. 2012; Venney et al. 2016; Berbel-Filho et al. 2019), and captive rearing and/or domestication (Nätt et al. 2012; Le Luyer et al. 2017; Rodriguez Barreto et al. 2019). In addition to its sensitivity to environmental changes, methylation can be inherited across generations (Kamstra et al. 2018; Fan et al. 2019; Santangeli et al. 2019). Methylation can exhibit additive (heritable) genetic variance (Hannon et al. 2018) and has been identified as a potential mechanism for the propagation of locus-specific maternal effects (Venney et al. 2020); both additive and maternal sources of variance are important components of the genetic architecture of traits. Due to its sensitivity to the environment and its transmission across generations, DNA methylation represents a possible novel mechanism behind environmentally labile genetic architecture and phenotypic plasticity.

Chinook salmon (*Oncorhynchus tshawytscha*) are an ideal species for the study of phenotypic plasticity and genetic architecture early in life. Chinook salmon undergo a single, terminal reproductive event and lack parental care (Heath et al. 1999), eliminating the confounding effects of parental care on offspring phenotype. External fertilization and the production of large numbers of gametes enable large-scale sophisticated breeding experiments. Salmon are sensitive to environmental changes, often exhibiting GxE effects on phenotype and fitness, consistent with other evidence for local adaptation (García de Leániz et al. 2007; Fraser et al. 2011). Many salmon species are economically and ecologically important with various supplementation and conservation efforts aimed at maintaining and supplementing Chinook salmon stocks (Fraser 2008). However,

hatchery rearing often results in reduced fitness and survival in salmon (Araki et al. 2007; Blouin et al. 2010; Fraser et al. 2011; Becker et al. 2014; Le Luyer et al. 2017), even after a single generation of hatchery rearing (Araki et al. 2007). Hatchery reared salmon exhibit altered DNA methylation patterns (Le Luyer et al. 2017; Rodriguez Barreto et al. 2019), transcription (Christie et al. 2016; Wellband et al. 2018), disease resistance (Becker et al. 2014), brain development (Kihslinger and Nevitt 2006), egg size (Heath et al. 1996), and reduced survival (Blouin et al. 2010; Becker et al. 2014). Differences in genetic architecture among salmon populations (Aykanat et al. 2012a; Houde et al. 2013; Houde et al. 2015) and among environments (Aykanat et al. 2012a; Wellband et al. 2018) have been reported, thus it is possible that rearing juveniles in uniform environments (hatcheries) as opposed to their natural environment influences the genetic architecture of DNA methylation in Chinook salmon. This hypothesis is supported by previous research which identified differentially methylated regions of the genome in hatchery reared compared to wild Coho salmon (Le Luyer et al. 2017), as well as differences in the genetic architecture of transcription in hatchery reared and semi-naturally reared Chinook salmon (Wellband et al. 2018).

Here we characterized the effect of rearing environment on the genetic architecture and plasticity of DNA methylation to determine the genetic basis of the effects of environment on DNA methylation. We created two 6x6 factorial (North Carolina II) breeding crosses using Chinook salmon and raised them in hatchery and semi-natural rearing environments to determine the effect of early rearing environment on (1) the role of DNA methylation in in plastic response to early life environmental conditions, (2) the extent of genotype-by-environment (GxE) interactions on methylation, and (3) the

genetic architecture of DNA methylation. We assayed methylation in Chinook salmon alevins (larval stage) and fry (post-exogenous feeding) at 13 genes involved in development, immune response, stress response, and metabolism using a PCR-based bisulfite sequencing protocol for Next-Generation sequencing (Venney et al. 2016). Since environmental differences induce changes in the genetic architecture of various traits (Holloway et al. 1990; Etterson 2004; Yeaman and Whitlock 2011; Parsons et al. 2016; Wellband et al. 2018), we predicted that different rearing environments would induce changes in the genetic architecture of DNA methylation, ultimately contributing to underlying changes in phenotype among environments. Based on previous research showing strong environmental effects on methylation (Fang et al. 2013; Morán et al. 2013; Anastasiadi et al. 2017; Le Luyer et al. 2017), we hypothesized that rearing environment would induce changes in DNA methylation at specific genes. Based on known transgenerational transmission of methylation (Kamstra et al. 2018; Fan et al. 2019; Santangeli et al. 2019) and interactions between transmitted methylation signals and the environment, we expected to observe GxE effects on methylation. Environmental conditions influence the phenotype of organisms (Scheiner and Lyman 1989; Uller 2008; Richards et al. 2010; Guillaume et al. 2016) as well as changes in the genetic architecture underlying phenotypic traits (Fang et al. 2013; Morán et al. 2013; Anastasiadi et al. 2017; Le Luyer et al. 2017). Understanding the mechanistic and molecular genetic basis of phenotypic variation among environments is critical to quantifying variation within natural populations and understanding how environmental fluctuations influence organismal phenotype, and often fitness, in a rapidly changing world. Quantifying the sources of phenotypic variation and environmental effects on phenotype is critical to

making informed conservation and management decisions, and to understanding the molecular basis of phenotype.

Methods

Breeding design and sampling

Two 6x6 North Carolina II breeding crosses were set up on October 31st, 2014, using three-, four-, and five-year old sexually mature male and female Chinook salmon at Yellow Island Aquaculture, Ltd (YIAL). The North Carolina II design allows for the estimation of additive (sire), maternal (dam – sire), and non-additive (dam x sire interaction) variance components. Replicated 6x6 factorial crosses were made using six males and six females, resulting in 36 families per cross (72 families total). Fertilized eggs from each family were split into two replicate cells and incubated in freshwater vertical incubators following standard procedures at YIAL. On December 19th, 2014, approximately 40 eyed eggs per replicate cell were transferred to a Whitlock-Vibert box and buried in the gravel substrate of an artificial seminatural channel at YIAL. The seminatural channel experienced greater temperature and environmental fluctuations and served as a proxy for a more variable, natural environment.

On March 2nd, 2015, alevins were collected from the hatchery incubators and semi-natural channels, humanely euthanized, and stored in a high salt buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) for later analysis. To minimize cumulative environmental effects across developmental stages, the semi-natural channel was restocked with alevin from the hatchery. This allowed us to test the effects of rearing environment on DNA methylation at both the alevin and fry stage while

eliminating the possibility that shifts in methylation are simply maintained through development. The two replicate incubation tray cells for each family in the hatchery were pooled to reduce replicate effects. Approximately 10 alevins per replicate were taken from the incubator trays in the hatchery and transferred to the artificial stream environment in one of 24 randomly assigned aluminum enclosures measuring 120 x 60 x 60 cm. The enclosures consisted of a bottom tray filled with coarse gravel, and a frame extending above the surface of the artificial stream with netting from the top of the frame to below the gravel. Each enclosure contained offspring from nine families of fish. The remaining alevins from each family were split between two 200 L flow-through barrels (144 barrels total) with adequate flow and oxygenation in the hatchery. All fry were humanely euthanized and sampled after 10 weeks of hatchery or seminatural channel rearing on May 11th, 2015. The fry were cut open to expose their body cavities and preserved in a high salt buffer as described above for alevin.

DNA extraction

Digestions for DNA extractions were performed as in Venney et al. (2020). Alevins were cut in half to aid in digestion and both halves were digested in 6000 µL of digestion buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS) with 10 µL of proteinase K. The fry had their livers removed for another experiment, were cut into three pieces (to help with digestion) and digested in 7000 µL of digestion buffer with 10 µL proteinase K. The liver represents a small portion of total somatic genomic DNA; thus, the removal of this organ is unlikely to significantly affect our results regardless of the metabolic importance of the liver. While studying average whole-body methylation masks potential tissue-specific methylation signals, it allowed us

to study both larval (alevin) and fully developed (fry) fish. All samples were digested overnight at 37°C before a 150 µL aliquot was used for DNA extraction via a high throughput plate-based protocol (Venney et al. 2020) based on a protocol by Elphinstone et al. (2003).

Parentage analysis

Since multiple families of fry were combined and reared in the seminatural channel enclosures, parentage assignment was performed using microsatellite genotyping (for detailed methods, see Wellband et al. (2018)). Fin clips were taken from all fry in the semi-natural channel and DNA was extracted using the high-throughput plate-based protocol (Elphinstone et al. 2003). Individuals were genotyped at five microsatellite loci by analyzing PCR fragments on a Licor 4300 DNA Analyzer. Genotypes were scored based on the sizes of parental alleles, and analyzed in Cervus v3.0.7 (Kalinowski et al. 2007) where parentage was determined using known parental pairs with an 80% confidence interval. Fish achieving a 95% confidence interval for parentage were preferentially used for further analyses.

Bisulfite conversion, PCR, and Next-Generation sequencing

DNA was quantified using a Quant-IT PicoGreen dsDNA Assay kit, an accurate plate-based DNA quantification method. Bisulfite conversion was performed using 500 ng of DNA and an EZ-96 DNA Methylation-Lightning kit following the manufacturer protocol.

PCR was performed using bisulfite sequencing primers for coding regions of 13 highly conserved genes involved in metabolism, stress response, and early development

(Venney et al. 2016). The selected genes span a broad range of functions, are important in early development, and/or are logical targets for maternal or environmental effects. Between 136 and 225 bp were amplified per gene (2371 bp total; Appendix 1) after primer sequences were removed. Bisulfite sequencing libraries were generated using a two-stage PCR approach and sequencing method (Venney et al. 2016) wherein the first stage amplified the targeted gene loci, and the second stage ligated barcode sequences, sequencing adaptors, and primers. Next-Generation sequencing was performed on the Ion Torrent Personal Genome Machine® (PGM™) using an Ion PGM™ Sequencing 400 kit (maximum length of 400 bp) with an Ion 318™ Chip. Samples were spread across four sequencing runs.

Data processing

Sequence data were demultiplexed using mothur (Schloss et al. 2009) to remove primer sequences and generate one sequence file per individual based on barcode sequences. Bisulfite sequence data were aligned to existing sequence data for the target loci using bwa-meth (Pedersen et al. 2014) with only two non-cytosine mismatches allowed to ensure high sequence fidelity due to short read length. A table with data on average percent methylation for each CpG site in each gene in each individual was generated using bwa-meth. Data tables were imported into R (R Development Core Team 2016) which was used for all downstream analyses unless otherwise stated. Additional quality assurance was performed to ensure that CpG sites with less than five reads per gene per individual, and those that were present in less than 70% of individuals, were excluded from the analysis (Venney et al. 2016). Rosner's test for extreme outliers was

used to identify outlier methylation estimates, which were likely due to low read depth rather than a true biological signal.

Genetic architecture of DNA methylation

To characterize the genetic basis behind variation in DNA methylation, we measured the genetic architecture of DNA methylation by estimating additive, non-additive, and maternal variance components. Additive genetic variance is calculated as 4 x (sire component of variance), non-additive genetic variance is calculated as 4 x (sire x dam interaction variance), and maternal variance is calculated as (dam – sire) components of variance (Lynch and Walsh 1998). We studied genetic architecture at two levels: (1) across all genes combined with environment as a factor to determine how environment influences the genetic architecture of DNA methylation across all genes, and (2) for each gene in each environment for the two developmental stages, to quantify changes in genetic architecture underlying variation in DNA methylation among loci, environments, and developmental stages.

First, we tested if rearing environment affected the genetic architecture of DNA methylation across all genes. For each developmental stage, we ran a linear mixed model (LMM) in lme4 (Bates et al. 2015) to estimate the fixed effects of environment and gene, random effects of dam and sire, and all two-, three- and four-way interactions, on DNA methylation across all genes. The significance of each term was tested using likelihood ratio tests starting with higher-order interaction terms, which were excluded when they did not significantly contribute to model fit.

To assess the locus-specific genetic architecture of DNA methylation in each developmental stage and rearing environment, restricted variance analyses (genetic variance components greater than zero) were performed in the R package fullfact (Houde and Pitcher 2016). Briefly, LMMs were used to estimate the random effects of dam, sire, and dam x sire interaction on DNA methylation at each locus. A restricted variance analysis was performed for each gene in each developmental stage in each rearing environment to estimate the gene-specific additive, non-additive, and maternal variance components contributing to the genetic architecture of DNA methylation. A Benjamini-Hochberg FDR correction was used to correct for multiple comparisons. Two-sided paired t-tests were used to determine whether there was a significant difference in the percent variance (additive, maternal, and non-additive) across all genes due to environmental effects on the genetic architecture of methylation in each developmental stage.

Plasticity and GxE interactions on DNA methylation

We tested for genotype, environment, and GxE effects on methylation using full-sibling unrelated families (diagonal cells in 6x6 crosses) as a proxy for genotype to prevent inflating similarity due to half-siblings from other crosses. The R package lme4 (Bates et al. 2015) was used for all LMMs. For each developmental stage, an LMM was run across all genes to test for overall effects of gene, genotype (family), environment, GxE interaction, and all other two- and three-way interaction terms on DNA methylation. For all models, gene and environment were included as a fixed effect, while genotype and GxE interaction were specified as random effects. Terms were excluded from the model

starting with higher-order interaction terms using likelihood ratio tests to assess the significance of individual terms.

To determine which genes were driving significant effects, an LMM was run for each gene in each developmental stage to determine whether genotype, environment, and GxE interaction significantly affect locus-specific methylation, and a Benjamini-Hochberg false discovery rate (FDR) correction was used to correct for multiple comparisons.

Results

Genetic architecture of DNA methylation between environments

LMMs testing for environmental, gene, dam, sire, and interaction effects across all genes in each developmental stage were simplified to exclude all three- and four-way interaction terms based on lack of statistical significance from likelihood ratio tests, except the environment x gene x dam effect was retained in the LMM for fry methylation. Environment, as well as environment x gene, gene x dam, and gene x sire interactions, all significantly affected methylation across genes at the alevin stage (all $p<0.001$). At the fry stage, gene x sire interaction ($p<0.001$), environment x gene and environment x gene x dam interactions (both $p<0.001$) significantly affected methylation.

Using LMMs for each gene in each rearing environment and developmental stage, we detected significant dam effects on methylation of GTIIBS ($p<0.05$), metA ($p<0.01$), hsc71, and itpa (both $p<0.001$) in hatchery alevins after FDR correction, as well as dam effects on GTIIBS and itpa in seminatural channel alevins ($p<0.01$) after FDR correction. We detected no significant effects in the fry stage except a dam effect on GTIIBS

methylation in seminatural channel fry ($p<0.01$) after FDR correction. We observed significant sire effects on GTIIBS in hatchery alevins, and no significant dam x sire effects. Rearing environment influenced the genetic architecture underlying DNA methylation in a gene-specific manner (Figure 4.1 and Appendix 3). In general, we observed increased additive and non-additive variation in hatchery-reared fish and increased maternal effects in seminatural channel-reared fish at both alevin and fry life stages (Figure 4.2). Two-sided paired t-tests testing for differences in the percent variance (additive, maternal, and non-additive) of methylation were non-significant except for maternal effects in the fry stage (Figure 4.2).

Genotype, environment, and GxE effects on methylation

LMMs for the effects of genotype (full-sibling family), environment, gene, and all two-way interactions were run in each developmental stage and environment. Likelihood ratio tests for LMMs allowed the exclusion of the three-way interaction effect due to non-significance. LMMs identified strong environment x gene interaction effects on DNA methylation across all genes in both the alevin and fry stages ($p<0.001$) indicating gene-specific methylation in response to rearing environment, as well as strong genotype x gene effects in the alevin stage ($p<0.001$) indicating variation in methylation among families. Genotype, environment, gene, and genotype x environment effects were not significant in either developmental stage.

When LMMs were run for each gene in each developmental stage, we detected strong environmental effects on DNA methylation at specific loci after FDR correction (Figure 4.3 and Appendix 4). We detected significant environmental effects on methylation at hsc71 and metA in alevin ($p<0.001$), as well as effects on fry DNA

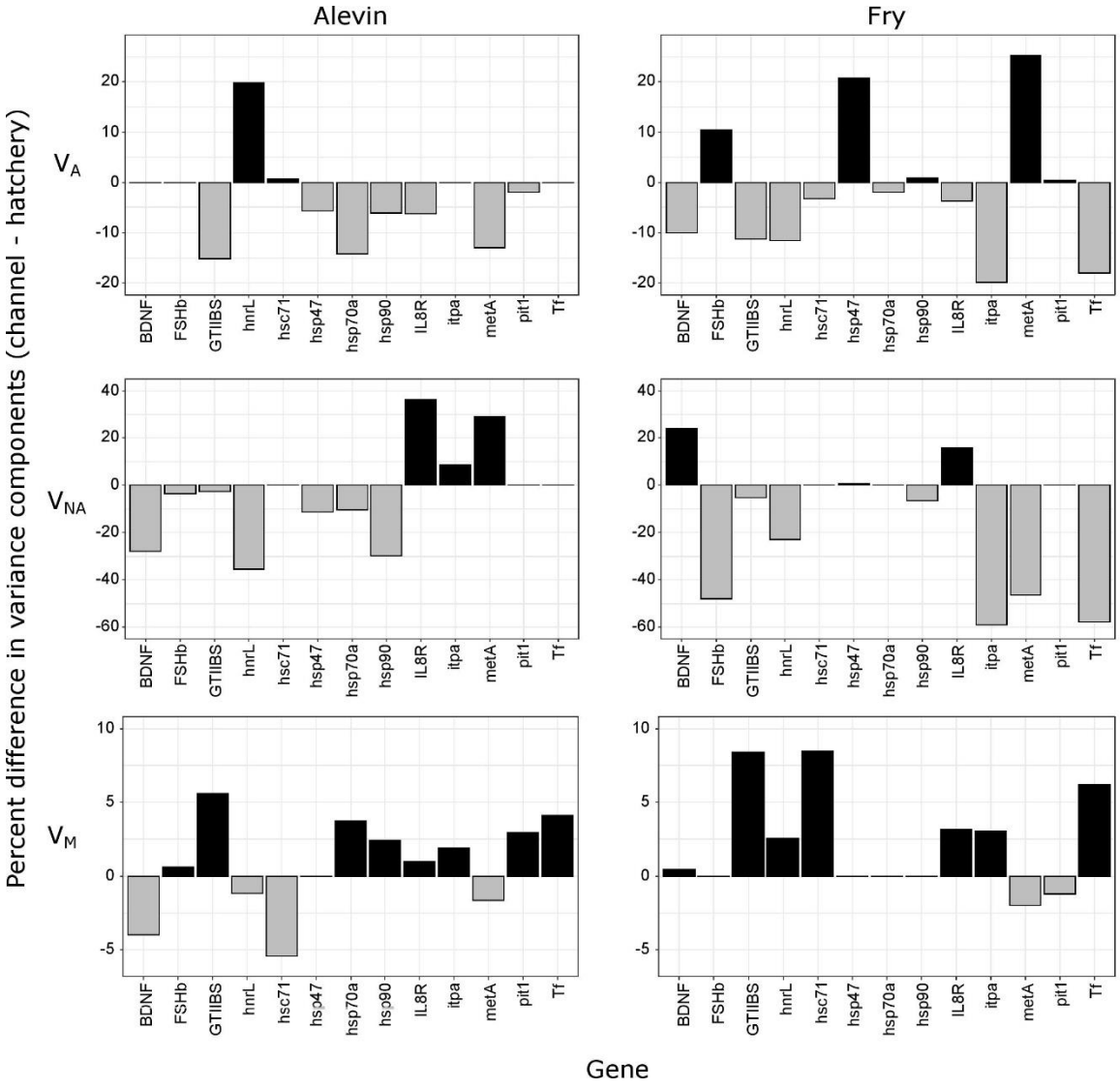

Figure 4.1: Bar graph showing the effects of rearing environment on additive (V_A), non-additive (V_{NA}), and maternal (V_M) variance components on gene-specific DNA methylation in Chinook salmon. Bars represent the percent difference in variance components (seminatural channel – hatchery) due to early rearing environment. Black bars indicate greater contributions of the variance component to methylation status of genes in the seminatural channel while grey bars indicate greater contributions of the variance component in the hatchery.

methylation at hsp47, hsp70a, and metA (all $p<0.001$), hsp90, and pit1 ($p<0.01$) after FDR correction. Genotype and GxE effects were not significant.

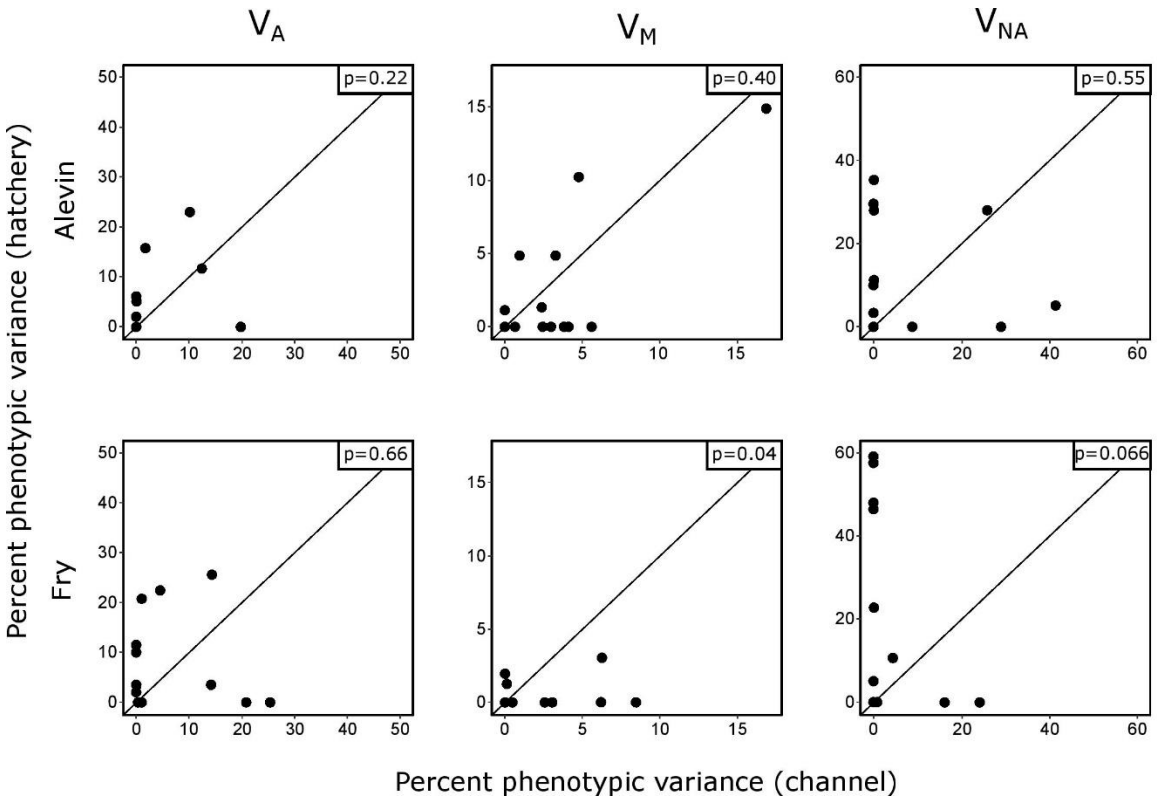

Figure 4.2: Scatterplot comparing additive (V_A), non-additive (V_{NA}), and maternal (V_M) variance components between rearing environments, with the 1:1 line plotted for reference. Each point represents a gene locus; points above the 1:1 line indicate that the methylation variance component is higher in hatchery-reared fish relative to seminatural channel-reared fish, while points below the line indicate the opposite. P-values from two-sided paired t-tests for each variance component in each developmental stage are reported, indicating whether rearing environment significantly affected the proportion of variance across all genes.

Discussion

To fully understand the genetic basis of phenotypic variation within and among natural populations, it is crucial to characterize the genetic architecture of traits of interest, as well as the effects of the environment on that genetic architecture (Holloway et al. 1990; Etterson 2004; Yeaman and Whitlock 2011; Parsons et al. 2016; Wellband et al. 2018). Numerous previous studies have reported evidence for environmental effects on phenotype which can influence individual survival and the persistence of populations

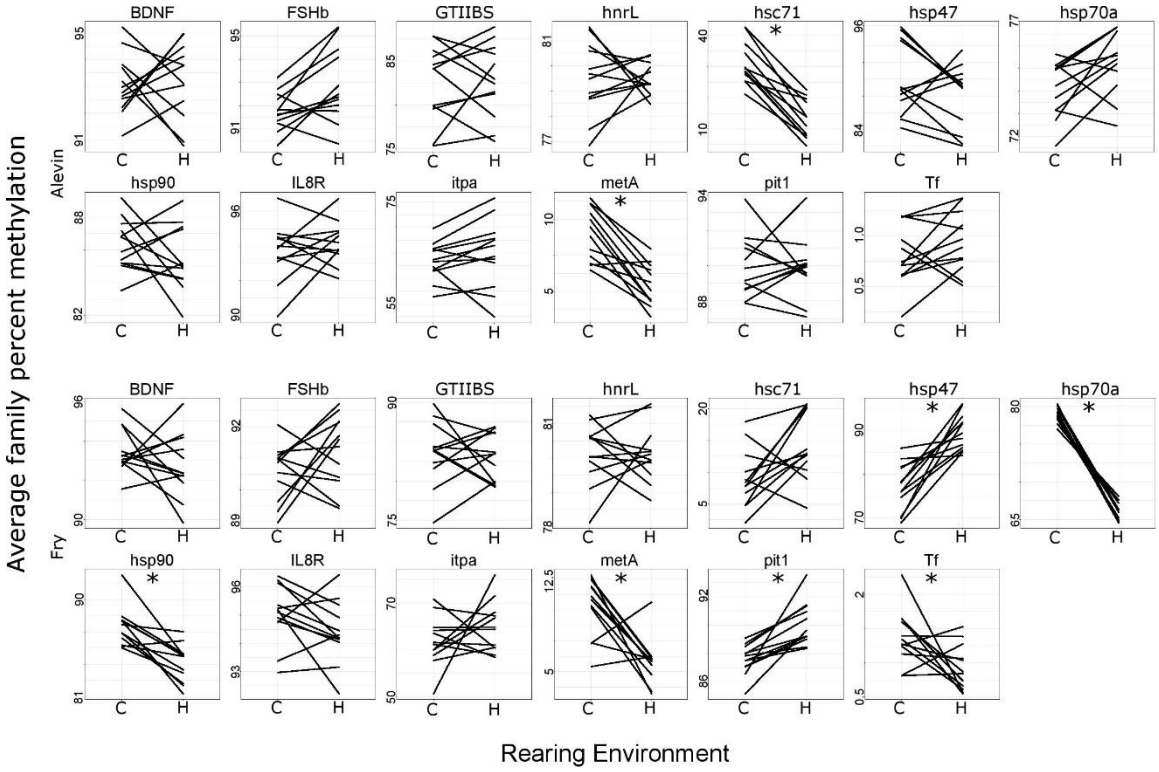

Figure 4.3: Reaction norm plots showing the effects of rearing environment on gene-specific DNA methylation in Chinook salmon alevin (top half) and fry (bottom half) full-sibling families. Each line represents the average percent methylation of a full-sibling family, while asterisks denote significant environmental effects on gene-specific methylation. Genotype x gene locus effects on methylation were significant across all genes in the alevin, but not the fry stage. Genotype x environment effects were not significant. C=semi-natural channel, H=hatchery.

in changing environments (reviewed in Angers et al. 2010; Savolainen et al. 2013; Bernatchez 2016; Sae-Lim et al. 2016; Sheriff et al. 2017). While many studies have shown that the environment influences DNA methylation (Ball et al. 2009; Angers et al. 2010; Nätt et al. 2012; Fang et al. 2013; Morán et al. 2013; Head 2014; Anastasiadi et al. 2017; Le Luyer et al. 2017; Metzger and Schulte 2017; Metzger and Schulte 2018; Olsvik et al. 2019; Li et al. 2020; Liew et al. 2020), the effects of environmental differences on the genetic architecture of DNA methylation have not been studied. However, previous studies have provided evidence for additive inheritance of methylation targeted to

specific regions of the genome (Hannon et al. 2018), a link between genotype and methylation status (Herrera and Bazaga 2010; Liu et al. 2012; Foust et al. 2016; Berbel-Filho et al. 2019), and family effects on methylation (Metzger and Schulte 2018). We observed differences in the genetic architecture of DNA methylation in Chinook salmon based on rearing environment and developmental stage, consistent with previous studies on environmental and developmental effects on genetic architecture (Etterson 2004; Aykanat et al. 2012b; Parsons et al. 2016; Wellband et al. 2018). We found significant dam x gene effects in alevin across all genes and both rearing environments, indicating locus-specific maternal effects at the alevin stage, consistent with previous research (Venney et al. 2020). Sire x gene interactions were significant at both the alevin and fry stage, indicating that additive genetic variation is targeted to specific genes. The environment x gene x dam interaction term significantly affected methylation across all genes in the fry stage, suggesting that rearing environments can facilitate or inhibit latent maternal effects. While most studies show a decline in maternal effects through development in salmon (Heath et al. 1999; Houde et al. 2015; Venney et al. 2020), parental effects have been shown to influence offspring at the fry stage in Chinook salmon (Falica and Higgs 2013). In general, we observed higher additive and non-additive variation and lower maternal effects in hatchery-reared fish relative to fish reared in the seminatural channel in both the alevin and fry stage (Figures 4.1 and 4.2). Control of methylation is a complicated process involving many proteins and pathways, some of which are still being identified (Grandjean et al. 2007), and methylation is inherently sensitive to environmental stimuli (e.g. Angers et al. 2010; Morán et al. 2013; Anastasiadi et al. 2017; Le Luyer et al. 2017). Thus, it makes sense that rearing

environment influences the genetic architecture of methylation through development, likely through multi-locus (i.e. epistatic) effects on methylation and demethylation processes (Grandjean et al. 2007). It remains unclear whether the emergence of additive and non-additive effects in hatchery fish, or of maternal effects in seminatural channel-reared fish, would be beneficial to offspring. Maternal effects prepare offspring for a predicted environment based on maternal genotype and environmental experience and thus have the potential to adaptively influence offspring fitness (Wolf and Wade 2009). However, additive and non-additive effects on offspring traits can also prove adaptive (Neff et al. 2011). Some traits exhibit additive genetic variation, allowing for selection for or against a given trait, whereas other traits exhibit non-additive variation due to improved fitness from the pairing of specific alleles or genetic factors with one another, resulting in a beneficial trait (Neff et al. 2011). It is unclear whether maternal effects, or additive and non-additive effects on DNA methylation will prove beneficial to offspring, though it is important to consider environmental context when studying the genetic architecture of DNA methylation, and in epigenetic studies in general. While hatchery reared salmon often exhibit reduced survival in the wild (Blouin et al. 2010; Becker et al. 2014), our study used a captive-bred population. Future studies on DNA methylation using wild-caught salmon as parents to quantify changes in the genetic architecture of DNA methylation in response to rearing environment may provide insights into the mechanisms behind reduced fitness of hatchery-reared salmon for applications in conservation efforts, and the relevance of GxE effects on methylation. Environmentally-induced shifts in the genetic architecture of DNA methylation could have important impacts on phenotype due to the effects of DNA methylation on gene expression

(Bossdorf et al. 2008) and phenotype (Cubas et al. 1999; Bossdorf et al. 2008; Ma et al. 2018). Thus, it is important to consider the environment in which an organism resides, as well as the effects of the environment on the genetic architecture of traits when studying interindividual variation.

Numerous studies have shown plasticity in methylation in response to environmental effects (Ball et al. 2009; Angers et al. 2010; Nätt et al. 2012; Fang et al. 2013; Morán et al. 2013; Head 2014; Anastasiadi et al. 2017; Le Luyer et al. 2017; Metzger and Schulte 2017; Metzger and Schulte 2018; Olsvik et al. 2019; Li et al. 2020; Liew et al. 2020). Hatchery rearing has become increasingly important in fish supplementation and conservation efforts (Fraser 2008), though the epigenetic effects of hatchery vs. (semi)natural rearing remain unclear due to conflicting results (Blouin et al. 2010; Le Luyer et al. 2017). However, rearing environment-induced plasticity in methylation has far-reaching implications in our understanding of how the environment shapes organismal function and development, particularly in stochastic environments and those influenced by climate change. In our study, rearing environment affected gene-specific methylation across genes in Chinook salmon as indicated by significant environment x gene locus interactions, as well as significant environmental effects. We observed substantial plasticity in methylation levels of heat shock proteins (hsc71 in alevin; hsp47, hsp70a, and hsp90 in fry) between rearing environments. Temperatures in the hatchery environment remain relatively stable with minor daily temperature fluctuations, whereas temperatures in the seminatural channel environment fluctuate with ambient temperature. Thus, short-term differences in seminatural channel temperature likely drive a gene-specific heat shock response (Basu et al. 2002; Lejeusne et al. 2006)

and can have long-lasting effects on DNA methylation states, gene expression (Anastasiadi et al. 2017), and heat shock protein expression (Basu et al. 2002). We also observed differences between hatchery and seminatural channel-reared fish in metA methylation in both life stages, as well as Tf and pit1 methylation in fry; these loci are involved in immune response and normal growth or metabolic functions (Berczi 1997; Stafford and Belosevic 2003; Vignesh and Deepe 2017). A previous study on hatchery-reared and wild Coho salmon identified differentially-methylated regions associated with immune response and metal ion processing (Le Luyer et al. 2017), consistent with our results. It is not surprising that fry exhibited more environmental effects on methylation than the alevins, as offspring experience more environmental variation over time as they develop and depart from maternal influences. Our results support DNA methylation as a mechanism for phenotypic plasticity due to its effects on gene expression (Bossdorf et al. 2008) and phenotype (Cubas et al. 1999; Bossdorf et al. 2008; Ma et al. 2018), consistent with previous research on environmental effects on methylation (e.g. Angers et al. 2010; Morán et al. 2013; Anastasiadi et al. 2017; Le Luyer et al. 2017). The capacity for plasticity of methylation in response to environmental change highlights the potential for downstream adaptive effects on phenotype and fitness without the long lag times associated with genotypic evolutionary change (Angers et al. 2010); thus, plasticity in methylation could aid organisms in responding to rapid environmental change, prolonging organismal survival in changing environments.

Genotype and environment both influence physiological and phenotypic traits, sometimes through GxE effects wherein the environment causes differences in phenotype due to genetic differences among individuals (Sae-Lim et al. 2016). Previous studies have

identified strong GxE effects on traits such as transcription in Chinook salmon (Wellband et al. 2018), survival in numerous fish species (Sae-Lim et al. 2016), and growth in transgenic Coho salmon (Sundström et al. 2007), European seabass, and other species (Dupont-Nivet et al. 2008). While methylation has been repeatedly shown to be influenced by underlying genetic factors (Herrera and Bazaga 2010; Fraser et al. 2012; Liu et al. 2012), it is unclear whether GxE interactions result in another layer of complexity underlying variation in DNA methylation. Genotype x gene interactions significantly affected methylation across all genes at the alevin stage, indicating that there is variation in gene-specific methylation among families irrespective of rearing environment. This could be due to underlying genetic control of or constraint in DNA methylation (Herrera and Bazaga 2010; Fraser et al. 2012; Liu et al. 2012), or due to significant dam (maternal) and sire (additive) genetic variation at the alevin stage. However, we found no evidence for significant GxE effects on DNA methylation in Chinook salmon, either across all genes or targeted to specific genes. While Figure 4.3 shows patterns of changing methylation rank among genotypes consistent with GxE interactions at several loci, we detected no significant GxE effects on DNA methylation, though GxE effects contributed a considerable amount of phenotypic variance to the methylation of certain genes (Appendix 4). It is possible that our relatively small sample size of four siblings per 12 unrelated families (versus 72 families in previous analyses) lacks sufficient power for the detection of GxE effects (Sae-Lim et al. 2016). DNA methylation is highly variable, even within lineages of clonal fish in the absence of genetic variation, thus substantial variation in DNA methylation can exist among closely related individuals (Massicotte et al. 2011). This inherent variability contributed to the

lack of significant GxE effects in our study due to low family number and high inter-individual variation. Consistent with the findings of Massicotte et al. (2011), genotype did not significantly affect methylation status in our study, though increased sample size in future studies may clarify whether there is genetic variation in the capacity for phenotypic plasticity of DNA methylation.

Environmental effects on DNA methylation have been extensively studied, yet few studies have focused on the genetic architecture or familial basis of epigenetic response to environmental differences. We show that early rearing environment influences the genetic architecture of DNA methylation at specific loci, with hatchery-reared offspring exhibiting higher additive and non-additive genetic variation and offspring reared in the semi-natural channel exhibiting higher maternal effects. Changes in the genetic architecture of traits can have significant effects on phenotype and fitness (Etterson 2004; Aykanat et al. 2012b; Parsons et al. 2016; Wellband et al. 2018), and thus are important considerations in evolutionary and conservation biology (Banta and Richards 2018). We show that DNA methylation exhibits phenotypic plasticity at specific loci in response to environmental change, consistent with previous studies on the effects of environment on DNA methylation (e.g. Angers et al. 2010; Morán et al. 2013; Anastasiadi et al. 2017; Le Luyer et al. 2017). We did not detect significant effects of genotype or GxE interactions on methylation when using full-sibling families as a proxy for genotype, likely due to high variance in methylation levels within full-sibling families. We present evidence for plasticity in methylation between environments, and changes in the genetic architecture of methylation which indicate that both parentage and rearing environment influence the methylation status of specific genes, consistent with

previous research (Metzger and Schulte 2018). Since environmental acclimation via DNA methylation has been proposed as a novel mechanism for coping with environmental stress (Angers et al. 2010; Massicotte et al. 2011; Varriale 2014), understanding the genetic and environmental basis of DNA methylation is critical for future study of DNA methylation as a potential mechanism for environmental acclimation and local adaptation.

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CHAPTER 5 - POPULATION DIFFERENCES IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) DNA METHYLATION: GENETIC DRIFT AND ENVIRONMENTAL FACTORS

Summary

Local adaptation and phenotypic differences among populations have been reported in many species, though most studies focus on either neutral or adaptive genetic differentiation. With the discovery of DNA methylation, questions have arisen about its contribution to individual variation in and among natural populations. Previous studies have identified differences in methylation among populations of organisms, although most to date have been in plants and model animal species. Here we obtained eyed eggs from eight populations of Chinook salmon (*Oncorhynchus tshawytscha*) and assayed DNA methylation at 23 genes involved in development, immune function, stress response, and metabolism using a gene-targeted PCR-based assay for Next-Generation sequencing. Evidence for population differences in methylation was found at eight out of 24 gene loci after controlling for developmental timing. However, we found no correlation between freshwater environmental parameters and methylation variation among populations at those eight genes. A weak correlation was identified between pairwise DNA methylation dissimilarity among populations and pairwise F_{ST} based on 15 microsatellite loci, indicating weak effects of genetic drift or geographic distance on methylation. The weak correlation was primarily driven by two genes, GTIIBS and Nkef, although single-gene Mantel tests were not significant after Bonferroni correction. Thus, population differences in DNA methylation are more likely related to oceanic environmental conditions or local adaptation, with genetic drift also playing a role. DNA methylation presents a novel mechanism that contributes to among population variation, with potential influences on organism phenotype, adaptive potential, and population resilience.

Introduction

Local adaptation occurs when organisms evolve in response to selective pressures in their immediate environment, resulting in increased individual fitness within their native habitat relative to non-native habitats (Kawecki and Ebert 2004; García de Leániz et al. 2007; Savolainen et al. 2013). Traditionally, the main mechanism underlying local adaptation has been the process of genetic adaptation: selection acts upon the phenotypes produced by standing genetic variation, resulting in increased frequency of beneficial alleles and thus evolution of populations over multiple generations (Bernatchez 2016). However, other mechanisms are now accepted as contributing to local adaptation, such as chromosomal translocations resulting in co-adapted gene complexes resistant to crossing-over (Kirkpatrick and Barton 2006; Barth et al. 2019; Lehnert et al. 2019; Kess et al. 2020), differences in organisms' capacity for phenotypic plasticity wherein organismal phenotypes are shifted towards an "ideal" phenotype based on their environment without underlying genetic changes (Pfennig et al. 2010; Hutchings 2011; Torres-Dowdall et al. 2012), maternal effects (Aykanat et al. 2012b; Galloway, 2005; Galloway & Etterson, 2007), and differences in gene expression (Fangue et al. 2006; Whitehead and Crawford 2006; Wellband and Heath 2013). However, phenotypic plasticity can also lead to population-level phenotypic responses to environmental conditions, eliminating selection on genetic variation, and thus acting in lieu of local adaptation. Adaptive population differences in gene expression have been reported in a broad variety of taxa. Studies have identified differences in gene expression among populations of killifish (*Fundulus heteroclitus*) across a natural thermal cline (Fangue et al. 2006), among rainbow trout (*O. mykiss*) from different tributaries subjected to stress challenges (Wellband and Heath

2013), between populations of the copepod *Tigriopus californicus* residing in different thermal regimes (Schoville et al. 2012), among populations of *Drosophila subobscura* across latitudinal and thermal clines in Europe (Porcelli et al. 2016), and both within and among populations of teleost fish from the genus *Fundulus* (Oleksiak et al. 2002). Further, patterns in gene expression variation may also reflect parallel evolution due to similar environmental conditions (reviewed in Fraser et al. 2011). While local adaptation through variation in gene expression has been frequently reported, the mechanisms underlying these differences in gene expression are poorly characterized, though environmental, genetic, and epigenetic variation could contribute to locally adapted gene expression profiles.

DNA methylation is one potential mechanism underlying transcriptional differences observed among populations in the context of local adaptation. DNA methylation is the addition of a methyl group to cytosine (C) bases that precede a guanine (G) in the DNA sequence, known as a CpG site (Head 2014). Numerous studies have shown that DNA methylation is highly sensitive to environmental signals (Bossdorf et al. 2008; Herrera and Bazaga 2010; Richards et al. 2010; Barfield et al. 2014; Foust et al. 2016) and is involved in acclimation to environmental stress (Morán et al. 2013; Metzger and Schulte 2017; Metzger and Schulte 2018). Due to the potential to modify methylation in response to environmental cues, methylation presents an important mechanistic intersection between acclimation and adaptation, particularly with extensive evidence for rapid (or "contemporary") evolution over short time scales (Stockwell et al. 2003). Methylation has been shown to be a highly targeted process (Venney et al. 2016; Venney et al. 2020). Therefore, short-term changes in methylation can occur that allow an

organism to cope with its environment, without the lag times associated with selection on standing genetic variation (Bossdorf et al. 2008; Richards et al. 2010; Hu and Barrett 2017), consistent with rapid evolution. Due to the sensitivity of methylation to environmental cues, it presents a novel mechanism for organisms to adapt to their environment and adds an additional level of complexity in organismal phenotypic variation and evolution (Bossdorf et al. 2008). Furthermore, methylation may respond to environmental stress, allowing for targeted short-term responses to environmental changes, which cannot occur through genetic adaptation (Hu and Barrett 2017). If methylation results in phenotypic plasticity, it may act in lieu of genetic adaptation, since the detrimental phenotype is no longer present to be selected against, or it may prolong the persistence of organisms in stressful environments until selection and genetic adaptation can occur (Crispo 2008).

Population-level variation in methylation has been reported in a variety of species and appears to have an underlying genetic basis. Several studies have identified a link between genetic and epigenetic variation (Herrera and Bazaga 2010; Fraser et al. 2012; Liu et al. 2012). For example, a study in Spanish violets (*Viola cazorlensis*) across an elevation gradient identified a strong correlation between methylation and genetic variation using pairwise distance-based AFLP analyses (Herrera and Bazaga 2010). Similar results were found using restriction enzyme-based methods for whole genome DNA methylation estimation and sequence polymorphism in female great roundleaf bat (*Hipposideros armiger*) populations (Liu et al. 2012), when comparing CpG-specific methylation and sequence variation in oak (*Quercus lobata* Née) populations (Platt et al. 2015), and for correlations between methylation differences and allele frequencies among

human ethnicities (Fraser et al. 2012). However, a study in salt marsh perennials (*Spartina alterniflora***)** was unable to link genetic differences with variation in methylation through AFLP-based approaches, and instead found a strong correlation with environmental variation (Foust et al. 2016). Thus, the relationship among epigenetic variation, genetic variation, and environmental heterogeneity is unclear, yet characterizing the interactions between these three drivers of population-level phenotypic variation is important in determining the role DNA methylation may play in driving local adaptation. While many studies have shown methylation differences among populations, most studies have focused on agriculturally important lab-reared species, while studies of natural populations are limited (Richards et al. 2010), making the role of DNA methylation in population differentiation unclear.

Chinook salmon (*Oncorhynchus tshawytscha*) are a culturally, ecologically, and economically important species of Pacific salmon. There is ample evidence for local adaptation based on functional differences among populations of Chinook salmon resulting in increased fitness in their native environments (Fraser et al. 2011). Adaptive genetic variation occurs at selected immune and growth-related candidate loci indicating genetic adaptation to their environment, while divergence at neutral (microsatellite) loci is related to isolation and genetic drift (Heath et al. 2006). Adaptation can occur within Chinook salmon stocks, for example, as evidenced by intrapopulation genetic differences in circadian clock genes based on migration timing, in the absence of neutral genetic variation (O'Malley et al. 2013). Variants impacting life history traits associated with environmental differences have also been reported in recently colonized Chinook salmon populations (Unwin et al. 2000), as well as differences in genetic variance components

and fitness-related traits (Aykanat et al. 2012a). Thus, there is abundant evidence for adaptive differences among populations of Chinook salmon, though most studies focus on genetic differences. While there have been studies documenting neutral and functional genetic variation among populations of Chinook salmon, it is unclear how rapid adaptation occurs when local conditions change or salmon colonize new habitats. However, studies have shown evidence for rapid adaptation to hatchery rearing, resulting in differences in gene expression (Christie et al. 2016), reproductive success (Christie et al. 2012), and DNA methylation (Le Luyer et al. 2017; Gavery et al. 2018). Due to the role of DNA methylation in rapid evolution of salmonids, it is possible that DNA methylation is important for responding to environmental changes, as well as maintaining standing genetic variation in salmon.

The goal of this study is to determine the role of DNA methylation in maintaining differences (adaptive or drift-related) among populations, and to assess genetic and environmental drivers of population-level differences in methylation. We characterize locus-specific population differences in DNA methylation in Chinook salmon and determine the influence of freshwater environment and genetic drift on levels of methylation at selected genes. We obtained eyed eggs from eight populations of Chinook salmon and measured DNA methylation using a gene-targeted PCR-based DNA methylation assay for Next-Generation sequencing. We expected that populations would exhibit different levels of DNA methylation at specific functional loci. Such patterns of methylation differences among populations could be due to environmental acclimation (Foust et al. 2016), underlying adaptive genetic variation (Herrera and Bazaga 2010; Fraser et al. 2012; Liu et al. 2012), or maternal effects at the eyed egg stage (Venney et

al. 2020). We hypothesized that population differences in methylation should be targeted to specific genes in response to unique environmental conditions and/or selective pressures among natural environments. We tested for correlations between locus-specific methylation and freshwater environmental variables from the native rivers of each population to determine whether local environmental factors influence gene-specific DNA methylation differences. We also tested for a correlation between genetic drift (variation at neutral marker loci) and methylation differences among populations to determine if methylation differences could be explained by population divergence due to genetic drift (and/or geographic isolation) distance. DNA methylation presents a novel evolutionary mechanism for populations to respond to their environments and cope with environmental stress. Due to the capacity for rapid DNA methylation changes in response to environmental cues, methylation represents a potential mechanism for organisms to locally adapt to their surrounding environment without the lag times associated with selection acting on standing genetic variation. Knowing the mechanisms involved in acclimation and local adaptation will impact how we manage and conserve natural populations, and therefore carries important implications for management and conservation of adaptive variation.

Methods

Eyed egg sampling and DNA extraction

Sampling adhered to Canadian Animal Care guidelines as approved by the University of Windsor (ACC #17-08). Eyed eggs (embryos) were sampled from eight populations of Chinook salmon from bulk incubators containing offspring from multiple mothers. Samples from seven populations were obtained from DFO Salmon

Enhancement Program hatcheries in November 2015 by hatchery staff while Quesnel River eggs were obtained from another project (Figure 5.1). Additional samples were obtained from Big Qualicum (BQ) and Harrison (Harr) populations in 2017 to test for interannual variation in methylation. Eggs were immediately preserved in a high salt buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) for future analysis. An estimate of ATUs (accumulated temperature units, a measure of developmental timing in salmon based on daily temperature) was obtained for each population based on water temperature from their resident hatchery.

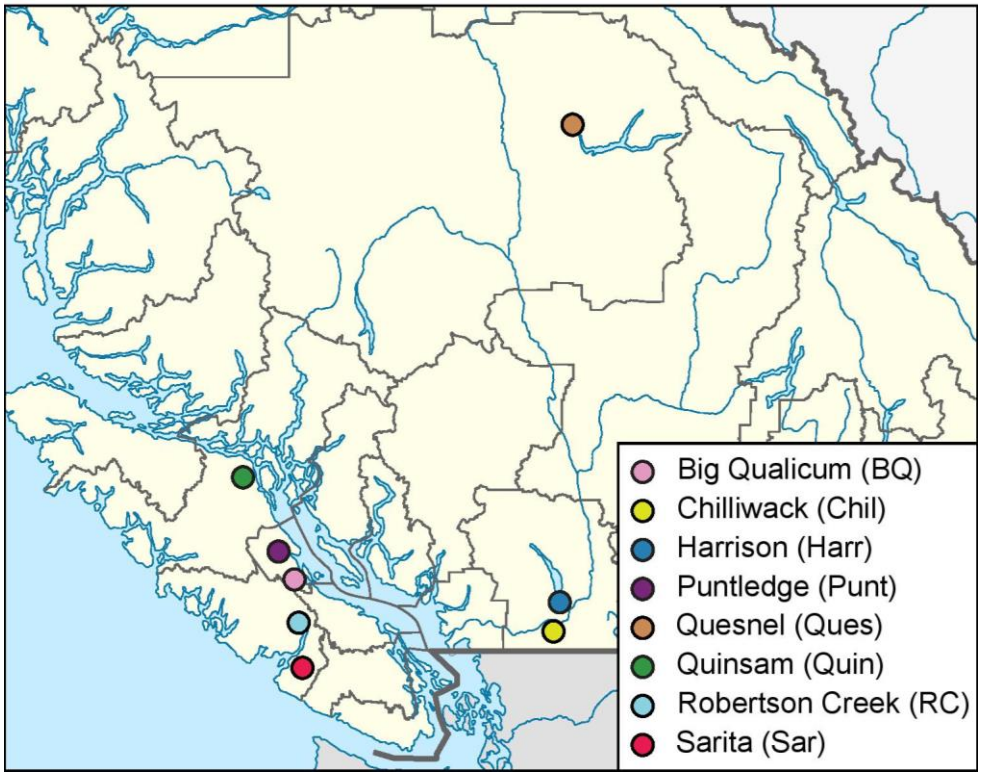

Figure 5.1: Locations of source populations of Chinook salmon eyed eggs sampled from DFO hatcheries in 2015. Eggs were obtained from Big Qualicum Hatchery (BQ), Chilliwack River Hatchery (Chil), Chehalis River Hatchery (Harr), Puntledge River Hatchery (Punt), Quinsam River Hatchery (Quin), Robertson Creek Hatchery (RC), and Nitinat River Hatchery (Sar). Quesnel River eggs were obtained from another project.

Embryos were dissected from 48 eyed eggs per population (n=10) and digested in 10 µL of 20 mg/mL proteinase K and 1000 µL of digestion buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS) at 37°C for 24 hours. We used 150 µL of the digested product for DNA extraction in a high-throughput automated plate-based DNA extraction protocol (Venney et al. 2020).

Bisulfite conversion and sequencing

DNA concentration was determined using a Quant-IT PicoGreen® dsDNA Assay Kit. Approximately 500 ng of DNA underwent bisulfite conversion using a Zymo EZ-96 DNA Methylation-Lightning kit following the manufacturer's protocol. Bisulfite conversion converts unmethylated cytosines to uracil while not affecting methylated cytosines, allowing for the determination of sites of methylation in the DNA sequence.

Methylation analysis was performed with 21 published bisulfite sequencing primers (Venney et al. 2016) and two novel bisulfite sequencing primer sets for growth hormone 2 (GH2, forward primer 5'-TTATTAAACCTTTCTAAAAACACAC-3', reverse primer 5'-ATTTAAATTTTAATTTTTTATAGGG-3', 241 bp fragment excluding primer sequences) and heat shock factor 1b (hsf1b, forward primer 5'-AGGATTAGGATTTTGAAGAGGATTT-3', reverse primer 5'-AATTAATTTTTCATCATCTACACATTAACA-3', 132 bp fragment excluding primer sequences). All primers were designed for gene regions with little to no sequence variation to minimize the effects of genetic variation on the interpretation of DNA methylation data. Assayed genes were selected for their roles in early development, stress and immune function, metabolism, early growth and differentiation. Amplicons ranged from 79 to 249 bp, with a total of 4111 bp sequenced excluding primer sequences

(Appendix 1). PCRs were performed using a two-stage PCR approach (Venney et al. 2016) where the first stage amplified the targeted gene region, and the second stage ligated sample barcode and adaptor sequences to the amplicon. Barcode sequences are 10-12 bp unique sequences that allow for the identification of individual samples in massively parallel (Next Generation) sequencing. Samples were split among three sequencing runs and sequenced with an Ion 318™ Chip using an Ion PGM™ Sequencing 400 bp kit on the Ion Torrent Personal Genome Machine® (PGM™) with an expected 500 reads per gene per sample.

Bisulfite sequencing data processing

Sequence data files were demultiplexed using mothur (Schloss et al. 2009), primer sequences were trimmed, and one fastq sequence file was created per individual. Bisulfite sequence data were aligned with known genomic sequences using bwa-meth (Pedersen et al. 2014) with a maximum of two mismatches per sequence to ensure sequences represented the target genes. Tabulated methylation data from bwa-meth were imported into R (R Development Core Team 2020) for quality filtering to ensure the same CpG sites were compared across all samples: CpG sites sequenced with (1) fewer than five reads per gene per sample, and (2) in less than 70% of individuals were excluded from the analysis. Rosner's test for extreme outliers was used to exclude significant outlier data points, which were likely reflections of low sequence depth rather than biologically meaningful variation. The final processed data provided average percent methylation for each individual in each gene that surpassed quality guidelines.

ATU and sampling year effects on methylation

Due to differences in ATUs (developmental timing) among populations, and within populations among sampling years, we tested for ATU effects on gene-specific methylation since developmental stage can have significant effects on methylation. Using the average percent methylation data, we determined the median methylation percentage for each gene in each population and used a linear regression per gene using the per population median methylation percentage to test for the effect of ATU on median methylation levels. We corrected for multiple comparisons using a Benjamini-Hochberg false discovery rate (FDR) correction. As developmental stage was found to be correlated with methylation (see Results), we used the residuals from linear models of ATU effects on single gene methylation instead of raw methylation data for all analyses to control for the effect of ATU on methylation.

We tested for the effect of sampling year on methylation using residuals generated from linear regressions for 20 loci for the BQ and Harrison 2015 and 2017 samples. For this analysis, we used only 20 loci due to three loci being excluded by quality filtering. An ANOVA was used for each gene to test for the effects of population, year, and their interaction using only BQ and Harrison gene methylation data to determine whether methylation changed from year to year. P-values were corrected using a Benjamini-Hochberg false discovery rate (FDR) correction.

Population effects on methylation

We tested for population-level effects across all genes using the 2015 samples (eight populations) to determine if overall methylation differs among populations using

an ANOVA for the effects of population, gene, and their interaction. An FDR-corrected ANOVA was used to test for the effect of population on individual gene methylation variation to determine which genes were driving population differences in methylation. Tukey's HSD posthoc test in the R package agricolae v1.3.2 (de Mendiburu 2020) was used to determine which populations were driving significant population-level effects on gene-specific methylation. R^2 values were obtained from all ANOVAs to estimate the methylation variance explained among populations, both across all genes, and for individual gene loci.

Principal component regressions for environmental effects on methylation

To determine whether environmental variation was driving population-level differences in methylation, we gathered data for 23 environmental variables from each natal river. In addition to longitude and latitude for each location, average temperature and precipitation were tabulated from the Government of Canada's historical climate database for the nearest available region (available at https://climate.weather.gc.ca/). Temperature and precipitation estimates were determined by averaging all available data from September to November (i.e. daily average temperature, and sum of precipitation). The Government of British Columbia's iMapBC app (https://www2.gov.bc.ca/gov/content/data/geographic-data-services/web-based-mapping/imapbc) was used to determine water turbidity, as well as concentrations of nitrite, nitrite + nitrate, chloride, and 14 metals in each river using water quality monitoring data (Appendix 5). Where possible, mean environmental data from several nearby monitoring stations was used. An estimate of pathogen diversity based on the number of diseases reported for fish from each population was included from the

Government of Canada's Fish Health Database (https://open.canada.ca/data/en/dataset/2ece9991-62aa-4b7a-bd7d-4f8f1052cd21).

Due to the large number of environmental variables collected, a principal component analysis (PCA) was used to reduce the dimensionality and autocorrelation of the environmental dataset. Principal components (PCs) were retained based on examination of a Scree plot and the eigenvalues of the PCs exceeding 1.0. To determine the effect of environmental factors on population differences in locus-specific methylation, a linear model was used to test the effects of each individual PC on methylation at each locus with a significant population effect on methylation (i.e. one linear model per PC per gene to avoid overfitting models for a small sample size). For all PC regressions, population medians from the residuals of ATU regressions on methylation were used instead of raw methylation data to minimize pseudoreplication and to control for the confounding effects of ATU. For each PC, a linear model was used to determine the effect of the PC on population-level differences in single gene methylation, and an FDR correction was used to correct for multiple comparisons.

Mantel tests comparing methylation data to microsatellite and SNP pairwise F_{ST}

Selected populations from the genetic baseline for Chinook salmon amplified by a microsatellite panel with 15 markers (Beacham et al. 2006) or a SNP panel with a minimum of 195 markers per sample and maximum of 369 markers (Beacham et al. 2018) were exported in genepop format from databases at the Molecular Genetics Lab (Fisheries and Oceans Canada). The SNP data specifically aimed to use fall populations when possible (i.e., Harrison, Puntledge, and Chilliwack River). The Chilliwack

population was restricted to the 2018 brood year. These datasets were analyzed using custom R scripts (R Development Core Team 2016; see Data Accessibility). In brief, datasets were loaded into R using adegenet v.2.1.1 (Jombart 2008), dendrograms were constructed using the aboot function of poppr v.2.8.3 (Kamvar et al. 2014) with the edwards.dist metric (Cavalli-Sforza and Edwards 1967) using 10,000 bootstraps. Data were then converted from genind format to hierfstat format using the genind2hierfstat function of hierfstat v.0.04-22 (Goudet 2005), and then pairwise F_{ST} values were calculated using the pairwise.WCfst (Weir and Cockerham 1984) function within hierfstat.

Pairwise distance matrices for microsatellite and SNP data were compared to methylation matrices to determine whether population-level differences in methylation corresponded with expected divergence due to isolation and genetic drift. A Euclidean distance matrix for population-level methylation variation was generated in the R package ade4 (Dray and Dufour 2007) using the medians of the residual methylation data across the eight genes showing significant population effects. The methylation distance matrix was compared to the pairwise microsatellite and SNP F_{ST} matrices using Mantel tests with 99 permutations in GenAlEx (Peakall and Smouse 2006; Peakall and Smouse 2012) to determine whether population differences in methylation across the eight genes were consistent with genetic divergence. A Euclidean distance matrix was generated for the median residual data of each gene to determine whether population differences in methylation at individual gene loci aligned with genetic drift expectations. We used a Bonferroni-corrected Mantel test with 99 permutations to determine whether divergence in methylation corresponded with genetic variation assessed by either microsatellite or

SNP variation (F_{ST}) for each of the eight gene loci that showed significant population effects. The resulting R^2 values were obtained with R^2 values from corresponding ANOVAs testing for population effects on methylation to estimate the relative contribution of genetic drift to the observed differences in DNA methylation. The best explanatory variable (drift or population effect) was determined based on relative R^2 values from the two models.

Results

ATU and sampling year effects on methylation

Linear regression results showed that accumulated temperature unit (ATU) significantly affected chemokine 1 (CK1) methylation before FDR correction ($p=0.0197$, $p=0.44$ after FDR, adjusted $R^2=0.56$), and approached statistical significance for four other loci: follicle stimulating hormone (FSHb), growth hormone 1 (GH1), heat shock protein 90 (hsp90), and metallothionein A (metA); $0.1>p>0.05$ before FDR correction). Thus, residuals from the linear regression for the effects of ATU on gene-specific methylation for all 48 individuals per population were used instead of raw methylation data to control for the potentially confounding effects of developmental timing.

We found no significant year effects on ATU-corrected methylation (after FDR correction) for the 2015 and 2017 BQ and Harrison samples. We did, however, find significant population effects on methylation between BQ and Harrison (2015 and 2017 samples) for gonadotropin II beta subunit (GTIIBS, $p<0.01$), natural killer enhancement factor (Nkef, $p<0.001$), hsp90 and CK1 ($p<0.05$) after FDR correction (Appendix 6). Before FDR correction, we found significant population x year interaction effects on five

genes, though after FDR we only detected a significant interaction effect on Nkef methylation ($p<0.01$ after FDR correction). Due to the significant Nkef population x year effect, as well as other significant interaction effects before FDR correction, only residuals from ATU models for the 2015 samples were used for downstream statistical analyses due to potential year effects on methylation. However, population and the population x year interaction contributed considerably more to variation in methylation than sampling year (Appendix 6).

Population differences in methylation

Population and the population x gene interaction significantly affected methylation levels across all genes combined (both $p<0.001$, $R^2=0.10$), indicating that while populations differ in overall methylation levels, they also differ in levels of gene-specific methylation. Direct between-gene differences in methylation were not quantifiable, as gene methylation values were standardized and centered around zero by using the ATU model residuals ($p=1.0$).

Population of origin significantly affected DNA methylation of eight genes: four heat shock proteins (all $p<0.01$ after FDR correction): heat shock protein 70 (hsp70), hsp90, heat shock protein 47 (hsp47), and heat shock cognate 71 (hsc71); GTIIBS, tumour suppressor protein 53 (p53), recombination activating gene 1 (RAG1), and Nkef (all $p<0.001$ after FDR correction, Figure 5.2, Appendix 7 for p-values and R^2 values). Tukey's HSD posthoc test identified similarities in Nkef, RAG1, and p53 methylation levels among BQ, Punt, Quin, and Sar (Figure 5.2), though no other patterns are apparent. Results from ANOVAs using raw methylation data instead of ATU-corrected data, which resulted in more significant population effects, are included in Appendix 7 for reference.

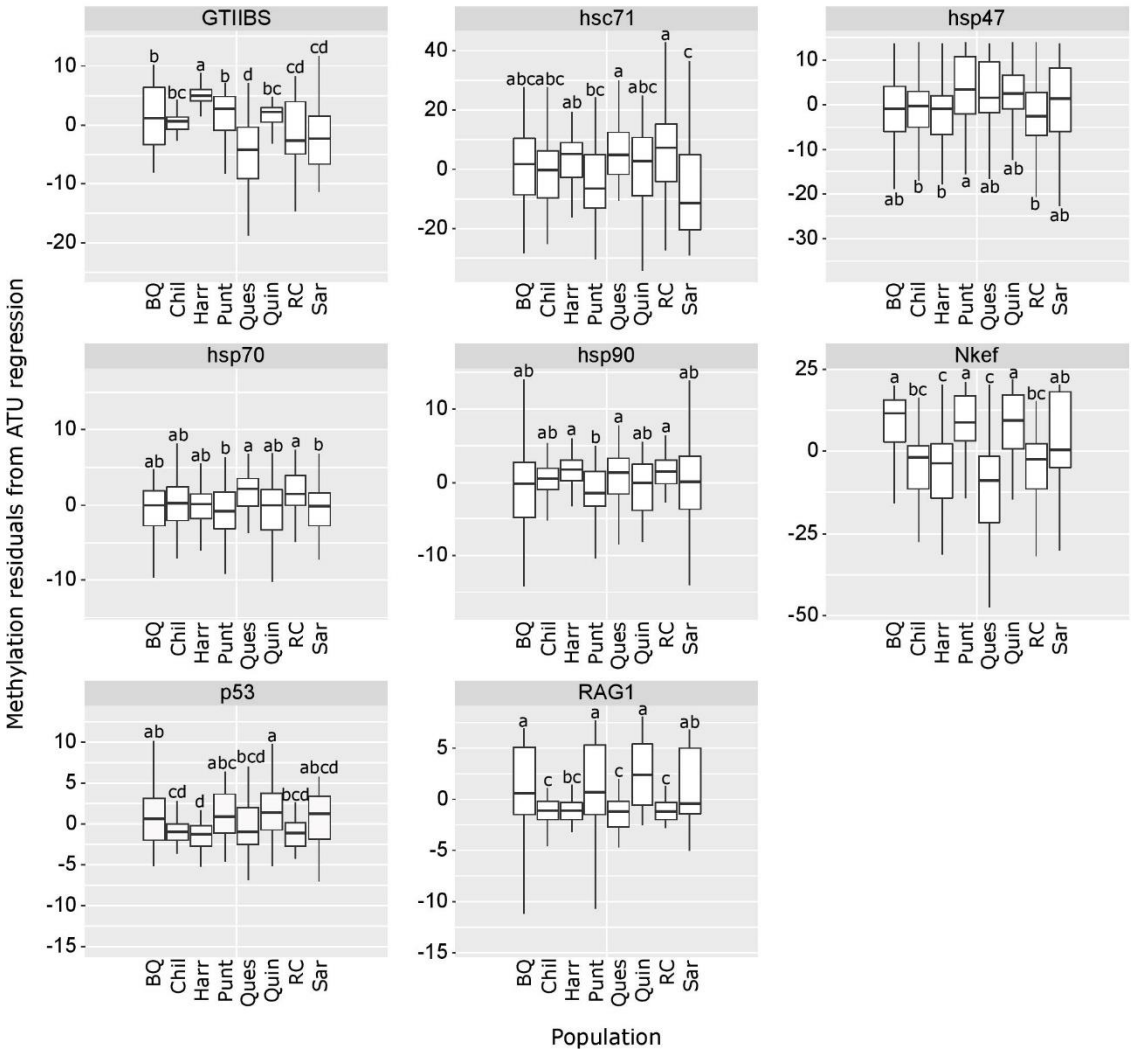

Figure 5.2: Box and whisker plots showing ATU-corrected methylation across all gene loci with significant population effects (N=8 loci) in Chinook salmon eyed eggs. Residuals from linear regressions for the effect of ATU were used to control for the confounding effect of ATU on methylation. Letters indicate significant differences identified by Tukey's HSD posthoc test.

Principal component regressions for environmental effects on methylation

Six principal components explaining 98.9% of variation in the environmental dataset were retained in the analysis based on PC eigenvalues greater than 1 and the Scree plot (Appendices 8 and 9). The results of this analysis showed that no environmental PC significantly affected population-level methylation at any of the eight

gene loci, except for Nkef. Nkef methylation was significantly affected by PC1 before FDR correction (p=0.029), though the effect was non-significant after correcting for multiple comparisons.

Mantel tests comparing methylation data to genetic differentiation (F_{ST})

Microsatellite pairwise F_{ST} values ranged from 0.00041 to 0.061 while SNP pairwise F_{ST} values ranged from 0.0032 to 0.19 (Appendix 10). Pairwise Euclidean dissimilarity values for methylation data ranged from 4.76 to 22.7 (Appendix 11). The Mantel test (Appendix 7) comparing microsatellite pairwise F_{ST} to median residual methylation data for all eight genes with a significant population effect showed a weak correlation between population-level differences in methylation and microsatellite genetic divergence (p=0.02, R^2=0.19, Figure 5.3), suggesting weak effects of genetic drift on methylation. The Mantel test comparing SNP pairwise F_{ST} to methylation data across all eight genes was not significant (p=0.10, R^2=0.064). Mantel tests correlating pairwise F_{ST} values with median residual methylation data for each gene were non-significant except for F_{ST} comparisons with GTIIBS (microsatellite analysis: p=0.02 before Bonferroni correction, R^2=0.25; SNP analysis: p=0.01 before Bonferroni correction, R^2=0.26) and Nkef (microsatellite analysis: p=0.03 before Bonferroni correction, R^2=0.20; SNP analysis: p=0.01, R^2=0.106 before Bonferroni correction), which became non-significant after correcting for multiple comparisons.

We compared the ANOVA results for population effects across all genes to the direct explanation of genetic differences (microsatellite and SNP F_{ST}) to determine whether population methylation effects were consistently explained by population of origin independent of genetic effects. The correlation between population differences in

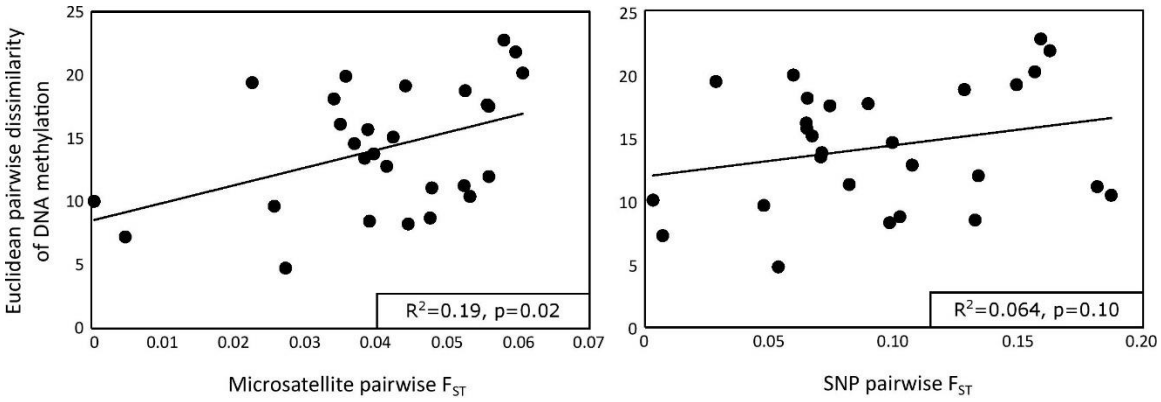

Figure 5.3: Scatterplots of pairwise Euclidean dissimilarity matrix for residual methylation medians (eight genes) versus (A) microsatellite FST values based on data from 15 loci, and (B) SNP FST values. The solid lines (and boxed statistics) show results of Mantel tests for correlation.

genetic variation and methylation variation was greater than the effect of population on methylation levels across all genes combined; however, this was only true for microsatellite genetic variation and not the SNP data. At the individual gene level, R^2 values from individual genes were greater from ANOVA models for five of eight genes (hsp90, hsc71, p53, RAG1, and Nkef) than from microsatellite and SNP Mantel tests. This indicates that while microsatellite genetic divergence explains more variation in methylation across all loci than population of origin alone, single-locus methylation status is more affected by population of origin.

Discussion

DNA methylation presents a novel evolutionary mechanism for individuals to rapidly respond to environmental changes and improve their survival in natural systems; in contrast, novel beneficial genetic mutations and natural selection acting upon existing variation are slow processes that take place over generations (Bossdorf et al. 2008; Richards et al. 2010; Hu and Barrett 2017). Rapid evolution has been shown to occur in a

variety of taxa and ecosystems, especially in response to increased environmental stress from human activities (reviewed in Stockwell et al. 2003), and DNA methylation has the capacity to serve as a mechanism facilitating rapid acclimation to local habitats. Rapid change in methylation has been observed due to habitat change (hatchery rearing) within a single generation (Le Luyer et al. 2017), with intergenerational effects on methylation passed on through the germline (Rodriguez Barreto et al. 2019). Previous epigenetic studies have primarily focused on sources of individual variation, rather than population-level differences in methylation (Hu and Barrett 2017), yet population-level differences in methylation could explain heritable variation among populations which cannot be explained solely by genetic variation (Bossdorf et al. 2008). We observed significant population differences in methylation across all genes combined, as well as a significant population x gene interaction, indicating that populations differ in overall methylation, as well as methylation targeted to individual genes. Methylation differences among populations have been reported in several other studies (Herrera and Bazaga 2010; Richards et al. 2010; Fraser et al. 2012; Liu et al. 2012; Barfield et al. 2014; Platt et al. 2015; Foust et al. 2016) with the potential to contribute to rapid acclimation and/or adaptation to stressors (Bossdorf et al. 2008; Richards et al. 2010; Hu and Barrett 2017). The population-level differences in methylation we report represent a novel evolutionary mechanism that may contribute to the extensive adaptive genetic variation observed in natural populations of Chinook salmon (Fraser et al. 2011). However, the patterns of broad population-level variation in DNA methylation reported here are of broad relevance when considering potential mechanisms of phenotypic differentiation in natural populations in general.

Population-level differences in methylation could reflect acclimation to the local environment, or local adaptation due to environmental selection on phenotypes. While several studies have identified population differences in methylation, most focus on methylation at the whole-genome level rather than using a candidate gene approach. We observed population-level differences in methylation at specific genes in Chinook salmon eyed eggs: four heat shock protein genes (hsc71, hsp47 hsp70, and hsp90), three immune genes (p53, RAG1 and Nkef), and one gene involved in endocrine function (GTIIBS), all of which are logical targets for differences in methylation among populations. Heat shock proteins have a variety of cellular roles and become upregulated in stressed organisms in response to a broad variety of stressors and environmental situations, often with clinal or population-level differences in heat shock protein expression (Sørensen et al. 2003; Tine et al. 2010). Previous studies in teleost fish have identified differences in immune response among populations (Evans et al. 1997; Evans et al. 2010; Fraser et al. 2011), as well as differences in hormone concentrations and endocrine function (Carr and Patiño 2011; Sopinka et al. 2017). Differences in gene methylation could reflect acclimation or adaptation to local environments. Transient environmental stressors such as temperature stress, pollutant exposure, and other acute stressors would likely induce an acclimation response, whereas exposure to long-term chronic stressors, such as differences in pathogen communities among populations, thermal regimes, and chronic pollution stress could result in local adaptation to cope with ongoing and predictable environmental stress. Further research is required to determine whether population-level differences in gene-specific methylation result from acclimation or adaptation, though significant differences in methylation between BQ and Harrison with no significant temporal effects

suggest local adaptation. Future research measuring methylation in reciprocal transplants or in common garden experiments with natural populations could determine whether population-level variation in methylation is retained, and whether it likely represents acclimation or adaptation. Regardless of the underlying process, the genes showing significant population effects are logical targets for differential DNA methylation due to differences in environmental context and stressors among populations.

DNA methylation is often influenced by environmental context (Bossdorf et al. 2008; Herrera and Bazaga 2010; Richards et al. 2010; Barfield et al. 2014; Foust et al. 2016). We used principal component analysis and regression to test for environmental effects on DNA methylation among populations using environmental data from the natal streams of the studied Chinook salmon populations. We found no significant effects after correcting for multiple comparisons, which was unexpected, as many studies have reported environmental effects on methylation (Angers et al. 2010; Morán et al. 2013; Dimond and Roberts 2016; Foust et al. 2016; Le Luyer et al. 2017). The lack of significant environmental correlates is likely due to our use of Chinook salmon eggs. At the egg stage, the embryo is isolated and protected from the environment, which may reduce its response to environmental variation, though it is still possible that eggs respond to local environmental conditions through changes in methylation. Additionally, Chinook salmon exhibit strong maternal effects on DNA methylation at the eyed egg stage (Venney et al. 2020) which may increase variation within a population and reduce correlations between gene-specific DNA methylation and environmental variables. Parents experience the freshwater environment prior to spawning, and thus could alter egg methylation signals in response to the offspring's predicted environment. Thus, the

population-level differences in methylation observed in Chinook salmon may be due to acclimation or adaptation to freshwater environmental signals from their parents, eyed egg acclimation to the environment, or due to genetic differences among populations (Fraser et al. 2012; Liu et al. 2012).

Population epigenetic studies vary in their conclusions as to the link between epigenetic differences among populations and genetic divergence. A study in salt marsh perennial plants found no link between genetic and epigenetic differences across environmental gradients, but a strong correlation with environmental conditions (Foust et al. 2016). However, the first true population epigenetic study linked DNA methylation differences in Spanish violets to genetic differences identified by AFLP in response to elevation (Herrera and Bazaga 2010). A significant correlation between genetic and epigenetic variation was also reported among female great roundleaf bat populations (Liu et al. 2012) and due to differences in allele frequency among human ethnic groups (Fraser et al. 2012). Here we compared epigenetic differences among populations to neutral genetic variation at microsatellite loci to determine whether differences in DNA methylation among populations align with genetic drift. The correlation between microsatellite F_{ST} and Euclidean pairwise dissimilarity in methylation among populations ($p=0.02$, $R^2=0.19$) was likely primarily driven by the significant correlation (before correction for multiple comparisons) between epigenetic differences at GTIIBS and Nkef and neutral genetic divergence. However, there was no significant correlation between SNP divergence and methylation pairwise dissimilarity across all eight genes ($p=0.12$, $R^2=0.064$), likely due to weaker single-gene correlations between GTIIBS and Nkef methylation and SNP divergence. Microsatellite genetic drift ($R^2=0.19$) explained more

variation in methylation data across all genes than the effect of population alone (ANOVA for population effect across all genes, R^2=0.10), though SNP divergence (R^2=0.064) explained less variation than population-level differences in methylation. However, for five of eight genes with a significant population effect on DNA methylation, the R^2 values from population effect ANOVAs were greater than those obtained from both the SNP and microsatellite Mantel tests. This suggests that while genetic drift may best explain DNA methylation across all loci, at specific loci, DNA methylation may be a result of both genetic drift and selection effects (i.e. local adaptation). While divergence in methylation among populations may be attributed in part to genetic drift, neutral genetic divergence in Chinook salmon is affected by geographic distance (Beacham et al. 2006; Heath et al. 2006). Given that geographic distance is expected to be related to ecosystem dissimilarity, it is possible that weak signals of drift may simply reflect environmental similarities among proximate populations. The weak correlation between neutral genetic markers and differences in methylation among populations suggests that while drift acts on methylation, mechanisms other than drift (such as selective mechanisms) likely also contribute to differences in methylation among populations. The lack of a strong correlation between methylation and neutral genetic divergence and the lack of consistent population-level similarities among proximal populations across genes (see groupings in Figure 5.2), coupled with extensive research showing local adaptation in salmonid fish populations (reviewed in Fraser et al. 2011) suggests that local adaptation is likely shaping population differences in methylation at key gene loci. However, drift effects or unmeasured environmental effects cannot be ruled out. This hypothesis is supported by a previous

study linking epigenetic and adaptive genetic variation (Herrera and Bazaga 2010), which found a significant correlation between methylation and genetic divergence in *Viola cazorlensis*. It is also possible that population differences in methylation are due to genetic control of methylation processes – in essence, different genotypes result in different methylation patterns (Liu et al. 2012). We show that differences in methylation among populations are not well explained by genetic drift alone, suggesting that methylation is likely also influenced by a combination of genomic differences among populations, environmental acclimation, and local adaptation.

We found that ATUs (a measure of developmental timing in salmon), and the interaction between population and sampling year influenced DNA methylation. DNA methylation patterns have been shown to change through development in fish (Fang et al. 2013; Fellous et al. 2018; Venney et al. 2020), thus we expected differences in methylation levels in the eyed eggs as they developed. In mangrove rivulus (*Kryptolebias marmoratus*), changes in methylation occurred during development throughout organogenesis leading up to hatch (Fellous et al. 2018). However, while developmental changes in methylation are well-characterized, interannual changes in methylation are not. We found a significant population x sampling year interaction on one gene after correcting for multiple comparisons when controlling for ATU in Harrison and BQ 2015 and 2017 samples, with four other genes showing significant effects before FDR correction. The significant population x year effect suggests that there is some inter-annual variation in methylation within populations which is likely due to acclimation, though population-level differences persist across years. These differences could be due to changes in freshwater and marine environments experienced by the parents and

offspring from year to year. This raises the question of whether the egg's freshwater environment, or the parental marine and/or freshwater environments are influencing offspring methylation patterns. Since the population of origin (Harr vs. BQ) significantly affected methylation of four genes after FDR correction, and sampling year explained very little phenotypic variation in methylation (Appendix 6), population clearly has a greater effect on methylation state than sampling year. Our results reinforce the importance of controlling for potential confounding variables such as organism age/developmental stage and year of sampling, since methylation is a highly sensitive and dynamic mechanism for controlling gene expression.

Population epigenetic status is an important new consideration in evolutionary and ecological studies (Bossdorf et al. 2008) since DNA methylation could act as a highly dynamic evolutionary mechanism upon which selection could act (Bossdorf et al. 2008; Hu and Barrett 2017). Unlike genetic adaptation, which requires standing variation and selection, methylation changes are rapid and dynamic, adding an additional layer of complexity and specificity for organisms to acclimate and adapt to their environment (Bossdorf et al. 2008; Hu and Barrett 2017). In this study, we provide evidence for differences in methylation among populations of Chinook salmon, consistent with previous population epigenetic studies (Herrera and Bazaga 2010; Richards et al. 2010; Fraser et al. 2012; Liu et al. 2012; Barfield et al. 2014; Platt et al. 2015; Foust et al. 2016). Despite reported strong environmental effects on DNA methylation (Bossdorf et al. 2008; Herrera and Bazaga 2010; Richards et al. 2010; Barfield et al. 2014; Foust et al. 2016), we found no link between freshwater environmental parameters and population differences in methylation. This may be due to (1) methylation corresponding to the

marine environment experienced by the parents rather than freshwater variables considered here; (2) strong maternal effects on methylation at the eyed egg stage in Chinook salmon (Venney et al. 2020), which could decrease DNA methylation-environment correlations due to varying environmental experiences of individual mothers; or (3) key environmental variables that affect methylation but were not included in our PCA. We identified weak correlations between genetic drift and DNA methylation, indicating that while some changes in methylation state among populations are likely due to drift, other differences could be the result of selection (Bossdorf et al. 2008) or are linked to underlying functional genetic differences (Fraser et al. 2012). Characterizing sources of phenotypic variation among natural populations is critical to understanding individual variation and the viability of natural populations. DNA methylation is an important novel source of phenotypic variation, and is an exciting and novel candidate for adaptive response in nature since an organism's environment and experiences can influence methylation levels (Bossdorf et al. 2008; Burggren 2014). Furthermore, methylation signals can be passed on to offspring generations and beyond (Kamstra et al. 2018; Santangeli et al. 2019), resulting in rapid adaptation and evolutionary change in response to changing environments.

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CHAPTER 6 – GENERAL DISCUSSION

The study of DNA methylation in an evolutionary context is in its infancy, though epigenetic mechanisms have been proposed to play a role in short-term evolutionary response before genetic evolution catches up (Bossdorf et al. 2008; Richards et al. 2010; Massicotte et al. 2011; Schrey et al. 2012; Herman et al. 2014). DNA methylation can affect gene expression (Jaenisch and Bird 2003; Varriale 2014), phenotype (Cubas et al. 1999), and potentially fitness (Rubenstein et al. 2016), and thus can have implications for the survival and persistence of organisms, populations, and species. Thus, characterizing variation in methylation from the single gene level all the way to the population level is important in understanding the complexity and highly regulated nature of DNA methylation, and its importance in natural systems. The aim of this dissertation was to characterize gene-specific methylation among tissues, in transmitting maternal effects, in plastic changes in the genetic architecture of traits in response to different rearing environments, and in population-level variation.

This dissertation began by establishing DNA methylation as a highly targeted mechanism within organisms. Many studies have hypothesized that DNA methylation could serve as a novel adaptive mechanism for dealing with environmental stress (Bossdorf et al. 2008; Angers et al. 2010; Dimond and Roberts 2016; Foust et al. 2016; Metzger and Schulte 2017). For methylation to be considered an adaptive mechanism for responding to environmental stimuli, it must be targeted to specific loci rather than occurring a whole genome level, as whole-genome methylation/demethylation would maladaptively affect the regulation of highly canalized genes critical for normal organismal function. While most methylation studies use whole-genome or reduced-representation sequencing techniques, I used a targeted candidate gene sequencing

method to allow the use of large experimental designs with lower costs than other methods. Using this gene-targeted approach, I provided evidence for the highly targeted, gene-specific nature of DNA methylation from the tissue level to the population level throughout this dissertation. The differences in gene-specific methylation observed at different levels of biological organization highlight the importance of considering and controlling for sources of variation in methylation in future studies. The targeted changes in methylation reported in this dissertation could represent either short-term acclimation responses to environmental stimuli, or longer-term adaptive responses based on selection. While the work presented in this dissertation cannot differentiate between the two mechanisms, it provides evidence for additive and non-additive effects on methylation, which could be subject to selection. This dissertation provides ample evidence for the highly targeted nature of DNA methylation, supporting methylation as a potential mechanism for adaptive responses.

This dissertation addressed the complicated role of DNA methylation during development. Since organisms require strict control of gene expression to ensure proper development and function (Zeitlinger and Stark 2010), it is logical that differences in methylation occurred among developmental stages. However, it was unexpected to find that DNA methylation may serve as a mechanism for the propagation of intergenerational plasticity, specifically maternal effects, due to the resetting of methylation shortly after fertilization in fish (Mhanni and McGowan 2004; Perez and Lehner 2019). Since maternal effects can play a role in optimizing offspring fitness based on the mother's genotype and experiences (Mousseau and Fox 1998; Green 2008; Wolf and Wade 2016), it is possible that methylation-based maternal effects fine-tune the gene expression of

offspring to help them cope with a predicted environment. Thus DNA methylation serves both as a tightly controlled mechanism for canalized gene expression during critical developmental periods, as well as a mechanism for maternal effects that act upon the offspring during the same developmental stages. Methylation-based maternal effects could underly previously reported phenotypic and physiological maternal effects and contribute to offspring fitness if they influence offspring phenotype. Since methylation is sensitive to the environment (e.g. Morán et al. 2013; Dimond and Roberts 2016; Foust et al. 2016; Metzger and Schulte 2017, 2018; Berbel-Filho et al. 2019; Li et al. 2020) and can be passed on through the germline (Zaghlool et al. 2016; Rodriguez Barreto et al. 2019; Santangeli et al. 2019; Liew et al. 2020), it presents a novel, complex mechanism that integrates signals from the parental environment and genome, and passes them on to offspring, with downstream effects on gene expression, phenotype, and physiology. This dissertation has shown that DNA methylation is important in organismal development and is a likely mechanism for the transmission of maternal effects, an important non-genetic source of variation during early development.

My doctoral research addressed the effects of rearing environment on the genetic architecture of DNA methylation. Genetic architecture, in this context, refers to additive (heritable), non-additive (dominance and epistasis), and maternal variance components (Lynch and Walsh 1998). Since DNA methylation serves as a mechanism for the transfer of maternal effects to offspring, and methylation is sensitive to the environment (e.g. Morán et al. 2013; Dimond and Roberts 2016; Foust et al. 2016; Metzger and Schulte 2017, 2018; Berbel-Filho et al. 2019; Li et al. 2020), it is likely that the environment will influence the genetic architecture of methylation. This dissertation provided evidence for

environmentally induced plasticity in methylation, as well as environmentally induced changes in the genetic architecture underlying methylation. Thus, while some environmental effects on methylation represent plastic responses to the environment, others likely represent complex interactions between the environment and the molecular machinery regulating DNA methylation, resulting in shifts in genetic architecture among environments. To fully understand how the environment influences methylation, it is therefore important to consider genetic (additive, non-additive, and maternal) sources of variation underlying differences in methylation. Variation in methylation is not simply the result of environmental context or genetic effects, but rather is the complex outcome of interactions between various developmental, environmental, and genetic inputs. Considering the genetic basis of variation in DNA methylation, rather than regarding environmental effects on methylation as a simple acclimation response, will improve our understanding of how methylation responds to environmental changes, and how individual variation arises as a result of environmental change and stress. Understanding sources of variation within populations is particularly important in a time of rapid environmental change, and methylation represents a potentially important mechanism for maintaining variation within a population and responding to environmental changes.

The final aim of this dissertation was to determine whether population-level differences in methylation exist, and to place them in the context of local adaptation to environmental context, and genetic drift. Populations often differ genetically and in terms of habitat, and since earlier work in this dissertation showed that methylation is influenced by both environmental and genetic differences, it seemed logical that populations would show potentially adaptive differences in methylation among

populations. While methylation showed a weak correlation with genetic drift and no correlation with the available freshwater environmental variables, it is likely that unmeasured environmental variables and functional genetic variation are also influencing population-level differences in methylation. Population-level differences in methylation are consistent with the idea that methylation could serve as an additional, rapid adaptive mechanism for coping with environmental stress (Bossdorf et al. 2008; Richards et al. 2010; Hu and Barrett 2017). Since methylation can change rapidly in response to the environment and can also be passed on to the next generation, methylation represents a potential novel evolutionary mechanism to bridge the gap between short-term acclimation responses and genetic adaptation. While this dissertation does not attempt to characterize DNA methylation as an adaptive mechanism, the results support the idea due to the highly targeted nature of methylation, its role in transmitting potentially adaptive maternal effects, and its potential role in local adaptation.

The research discussed in this dissertation evaluates variation in DNA methylation from the tissue to the population level and addresses important questions about the role of DNA methylation in evolutionary mechanisms. The findings detailed in this dissertation advance our understanding of the role DNA methylation plays in transgenerational signaling and local adaptation, and raises new and exciting questions about DNA methylation in evolutionary biology. Based on the work in this dissertation, I suggest future research on (1) the effects of methylation on the fitness of organisms; (2) intergenerational plasticity through methylation; (3) population-level differences in the plasticity of DNA methylation; and (4) the evolutionary implications of other epigenetic mechanisms. Future research should attempt to quantify whether differences in

methylation influence organism fitness (a) in response to environmental change, and (b) due to intergenerational transmission of DNA methylation-based maternal effects. While many studies have hypothesized about the adaptive role of DNA methylation in organisms, an estimate of the fitness effects of methylation is lacking in the literature. Future research on intergenerational transfer of DNA methylation should compare parental effects on methylation in species with different investment in offspring care (e.g. investment into gametes, semelparity vs. iteroparity, presence and absence of parental care) to determine the importance of methylation-based parental effects in different species. Methylation studies on intergenerational effects should also assess the persistence of methylation-based parental effects by tracing maternal effect signals through multiple generations, since multigenerational effects are less likely to accurately predict offspring environment and prove adaptive. Since both the methylation state of genes, as well as the machinery controlling methylation, could be subject to selection, future research should expand the work presented in this dissertation on population-level variation in methylation. Future studies should determine if there are population-level differences in the plasticity of methylation in response to environmental stress. This would improve our understanding of the mechanisms through which organisms respond to environmental change, which is of particular importance in the midst of human-induced rapid environmental change. Finally, while DNA methylation is the most studied epigenetic mechanism, future studies should determine the evolutionary significance of other epigenetic mechanisms (e.g. non-coding RNA molecules such as microRNAs). The research in this dissertation improves our understanding of the role of DNA methylation in an evolutionary context, and expands our knowledge of the mechanisms underlying

plasticity, the genetic architecture of traits, intergenerational plasticity, and variation among populations.

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APPENDICES

Appendix 1: Bisulfite sequencing primer sequences for Chinook salmon.

Gene	Forward primer	Reverse primer
	Growth genes	
Brain-derived neurotrophic factor (BDNF)	GATTAAGGATGTTGATTTGT	TAACAATCTACCCAAACATATCTAT
Follicle stimulating hormone beta (FSHb)	TGTGTAATTTTAAGGAGTGGTTTTA	ACATTTCTAATAAATTTACTATACAACTAA
Growth hormone 1 (GH1)	TTTAGTTAGAAAGTATAGTGTAAGGATTA	TTATTAAACCTTTCTAAAAACACAC
Growth hormone 2 (GH2)	ATTTAAATTTTAATTTTTTATAGGG	CAATCAATAAAATAAATTACCCCATCAC
Gonadotropin II beta subunit (GTIIBS)	TTTTGTGTATTTATTTATTAGGAGT	ATACAAAAATCTAACTACAAACTCTC
Pituitary-specific transcription factor (pit1)	GAGAATTTGTAGTTGAGTTTTAAGA	AAAATAAAAACTTAATCTTCTCCCC
	Immune and stress-related genes	
Antithrombin (anthr)	TTAAATATTTTTATGTTTTTTATTA	TCTCAATCTTAATTTTATATTTT
Chemokine 1 (CK1)	TTTTTTTTTTTTATTATTATTTTTA	CTAAATAAACTTCAAACAACAATC
Heterogeneous nuclear ribonucleoprotein L (hnrL)	TATATTTGAGTTTAATTTTGGAAT	CACACCATTTAAATAAAACCATAAT
Interleukin 8 receptor (IL8R)	TTTGTTTTTATTATTTATTATGGTGG	AAATACACCAACTTAACCCTCATC
Natural killer enhancement factor (Nkef)	TAGAATAATATTTTTAGTATTTTTT	TTCCTCATTTCAAACTATCCCATCT
Recombination activating gene 1 (RAG1)	TTTAAGTTTAATTTAGAGATGTTTT	CCTCCAAACCCTCCATCTCTCACAC
Transferrin (Tf)	ATAGTATTTATTTTGTTTTTAGTTA	CTCACCTTAATAACTTTAATACATTCAAAA
	Metabolic genes	
Heat shock cognate protein 71 (hsc71)	TTGATTTTGGTTTAATTATTTGAGGA	TCAAACACTCCCTAATACCATTTAC

Heat shock factor 1b (hsf1b)	AGGATTAGGATTTTGAAGAGGATTT	AATTAATTTTTCATCATCTACACATTAACA
Heat shock protein 47 (hsp47)	AAGTATTTTTAGGGAATAGGAGTGTATATA	TATCTAATTTTATAAAAAACAAAAATCAAA
Heat shock protein 70 (hsp70)	TAGTTGTTAAGAATTTTTTGGAGT	AACTAATACTCATACTCCTCTTTATC
Heat shock protein 70a (hsp70a)	GTAGGGAAATTTTTGTTTTATTG	CCAATTATTTTAATAACTACTATCTTATCT
Heat shock protein 90 (hsp90)	ATGAGATTTTATTTTTAGAGGGAGA	CCATAAAAAAACACTAACCAAATTACC
Inosine triphosphatase (itpa)	TTGTGTAGATTAGATAGTTTTATAT	AATCCAAATTTAATAAACTCTATCAATTTA
Metallothionein A (metA)	TTTATGGTAAATTTAATTAATTTTAATTGT	AACCTAAAACACACTTACTACAACC
Myosin 1A (Myo1A)	TGTAGGAGTTAGTTTTTGGTAAAGTAT	AAAAATCAATCTAAACTCACCAATC
Tumour antigen P53 (P53)	GGTTTTGGGTTGATTTTTAATTAAT	ATTAATCTCCTCTATCTTCCTATCTC

Appendix 2: Results from LMMs from each gene in each developmental stage. Variance components were used for an unrestricted variance analysis. Average read depth was calculated across all assayed CpG sites for each gene in each developmental stage.

Gene	Variable	Variance	Chi-square	P-value	Significance (Bonferroni correction)
			Egg		
FSHb	cross		0.021	0.886	
	dam	0.455	0.001	0.975	
	sire	0.000	0.000	1.000	
	dam x sire	6.830	15.759	0.000	**
GTIIBS	cross		3.012	0.083	
	dam	5.121	22.267	0.000	***
	sire	4.333	18.840	0.000	***
	dam x sire	0.030	0.002	0.966	
GH1	cross		2.716	0.099	
	dam	0.000	0.000	1.000	
	sire	0.322	1.000	0.655	
	dam x sire	0.099	0.013	0.910	
hsp90	cross		1.318	0.251	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	1.220	1.423	0.233	
pit1	cross		0.736	0.391	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
metA	cross		0.041	0.839	
	dam	0.055	0.198	0.656	
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
Tf	cross		1.127	0.288	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
IL8R	cross		0.171	0.680	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
hsc71	cross		0.331	0.565	
	dam	43.755	16.250	0.000	***
	sire	27.816	9.834	0.002	*
	dam x sire	0.148	0.001	0.970	

hsp70a	cross		0.167	0.683	
	dam	0.589	4.228	0.040	
	sire	0.000	0.000	1.000	
	dam x sire	0.213	0.316	0.574	
itpa	cross		4.620	0.032	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	20.100	2.042	0.153	
CK1	cross		2.605	0.107	
	dam	1.981	0.988	0.320	
	sire	0.000	0.000	1.000	
	dam x sire	4.574	3.577	0.059	
BDNF	cross		0.328	0.070	
	dam	0.000	0.000	1.000	
	sire	0.448	0.047	0.828	
	dam x sire	0.000	0.000	1.000	
hnrL	cross		1.339	0.247	
	dam	3.910	2.285	0.131	
	sire	0.000	0.000	1.000	
	dam x sire	3.507	1.008	0.315	
			Alevin		
FSHb	cross		0.828	0.363	
	dam	0.000	0.000	1.000	
	sire	0.120	0.000	1.000	
	dam x sire	0.957	1.100	0.294	
GTIIBS	cross		3.230	0.073	
	dam	3.350	7.090	0.008	
	sire	3.710	8.071	0.005	
	dam x sire	2.400	2.736	0.098	
GH1	cross		2.331	0.127	
	dam	2.010	8.805	0.003	*
	sire	0.359	0.460	0.498	
	dam x sire	0.000	0.000	1.000	
hsp90	cross		0.153	0.696	
	dam	0.158	0.026	0.873	
	sire	0.000	0.000	1.000	
	dam x sire	0.896	2.656	0.103	
pit1	cross		3.366	0.067	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
metA	cross		0.015	0.903	

	dam	0.459	10.988	0.001	*
	sire	0.244	4.947	0.026	
	dam x sire	0.000	0.000	1.000	
Tf	cross		0.117	0.733	
	dam	0.000	0.000	1.000	
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
IL8R	cross		0.127	0.722	
	dam	0.090	0.926	0.336	
	sire	0.050	0.242	0.623	
	dam x sire	0.027	0.086	0.770	
hsc71	cross		0.853	0.356	
	dam	7.490	15.061	0.000	**
	sire	1.640	1.754	0.185	
	dam x sire	0.000	0.000	1.000	
hsp70a	cross		0.364	0.546	
	dam	0.050	0.039	0.843	
	sire	0.201	1.355	0.244	
	dam x sire	0.105	0.285	0.594	
itpa	cross		2.896	0.089	
	dam	15.200	14.596	0.000	**
	sire	0.000	0.000	1.000	
	dam x sire	0.000	0.000	1.000	
CK1	cross		1.119	0.290	
	dam	3.640	3.132	0.077	
	sire	3.220	2.104	0.147	
	dam x sire	1.810	0.421	0.517	
BDNF	cross		3.107	0.078	
	dam	0.245	0.710	0.400	
	sire	0.000	0.000	1.000	
	dam x sire	0.290	0.609	0.435	
hnrL	cross		0.112	0.738	
	dam	0.075	0.188	0.665	
	sire	0.000	0.000	1.000	
	dam x sire	0.206	1.205	0.272	
			Fry		
FSHb	cross		1.325	0.250	
	dam	0.000	0.000	1.000	
	sire	0.086	0.000	1.000	
	dam x sire	0.818	2.679	0.102	
GTIIBS	cross		0.686	0.408	
	dam	0.000	0.000	1.000	

	sire	2.240	3.543	0.060
	dam x sire	0.438	0.065	0.799
GH1	cross		1.665	0.197
	dam	0.000	0.000	1.000
	sire	0.007	0.000	1.000
	dam x sire	0.000	0.000	1.000
hsp90	cross		0.159	0.690
	dam	0.023	0.000	1.000
	sire	0.000	0.000	1.000
	dam x sire	0.000	0.000	1.000
pit1	cross		0.712	0.399
	dam	0.138	0.067	0.796
	sire	0.000	0.000	1.000
	dam x sire	0.000	0.000	1.000
metA	cross		0.074	0.785
	dam	0.146	0.177	0.674
	sire	0.000	0.000	1.000
	dam x sire	0.585	3.335	0.068
Tf	cross		0.056	0.812
	dam	0.010	0.156	0.693
	sire	0.024	1.054	0.305
	dam x sire	0.050	4.514	0.034
IL8R	cross		0.835	0.361
	dam	0.162	1.128	0.288
	sire	0.033	0.007	0.935
	dam x sire	0.000	0.000	1.000
hsc71	cross		0.918	0.338
	dam	0.000	0.000	1.000
	sire	0.568	0.000	1.000
	dam x sire	0.000	0.000	1.000
hsp70a	cross		0.459	0.498
	dam	0.071	0.000	1.000
	sire	0.220	0.079	0.778
	dam x sire	0.029	0.013	0.909
itpa	cross		5.877	0.015
	dam	0.000	0.000	1.000
	sire	1.900	0.019	0.890
	dam x sire	14.900	4.932	0.026
CK1	cross		0.515	0.473
	dam	0.000	0.000	1.000
	sire	0.666	0.043	0.836
	dam x sire	0.000	0.000	1.000

BDNF	cross		0.115	0.734
	dam	0.071	0.000	1.000
	sire	0.248	0.313	0.576
	dam x sire	0.198	0.313	0.576
hnrL	cross		0.071	0.789
	dam	0.000	0.000	1.000
	sire	0.149	0.039	0.843
	dam x sire	0.000	0.000	1.000

Appendix 3: Results from LMMs assessing the genetic architecture of DNA methylation in Chinook salmon. For each developmental stage and rearing environment, we report (1) p-values corrected for multiple comparisons using a Benjamini-Hochberg FDR correction (significant p-values are bolded and italicized), (2) results from the restricted variance analysis, and (3) results from the restricted variance analysis expressed as the percent phenotypic variance.

Group	Statistic	Term	BDNF	FSHb	GTIIBS	hnrL	hsc71	hsp47	hsp70a	hsp90	IL8R	itpa	metA	pit1	Tf
Hatchery alevin	**LMM p-values (FDR corrected)**	dam:sire	0.57	1.00	0.53	0.53	1.00	1.00	1.00	0.59	1.00	1.00	1.00	1.00	1.00
		sire	1.00	1.00	***0.01***	1.00	0.53	1.00	0.53	1.00	1.00	1.00	0.14	1.00	1.00
		dam	0.53	1.00	***0.02***	1.00	***0.00***	1.00	1.00	1.00	0.66	***0.00***	***0.01***	1.00	1.00
	Restricted variance analysis	additive	0.00	0.00	17.28	0.00	6.49	4.52	0.71	0.54	0.16	0.00	0.91	0.24	0.00
		non-additive	1.27	0.51	9.40	0.97	0.00	8.86	0.45	2.65	0.13	0.00	0.00	0.00	0.00
		maternal	0.22	0.00	0.00	0.03	5.67	0.00	0.00	0.00	0.04	18.21	0.19	0.00	0.00
	Percent phenotypic variance	additive	0.00	0.00	51.76	0.00	11.65	5.69	15.93	6.02	6.24	0.00	23.01	2.06	0.00
		non-additive	28.01	3.36	28.17	35.47	0.00	11.14	10.18	29.66	5.14	0.00	0.00	0.00	0.00
		maternal	4.90	0.00	0.00	1.15	10.18	0.00	0.00	0.00	1.38	14.93	4.87	0.00	0.00
		residual	67.09	96.64	20.07	63.38	78.17	83.18	73.89	64.32	87.24	85.07	72.12	97.94	100.00
Hatchery fry	**LMM p-values (FDR corrected)**	dam:sire	1.00	0.43	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.43	0.52	1.00	0.43
		sire	1.00	1.00	0.43	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		dam	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Restricted variance analysis	additive	0.47	0.31	8.50	0.44	2.16	0.00	0.19	0.00	0.13	17.19	0.00	0.00	0.08
		non-additive	0.00	4.08	1.71	0.87	0.00	0.00	0.00	1.72	0.00	48.60	2.87	0.00	0.21
		maternal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.12	0.12	0.00
	Percent phenotypic variance	additive	10.03	3.67	25.65	11.63	3.22	0.00	2.06	0.00	3.64	20.91	0.00	0.00	22.41
		non-additive	0.00	47.98	5.16	23.02	0.00	0.00	0.01	10.74	0.00	59.09	46.58	0.00	57.70
		maternal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.08	0.00	1.98	1.30	0.00
		residual	89.97	48.35	69.19	65.34	96.78	100.00	97.94	89.26	93.28	20.00	51.43	98.70	19.89
Channel alevin	**LMM p-values (FDR corrected)**	dam:sire	1.00	1.00	0.56	1.00	1.00	1.00	1.00	1.00	0.38	1.00	0.56	1.00	1.00
		sire	1.00	1.00	0.08	0.35	0.56	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00

		dam	1.00	1.00	***0.01***	0.59	0.08	1.00	0.38	0.75	0.99	***0.00***	0.38	0.56	0.38
	Restricted variance analysis	additive	0.00	0.00	11.05	0.85	16.20	0.00	0.11	0.00	0.00	0.00	1.30	0.00	0.00
		non-additive	0.00	0.00	7.78	0.00	0.00	0.00	0.00	0.00	1.75	5.28	3.72	0.00	0.00
		maternal	0.04	0.04	1.68	0.00	6.22	0.00	0.25	0.23	0.10	10.26	0.41	0.34	0.01
	Percent phenotypic variance	additive	0.00	0.00	36.56	19.76	12.36	0.00	1.67	0.00	0.00	0.00	10.11	0.00	0.00
		non-additive	0.00	0.00	25.72	0.00	0.00	0.00	0.00	0.00	41.44	8.67	29.03	0.00	0.00
		maternal	0.92	0.63	5.57	0.00	4.75	0.00	3.79	2.40	2.39	16.85	3.23	2.96	4.08
		residual	99.08	99.37	32.15	80.24	82.89	100.00	94.55	97.60	56.16	74.48	57.63	97.04	95.92
Channel fry	**LMM p-values (FDR corrected)**	dam:sire	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		sire	1.00	0.73	0.73	1.00	1.00	0.73	1.00	1.00	1.00	1.00	0.41	1.00	1.00
		dam	1.00	1.00	***0.01***	1.00	0.07	1.00	1.00	1.00	0.41	0.78	1.00	1.00	0.41
	Restricted variance analysis	additive	0.00	0.41	4.83	0.00	0.00	16.19	0.00	0.08	0.00	0.92	2.63	0.02	0.02
		non-additive	0.89	0.00	0.00	0.00	0.00	0.65	0.00	0.35	0.64	0.00	0.00	0.00	0.00
		maternal	0.02	0.00	2.83	0.07	3.37	0.00	0.00	0.00	0.25	2.64	0.00	0.01	0.03
	Percent phenotypic variance	additive	0.00	14.07	14.30	0.00	0.00	20.70	0.00	0.94	0.00	1.06	25.31	0.33	4.43
		non-additive	24.10	0.00	0.00	0.00	0.00	0.84	0.00	4.25	16.07	0.00	0.00	0.00	0.00
		maternal	0.46	0.00	8.39	2.55	8.43	0.00	0.00	0.00	6.22	3.03	0.00	0.07	6.20
		residual	75.44	85.93	77.31	97.45	91.57	78.46	100.00	94.81	77.71	95.91	74.69	99.60	89.38

Appendix 4: Genotype, environment, and GxE LMM results for gene-specific GxE analysis in Chinook salmon. For each developmental stage, the FDR-corrected p-values, mean squared error estimates, and percent phenotypic variance (calculated from mean squared error) are reported. Significant p-values are bolded and italicized.

Group	Statistic	Term	BDNF	FSHb	GTIIBS	hnrL	hsc71	hsp47	hsp70a	hsp90	IL8R	itpa	metA	pit1	Tf
Alevin	**FDR-corrected p-values**	genotype	1.00	1.00	0.49	1.00	0.49	0.88	0.96	1.00	0.96	0.41	0.87	1.00	1.00
		environment	1.00	1.00	1.00	1.00	***0.00***	0.88	0.25	0.88	0.88	0.94	***0.00***	1.00	0.81
		GxE	0.87	1.00	0.87	0.96	1.00	1.00	0.88	1.00	0.61	1.00	1.00	1.00	1.00
	Mean squared error	genotype	4.45	7.54	110.47	3.48	254.00	68.46	9.08	12.21	9.33	193.48	13.40	12.78	0.39
		environment	0.61	26.79	0.08	0.22	6947.00	55.29	32.02	22.03	5.42	37.24	296.92	0.08	0.41
		GxE	7.50	4.00	36.89	5.60	77.00	44.03	5.07	12.36	6.62	41.60	6.69	7.47	0.21
		Residual	3.80	12.64	21.32	3.13	78.00	39.29	3.45	10.78	3.03	75.45	4.47	10.43	0.26
		total	16.35	50.98	168.76	12.44	7356.00	207.07	49.62	57.38	24.39	347.77	321.48	30.76	1.27
	Percent phenotypic variance	genotype	27.21	14.79	65.46	27.96	3.45	33.06	18.30	21.28	38.24	55.63	4.17	41.56	30.35
		environment	3.71	52.55	0.05	1.78	94.44	26.70	64.53	38.39	22.20	10.71	92.36	0.26	32.44
		GxE	45.83	7.85	21.86	45.06	1.05	21.26	10.22	21.54	27.14	11.96	2.08	24.27	16.37
Fry	**FDR-corrected p-values**	genotype	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		environment	0.96	0.79	1.00	1.00	0.09	***0.00***	***0.00***	***0.00***	0.50	0.86	***0.00***	***0.00***	***0.01***
		GxE	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Mean squared error	genotype	3.11	3.18	42.94	2.47	69.00	85.00	12.20	11.57	4.19	62.34	12.30	7.74	0.21
		environment	8.39	7.47	6.63	0.01	428.90	2487.20	3010.30	241.46	7.13	97.16	431.90	132.06	3.12
		GxE	7.22	4.14	39.02	2.27	76.80	87.30	5.10	9.54	2.52	125.34	15.60	4.96	0.57
		Residual	5.00	4.72	36.38	3.26	47.20	64.70	8.60	15.53	2.44	85.04	9.40	6.28	0.24
		total	23.71	19.51	124.97	8.00	621.90	2724.20	3036.20	278.10	16.29	369.88	469.20	151.04	4.15
	Percent phenotypic variance	genotype	13.10	16.29	34.36	30.81	11.10	3.12	0.40	4.16	25.72	16.85	2.62	5.12	5.18
		environment	35.37	38.28	5.31	0.09	68.97	91.30	99.15	86.82	43.79	26.27	92.05	87.43	75.31
		GxE	30.47	21.23	31.22	28.31	12.35	3.20	0.17	3.43	15.50	33.89	3.32	3.28	13.65

Appendix 5: Climate data (https://climate.weather.gc.ca/) and water quality data (https://www2.gov.bc.ca/gov/content/data/geographic-data-services/web-based-mapping/imapbc) for natal streams of eight populations of Chinook salmon.

Population	BQ	Chil	Harr	Punt	Ques	Quin	RC	Sar
Latitude	49.393902	49.08082	49.27145	49.68617	52.65973	50.01665	49.33967	48.89538
Longitude	-124.618084	-121.704959	-121.91462	-125.03228	-121.69789	-125.30218	-124.98791	-124.96138
Average Temperature (°C, Sept-Nov)	9.47	9.57	11.00	9.43	4.87	9.40	9.90	10.13
Precipitation (mm, Sept-Nov)	411.60	477.60	564.30	431.10	157.00	497.70	634.40	869.30
Turbidity (NTU)	0.59	4.59	2.15	1.06	1.32	1.35	0.29	0.60
Al (mg/L)	0.05	0.00	0.63	0.08	0.04	0.05	0.06	0.12
As (mg/L)	0.25	0.00	0.06	0.05	0.00	0.09	0.04	0.00
Ca (mg/L)	10.13	26.68	11.10	5.91	16.88	13.39	4.95	2.79
Cd (mg/L)	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Co (mg/L)	0.10	0.00	0.01	0.04	0.00	0.03	0.00	0.00
Cr (mg/L)	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cu (mg/L)	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Fe (mg/L)	0.10	0.02	0.31	0.17	0.05	0.14	0.05	0.07
Mg (mg/L)	1.78	5.48	3.10	1.20	1.91	3.04	0.39	0.56
Mn (mg/L)	0.01	0.00	0.01	0.01	0.00	0.01	0.00	0.01
Mo (mg/L)	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Ni (mg/L)	0.05	0.00	0.02	0.02	0.00	0.01	0.01	0.00
Pb (mg/L)	0.10	0.00	0.03	0.02	0.00	0.03	0.00	0.00
Zn (mg/L)	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.00
Chloride (mg/L)	5.00	0.50	0.20	1.60	0.51	2.20	0.87	3.80
Nitrate + Nitrite (mg/L)	0.05	0.06	0.07	0.06	0.10	0.13	0.02	0.04
Nitrite (mg/L)	0.01	0.00	0.01	0.00	0.00	0.01	0.01	0.00

Appendix 6: ANOVA results for the effects of population, year, and population x year interaction on methylation residuals from ATU regressions for 20 genes in Chinook salmon. Fish were sampled from Big Qualicum and Harrison River in 2015 and 2017 to test for an interannual effect on methylation. Presented are (1) FDR-corrected p-values, (2) mean square estimates, and (3) percent phenotypic variance attributed to each term. Significant p-values are bolded and italicized.

	P-value (FDR corrected)			Mean squares					Percent phenotypic variance			
Gene	Population	Year	Population x Year	Population	Year	Population x Year	Residual	Total	Population	Year	Population x Year	Residual
FSHb	0.998	0.998	0.960	1.8	0.0	3.8	3.8	9.4	19.3	0.3	40.1	40.3
GTIIBS	***0.002***	0.998	0.560	346.1	0.2	46.4	21.1	413.8	83.6	0.0	11.2	5.1
GH1	0.998	0.998	0.998	6.7	0.0	0.2	13.3	20.1	33.1	0.0	0.9	66.0
GH2	0.960	0.998	0.865	35.2	1.1	46.0	33.9	116.1	30.3	0.9	39.6	29.2
hsf1b	0.998	0.998	0.151	2.7	1.6	217.1	41.1	262.4	1.0	0.6	82.7	15.7
hsp90	***0.048***	0.998	0.986	123.6	0.0	12.8	14.3	150.7	82.0	0.0	8.5	9.5
metA	0.998	0.998	0.998	1.0	0.0	0.0	2.6	3.7	28.3	0.0	0.1	71.6
pit1	0.998	0.998	0.998	0.0	0.0	0.3	8.0	8.4	0.5	0.0	3.4	96.1
IL8R	0.957	0.998	0.998	3.7	0.0	0.8	3.3	7.8	47.8	0.2	10.1	41.9
Tf	0.998	0.998	0.998	0.1	0.0	0.0	0.2	0.3	20.2	0.0	0.1	79.7
p53	0.077	0.998	0.077	79.1	0.3	79.3	11.4	170.1	46.5	0.2	46.6	6.7
hsc71	0.998	0.998	0.160	147.6	8.3	945.6	189.1	1290.6	11.4	0.6	73.3	14.7
hsp47	0.998	0.998	0.104	12.4	1.6	195.6	31.7	241.3	5.1	0.7	81.1	13.1
hsp70a	0.998	0.998	0.998	0.2	0.0	0.1	5.7	5.9	3.5	0.0	1.3	95.2
CK1	***0.048***	0.998	0.803	146.2	1.2	26.6	17.1	191.1	76.5	0.6	13.9	8.9
ITPA	0.252	0.998	0.424	182.8	0.5	136.3	45.4	365.0	50.1	0.1	37.3	12.4
BDNF	0.560	0.998	0.998	10.9	0.0	0.0	4.8	15.8	69.2	0.0	0.3	30.5
hnrL	0.998	0.998	0.560	0.3	0.1	12.4	5.3	18.1	1.9	0.5	68.5	29.1
anthr	0.998	0.998	0.998	0.1	0.0	1.6	7.8	9.6	0.7	0.1	17.2	82.0
Nkef	***0.000***	0.998	***0.001***	2951.3	8.7	1966.7	110.1	5036.8	58.6	0.2	39.0	2.2
								Average:	33.5	0.3	28.8	37.5

Appendix 7: P-values and R^2 values from ANOVAs and Mantel tests for population effects on DNA methylation in Chinook salmon. ANOVAs tested for significant population effects on methylation. Mantel tests tested for a correlation between a Euclidian distance matrix for DNA methylation and microsatellite pairwise FST divergence to determine if differences in DNA methylation among populations were explained by genetic drift (critical p-value=0.006 for Bonferroni correction). Significant p-values are bolded and italicized. All analyses use ATU-corrected data, except the ANOVA analysis for "raw" methylation data.

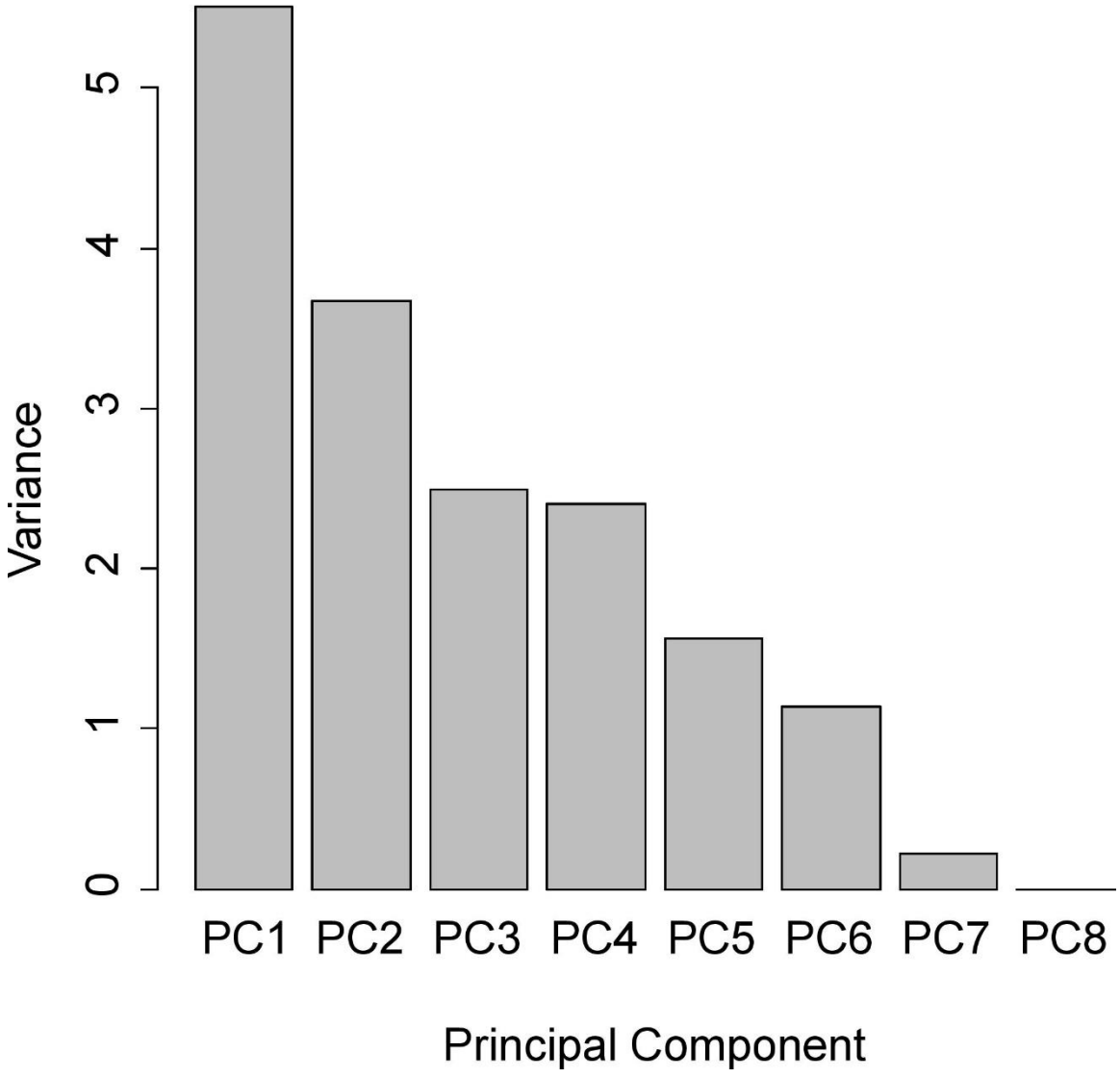
	ANOVA for population effect (ATU residual data)		ANOVA for population effect (raw data)	Mantel test for correlation with microsatellite F_{ST}		Mantel test for correlation with SNP F_{ST}	
Gene	P-value (FDR correction)	Adjusted R^2	P-value (FDR correction)	P-value	Adjusted R^2	P-value	Adjusted R^2
FSHb	0.646	-0.003	0.356				
GTIIBS	***0***	0.192	***0.000***	0.02	0.245	0.254	0.01
GH1	0.429	0.005	0.141				
GH2	0.799	-0.009	0.497				
hsf1b	0.175	0.016	***0.046***				
hsp70	***0.003***	0.048	***0.001***	0.1	0.102	0.2522	0.05
hsp90	***0.005***	0.042	***0.000***	0.33	0.009	0.0199	0.35
metA	0.45	0.003	***0.046***				
pit1	0.32	0.008	0.187				
IL8R	0.646	-0.002	0.497				
Tf	0.2	0.014	0.156				
p53	***0***	0.091	***0.000***	0.31	0.027	0.0053	0.3
Myo1A	0.263	0.011	0.057				
hsc71	***0***	0.063	***0.000***	0.38	0	0.0237	0.3
hsp47	***0.003***	0.047	***0.002***	0.15	0.058	0.0537	0.17
hsp70a	0.646	-0.003	0.537				
RAG1	***0***	0.172	***0.000***	0.19	0.047	0.0015	0.57
CK1	0.066	0.024	***0.000***				
ITPA	0.287	0.01	0.187				
BDNF	0.786	-0.008	0.497				
hnrL	0.767	-0.006	0.659				

anthr	0.646	-0.003	0.469				
Nkef	***0***	0.227	***0.000***	0.03	0.201	0.106	0.01

Appendix 8: PCA loadings for 23 environmental variables gathered for natal streams of eight Chinook salmon populations.

Variable	PC1	PC2	PC3	PC4	PC5	PC6
Latitude	0.112	0.035	-0.258	0.440	-0.163	0.074
Longitude	0.200	0.314	0.065	0.091	-0.134	0.302
Pathogen diversity	-0.017	0.117	0.407	0.240	0.042	0.274
Average Temperature (°C, Sept-Nov)	-0.129	-0.014	0.342	-0.342	0.088	-0.190
Precipitation (mm, Sept-Nov)	-0.032	-0.256	0.308	-0.275	0.222	-0.025
Turbidity (NTU)	0.196	0.335	0.047	-0.188	0.175	-0.027
Al (mg/L)	-0.037	0.129	0.459	0.140	-0.187	0.053
As (mg/L)	-0.318	0.141	-0.115	-0.019	0.011	0.001
Ca (mg/L)	0.176	0.365	-0.156	-0.058	0.126	-0.038
Cd (mg/L)	-0.313	0.137	-0.069	-0.055	-0.120	0.177
Co (mg/L)	-0.308	0.111	-0.184	-0.015	0.083	0.033
Cr (mg/L)	-0.318	0.150	-0.099	0.019	-0.079	0.036
Cu (mg/L)	0.077	0.342	-0.072	-0.344	0.156	0.027
Fe (mg/L)	-0.120	0.148	0.386	0.204	-0.111	-0.161
Mg (mg/L)	0.127	0.395	0.015	-0.117	0.224	-0.155
Mn (mg/L)	-0.240	-0.149	0.035	0.193	0.417	0.104
Mo (mg/L)	-0.251	0.251	0.168	-0.033	-0.222	-0.017
Ni (mg/L)	-0.322	0.153	-0.043	-0.014	-0.086	0.051
Pb (mg/L)	-0.310	0.179	-0.083	0.022	0.036	0.083
Zn (mg/L)	-0.050	-0.084	-0.192	-0.340	-0.490	-0.165
Chloride (mg/L)	-0.254	-0.125	-0.111	-0.058	0.392	0.218
Nitrate + Nitrite (mg/L)	0.035	0.166	-0.101	0.372	0.271	-0.438
Nitrite (mg/L)	-0.195	-0.005	0.037	0.126	-0.037	-0.644

Appendix 9: Scree plot showing importance of the first eight PCs in the PCA for environmental variables. PCs 1-6 were retained based on examination of the Scree plot and associated eigenvalues.



Appendix 10: Pairwise FST estimates for SNP (above diagonal) and microsatellite (below parallel) markers estimating divergence among populations of Chinook salmon. Microsatellite data from Beacham et al. (2006).

	BQ	**Chil**	**Harr**	**Punt**	**Ques**	**Quin**	**RC**	**Sar**
BQ		0.071	0.065	0.003	0.163	0.054	0.065	0.089
Chil	0.040		0.007	0.071	0.187	0.107	0.102	0.134
Harr	0.035	0.005		0.065	0.182	0.099	0.098	0.128
Punt	0.000	0.038	0.034		0.159	0.048	0.060	0.082
Ques	0.060	0.053	0.048	0.058		0.157	0.132	0.149
Quin	0.027	0.041	0.037	0.026	0.061		0.067	0.074
RC	0.039	0.048	0.045	0.036	0.039	0.042		0.029
Sar	0.056	0.056	0.053	0.052	0.044	0.056	0.023	

Appendix 11: Pairwise Euclidean dissimilarity matrix for population-level differences in methylation data across eight genes showing a significant population effect.

	BQ	**Chil**	**Harr**	**Punt**	**Ques**	**Quin**	**RC**	**Sar**
BQ								
Chil	13.76							
Harr	16.12	7.23						
Punt	10.03	13.44	18.09					
Ques	21.81	10.41	11.09	22.74				
Quin	4.76	12.80	14.58	9.63	20.15			
RC	15.68	8.72	8.25	19.89	8.45	15.08		
Sar	17.65	11.96	18.74	11.25	19.12	17.49	19.40	

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SPRINGER NATURE

Rearing environment affects the genetic architecture and plasticity of DNA methylation in Chinook salmon

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