ENVIRONMENTAL EPIGENOMICS IN STICKLEBACK: PLASTICITY OF DNA METHYLATION AND GENE EXPRESSION PATTERNS ACROSS TIME SCALES

by

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Abstract

Epigenetic mechanisms such as DNA methylation have been proposed as an important source of variation that can influence phenotypic plasticity and adaptive evolutionary processes, yet little is known about the role of DNA methylation in an ecological or evolutionary context in vertebrates. In this thesis I examine the effects of the environment and sex on DNA methylation and gene expression patterns in the threespine stickleback fish (*Gasterosteus aculeatus*), an ecological and evolutionary model system that has been used to study mechanisms involved in the evolution of adaptive phenotypes in novel environments.

The dynamic regulation of DNA methylation and gene expression patterns during early developmental periods plays an important role in cell differentiation and establishing adult phenotypes. Here I demonstrate that adult DNA methylation and gene expression patterns are modified in response to the temperature and salinity experienced during development. Similarly, maternal stress can have long-term effects on neurodevelopment and the behavior of offspring that can influence offspring performance and population evolutionary trajectories. I demonstrate that the effects of maternal stress on the brain transcriptome differ between adult male and female stickleback offspring. These sex-specific effects of maternal stress suggest that male and female offspring may respond differently to maternal stress exposure, which could have important implications when assessing the long-term ecological and evolutionary impacts of stress across generations.

DNA methylation has also been proposed to play a key role in regulating sexually dimorphic phenotypes and in the evolution of sex determination mechanisms. I compare genome-wide DNA methylation patterns between male and female stickleback and identify apparent differential methylation on the stickleback sex chromosome that correspond to the

regions of genetic divergence between the X and Y chromosome. These data provide evidence of a potential role of DNA methylation in the evolution of sex chromosomes in vertebrates.

Taken together, these data demonstrate that there is a complex relationship between genetic, epigenetic, and transcriptomic processes that are dynamically regulated during development and in response to environmental cues, and that epigenetic processes may be involved in regulating evolutionary processes.

Lay Summary

Epigenetic mechanisms such as DNA methylation are thought to play key roles in an organism's to response to changes in the environment by regulating the expression of genes in different environmental conditions. However, little is known about how DNA methylation patterns are modified in response to the environment. In this thesis I demonstrate that differences in environmental temperature, salinity, and maternal stress have widespread effects on DNA methylation levels and gene expression in threespine stickleback (*Gasterosteus aculeatus*), and demonstrate that sex is an important factor in determining the effects of maternal stress. I also identified sex-specific DNA methylation patterns on the stickleback sex chromosome that are associated with the evolution of this chromosome. These data suggest that DNA methylation is likely involved in both the dynamic regulation of gene expression in response to environmental change, and in regulating evolutionary processes such as the evolution of sex chromosomes.

Preface

Sections of the introduction (chapter one) and discussion (chapter six) of this thesis have been published as: Metzger DCH, Schulte PM (2016) Epigenomics in marine fishes. *Marine Genomics*, 30, 43–54. Dr. Schulte and I contributed equally to the writing of this manuscript.

A version of chapter two has been published as: Metzger DCH, Schulte PM (2017) Persistent and plastic effects of temperature on DNA methylation across the genome of threespine stickleback (*Gasterosteus aculeatus*). *Proceedings of the Royal Society B: Biological Sciences*, 284, 20171667. I designed and conducted the experiments for this study, performed the data analysis, and wrote the manuscript under the supervision of Dr. Schulte.

A version of chapter three has been published as Metzger DCH, Schulte PM (2018) Similarities in temperature-dependent gene expression plasticity across time-scales in threespine stickleback (*Gasterosteus aculeatus*). *Molecular Ecology.* I designed and conducted the experiments for this study, performed the data analysis, and wrote the manuscript under the supervision of Dr. Schulte.

A version of chapter four has been accepted for publication as: Metzger DCH, Schulte PM (2018) The DNA methylation landscape of stickleback reveals patterns of sex chromosome evolution and effects of environmental salinity. *Genome Biology and Evolution*, 10, 775-785. I designed and conducted the experiments for this study, performed the data analysis, and wrote the manuscript under the supervision of Dr. Schulte.

A version of chapter five has been published as: Metzger DCH, Schulte PM (2016) Maternal stress has divergent effects on gene expression patterns in the brains of male and female threespine stickleback. *Proceedings of the Royal Society B: Biological Sciences*, 283,

20161734. I designed and conducted the experiments for this study, performed the data analysis, and wrote the manuscript under the supervision of Dr. Schulte.

All animal use was conducted under approved University of British Columbia animal care protocols A11-0372 and A10-0285, and adhered to all guidelines and policies of the Canadian Council on Animal Care.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

14:10 h	14 hours of light followed by 10 hours of dark
5caC	5-carboxyl-cytosine
5fC	5-formyl-cytosine
5hmC	5-hydroxy-methyl cytosine
5mC	methylated cytosine nuceotide
bp	base pairs
BSradSeq	bisulfite restriction site associated sequencing
C	cytosine nucleotide
°C	degrees celsius
cDNA	complementary DNA sequence
CH3	methyl group
chr	chromosome
cm	centimeter
CpG	cytosine-phosphate-guanine dinucleotide
CpHpG	cytosine-phosphate-(A,C or T nucleotide)-phosophate-Guanine nucleotide
CpHpH	cytosine-phosphate-(A,C or T nucleotide)-phosophate--(A,C or T nucleotide) nucleotide
DE	differentially expressed
DMC	differentially methylated cytosine
DMR	differentially methylated region
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
E2	estradiol
e.g.	exempli gratia
endo-siRNA	endogenous small interfering RNA
EpiRADseq	methylation-sensitive restriction site associated DNA sequencing
ESD	environmental sex determination
et al.	et alia
F	filial generation
FDR	false discovery rate
G	guanine nucleotide
gDNA	genomic DNA
Gb	giga base pairs
GO	gene ontology
GPS	global positioning system
GSD	genotypic sex determination
HBCD	hexa-bromo-cyclododecane
HPI	hypothalamic-pituitary-interrenal
HPLC	high-performance liquid chromatography
i.e.	id est
IQR	interquartile range

K^+	potassium
kb	kilobase pair
KEGG	Kyoto encyclopedia of genes and genomes
K-S	Kolmogorov-Smirnov
L	litre
Ma	mega-annum
MBDSeq	Methyl-CpG binding domain protein sequencing
MeDIP-seq	methylated DNA immunoprecipitation sequencing
mg	milligrams
miRNA	micro RNA
mL	milliliter
µL	microliter
mOsm	milliosmoles
mRNA	messenger RNA
MS-AFLP	methylation-sensitive amplified fragment length polymorphism
n	number
Na^+	sodium
NIH	National Institute of Health
NKA	sodium potassium ATPase
PAR	pseudo autosomal region
PC	principal component
PCR	polymerase chain reaction
piRNA	PIWI-interacting ribonucleic acids
ppt	parts per thousand
RIN	RNA integrity number
RLE	relative log expression
RNA	ribonucleic acid
RNA-Seq	RNA-sequencing
RP-HPLC	reverse phase high-performance liquid chromatography
RRBS	reduced representation bisulfite sequencing
s	seconds
SD	standard deviation
SLIM	sliding linear model
SNP	single nucleotide polymorphism
T	thymine nucleotide
TET	ten-eleven translocation enzyme
TpG	cytosine-phosphate-thymine dinucleotide
TSD	temperature dependent sex determination
U	uracil nucleotide
UBC	University of British Columbia
W	W chromosome
WGBS	whole genome bisulfite sequencing
X	X chromosome
Y	Y chromosome
Z	Z chromosome

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I would also like to take this opportunity to thank not only those that have supported me during my PhD but to also thank all of my teachers and mentors that have helped me get to this point. I am fortunate to have experienced working in many different laboratories with many excellent people who have all played important roles in shaping my views on scientific research.

Finally, completing this degree would not have been possible without the support of my family and friends. The trips back east, weekends at Galiano, time in the mountains, and nights spent sharing bottles filled with the wonders of fermentation kept my batteries fully charged. I am, and will forever be, grateful for their unwavering support.

Chapter 1: Introduction

1.1 Goals of this thesis

Changes in gene expression regulate many of the cellular processes that allow organisms to respond to environmental change (Gracey 2007; Somero 2010). Epigenetic mechanisms, such as DNA methylation, have a well characterized role in regulating gene expression activity (Jaenisch & Bird 2003) yet we know very little about how these mechanisms are involved in responses to environmental change, particularly in an ecologically relevant context (Bossdorf *et al.* 2008; Verhoeven *et al.* 2016). The goal of my thesis was to examine plasticity of DNA methylation patterns and gene expression across different timescales in response to environmental change. Using the threespine stickleback (*Gasterosteus aculeatus*) as a model system, I examined how temperature, salinity, and maternal stress affect DNA methylation and gene expression patterns because these are ecologically relevant stressors in this species. By examining the effects of these environmental factors on DNA methylation and gene expression, the studies outlined in this thesis contribute to our fundamental understanding of epigenetic processes in fish by 1) characterizing how DNA methylation and gene expression are regulated in response to changes in the environment, 2) investigating the persistent effects of developmental conditions on DNA methylation and gene transcript levels, and 3) assessing the effects of sex on differences in DNA methylation patterns among individuals. In the remainder of this chapter, I provide the background required to place my studies in the context of the biological literature and the current state of understanding of the role of epigenetic mechanisms in responses to environmental change, with particular reference to our knowledge of these processes in fish.

1.2 Epigenetics and plasticity

Environmentally induced phenotypic plasticity is a critical component of organismal responses to a changing environment (Ghalambor *et al.* 2007; Donelson *et al.* 2017). Understanding the molecular mechanisms underlying this plasticity is an important question in basic biology that also has applications in areas as diverse as aquaculture and environmental monitoring (Somero 2012; Pittman *et al.* 2013). Much of the work on the mechanisms of environmentally-induced plasticity in fishes has focused on the reversible phenotypic plasticity that is characteristic of acclimation/acclimatization responses, with many studies using techniques such as cDNA microarray to examine changes in RNA levels in response to environmental change (Gracey *et al.* 2004; Cossins 2006; Kalujnaia *et al.* 2007; Logan & Buckley 2015). Less well understood are the longer-lasting phenotypic changes that occur in response to environmental changes that are experienced during development, a phenomenon known as developmental plasticity (Pfennig *et al.* 2010; Scott & Johnston 2012; Alvarado *et al.* 2015). Some of these acquired phenotypes can even last for multiple generations, in what has been termed transgenerational plasticity (Agrawal *et al.* 1999; Salinas *et al.* 2013).

The mechanisms that regulate these longer-lasting forms of plasticity are often grouped together under the umbrella term of "epigenetics". Much of the research on epigenetic mechanisms has been performed in model systems (Bird 2002; Li & Zhang 2014), but recent epigenetic studies in ecologically relevant non-model organisms have begun to demonstrate the promise of epigenetic and epigenomic approaches in fields such as ecology, evolutionary biology and environmental biology (Bossdorf *et al.* 2008; Ledon-Rettig 2013; Bonasio 2015).

1.2.1 What is epigenetics?

Conrad Waddington was first credited with using the term "epigenetics" in the 1940s to describe the developmental mechanisms that can give rise to alternative phenotypes (Waddington 1942), but since this time there has been almost continuous refinement of this concept (Deans & Maggert 2015). More contemporary definitions are intrinsically tied to aspects of phenotypic plasticity, or how a single genotype can produce multiple phenotypes in response to changes in the environment. Thus, the broadest definitions of epigenetics include any process that results in heritable phenotypic changes such as variation in gene expression. Several attempts have been made, however, to refine the definition of epigenetics in a way that distinguishes epigenetic mechanisms from broader processes that regulate gene expression and phenotypic plasticity. The most widely accepted contemporary definitions of epigenetics echo the one put forward by Deans and Maggert (2015) who define epigenetics as "The study of phenomena and mechanisms that cause chromosome-bound, heritable changes to gene expression that are not dependent on changes to DNA sequence." In this context, the word heritable is defined as involving both meiotic and mitotic inheritance (Wu & Morris 2001), and thus according to these definitions, epigenetic mechanisms need not be confined to processes that are inherited across generations, but must at least be inherited across cell divisions. However, not all epigeneticists subscribe to this definition. For example, the field of epigenomics (or the study of epigenetics at a whole-genome level) tends to follow a broader definition that includes additional forms of phenotypic plasticity. For example, the NIH epigenomic roadmap program uses the following definition: "epigenetics refers to both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the

transcriptional potential of a cell that are not necessarily heritable" (http://www.roadmapepigenomics.org/overview).

Because there is still disagreement about how to most appropriately define the field of epigenetics, the mechanisms that constitute an epigenetic signal or process are also controversial (Schaefer & Nadeau 2015; Deans & Maggert 2015). Depending on the author, the list of potential epigenetic mechanisms may include covalent modification of DNA through methylation of cytosines (Lister *et al.* 2009), covalent modification of histones (Bannister & Kouzarides 2011) via acetylation, phosphorylation, methylation and other mechanisms, as well as gene regulation by non-coding RNAs (as microRNAs (miRNA), endogenous small interfering RNAs (endo-siRNA), and PIWI-interacting RNAs (piRNA) (Costa 2008).

1.3 Ecological epigenetics

The field of "ecological epigenetics" aims to address the potential role of epigenetic mechanisms in ecological and evolutionary processes (Bossdorf *et al.* 2008; Kilvitis *et al.* 2014). In an ecological context, epigenetic mechanisms could be involved in regulating phenotypic plasticity in response to changes in the environment (Bossdorf *et al.* 2008; Castonguay & Angers 2012; Zhang *et al.* 2013) and these effects can persist across a wide range of timescales. The persistence of epigenetic marks could have heritable effects on fitness that are not entirely dependent on the underlying DNA sequence, and thus epigenetic changes have the potential to influence microevolutionary processes (Bossdorf *et al.* 2008).

The field of ecological epigenetics is closely parallel to that of ecological genomics (Figure 1.1). A key objective of both fields is to understand how heritable variation (i.e. epigenetic or genetic) translates into phenotypic variation and fitness differences on which

selection can act and influence evolutionary trajectories (Bossdorf *et al.* 2008; Kilvitis *et al.* 2014; Verhoeven *et al.* 2016). The major difference between epigenetic and genetic processes is that epigenetic processes can be directly modified in response to environmental signals (e.g. Verhoeven *et al.* 2010; Dowen *et al.* 2012), which adds an additional level of complexity to understanding the role epigenetic processes in ecology and evolution. While genetic variation can also accumulate in populations from cellular and environmental processes (i.e. genetic recombination and DNA damage), changes to epigenetic processes can occur much more rapidly and could therefore provide an accelerated pathway for evolutionary change (Kilvitis *et al.* 2014). For example, epigenetic variation could facilitate the exploration of phenotypic space and maintain a particular phenotype as it becomes integrated into the genome through genetic assimilation (Verhoeven *et al.* 2016). However, ecological epigenetic and ecological genomic processes are not mutually exclusive. Broadly speaking, epigenetic mechanisms have the potential to regulate the rate at which genetic mutations accumulate in the genome and can regulate the expression of genetic variation while the DNA sequence can determine which regions of the genome are affected by epigenetic processes (Verhoeven *et al.* 2016).

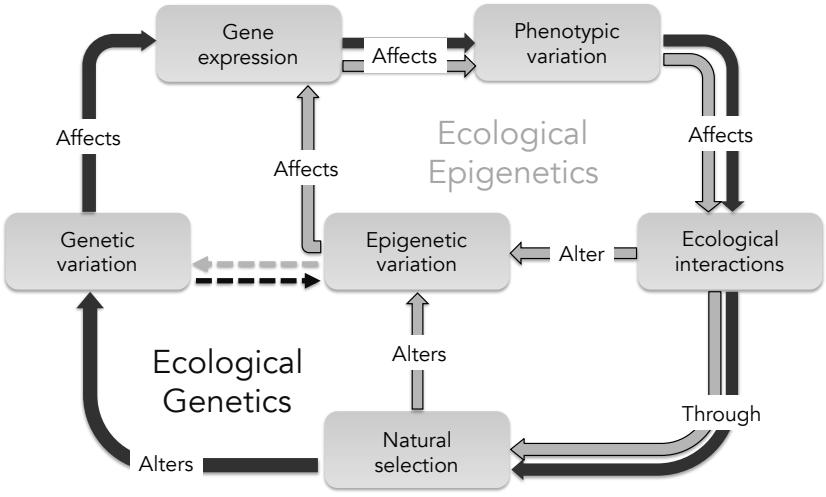

Figure 1.1: Flow diagram illustrating parallel nature of ecological genetics (black lines) and ecological epigenetics (grey lines) (adapted from Bossdorf *et al.* 2008).

Despite the widely acknowledged potential for epigenetic mechanisms to be important in an ecological and evolutionary context (Jablonka & Lamb 2005; Bossdorf *et al.* 2008; Ledon-Rettig 2013; Burggren & Crews 2014; Verhoeven *et al.* 2016; Hu & Barrett 2017), there is very little empirical evidence to support the ecological and evolutionary roles of epigenetic mechanisms. Most of what is currently known about how epigenetic processes are regulated and how epigenetic processes are involved in regulating cellular functions have come from studies in model systems investigating the role of epigenetics in the development of chronic diseases such as cancer. Given the limited amount of empirical evidence regarding the role epigenetic mechanisms in ecology and evolution, there are several key questions that should be addressed in order to begin to understand the potential ecological and evolutionary role of epigenetic processes including how different environments effect epigenetic variation, how epigenetic variation is influenced by genetic variation and vice versa, and how epigenetic variation influences phenotypes (from gene expression to whole animal phenotypes). My thesis aims to begin to address these issues.

1.4 DNA methylation as an epigenetic mark

DNA methylation, which fulfills the criterion of being a chromosome-bound, potentially heritable change that is not dependent on a change to DNA sequence, was the first epigenetic mark to be discovered and has become one of the most widely studied of the potential epigenetic mechanisms (Deans & Maggert 2015). DNA methylation is a covalent modification in which a methyl group (CH_3) is added to position 5 of the pyrimidine ring of a cytosine (5mC). In eukaryotic cells, the majority of methylated cytosines occur on cytosine-phosphate-guanine

(CpG) dinucleotides resulting in complementary methylation of CpG motifs on both strands of DNA. Methylated cytosines can also occur on non-CpG sites such as CpHpG and CpHpH motifs (where H = A, C or T). However, these epigenetic marks are fundamentally different from CpG methylation because they are strand-specific. These strand-specific marks are rare in animals, although they are prominent in the epigenetic landscape of plants (Law & Jacobsen 2010).

The addition or removal of methyl groups can regulate the transcriptional activity of neighbouring genes by altering the structure and function of chromatin. High levels of methylation at the promoter of a gene are associated with repression of transcription (Li & Zhang 2014). This repression of transcription by promoter methylation is thought to be due to the formation of inactive heterochromatin (Razin 1998; Martinowich *et al.* 2003). In addition, methylation of enhancer elements can also repress transcription by preventing the binding of the transcription factors that would otherwise induce expression. Alternatively, methylated DNA can recruit methyl-binding proteins that repress transcription (Klose & Bird 2006). DNA methylation of intragenic regions, however, is associated with active expression of nearby genes (Zemach *et al.* 2010; Jjingo *et al.* 2012) and the regulation of alternative splice variants (Laurent *et al.* 2010; Lyko *et al.* 2010; Park *et al.* 2011).

There is dramatic variation in the prevalence and patterns of DNA methylation among taxa (Feng *et al.* 2010). For example, in Arabidopsis only ~22% of CpG sites are methylated, while the CpG sites in vertebrate genomes are generally methylated to high levels (>80%). Invertebrates exhibit a wide range of CpG methylation levels, with the "model" invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans* essentially lacking DNA methylation and the honeybee having very low CpG methylation levels (~0.7%; Lyko *et al.* 2010). Many other invertebrates have intermediate levels of CpG methylation (around 50%) with a "mosaic" pattern

of methylation such that the genome consists of interspersed regions of methylated and unmethylated DNA of similar length (Feng *et al.* 2010). In contrast, vertebrate genomes consist of long tracts in which the CpG dinucleotides are methylated to high levels (>80%) punctuated by short unmethylated regions. These unmethylated regions typically fall in areas called CpG islands (CGI), which have a GC-rich base composition with a CpG dinucleotide approximately every 10 base pairs. CpG islands tend to be preferentially located in the proximal promoter regions of genes (Figure 1.2; Deaton & Bird 2011), and this accounts for an observed decrease in the levels of DNA methylation around the transcription start sites of actively transcribed genes (Zemach *et al.* 2010; Jones 2012).

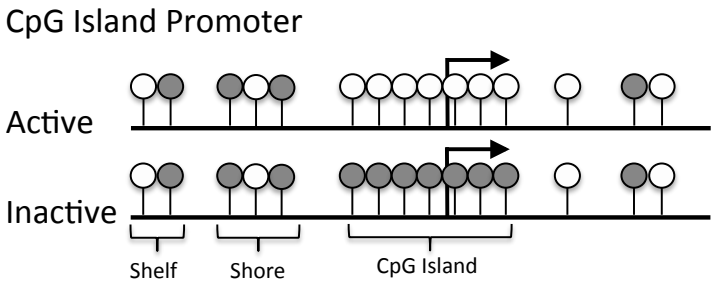

Figure 1.2: Patterns of CpG island methylation in proximal promoter regions. CpG islands are regions with high concentrations of CpG motifs. Shore and shelf regions are located at immediately flanking CpG islands. A high level of methylation in a CpG island associated with the transcription start site of a gene is associated with inactivation of transcription. Bent arrow indicates transcription start site; empty circles indicate umethylated CpG sites; filled circles indicate methylated CpG sites (reproduced from Metzger & Schulte 2016).

There is also epigenetic variation among individuals within a species in DNA methylation patterns (e.g. Massicotte *et al.* 2011; Liebl *et al.* 2013). This individual-level variation in DNA methylation can be maintained throughout the lifespan of an organism and can even be transmitted across generations in at least some species (e.g. Johannes *et al.* 2009; Verhoeven *et al.* 2010), providing an added source of heritable variation that is independent of genetic variation. Thus, the potential impact of epigenetic variation has become an important topic in the fields of ecology and evolution (Bossdorf *et al.* 2008; Ledon-Rettig 2013), as it has

been suggested to be important for evolutionary adaptation to altered environments (Flores *et al.* 2013).

DNA methylation patterns can also vary within a single individual across life stages or among cell types (Richardson 2003; Flanagan *et al.* 2006; Massicotte *et al.* 2011). DNA methylation during development is thought to play an important role in cellular differentiation and is involved in maintaining cell-type specific transcriptional activity in the genome (Monk *et al.* 1987; Li 2002). Variation in DNA methylation patterns that are induced by environmental factors can alter the developmental trajectory of an individual resulting in the expression of alternative phenotypes from a single genotype (i.e. developmental plasticity) (e.g. Wolff *et al.* 1998; Kucharski *et al.* 2008; Shao *et al.* 2014).

Most of what is known about the mechanisms that establish and maintain DNA methylation patterns has been derived from studies in mammals. In mammals, DNA methylation patterns are established by the de novo DNA methylation activity of the DNA methyltransferases, DNMT3A and DNMT3B, which add methyl groups to previously unmethylated cytosines (Law & Jacobsen 2010). Maintenance of DNA methylation across cell divisions is achieved by the addition of methyl groups to the newly synthesized strand of DNA by the DNA methyltransferase DNMT1 which is recruited to DNA by the SET- and RING-associated domain contain protein UHRF1 (Sharif *et al.* 2007; Bostick *et al.* 2007). Two other genes in the mammalian genome are related to the described DNA methyltransferase genes but do not possess DNA methyltransferase activity. DNMT3L is a methylation cofactor that has been associated with genomic imprinting (Suetake *et al.* 2004). Another methyltransferase, DNMT2, predominantly methylates RNA molecules, specifically tRNA, as opposed to DNA (Goll *et al.* 2006).

While the addition of DNA methylation marks in the genome is an active process, de-methylation can occur via both active and passive processes. Passive de-methylation occurs when DNA methylation is not maintained through DNA replication and cell division as a result of the lack of DNMT1 activity (Li & Zhang 2014). Active de-methylation requires the recruitment of protein complexes that convert methylated cytosines to the methylation intermediates that are then removed by base excision repair mechanisms. The intermediate forms of methylated cytosines include 5-hydroxy-methyl cytosine (5hmC), 5-formyl-cytosine (5fC), and 5-carboxyl-cytosine (5caC) (Shen *et al.* 2014). A family of enzymes known as the ten-eleven translocation (TET) enzymes catalyzes the stepwise oxidation of 5mC to 5fC and then 5caC, which can then be converted to unmethylated cytosines (Shen *et al.* 2014). These oxidized derivatives were originally thought to be transient demethylation intermediates, but recent studies suggest that these marks are more persistent in the genome than originally thought, and may have functional significance in their own right (Branco *et al.* 2011; Véron & Peters 2011; Kroeze *et al.* 2015).

1.4.1 Methods for assessing DNA methylation

There are a number of reviews summarizing the major techniques available for assessing levels of DNA methylation (Shen & Waterland 2007; Laird 2010; Harrison & Parle-McDermott 2011; Umer & Herceg 2013; Plongthongkum *et al.* 2014; Metzger & Schulte 2016b). In general, different techniques offer different levels of resolution from low-resolution information about whole-genome methylation levels to techniques that provide information about methylation status at the single base-pair level. Each of these approaches has advantages and disadvantages which are summarized in Table 1.1. Choosing the method that is best suited for a particular study

generally comes down to making a complex trade-off that weighs the cost, the resolution of the assay, and the extent of the genome that is monitored. In this thesis I use techniques that provide DNA methylation information at single base-pair resolution. In the following section I briefly describe different methods that can be used to obtain DNA methylation information at the level of individual nucleotides and discuss the advantages and disadvantages of the different methods.

Most methods for measuring DNA methylation with single base-pair resolution rely on bisulfite conversion of genomic DNA. When DNA is treated with sodium bisulfite or metabisulfite, the unmethylated cytosine bases (C) in the DNA are chemically converted into the base uracil (U), whereas methylated cytosines (5mC) are protected from this bisulfite conversion. During subsequent PCR amplification, a thymine (T) base is incorporated at any position that has been converted to a U, which changes the original sequence from a C to a T in unmethylated positions, whereas methylated positions remain as C's (Figure 1.3).

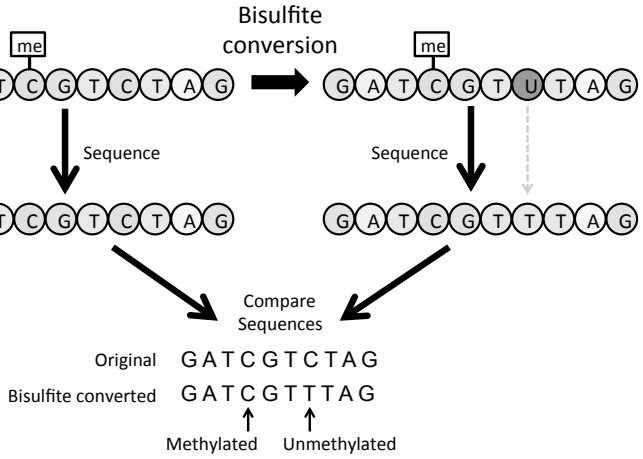

Figure 1.3: Bisulfite sequencing. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (C) into uracil (U), while methylated cytosines are protected. During PCR and sequencing uracil is converted to thymine (T). Sequence comparison of untreated and bisulfite converted DNA sequences allows the detection of methylated sites (reproduced from Metzger & Schulte 2016).

By comparing the bisulfite converted and untreated genomic DNA sequences it is possible to detect the location of methylated cytosines. Individual cells within a tissue can vary in whether a

particular position is methylated or not, and the initial DNA isolation step that is the starting point of bisulfite conversion is usually a sample that contains multiple cells. Thus, results from these assays provide information on the percentage of cells that have a methylated cytosine at a particular position.

The most comprehensive method for assessing patterns of DNA methylation at single base-pair resolution is bisulfite conversion of genomic DNA followed by whole-genome shotgun sequencing (whole-genome bisulfite sequencing WGBS; Laird 2010). However, the cost of comprehensive sequencing remains prohibitive for organisms with large genomes, such as most fishes, and thus WGBS is usually only applied to questions that can be answered using small numbers of biological replicates. For example, current recommendations are that 10X genome coverage and at least two biological replicates should be used at a minimum for bisulfite sequencing (Ziller *et al.* 2015). As the costs of genome sequencing decrease, however, WGBS has become more accessible, and this technique is likely to have much wider application. An additional barrier to applying this method stems from the challenges of the associated bioinformatics, which can be difficult even in model organisms (Bock 2012; Adusumalli *et al.* 2015) and are a particular barrier in species that lack a sequenced genome, such as many fishes. However, there has been some recent development of bioinformatics methods that should allow the application of these techniques even in the absence of a sequenced genome (Bewick *et al.* 2015).

A variety of methods have been devised to mitigate the high cost of WGBS. In general, these methods reduce costs by focusing sequencing effort on the methylated portions of the genome. This enrichment is performed either using affinity-based or restriction-enzyme based approaches. Reduced representation bisulfite sequencing (RRBS; Jeddeloh *et al.* 2008; Gu *et al.*

2011) is a restriction-enzyme based approach where genomic DNA is first digested using a methylation-insensitive restriction enzyme such as *Msp*I that specifically target CpG motifs. These fragments are then sequenced. This allows sequencing efforts to be concentrated primarily on regions of the genome that have the potential to be methylated (Jeddeloh *et al.* 2008; Gu *et al.* 2010). Although the RRBS technique helps to mitigate the cost of sequencing per individual, the downstream analysis of RRBS data in the absence of a reference genome can be complicated. However, there are currently a variety of tools that are available to help perform these analyses in species without a reference genome (e.g. Chen *et al.* 2010; Stockwell *et al.* 2014).

Table 1.1: Advantages and disadvantages of selected methods for assessing DNA methylation

Method	Resolution	Advantages	Disadvantages
Reverse Phase HPLC (RP-HPLC)	Low (whole genome)	• No sequence information required • Inexpensive	• Only very large differences can be detected • Very low resolution
ELISA	Low (whole genome)	• No sequence information required • High throughput • Inexpensive	• Only very large differences can be detected • Very low resolution
MS-AFLP	Low-Moderate	• No sequence information required • Inexpensive • Some information about location of methylated sites can be obtained (if candidate loci sequenced)	• Low resolution • Provides +/- information (qualitative) • Screens anonymous loci (candidates can be sequenced) • Reproducibility can be poor
Bisulfite sequencing of a candidate locus	Single base pair	• All CpG sites in targeted region can be analyzed • Generates information about location of methylated sites • Inexpensive	• Low coverage of the genome • Low throughput • Identifying appropriate candidate regions is challenging • Sequence information is required for primer design
EpiRADseq	Single base pair	• High resolution • Quantitative • Genome-wide (~ 2 million CpG sites examined per sample; ~6% of total sites)*	• Expensive • Requires genomic sequence information • Only examines methylation at *HpaII* sites • Fully methylated sites are not captured
Reduced Representation bisulfite sequencing (RRBS)	Single base pair	• High resolution • Quantitative • Genome-wide (~3 million CpG examined per sample; ~10% of total sites)*	• Expensive • Requires genomic sequence information • Only examines sites within ~100bp of an enzyme recognition site
MeDIP-Seq/MBDSeq	100-300bp (single base pair when combined with bisulfite sequencing)	• Fairly high resolution • Quantitative • Genome-wide (~6 million CpG examined per sample; ~20% of total sites)*	• Expensive • Requires genomic sequence information • Quantification complex
Whole-genome bisulfite sequencing (WGBS)	Single base pair	• High resolution • Quantitative • Genome-wide (all CpG sites examined)	• Extremely expensive • Requires sophisticated bioinformatics • Requires assembled genome sequence

*Based on a 3Gbp genome size

1.5 DNA methylation in fish

Much of what is currently understood about mechanisms and patterns of DNA methylation in fish comes from studies conducted in zebrafish (Goll & Halpern 2011; Kamstra *et al.* 2015). Proteins involved in DNA methylation in mammals are also present in fish including the DNA methyltransferases (DNMTs), CXXC finger protein 1 (*cfp1*), methyl binding proteins *mecp2* and *mbd2*, the Piwi-related proteins *ziwi* and *zili*, and the *dnmt1* cofactor *uhrf1,* which is required for the maintenance of DNA methylation patterns (Goll & Halpern 2011).

Overall levels of DNA methylation appear to be similar among vertebrates, with 80% of CpG dinucleotides in the larval zebrafish genome being methylated compared to about 74% in the mouse (Feng *et al.* 2010). Similarly, as is the case for other vertebrates, zebrafish genomes have prominent unmethylated CpG islands located near the transcription start sites of actively transcribed genes. Differential methylation of distal enhancer elements has also been shown to play a biologically important role in zebrafish (Lee *et al.* 2015). The de-methylation intermediate 5hmC has also been observed in zebrafish, with the highest levels being observed in brain tissue (Kamstra *et al.* 2015). Non-CpG methylation, such as CpHpG and CpHpH, has also been detected in zebrafish but at low levels (2.13% in embryos) and these sites are evenly distributed throughout the genome. By comparison, non-CpG methylation in mouse embryos is 0.59% (Feng *et al.* 2010).

In mammals, methylation patterns are thought to be globally erased and reset soon after fertilization. Whether or not this occurs in zebrafish is not clear. The earliest studies of this phenomenon in zebrafish yielded contradictory results (Macleod *et al.* 1999; Mhanni & McGowan 2004), whereas the most recent work suggests that intermediate levels of erasure occur (Jiang *et al.* 2013; Potok *et al.* 2013). Zebrafish sperm DNA is hypermethylated (95%)

relative to oocyte DNA (75%). Following fertilization, 16-cell embryo DNA methylation levels are intermediate to those of sperm and oocytes, but whether this is the result of erasure is unclear (Jiang *et al.* 2013; Potok *et al.* 2013). The lack of clarity regarding the level of erasure of methylation may be due, at least in part, to the asynchronous development of zebrafish oocytes (Kimmel *et al.* 1995) and the associated difficulty of obtaining enough developmentally synchronized embryos to obtain the quantity of genomic DNA necessary for this type of analysis. Perhaps using a fish species in which more synchronous fertilization can be achieved would allow for a more detailed analysis of whole genome DNA methylation levels earlier in development.

After the 16-cell stage, whole-embryo DNA methylation levels increase at each cell division, approaching the methylation levels observed in sperm DNA by the time the embryo reaches gastrulation. It has been proposed that patterns of DNA methylation in developing embryos are reset to the patterns observed in sperm. However, parthenogenic fertilization of zebrafish oocytes results in the same whole-genome DNA methylation patterns as traditional fertilization (Potok *et al.* 2013), suggesting that components within the oocyte provide all of the information required to establish methylation patterns in the offspring. It is possible that the demethylated state of the oocyte is indicative of a more totipotent state of the oocyte compared to the more differentiated state of the embryo, with increased whole genome methylation levels. Alternatively, piRNAs could survive the UV irradiation treatment of sperm DNA prior to gynogenic fertilization acting as the primary regulators of DNA methylation reprograming to a paternal pattern (Potok *et al.* 2013). Whether the changes in methylation levels and patterns during development are conserved across fishes or are unique to zebrafish remains largely unexplored.

1.5.1 Effects of environmental stressors on DNA methylation in fish

Epigenomic changes have the potential to play an important role in the response of fishes to environmental change, and may influence adaptation to new environments (Franks & Hoffmann 2012). Understanding responses to environmental change is of increasing importance in the context of global environmental change and other human impacts on aquatic environments. For example, multiple studies have shown that the DNA methylation patterns of fishes change following exposure to environmental toxicants (Timme-Laragy *et al.* 2005; Aniagu *et al.* 2008; Wang *et al.* 2009; Pierron *et al.* 2014), highlighting an underappreciated impact of environmental toxicants on organismal biology. In the remainder of this section, I focus on what is known about the effects of temperature and salinity on DNA methylation in fishes because these environmental factors are directly assessed in this thesis.

Changes in environmental temperature has pervasive effects on the biology of fishes, yet there are surprisingly few studies investigating its effects on the epigenome (Varriale & Bernardi 2006; Campos *et al.* 2013; Shao *et al.* 2014). A particularly intriguing study (Varriale & Bernardi 2006) found that whole-genome methylation levels (as assessed using RP-HPLC) were inversely correlated with temperature, with the genomes of fish from cooler habitats exhibiting higher levels of whole-genome DNA methylation compared to fish from warmer habitats. Several subsequent studies have also identified temperature as an important factor that may modify methylation levels in fish (Campos *et al.* 2013; Shao *et al.* 2014; Anastasiadi *et al.* 2017). For example, temperature has been shown to affect methylation levels in the promoter of the myogenin gene in Senegalese Sole (*Solea senegalensis*), as detected using candidate-gene bisulfite sequencing (Campos *et al.* 2013). These changes were correlated with changes in myogenin expression and muscle cellularity, clearly demonstrating the linkages between changes

in DNA methylation and important organismal phenotypes. In Atlantic salmon, changes in the expression of genes known to be involved in DNA de-methylation have also been demonstrated in response to changes in embryonic temperature and myogenin expression (Burgerhout *et al.* 2017).

Similar to studies investigating the effects of environmental temperature on DNA methylation patterns, most of the studies conducted to date that have investigated the effects of environmental salinity on DNA methylation patterns have used low resolution techniques (e.g. Morán *et al.* 2013; Li *et al.* 2017). From these studies, it is clear that fish modify genomic DNA methylation levels in response to changes in environmental salinity; however, it is unknown which regions of the genome are affected by these changes in DNA methylation levels. A candidate gene study that measured DNA methylation levels in the *igf1* gene in the liver tissue of half smooth tongue sole (Li *et al.* 2017) found an inverse correlation between DNA methylation levels of the *igf1* gene and expression of *igf1* 7 days after transfer from 30 ppt to 15 ppt and no differences in DNA methylation levels were detected after 30 days suggesting that DNA methylation levels are dynamically regulated in response to changes in environmental salinity.

1.5.2 DNA methylation and sex determination in fish

Gorelick (2003) presents a hypothesis for how differences in DNA methylation patterns could provide a mechanism of sex determination in both environmental sex determination (ESD) and genotypic sex determination (GSD). In ESD, DNA methylation is involved in the sex-specific expression of key genes involved in sex determination such as genes that regulate male and female gonad development. In GSD, DNA methylation is hypothesized to be involved in both suppressing recombination between sex chromosomes by regulation the formation of

heterochromatin, and in increasing mutation rates via the deamination of methylated cytosines to thymines. Together, recombination suppression and increased mutation rates could initiate and accelerate the speed of Muller's ratchet thus leading to the degradation and divergence of the heterogametic sex chromosome pairs (Gorelick 2003).

Fishes demonstrate a wide range of sex-determining mechanisms, including both ESD and GSD, but the details of these sex determining mechanisms remain poorly understood (Devlin & Nagahama 2002; Mank *et al.* 2006). There is accumulating evidence that DNA methylation likely plays a key role in temperature dependent sex determination (TSD) mechanisms, a form of ESD (e.g. Navarro-Martín *et al.* 2011; Chen *et al.* 2014; Shao *et al.* 2014; Wen *et al.* 2014). For example, the *cyp19a* gene is an aromatase gene that converts androgens into estrogens and is thought to play an important role in gonad differentiation. Hypermethylation of the *cyp19a* gene in male gonad tissue compared to female gonad tissue in the European Sea Bass reduces expression of *cyp19a* and causes a reduction in the synthesis of estrogen in male gonad tissue (Navarro-Martín *et al.* 2011). Interestingly, exposure of European Sea Bass larvae to elevated temperatures results in increased methylation of the *cyp19a* promoter and a masculinization of female gonad tissue.

The role of epigenetics in sex determination has also been investigated in the Half-Smooth Tongue Sole (*Cynoglossus semilaevis*), which exhibits TSD and GSD. This species has a ZZ/ZW sex determination system in which it has been previously demonstrated that male-specific expression of the Z-linked *dmrt1* gene is associated with male gonad development (Chen *et al.* 2014). Shao et al. (2014) used WGBS to determine whole-genome DNA methylation patterns in male, female, and pseudo-male gonads. They found that genes associated with sex-determination pathways are differentially methylated between male and female gonad tissue.

Specifically, the authors discovered that the *dmrt1* gene has high levels of DNA methylation in female gonads, whereas ZZ males and ZW pseudo-males were hypomethylated at the *dmrt1* locus suggesting that demethylation of dmrt1 is necessary for male gonad development. Interestingly, although ZW pseudo-males can be produced by exposing ZW females to high environmental temperatures, pseudo-males can also give rise to ZW pseudo-male offspring in the absence of a high-temperature environmental cue (Chen *et al.* 2014). This suggests that there may be a transgenerational epigenetic mechanism of sex determination in this species.

A study by Wen et al. (2014) measured DNA methylation and gene expression levels of both *cyp19a* and *dmrt1* in Japanese Flounder (*Paralichthys olivaceus*). They found that high levels of *dmrt1* expression in male gonad tissue are correlated with low levels of DNA methylation in the *dmrt1* promoter, whereas low levels of expression are correlated with high levels of DNA methylation. In contrast, the promoter of *cyp19a* was hypermethylated in testes compared to ovaries, which corresponded to lower levels of *cyp19a* mRNA in testis tissue compared to ovaries. Subsequent studies in Japanese flounder have observed similar patterns in which DNA methylation patterns of *cyp19a1a* and *foxl2* are inversely correlated with mRNA expression levels during ovarian development (Si *et al.* 2016). Taken together, these studies clearly demonstrate that the methylation of the *dmrt1* and *cyp19a* genes is important for sex determination in a variety of fish species.

For species that use GSD, sex is determined by the inheritance of sex specific factors such as male and female specific alleles or heteromorphic sex chromosomes. Different forms of GSD appear to have repeatedly evolved in different fish species, which makes it difficult to conclusively determine the ancestral GSD system in fish (Mank *et al.* 2006); however, some studies suggest that autosomal sex determination is the ancestral state (Ohno 1967; Traut &

Winking 2001). Sex chromosome evolution from autosomes is thought to involve recombination suppression of sex determining regions during meiosis followed by the accumulation of deleterious mutations in these regions leading to degeneration of the chromosome in one sex but not the other (the male Y in an XY sex chromosome system or the female W in a ZW sex chromosome system; Wright *et al.* 2016; Graves 2016).

In more derived heteromorphic sex chromosome systems (such as those found in mammals), DNA methylation is thought to be involved in regulating gene expression of dosage sensitive genes on heteromorphic sex chromosomes (Graves 2016), and it is also thought to play a role in the initial establishment of heteromorphic sex chromosomes (Gorelick 2003). Differences in DNA methylation levels between sex chromosomes have been hypothesized to play a role in this initial recombination suppression, and there are some data supporting this idea from studies in plants (Zhang *et al.* 2008). However, nothing is known about the potential role of DNA methylation in the establishment of heteromorphic sex chromosomes in vertebrates. Fish are an excellent model system for studying the evolution of heteromorphic sex chromosomes because various fish species have heterogametic sex chromosomes that are at different evolutionary stages of differentiation. For example, sex determination in the tiger pufferfish (*Takifugu rupripes*) is controlled by a single missense mutation in the *amhr2* gene (Kamiya *et al.* 2012) with no evidence of suppressed recombination or additional genetic divergence between the proto-X and proto-Y chromosomes. In contrast, threespine stickleback have a heteromorphic XY sex chromosome pair at the early stages of differentiation (Peichel *et al.* 2004; White *et al.* 2015). However, to date, no studies have investigated patterns of DNA methylation in young heteromorphic sex chromosome systems.

1.6 Research organism: threespine stickleback (*Gasterosteus aculeatus*)

The development of genomic resources for threespine stickleback has created a powerful system in which to study how genetic variation and phenotypic plasticity contribute to the evolution of adaptive phenotypes, and the evolution of heteromorphic sex chromosomes and sex determination in vertebrates. The utility of stickleback as a model system is due in large part to the ability to capitalize on the extensive efforts to study the natural history of this species and characterize the behavioral and morphological variation among marine and freshwater populations. In this thesis, I examine how three environmental factors that differ between marine and freshwater environments (salinity, temperature, and maternal stress) affected DNA methylation and gene expression patterns in stickleback form a marine population, and explore the relationship between DNA methylation and the evolution of heteromorphic sex chromosomes. Previous studies in stickleback have demonstrated a great deal of plasticity with respect to coping with changes in these environmental factors. In addition, differences in these environmental factors between marine and freshwater environments are thought to impose different selective pressures, which have contributed to the rapid divergence of marine and freshwater stickleback populations. Therefore, examining DNA methylation patterns in stickleback and how transcriptomic and epigenomic mechanisms respond to environmental changes will provide insight into the relationship between epigenomics and phenotypic plasticity in the context of ecologically relevant evolutionary processes. In this section I discuss how threespine stickleback have been used as a model system for studying mechanisms of adaptive evolution and how our current understanding of stickleback evolution can be used as a powerful foundation from which to investigate the ecological and evolutionary roles of epigenetic processes.

1.6.1 Threespine stickleback taxonomy and evolution

Populations of threespine stickleback (*Gasterosteus aculeatus*) are found in both marine and freshwater environments throughout their Holarctic distribution. Analysis of mitochondrial DNA, allozyme and microsatellite data indicates that current stickleback populations can be divided into three distinct clades termed the Pacific, Euro-North American, and Japan sea clades (Haglund *et al.* 1992; Higuchi & Goto 1996; Yamada *et al.* 2001; Kitano *et al.* 2007). Post glacial rebound following the retreat of the Pleistocene glaciers ~10-12 thousand years ago resulted in the reproductive isolation of marine stickleback in newly formed freshwater habitats across the northern species range (Haglund *et al.* 1992; Bell & Foster 1994; Orti *et al.* 1994; Taylor & McPhail 1999). With the exception of the anadromous Japan sea populations (Orti *et al.* 1994; Higuchi & Goto 1996; Kitano *et al.* 2007), much of the phenotypic diversity that has been observed among threespine stickleback populations is found among the freshwater populations that have evolved post glacially (Colosimo *et al.* 2005; Marchinko 2009).

Reproductive isolation of freshwater populations combined with different selective pressures in freshwater environments compared to the ancestral marine environment has resulted in the rapid, parallel divergence of numerous morphological and behavioral phenotypes between freshwater populations and the ancestral marine populations (Boughman 2007). The amount of phenotypic diversity observed among threespine stickleback populations initially led to the characterization of as many as 30 species of threespine stickleback (Mattern 2007). More recent classifications based on studies using current molecular and phylogenetic tools now classify threespine stickleback as a single species, *Gasterosteus aculeatus* (Kawahara *et al.* 2009) stemming from the greek etymology combining "gaster", meaning stomach, "osteon" meaning

bone, and "aculeatus*"* meaning spiny or prickly, an apt description of their boney lateral plates that are present in various numbers depending on the population, and three prominent dorsal spines. While only a single species of threespine stickleback is officially recognized, populations are often distinguished from one another based on their lateral plate morphology and the environments in which they are found, and these differences were often the basis of earlier classification schemes (eg. *G. trachurus* for fully plated marine populations and *G. leiurus* for low plated freshwater populations; Mattern 2007). Reproductively isolated populations of freshwater stickleback have been further resolved into benthic and limnetic sympatric species pairs, which inhabit the littoral and pelagic zones respectively (Boughman 2007). While a comprehensive review of stickleback phylogeny is beyond the scope of this thesis, several excellent books and review papers that have been written on this topic which illustrate the diversity of morphological and behavioral traits that are contained within the threespine stickleback species complex and which have been used to study the parallel evolution of adaptive morphological and behavioral traits (e.g. Bell & Foster 1994; Östlund-Nilsson *et al.* 2007).

1.6.2 Threespine stickleback genomic tools

One of the most significant contributions that has catapulted threespine stickleback into a prominent vertebrate model systems for studying the molecular basis of adaptive evolutionary processes was the completion of the stickleback genome sequence in 2006 by the Broad Institute. The stickleback genome is ~446 megabases encoding 20, 787 genes assembled into 21 chromosomes and several unassembled scaffolds and it is publically available at the Ensembl genome database (Zerbino *et al.* 2018). Due to the intense research interest in stickleback genomics and genome evolution, the stickleback genome assembly continues to be updated

(Glazer *et al.* 2015; Peichel *et al.* 2017). This wealth of genomic information, combined with the extensive literature on the parallel evolution of adaptive phenotypes among threespine stickleback populations resulted in a rapid increase in the number of studies investigating the genetic mechanisms of adaptive evolution in stickleback (e.g. Jones *et al.* 2012).

1.6.3 Physiological divergence of halotolerance between stickleback populations

Teleosts are osmoregulators and maintain an internal osmolarity of approximately 250-350 mOsm. Transitioning from marine to freshwater environments requires a major shift in osmoregulatory physiology to cope with the challenges posed the hyper-osmotic marine environment with an osmolarity of approximately 1,000 mOsm where stickleback must actively expel ions and drink water in order to balance water lost by diffusion, to a hypo-osmotic freshwater environment with an osmolarity of approximately 0.5-15 mOsm where they must actively uptake ions and expel water (Hill *et al.* 2008). Threespine stickleback are euryhaline and can tolerate a wide range of both hyper and hypo-osmotic conditions by restructuring the proteins involved in ion transport in the mitochondrial-rich ionocytes of the gill epithelium from conditions that favor ion uptake and inhibit diffusive ion losses in fresh water to ion excretion and water retention in marine environments (Edwards & Marshall 2013). The energy required to actively uptake ions from fresh water or actively expel them in seawater comes from the electrochemical gradient that is established by the basolateral Na^+/K^+ - ATPase (NKA). Ion co-transporters located on the apical and basolateral membranes of the ionocytes utilize the potential energy created by this electrochemical gradient to transport ions against their electrochemical gradient. The composition of ion transporters and ion transporter isoforms that are expressed by ionocytes can vary among species and can be modified by the individual depending on whether

the primary objective is ion-uptake in freshwater environments or ion excretion in marine environments (Richards *et al.* 2003).

Diffusive ion movement across the large surface area of the gill epithelium creates another potential challenge to ionoregulation in teleosts. To cope with this challenge, fish can alter the expression of proteins involved in the formation of tight junctions to modify the paracellular ion permeability of the gill epithelium. Fish can also reduce the amount of "leakiness" of the gill by modifying the size of the gill interlamellar cell mass, which effectively increases the diffusive distance across the gill and reduces the amount of ion loss due to passive ion flux. Recent studies have shown that this type of gill remodeling occurs in response to a range of environmental stressors including salinity, temperature, oxygen levels, and exercise (Sollid 2005; Mitrovic & Perry 2009; LeBlanc *et al.* 2010; Brauner *et al.* 2011; Fu *et al.* 2011; Nilsson *et al.* 2012; Perry *et al.* 2012; Barnes *et al.* 2014).

Stickleback from marine populations reared in freshwater conditions for several generations exhibit a similar divergence rate in halotolerance (0.569 haldanes; Divino *et al.* 2016) and cold tolerance (0.63 haldanes; Barrett *et al.* 2011) that are more rapid than the divergence in many of the morphological traits between marine and freshwater stickleback (Bell & Aguirre 2013). The physiological divergence in halotolerance and thermal tolerance suggests that these abiotic factors impose a strong selective pressure on the physiological mechanisms underlying these traits.

Salinity transfer experiments of marine and resident freshwater stickleback have demonstrated reduced tolerance to hyper-saline conditions in derived freshwater populations (Heuts 1947; Marchinko & Schluter 2007; McCairns & Bernatchez 2010; DeFaveri & Merilä 2014). Consistent with these findings, analysis of marine stickleback that had been reared in

freshwater for two generations exhibit increased tolerance to freshwater suggesting that rapid evolution of physiological processes involved in ion regulation (Divino *et al.* 2016). Alternatively, epigenetic mechanisms and transgenerational plasticity could also be playing an important role in modulating physiological adaptions to freshwater environments.

Recent studies have investigated how changes in environmental salinity between marine populations and resident freshwater populations of stickleback have shaped the physiological evolution of stickleback. These studies have identified regions of genetic divergence that are associated with osmoregulatory genes (Shimada *et al.* 2011; DeFaveri *et al.* 2011, 2013; Jones *et al.* 2012a; Kusakabe *et al.* 2017), as well as divergence in the regulation of the expression of osmoregulatory genes (McCairns & Bernatchez 2010; Taugbøl *et al.* 2014; Gibbons *et al.* 2017; Kusakabe *et al.* 2017).

1.6.4 Evolution of thermal tolerance in threespine stickleback

Temperature, a measure of average kinetic energy, has profound effects on the performance and distribution of organisms (Fry 1947; Hochachka & Somero 2002; Somero 2005; Sunday *et al.* 2012). Increases in temperature can reduce the stability of biological structures such as proteins, and increase the rate at which biological reactions occur (Hochachka & Somero 2002), which in turn can influence higher order processes such as metabolism and growth. Therefore, organisms must actively regulate biochemical processes in order to maintain performance in response to changes in temperature.

Biochemical processes of ectothermic poikilotherms, such as most fishes, are particularly vulnerable to changes in environmental temperatures as their body temperature closely matches that of the surrounding environment (Hochachka & Somero 2002; Schulte 2011). Observed

variation in thermal tolerance among different species and among different populations of the same species is the result of variation in plasticity and genetic divergence that impact lower level processes such as gene expression and protein function (Dalziel *et al.* 2009). Stenotherms have a fairly narrow thermal tolerance window while eurytherms can tolerate a wider range of temperatures. Differences in the thermal limits among organisms is often associated with variation in the thermal environment such that organisms that inhabit regions that experience fairly stable thermal conditions, such as polar and equatorial regions, are often stenothermic, while organisms that experience larger variation in environmental temperatures, such as large seasonal changes, are often eurythermic (Stillman 2002; Chown *et al.* 2004, 2010; Sunday *et al.* 2011; Clusella-Trullas *et al.* 2011).

Threespine stickleback are eurytherms, but freshwater populations in temperate regions experience more variation in environmental temperature compared to marine populations (Lee & Bell 1999). Adaptation of marine stickleback to freshwater habitats is associated with a rapid divergence in thermal performance at low temperatures, with freshwater populations having increased tolerance to cold conditions (Barrett *et al.* 2011). The rapid acquisition of thermal tolerance traits in stickleback may be associated with transgenerational plasticity, as the maternal and grand-maternal environment has been observed to impact thermal performance of offspring (Shama & Wegner 2014; Shama *et al.* 2016). However, the mechanism(s) through which information from the parental and grand-parental thermal environment is transmitted to subsequent generations is not well understood.

1.6.5 Evolution of heteromorphic sex chromosomes in threespine stickleback

In the family Gasterosteidae, in which at least six species are recognized, at least five different sex-determining systems have been identified, two of which are found among populations of *G. aculeatus.* Threespine stickleback (*G. aculeatus*) from Pacific and Euro-North American clades which have a relatively young XY sex chromosome pair that has evolved since the species first arose at least ~13-16 Ma (Kawahara *et al.* 2009; Ross *et al.* 2009; Bell *et al.* 2009). In contrast, *G. aculeatus* from the Sea of Japan have an X_1X_2Y sex chromosome system which is thought to have been generated from a fusion between chr19, an ancestral Y, with an autosomal copy of chr09 and arose independently from the X_1X_2Y sex chromosome system in black-spotted stickleback, *G. wheatlandi* (Ross *et al.* 2009) which is thought to have been generated from a fusion between chr19 and chr12 (Kawahara *et al.* 2009).

Divergence between the X and Y chromosome in *G. aculeatus* is thought to have involved at least three pericentric inversions on the Y followed by a large deletion (Ross & Peichel 2008) generating three evolutionary strata consisting of the pseudo-autosomal region (PAR), a younger evolutionary strata (stratum two), and an older evolutionary strata (stratum one; White *et al.* 2015). However, relatively little is known about the mechanisms that are involved in the recombination suppression on sex chromosome pairs in vertebrates. In plants, epigenetic modifications such as DNA methylation are thought play a role in regulating the formation of heterochromatin (Zhang *et al.* 2008), which is thought to regulate sex chromosome recombination (Gorelick 2003). The diversity of sex chromosome systems found among closely related members of the Gasterosteidae family, combined with the relatively recent evolutionary history of these systems makes *G. aculeatus* a particularly interesting system in which to study the evolution of sex chromosomes and sex determining systems.

1.6.6 Effects of maternal stress on offspring phenotypes in threespine stickleback

Much of what is known regarding developmental effects of maternal stress comes from mammals where the placenta is involved in regulating the fetal environment. Whether maternal stress has similar effects on offspring development in non-placental organisms is not well understood. Unlike mammalian systems, where complex maternal-fetal interactions via the placenta are thought to regulate the sexually dimorphic effects of maternal stress (Nugent & Bale 2015; Bronson & Bale 2016), oviparous embryos develop outside the body, separated from the further influence of maternal conditioning. In oviparous fish, females deposit nutrients and proteins into developing eggs during a process known as vitellogenesis (Tyler & Sumpter 1996). Variation in maternal condition such as nutritional state or circulating stress hormone levels can affect the nutrient and hormone composition of the eggs, which can have persistent effects on adult offspring phenotypes (e.g. Marteinsdottir & Steinarsson 1998; Mccormick 1998; Giesing *et al.* 2011). Despite differences in the developmental environment of mammalian and teleost embryos, several studies have observed effects of maternal stress on offspring behavior and the regulation of the hypothalamic-pituitary-interrenal (HPI) axis in teleost fishes (Mommer & Bell 2013; Bell *et al.* 2016; Sopinka *et al.* 2017).

Threespine stickleback are an interesting system in which to investigate the effects of parental stress on offspring phenotypes and the mechanisms underlying the transmission and development of these traits. Variation in the number and type of predators present between marine and freshwater environments, and variation among freshwater environments is thought to exhibit a strong selective pressure in stickleback (Boughman 2007). This divergent selection has

resulted in differentiation of behavioral traits such as variation in schooling behavior between benthic and limnetic stickleback (Boughman 2007).

Changes in predatory stressors among different environments have the potential to result in changes in maternal responses to the novel stressors, which could affect their offspring. For example, exposure of female stickleback to simulated predatory stressors increases the amount cortisol levels and the size of their eggs (Giesing *et al.* 2011). Cortisol is a glucocorticoid stress hormone that is part of the HPI stress response axis in (Bonga 1997). While the concentration of maternally derived cortisol has been shown to decrease rapidly following fertilization (Alsop & Vijayan 2009; Paitz *et al.* 2015), reducing the concentration of maternally derived cortisol prior to fertilization has been shown to disrupt development of the HPI axis in zebrafish (Nesan & Vijayan 2016). Increased levels of cortisol are often implicated in modulating the effects of maternal stress on offspring phenotypes, but the artificial elevation of exogenous cortisol levels often has little to no effect on offspring phenotypes (Paitz *et al.* 2016) suggesting that cortisol levels may not be directly responsible for inducing many of developmental effects on offspring phenotypes. Thus, the mechanisms through which maternal stress impacts offspring development is complex and likely includes other factors in addition to components of the HPI axis (Beijers *et al.* 2014).

1.6.7 Epigenomics in stickleback

At the time I started the experiments described in the following chapters of this thesis, only a single study had examined DNA methylation patterns in stickleback and this study identified significant changes in whole-genome DNA methylation levels in gonad tissue

following environmental exposure to estradiol (E2) were detected using HPLC, but no changes in response to hexa-bromo-cyclododecane (HBCD) exposure in liver (Aniagu *et al.* 2008).

Over the past five years, three studies have been published that used reduced representation sequencing techniques (RRBS and BSradSeq) to characterize DNA methylation patterns in threespine stickleback. These studies investigated differences in DNA methylation between stickleback that differed in the number of lateral plates (Smith *et al.* 2015), between stickleback from a marine and a freshwater population (Trucchi *et al.* 2016), and between stickleback from a marine and a freshwater population at different salinities (Artemov *et al.* 2017). These papers revealed substantial differences in putative methylation patterns between stickleback populations or morphs (Smith *et al.* 2015) and suggest that DNA methylation patterns are modified following transfer between high and low salinity environments (Artemov *et al.* 2017). However, each of these studies has limitations that reduce our ability to fully interpret the reported data, in part because of the limitations on our current understanding of epigenomics in an environmental and ecological context.

The first limitation on the interpretation of these studies is that each study involved aligning the bisulfite treated DNA sequences from multiple wild populations or morphs to the single reference genome available from the Ensembl genome database. This creates a potential issue in the interpretation of the datasets if there are DNA polymorphisms between the populations or morphs. Unmethylated CpG loci are identified when a CpG dinucleotide is converted to a TpG dinucleotide following treatment with sodium bisulfite. Therefore, naturally occurring C to T polymorphisms between bisulfite sequences and the reference sequence can affect the calculation of DNA methylation levels. This complicates the interpretation of these data because one population might have the same CpG sequence as the reference genome while

the other population has a TpG at that location. These would then be interpreted as methylated and unmethylated loci respectively, when in fact the detected difference is a genetic polymorphism, not a difference in methylation. Because each of these studies investigated DNA methylation patterns in stickleback between genetically divergent populations, it is possible that the patterns described in these data are confounded by genetic polymorphisms both between populations and between the populations and the reference sequence.

A second potential limitation of these studies is that they used wild-caught individuals. Therefore, it cannot be determined if the differential methylation described in these studies are fixed differences between populations or changes in response to environmental differences. This is particularly true for the study by Artemov *et al.* (2017) because DNA methylation patterns were compared between stickleback from a marine and a freshwater population acclimated to their native salinity. Therefore, the differential methylation patterns described in this study could represent DNA methylation patterns in stickleback that are caused by exposure to different salinities instead of evolved differences between marine and freshwater populations as suggested in the manuscript.

However, despite their limitations, these studies illustrate how modern advances in sequencing technology have enabled the use of previously cost-prohibitive techniques to address questions in ecology and evolution, highlighting the interest in using threespine stickleback as a model system for studying epigenetic processes in an ecological and evolutionary context. To fully interpret these studies, however, it is critical to understand how DNA methylation patterns in stickleback are affected by changes in the environment, and how plasticity at different stages in development influences DNA methylation patterns. Therefore, a first step towards understanding whether DNA methylation has an important role in regulating the expression of

the phenotypic diversity observed among divergent populations of stickleback is to understand the plasticity of DNA methylation patterns in response to different environments and whether exposure to different environments at different developmental stages are temporary or if they have persistent effects on DNA methylation patterns. My thesis aims to fill this knowledge gap.

1.7 Thesis organization

My thesis is organized around four data chapters addressing the key questions that I posed that the beginning of this introductory chapter. Below I outline the methodological approaches and questions addressed in each of these chapters.

1.7.1 Chapter two

Chapter two of my thesis investigates the persistent effects of warm and cold developmental temperature on DNA methylation patterns in adult stickleback muscle tissue using an RRBS approach. I then compare these effects to changes in the DNA methylation patterns in the muscle tissue of adults acclimated to warm and cold temperatures. Comparing changes in DNA methylation patterns across these two timescales provides insight into the potential effects of the environment on epigenetic variation and into how epigenetic responses might influence the capacity of organisms to cope with environmental change.

1.7.2 Chapter three

Chapter three of this thesis uses the same experimental design and individual organisms used in chapter two to investigate the effects of developmental temperature and adult thermal acclimation on gene expression in adult muscle tissue using an RNA-Seq approach. By

comparing developmental effects on adult gene expression to adult thermal acclimation, I investigate whether there are potential mechanistic connections between gene expression plasticity across timescales. Because the same individuals used in chapter two to investigate temperature effects on DNA methylation patterns were also used in chapter three, I was able to directly compare temperature dependent changes in gene expression to changes in DNA methylation.

1.7.3 Chapter four

Chapter four examines DNA methylation patterns in the young XY heteromorphic sex chromosome system of threespine stickleback using a whole genome bisulfite sequencing approach to determine whether DNA methylation patterns differ between male and female threespine stickleback. In this chapter I also characterize the effects of salinity on DNA methylation in stickleback and determine whether regions known to be under differential selection between marine and freshwater environments and genes whose expression is salinity responsive are differentially methylated in response to environmental salinity.

1.7.4 Chapter five

In chapter five I investigate whether stress experienced during vitellogenesis in female stickleback has a detectable effect on gene expression patterns in the brain tissue of adult stickleback offspring. This experiment also examined whether maternal stress has similar effects on male and female offspring.

Chapter 2: Persistent and plastic effects of temperature on DNA methylation across the genome of threespine stickleback (*Gasterosteus aculeatus*)

2.1 Introduction

Environmental temperature is a critical factor that determines the distribution and abundance of organisms (Angilleta 2009; Somero 2010; Schulte *et al.* 2011; Sunday *et al.* 2012), and the rapidly changing thermal environment due to global climate change is projected to have a particularly pronounced effect on ectothermic species such as fishes (Somero 2010; Sunday *et al.* 2012). Phenotypic plasticity plays an important role in the responses of organisms to rapid changes in the environment and could be critical in determining the ability of species to cope with climate change (Charmantier *et al.* 2008; Somero 2010; Seebacher *et al.* 2015). Plastic responses can persist across multiple time scales, from short-term and largely reversible acclimation responses, to longer-term developmental plasticity, to transgenerational responses (Beaman *et al.* 2016), but plasticity at these different timescales often impacts similar physiological processes. For example, in fishes, temperature changes during development can have persistent effects on sex determination, metabolism, thermal performance, muscle phenotypes, and gene expression (Devlin & Nagahama 2002; Schaefer & Ryan 2006; Scott & Johnston 2012; Schnurr *et al.* 2014). While exposure of adults to short term temperature change can induce temporary plastic responses in many of these traits (Guderley 2004; Schulte *et al.* 2011; Scott & Johnston 2012; Morris *et al.* 2014b). Physiological plasticity is often modulated at the level of gene expression, thus investigating regulatory mechanisms controlling gene

expression is fundamental to understanding how species cope with climate change (Somero 2010).

Epigenetic modifications can influence the plasticity of organisms by regulating gene expression without modifying the DNA sequence (Deans & Maggert 2015). DNA methylation, the addition of a methyl group to a cytosine nucleotide, is one of the best-characterized epigenetic processes. The addition or removal of DNA methylation can be dynamic, occurring rapidly in response to environmental cues (Bossdorf *et al.* 2008; Angers *et al.* 2010; Dowen *et al.* 2012). DNA methylation patterns can also persist through cell division and be passed on through multiple generations potentially influencing evolutionary processes and fitness (Jablonka & Raz 2009; Flores *et al.* 2013). The intra and intergenerational effects of DNA methylation and the role of DNA methylation in regulating plasticity is an intriguing system in which to examine how epigenetic processes modulate plastic and persistent phenotypic variation in response to changing environments. How this epigenetic system of "soft inheritance" (Mayr 1982; Dickins & Rahman 2012) functions as a response to changes in temperature has been highlighted by studies in plants (Dubin *et al.* 2015; Nicotra *et al.* 2015; Song *et al.* 2015; Keller *et al.* 2016; Rakei *et al.* 2016; Gugger *et al.* 2016; Kawakatsu *et al.* 2016; Li *et al.* 2016), yet little is known about these processes in ectothermic vertebrates (Hofmann 2017).

To understand how DNA methylation is modified in response to environmental temperature in vertebrates, we used threespine stickleback (*Gasterosteus aculeatus*), an important vertebrate model for studies of ecological adaptation (Bell & Foster 1994; Jones *et al.* 2012b), in which phenotypic effects of both developmental temperature and adult acclimation temperature have been clearly demonstrated (Jordan & Garside 1972; Guderley *et al.* 2001; Shama *et al.* 2014; Ramler *et al.* 2014; Morris *et al.* 2014b; Shama & Wegner 2014; Teigen *et al.*

2015). By comparing DNA methylation patterns between threespine stickleback with different thermal histories during development to methylation patterns in adult threespine stickleback acclimated to differing temperatures, we demonstrate that epigenetic mechanisms are a component of both persistent and plastic responses to environmental change.

2.2 Materials and methods

2.2.1 Experimental design

Adult threespine stickleback (*G. aculeatus*) were collected from a fully plated "marine" population in Oyster Lagoon (British Columbia, Canada, GPS: 49.6121-124.0314) in June 2014. Stickleback were separated into six 110-litre glass tanks (20 stickleback per tank) and acclimated for three weeks to 20 ppt salt water (dechlorinated Vancouver municipal tap water supplemented with Instant Ocean Sea Salt), 18 °C and 14:10 h light:dark photoperiod, which mimics the natural environmental conditions at the collection location at the time of collection. Stickleback were fed daily to satiation with Hakari Bio-Pure frozen Mysis Shrimp.

After the three weeks of laboratory acclimation, eggs were collected from six gravid females and testes were dissected from six males and individually macerated in a 1.75 mL microcentrifuge tube containing 150 μL Ginzberg's fish Ringer's solution. Eggs collected from a single female were arranged as a monolayer in petri dishes containing 5 mL of 20 ppt salt water. 50 μL of the sperm solution from a single male was applied directly on the egg mass and left for 30 min at the acclimation conditions to allow fertilization to occur. This process was repeated six times using six different females and six different males to generate six independent families. Following fertilization, an additional 10 mL of 20 ppt saltwater was added to each petri dish. Each clutch was then split across three separate petri dishes. A single petri dish from each family

was transitioned to a developmental temperature of 12, 18, or 24 °C (such that all six families were represented in each developmental temperature treatment). Petri dishes were partially covered to prevent water loss via evaporation and to allow for surface gas exchange. Eggs were monitored twice daily during which time any unfertilized eggs were removed and 10 mL of water was changed to prevent mold growth. Hatching time differed by several days between stickleback reared at different development temperatures (Appendix A, Figure A.1). After hatching, embryos were moved to 110 L glass aquaria at the original acclimation conditions of 18 °C with a 14:10 h light:dark photoperiod. Each family was held in a separate mesh breeding box, with two breeding boxes (i.e. two families) per glass aquaria. Aquaria were equipped with hanging box filters (Aquaclear) and sponge filters for filtration and aeration. Larvae were fed live brine shrimp nauplii twice daily ad libitum until they were large enough to feed on frozen Mysis shrimp. Once juvenile stickleback reached a size of approximately 1 cm they were released from the breeding boxes into the aquaria. For the experiment on the effects of developmental temperature on DNA methylation, families were kept separate (two families per split aquarium) until sampling, which occurred at 10 months of age. A total of six stickleback from each development temperature treatment were euthanized and muscle tissue samples (from behind the dorsal fin to the base of the tail) were taken from each stickleback (four females and two males from each treatment except for the 12 °C development group which had five females and one male), with sampling distributed across families (Appendix A, Table A.1). This experimental design minimizes the potential for effects of genetic variation and sex on DNA methylation patterns (Liu *et al.* 2010; Gertz *et al.* 2011).

For the experiment on the effects of adult thermal acclimation on DNA methylation patterns, after nine months of development, a random sample of stickleback from the six families

that had developed at 18 °C were mixed together and then split between three different acclimation temperatures (5, 18, or 25 °C). Following four weeks of thermal acclimation, a total of six sticklebacks from each acclimation temperature treatment were euthanized and muscle tissue was sampled as described above (from four females and two males from each treatment). Muscle tissue was immediately snap frozen in liquid nitrogen and stored at -80 °C until further use.

The developmental and acclimation temperature protocols described above created 5 different groups (4 treatments and 1 control) as follows: stickleback developed at 12 °C and acclimated to 18 °C, stickleback developed at 24 °C and acclimated to 18 °C, stickleback developed at 18 °C and acclimated to 5 °C, and stickleback developed at 18 °C and acclimated to 25 °C and stickleback developed at 18 °C and acclimated to 18 °C that were treated as the control group (Figure 2.1).

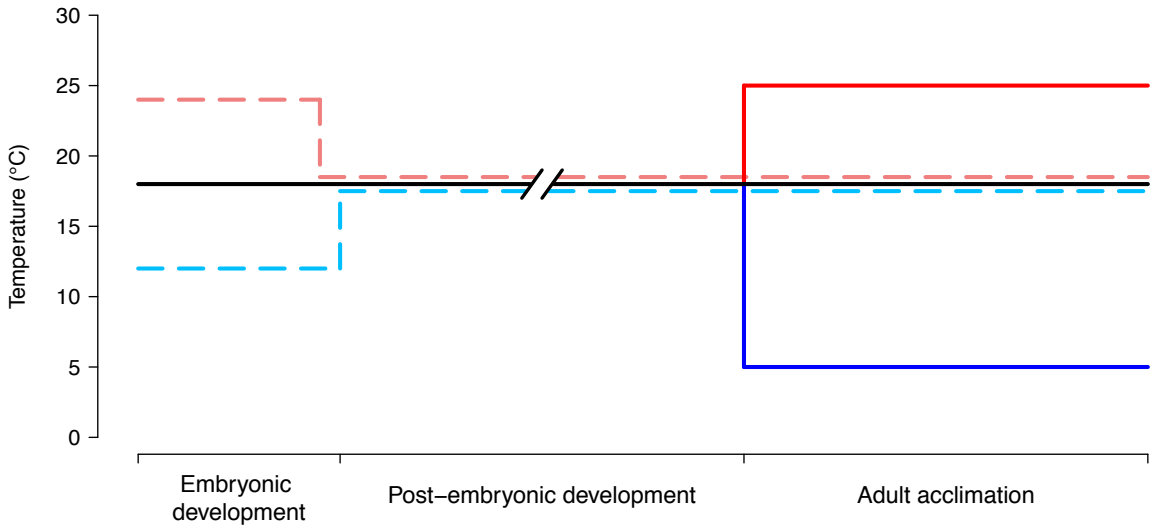

Figure 2.1: Experimental design. Solid lines represent stickleback that were developed and acclimated to 18 °C (black) or that developed at 18 °C and were acclimated to 25 °C (red) or 5 °C (dark blue). Dashed lines represent the developmental temperature treatments of 24 °C (pink), or 12 °C (light blue). Note that altered development temperature changes time to hatch by several days. Post-hatch, stickleback were transferred to 18 °C where they developed to adults for the remainder of the experiment (10 months).

2.2.2 Reduced representation bisulfite sequencing

We chose to examine methylation patterns in muscle tissue because previous work (Scott & Johnston 2012; Morris *et al.* 2014b) has indicated that both developmental temperature and acclimation temperature alter muscle gene expression in fish. Genomic DNA was isolated from muscle tissue using a *Quick*-DNA™ Miniprep kit (Zymo Research). Bisulfite-treated reduced representation genomic DNA libraries were prepared by the UBC Nucleic Acid and Protein Service core facility and sequenced at the UBC Biodiversity Centre sequencing facility. Libraries were created using a Bio-O NEXTflex Bisulfite-Seq Kit on MspI digested gDNA. Samples were barcoded using NEXTflex Bifulfite-seq Barcodes. Purified, adapter-ligated DNA was then bisulfite treated using an EZ DNA Methylation Gold Kit (Zymo Research). Samples were split between two lanes (3 samples per treatment per lane for 15 samples total per lane) of an Illumina HiSeq 2000. Average sequencing library size was 19,900,578 ± 3,880,665 (mean ± SD) million reads and covered an average of 12,901,548 CpG sites per sample.

2.2.3 Quantification and statistical analysis

Sequences from the bisulfite treated samples were aligned to the stickleback genome (Ensembl release 87) and annotated using CLC genomics workbench v9.5 with the bisulfite sequencing plugin v1.1.1. Average mapping efficiency was 88.3%. Total coverage and methylated cytosine coverage data were exported and analyzed for differential methylation in R v3.3.1 using *methylKit* package v1.1.3 (Akalin *et al.* 2012). Prior to global DNA methylation analysis, CpG loci were filtered so that only sites with at least 8 reads were retained in each sample. Sites that were in the 99.9th percentile of coverage were also removed from the analysis

to account for potential PCR bias. After filtering, an average of 137,954 CpG sites (1% of all CpG sites in the genome) were retained per library with a mean coverage depth of 15 reads.

To test for differential methylation at specific loci an additional filter was applied such that only CpG loci with at least 8 reads in at least 4 of the 6 samples within a treatment were kept. Threespine stickleback that were kept at 18 °C for the duration of the experiment were used as a reference group for a pairwise comparison between each of the developmental and acclimation temperature treatments.

Mean and median DNA methylation values for each individual were compared using a non-parametric Kruskal-Wallis Test. A Kolmogorov-Smirnov test was performed to test for differences in the shape of the cumulative methylation distributions between treatments. All statistical analyses were performed in R v3.3.1.

To identify differentially methylated regions (DMRs), the genome was divided into 100bp regions using the tileMethylCounts() function in *methylKit* v 1.1.3 with a window size of 100 and a step size of 100. Logistic regression was implemented using the calculateDiffMeth() function to identify differentially methylated loci and DMRs. P-values were false discovery rate corrected to Q-values using the sliding linear model (SLIM) method (Wang *et al.* 2011).

To determine the common and unique DMRs among treatment groups, the data were first filtered to include only those regions that were present in the reduced representation sequencing data with at least 8 reads in 4 of the 6 individuals in every group. Unique and overlapping DMRs were then visualized using the R-package *VennDiagram*. Annotations for DMRs were obtained using the annotatePeakInBatch() function in the R package *ChIPpeakAnno* v3.6.5 from Bioconductor (Zhu *et al.* 2010). GO annotations were obtained from a previous study (Metzger & Schulte 2016a). Enrichment analysis was performed using *goseq* v1.24 (Young *et al.* 2010)

and p-values were false discovery corrected for multiple comparisons (Benjamini & Hochberg 1995).

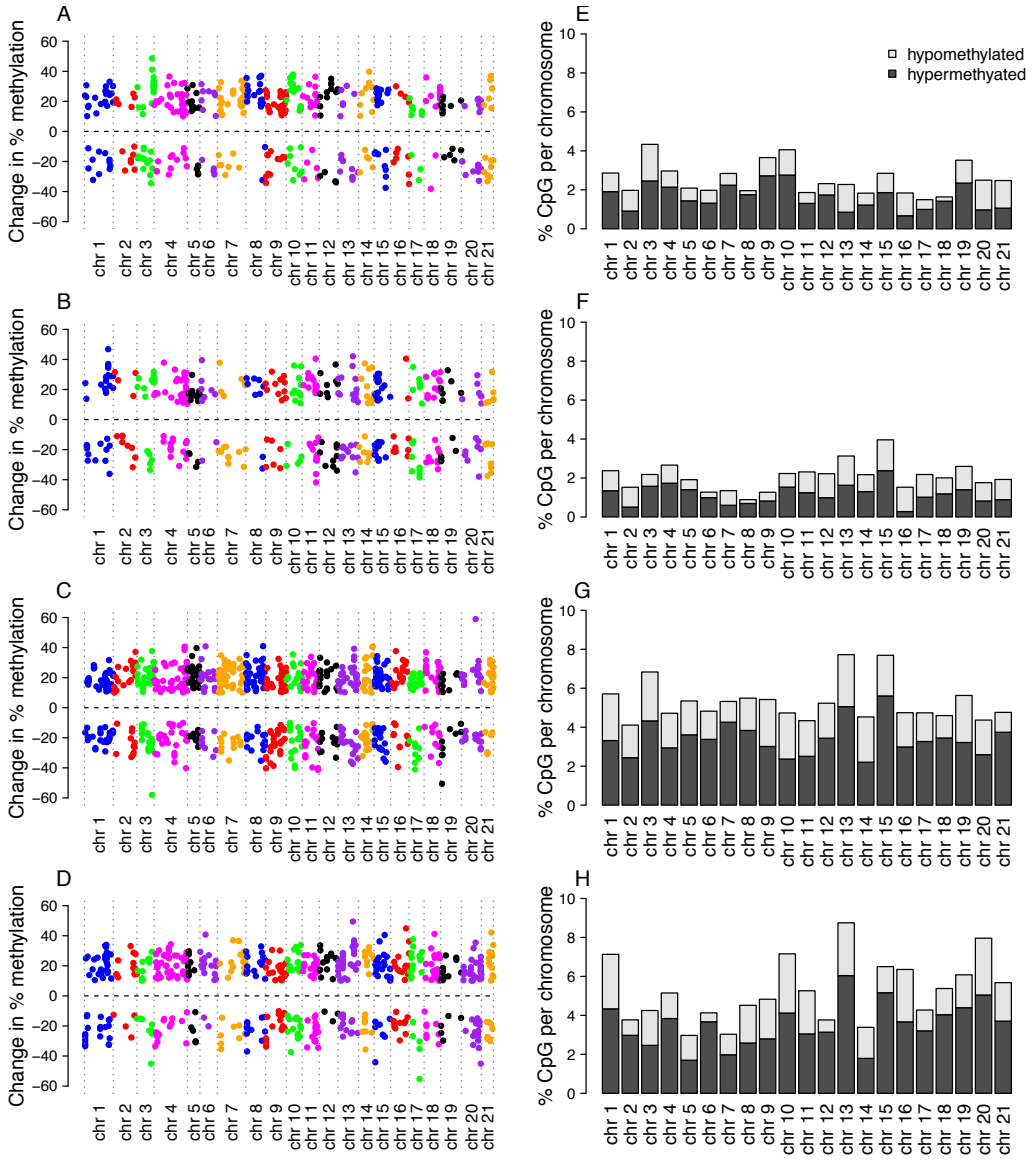

Figure 2.2: (A-D) Manhattan plots of the chromosomal positions of methylated CpG loci that differed significantly between stickleback that were developed at and acclimated to 18 °C, stickleback developed at 12 °C (A) or at 24 °C (B) then reared at 18 °C, or that were developed at 18 °C then acclimated to 5 °C (C) or 25 °C (D). Each point represents a single differentially methylated cytosine (DMC). The y-axis presents the difference in percent methylation for that DMC relative to the stickleback developed and acclimated at 18 °C. Only DMCs with >10% change in methylation are shown. Points above and below the horizontal dashed line are hypermethylated and hypomethylated loci, respectively. Points indicate chromosomal locations. Vertical dashed lines and colors indicate different chromosomal regions. (E-H) Chromosomal frequency distribution of hypermethylated (dark grey) and hypomethylated (light grey) DMCs in stickleback that developed at 12 °C (E) developed at 24 °C (F) or that were acclimated to 5 °C (G) or acclimated to 25 °C (H). The vertical axis represents the percent of CpGs that were sequenced that were differentially methylated.

2.3 Results and discussion

2.3.1 Temperature change induces genomic hypermethylation

We identified a total of 2,130 CpG loci in the stickleback genome that were differentially methylated in response to temperature in at least one treatment. Pairwise comparison of DNA methylation levels at individual CpG loci in stickleback from developmental and adult acclimation treatments to the control (18 °C) group identified 554 differentially methylated cytosines (DMCs) in stickleback that developed at 12 °C, 480 DMCs in stickleback that developed at 24 °C, 1150 DMCs in stickleback that were acclimated to 5 °C and 778 DMCs in stickleback acclimated to 25 °C. There was no apparent clustering of DMCs on a specific chromosome or chromosomal region (Figure 2.2A-D), and DMCs were distributed across promoters, introns, exons, and intergenic regions proportionally to the distribution of these features within the genome (Appendix A, Figure A.2).

Approximately 2-8% of the sequenced CpG sites on each chromosome exhibited differential methylation in response to altered temperature (Figure 2.2E-F). More DMCs were hypermethylated than were hypomethylated in all treatments suggesting that an increase in genomic DNA methylation levels is a general response to changes in environmental temperature (Figure 2.2E-F).

An increase in genomic DNA methylation levels was expected in stickleback exposed to cooler temperatures based on previous reports of latitudinal variation in genomic DNA methylation levels in fishes, in which polar fishes exhibit higher global DNA methylation levels than do equatorial fish (Varriale & Bernardi 2006). However, the observed increase in global DNA methylation levels in stickleback exposed to higher temperatures is in contrast to the inter-

specific correlations between DNA methylation levels and ambient temperature (Varriale & Bernardi 2006).

To further investigate the effects of temperature on genomic DNA methylation levels we examined the mean DNA methylation level for all individuals. Mean genomic methylation values ranged from 72.03-73.96%, and median methylation levels ranged from 80.3%-84.12% (Appendix A, Table A.2), but the treatment groups were not detected as significantly different from the controls. We also compared the cumulative distribution curves of genomic DNA methylation levels for each of the treatment groups to those of the 18 °C control group using the Kolmogorov-Smirnov (K-S) test. Results from the K-S test indicate that the distribution of genomic DNA methylation levels for all treatments was significantly different compared to stickleback from control conditions ($p < 2.2e^{-16}$, Figure 2.3 A/B) and that the distribution was the most different in stickleback acclimated to 25°C, which had the largest K-S test D statistic (0.05126, Appendix A, Table A.3). Visual inspection of the violin plots and cumulative distribution curves for DNA methylation (Figure 2.3A/B) suggest that, in general, these differences are driven by shifts towards increased proportions of highly methylated CpG loci relative to control conditions, which is consistent with the biases towards hypermethylation that we observed among DMCs for all treatments.

Taken together these data clearly illustrate that both developmental temperature and acclimation temperature alter the methylome of threespine stickleback. Changes in DNA methylation have the potential to causes changes in transcriptional regulation, and thus in cellular and organismal phenotypes, but changes in DNA methylation can also be the result of changes in cellular phenotype, which makes interpreting the physiological significance of changes in DNA methylation challenging (Lappalainen & Greally 2017). For example, both developmental

temperature and thermal acclimation have been shown to cause changes in the proportion of oxidative and glycolytic muscle fibres in fish (Egginton & Sidell 1989; Scott & Johnston 2012). Thus, the changes in DNA methylation that we observe could be either a cause or a consequence of changes in cell type.

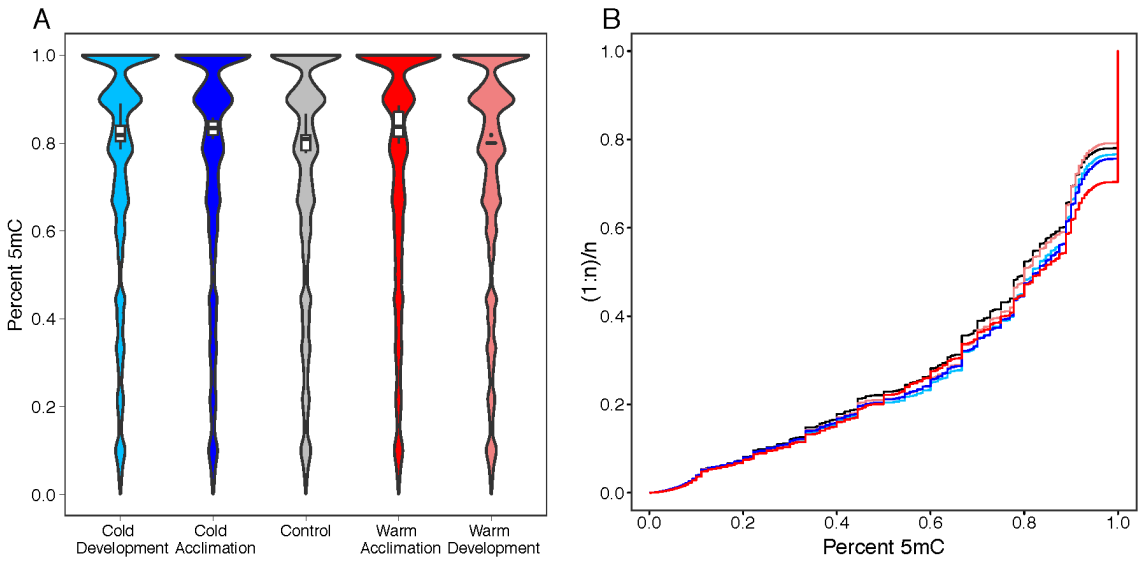

Figure 2.3: (A) Violin plot of genomic DNA methylation levels for each treatment condition and control condition. Colors depict different treatments. Stickleback developed and acclimated to 18 °C (grey/black), stickleback that developed at 18 °C and were acclimated to 25 °C (red) or acclimated to 5 °C (dark blue), and stickleback with a developmental temperature of 24 °C (pink), or 12 °C (light blue). Width indicates the pooled distribution density of percent methylation of CpG loci in a given treatment. Embedded box plots summarize variation in the median methylation level across the six samples in each treatment. The line indicates the median of these medians, the box defines the interquartile range (IQR), and the whiskers represent the maximum and minimum values, excluding values greater than 1.5x IQR (which are shown as individual points). (B) Cumulative distribution frequency plot of pooled DNA methylation levels for each treatment. Colors as in panel A and Figure 2.1.

Inter-specific variation in genomic DNA methylation levels with body temperature has also been hypothesized to be the result of temperature effects on the rate of deamination of methylated cytosines (Varriale & Bernardi 2006). This "methylation-temperature-deamination hypothesis" posits that the relatively low methylation levels of endotherms and tropical fishes compared to ectotherms in cooler climates could be the result of reduced genome GC content in animals with higher body temperatures, and further suggests that this reduced GC content is the

result of increased rates of deamination of methylated cytosines (causing increased mutation rate from C to T) at higher body temperatures (Varriale 2014). In the context of this hypothesis, our observation of increased genomic DNA methylation levels in stickleback exposed to elevated temperatures could ultimately result in accelerated mutation rates over evolutionary timescales, due to the potential for a positive feedback between the increased rate of deamination due to elevated environmental temperatures and the increased genomic DNA methylation levels.

2.3.2 Conserved differential methylation between temperature treatments

To determine whether there is a common core response of the epigenome to thermal change during both development and adult thermal acclimation, we summarized DNA methylation information across 100bp windows, and identified 1,206 differentially methylated regions (DMRs). In order to assess the potential functional significance of these DMRs we identified the nearest neighbor genes for each DMR. We then performed functional (Gene Ontology; GO) enrichment analysis for annotated genes located within 2kb of a DMR. There was no significant enrichment of GO-categories for any treatment group.

Approximately 25% of the DMRs associated with variation in developmental temperature were also differentially methylated as part of the thermal acclimation response in adults (Figure 2.4). We identified 172 DMRs that overlapped between stickleback that developed at cold temperatures and adult stickleback acclimated to cold temperatures, and 146 DMRs that overlapped between stickleback that developed at warm temperatures and adult stickleback that were acclimated to warm temperatures. Comparison of all DMRs identified 50 DMRs that were differentially methylated in response to all four treatments (Figure 2.4), and only two of these DMRs showed different directions of response to temperature across treatment while the other

48 regions all exhibited the same direction of change in DNA methylation level in all treatments (Appendix A, Table A.4). These regions may represent candidates for a generalized response to thermal change. One of these common DMRs was located in the TNF receptor-associated factor 7 gene, an E3 ubiquitin ligase that has been previously identified as a target for natural selection with latitude or temperature in sea urchins (Pespeni *et al.* 2012). These data suggest that there is a common core response of the epigenome to thermal change, and highlights the possibility that developmentally induced variation in DNA methylation patterns could influence plasticity in adult acclimation responses.

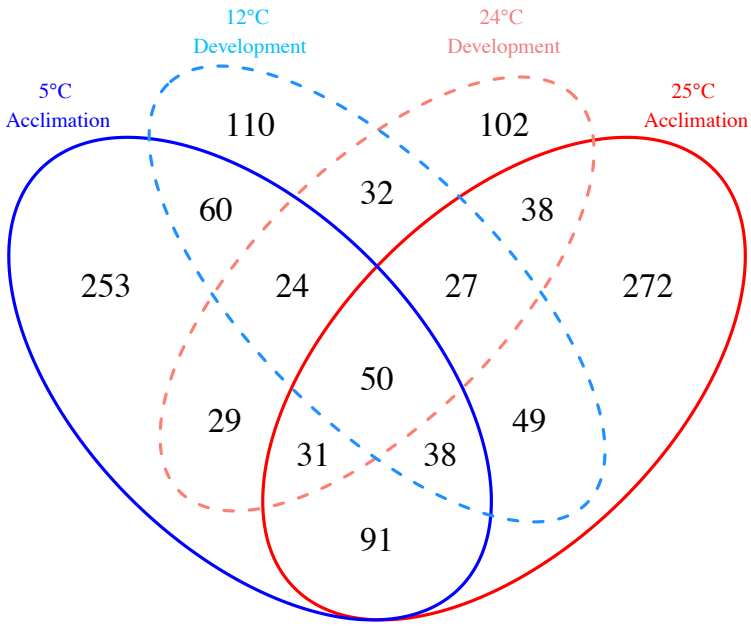

Figure 2.4: Venn Diagram depicting the number of common and unique differentially methylated regions identified between stickleback that were developed at and acclimated to 18 °C and stickleback that developed at 12 °C (dashed light blue line) or at 24 °C (dashed pink line) and then were acclimated to 18 °C, or that developed at 18 °C and were then acclimated to 5 °C (solid dark blue line) or 25 °C (solid red line).

The changes in methylation patterns that we detect in response to thermal change may point toward mechanisms underlying the transgenerational effects of temperature in stickleback (Shama *et al.* 2014, 2016; Ramler *et al.* 2014; Shama & Wegner 2014; Shama 2015), which have

been suggested to buffer the short-term effects of climate warming. Whether the effects of temperature on stickleback methylation levels persist through multiple generations requires further investigation; however, the abundance of distinct differentially methylated loci in each treatment suggests that variability in environmental temperatures could contribute to the observed accumulation of epigenetic variation between stickleback morphotypes (Smith *et al.* 2015).

2.4 Conclusions

Understanding the mechanisms regulating the capacity of organisms to respond to environmental variation is a critical aspect of determining the impacts of environmental change on populations (Somero 2010). In this study we have demonstrated that altered temperature during development has prolonged effects on DNA methylation levels in an ectothermic vertebrate and that modifications to DNA methylation levels are also associated with the plastic adult acclimation response to environmental temperatures. In addition, we have demonstrated that the persistent effects of developmental plasticity on DNA methylation patterns affect regions of the genome where DNA methylation patterns are also modified during adult acclimation. These data illustrate the profound effect of temperature on DNA methylation patterns across multiple time scales, which has important implications for elucidating the underlying mechanisms that may modulate the capacity of organisms to cope with environmental change.

Chapter 3: Similarities in temperature-dependent gene expression patterns between developmental plasticity and phenotypic flexibility in threespine stickleback (*Gasterosteus aculeatus*)

3.1 Introduction

Many organisms are able to respond to changes in environmental conditions by altering their phenotype, a phenomenon known as phenotypic plasticity. Phenotypic plasticity can be adaptive, maladaptive, or neutral (Ghalambor *et al.* 2007), and both maladaptive and adaptive phenotypic plasticity are thought to be important factors that can influence evolutionary trajectories (Ghalambor *et al.* 2015; Hendry 2016). However, there remains substantial debate as to whether phenotypic plasticity typically impedes or accelerates evolutionary change (Hendry 2016). The majority of phenotypic plasticity is ultimately the result of the differential regulation of gene expression (Schlichting & Smith 2002), and it has been suggested that understanding the molecular processes underlying this phenotypic plasticity is an important step in evaluating its effects in an evolutionary context (Schneider *et al.* 2014; Pfennig & Ehrenreich 2014).

Plastic responses can occur over a wide range of time-scales both across generations and within an individual organism's lifetime. Within-individual plasticity can, in turn, be divided into two broad classes acting at different life-history stages (Beaman *et al.* 2016). In the first class, which is typically termed developmental plasticity, the environment encountered during early life alters developmental trajectories. This type of plasticity is considered to result in a stable change in phenotype that lasts for the duration of the organism's lifetime. The second class of phenotypic plasticity, which is often termed phenotypic flexibility (Piersma & Drent 2003),

involves rapid and reversible changes in phenotype. This rapid and reversible plasticity includes processes such as physiological acclimation. There is substantial debate in the literature as to whether plasticity operating at different life stages operates via similar or different mechanisms (Kingsolver *et al.* 2002; Shintani & Ishikawa 2007; Kristensen *et al.* 2008; Colinet & Hoffmann 2012; Teets & Denlinger 2013). Thus, it is unclear whether plastic responses at different life stages are mechanistically linked and whether plastic responses across different life stages are under similar selective constraints (Gerken *et al.* 2015; Beaman *et al.* 2016). The ability to comprehensively measure changes in gene expression through the use of high throughput sequencing technologies provides an unbiased and powerful approach to better understand the mechanistic relationship between plasticity at different life stages (Aubin-Horth & Renn 2009).

In ectothermic organisms such as fishes, body temperature closely mimics that of the environmental temperature. Consequently, changes in environmental temperature have been shown to have pervasive effects on biochemical and physiological processes, including profound changes in gene expression. In fishes, gene expression plasticity in response to changes in environmental temperature has been predominately investigated by manipulating juvenile or adult thermal environments and measuring gene expression patterns to detect thermal acclimation responses (Gracey *et al.* 2004; Scott & Johnston 2012; Morris *et al.* 2014b; Shama *et al.* 2016; Kim *et al.* 2017a; Healy *et al.* 2017). A few studies have also examined the transgenerational effects of thermal exposure on the transcriptome of offspring, detecting effects of maternal or grandmaternal thermal exposure on offspring gene expression (Veilleux *et al.* 2015; Shama *et al.* 2016). Similarly, persistent effects of the temperatures experienced during early development (prior to hatch) on gene expression have been detected in adult fish held under common conditions (Scott & Johnston 2012; Oomen & Hutchings 2017). However, very

little is known about whether plasticity at different life stages affects similar or different processes.

Epigenetic processes, such as DNA methylation, which result in chromosome bound, heritable changes to gene expression patterns that are not dependent on changes to the underlying DNA sequence (Deans & Maggert 2015), are thought to be important mechanisms regulating gene expression plasticity (Hu & Barrett 2017). For example, changes in DNA methylation patterns during development are thought to play an important role in cellular differentiation and in maintaining cell-type specific transcriptional activity through mitosis (Monk *et al.* 1987; Li 2002). Although epigenetic effects are often considered to be relatively stable across the lifespan, or even heritable, recent studies (Baránek *et al.* 2015; Viggiano & de Pinto 2017; Metzger & Schulte 2017) indicate that DNA methylation can be rapidly altered by environmental change, and could be implicated in modulating plasticity at different life-history stages (Bird 2007; Deans & Maggert 2015; Huang *et al.* 2017a).

Understanding the relationship between plasticity in response to thermal change at different life-history stages has important implications for predicting the resilience of populations to anticipated environmental change (Somero 2010). Within the next century climate change is expected to result in an increase in mean temperatures as well as an increase in the magnitude, frequency, and duration of extreme temperature events and these changes in environmental thermal regimes are predicted to impact the distribution and abundance of ectothermic organisms (Sunday *et al.* 2012; Bauerfeind & Fischer 2014; Thornton *et al.* 2014; Seebacher *et al.* 2015; Frainer *et al.* 2017). Understanding the relationship between gene expression plasticity at different life stages has the potential to be important in determining the capacity of organisms to cope with these changes (Donelson *et al.* 2017)

Here, we examine changes in gene expression in response to altered developmental temperature and adult thermal acclimation in the threespine stickleback fish (*Gasterosteus aculeatus*). Stickleback populations are found in both marine and freshwater environments throughout their Holarctic distribution. Post-glacial rebound following the retreat of the Pleistocene glaciers ~10-12 thousand years ago resulted colonization of newly formed freshwater habitats (followed by adaptation and reproductive isolation). Differences in the selective pressures of freshwater environments have resulted in the rapid, parallel morphological and behavioral divergence of freshwater populations from ancestral marine populations (eg. Jones *et al.* 2012b), including variation in DNA methylation patterns (Smith *et al.* 2015; Artemov *et al.* 2017). Characterization of the underlying genetic divergence of marine and freshwater stickleback populations has established stickleback as a powerful system in which to investigate the genetic basis of adaptive evolution (Jones *et al.* 2012b). In addition, there is accumulating evidence of phenotypic plasticity in stickleback in ecologically relevant traits. For example, gene expression plasticity in response to temperature has been investigated in adult stickleback (Morris *et al.* 2014b), and temperature-dependent transgenerational and developmental plasticity in body shape (Ramler *et al.* 2014), hatching success (Shama *et al.* 2014), growth (Shama & Wegner 2014), DNA methylation (Metzger & Schulte 2017), and reproductive strategies (Kim *et al.* 2017b; Hovel *et al.* 2017) have been observed. Thus, stickleback not only present an ideal system in which to investigate the mechanisms underlying plasticity a different life stages, but to also help better understand the effects of plasticity on evolutionary processes.

Specifically, the objectives of this study were to 1) examine the persistent effects of temperature manipulations during development (from fertilization until hatch) on gene expression patterns in the muscle tissue of adults held under common conditions, 2) assess

mechanisms associated with phenotypic flexibility by acclimating adult stickleback to warmer and colder temperatures and measuring the temperature-dependent effects of thermal acclimation on gene expression patterns in muscle tissue, 3) examine whether the persistent effects of thermal manipulations during development on gene expression are similar to adult acclimation effects on gene expression patterns, and 4) assess whether gene expression plasticity in response to developmental and adult temperature manipulations are associated previously characterized changes in DNA methylation patterns (Metzger & Schulte 2017; Chapter two of this thesis).

3.2 Materials and Methods

3.2.1 *Gasterosteus aculeatus* rearing conditions

Adult threespine stickleback (*G. aculeatus*) of the fully plated "marine" ecotype were collected from Oyster Lagoon (British Columbia, Canada, GPS: 49.6121, -124.0314) in June 2014. In the lab, stickleback were separated into six 110-litre glass aquarium tanks (20 stickleback/tank) and acclimated to 20 ppt salt water (instant ocean), 18 °C and 14:10h light:dark photoperiod, and this photoperiod was held constant throughout the experimental period. These conditions are similar to summer conditions in Oyster Lagoon (20-30 ppt) at the time of collection. Stickleback were fed daily to satiation with Hakari Bio-Pure frozen Mysis Shrimp for three weeks prior to initiation of the breeding protocol.

Eggs were collected from a total of six different females as they became gravid. Testes were dissected from six different males, and used to generate six independent (unrelated) families. Fertilization and stickleback rearing were conducted as previously described (Metzger & Schulte 2017; Chapter two of this thesis) and are summarized in Figure 3.1.

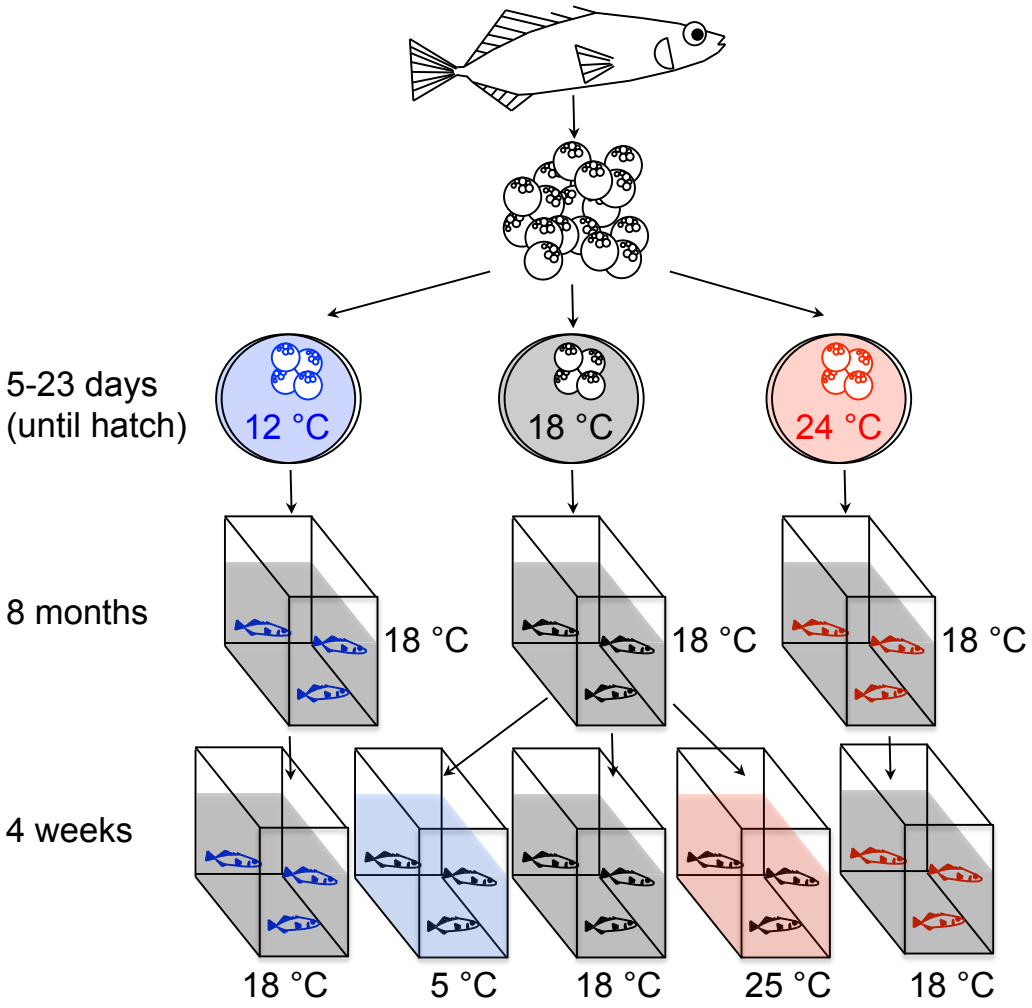

Figure 3.1: Experimental design. Six stickleback families were generated for use in this experiment. Each clutch was split between three developmental temperatures of 12 °C (blue), 18 °C (black), and 24 °C (red). Following hatch, all stickleback were transferred to aquaria at 18 °C and reared for 8 months. At this point, fish from the 18 °C development treatment were acclimated to either 5 °C (blue shaded aquaria), 18 °C (grey shaded aquaria), or 25 °C (red shaded aquaria) for 4 weeks, while fish from the other developmental treatments were maintained at 18 °C. This experimental design resulted in stickleback with 5 different thermal histories. At an age of nine months, muscle tissue was sampled for RNA-seq analysis (n=6 per treatment).

Briefly each clutch was split across three separate 10 cm petri dishes. A single petri dish from each of the six families was held at 12, 18, or 24 °C until hatch. We chose these temperatures (hereafter, developmental temperatures) because they span the temperature range that might be

experienced during the peak of breeding season of this population which is typically May through July (Saimoto 1993; Barrett *et al.* 2011).

Fertilized eggs were kept in a monolayer and submerged in 15 mL of 20 ppt seawater. Petri dishes were partially covered to decrease water loss from evaporation while still allowing for surface gas exchange to insure adequate oxygenation. Eggs were monitored twice daily during which time unfertilized eggs and mortalities were removed and 10 mL of water was changed to prevent mold growth. Hatching success was determined based on the number of fertilized embryos that hatched compared to the total number of fertilized embryos.

Developmental temperature did not have a significant effect on survival until hatch (Appendix B; Figure B.1). Embryos that developed at the coolest temperature took approximately 13 days longer to hatch than embryos that developed at the warmest temperature (Metzger & Schulte 2017; Chapter two of this thesis). Once all the embryos in a given family had hatched they were transferred to 110-litre glass aquarium tanks and maintained at 18 °C at a salinity of 20 ppt. At eight months post-hatch, a random sample of stickleback that developed at 18 °C were mixed together from the six different families and then split between three different acclimation temperatures (5, 18, or 25 °C) and held at these temperatures for four weeks.

These acclimation temperatures were chosen because they represent the ecologically relevant extremes that populations in this region would experience as adults in the wild in the winter and summer respectively (Barrett *et al.* 2011), and because they are close to the maximum and minimum temperatures to which threespine stickleback can be acclimated for extended periods (Wootton 1984; Lefébure *et al.* 2011). We selected 18 °C as the "control" temperature because it is the typical temperature at which stickleback are held in the lab. The two "experimental" acclimation temperatures differ in their magnitude of temperature change from

the "control" group of 18 °C because thermal performance curves are typically asymmetric, increasing gradually until reaching a peak and then rapidly decreasing (Dowd *et al.* 2015). Thus increasing and decreasing temperature by the same magnitude does not result in the same shift in thermal performance, a phenomenon known as Jensen's inequality (Denny 2017). Therefore, these acclimation temperatures were chosen to minimize the effects of Jensen's inequality by accounting for differences in slope along a thermal performance curve and choosing temperatures that would result in similar effects on performance rather than similar changes in temperature. We chose to use different thermal ranges at the two different life stages because these stages have different thermal sensitivities, and also encounter different temperatures in nature. The selected temperatures represent similar extents of the thermal tolerance breadth at each life stage.

Following four weeks of acclimation to these temperatures, a random sample of six stickleback from each acclimation temperature were euthanized and muscle tissue samples were snap frozen in liquid nitrogen and stored at -80 °C until further use. A total of six stickleback from each development temperature treatment were also euthanized at nine months post hatch, and muscle tissue samples were taken from each stickleback with sampling distributed across families (Appendix A; Table A.1). One-way ANOVAs were used to determine whether there was a main effect of either developmental temperature or adult acclimation temperature on the stickleback length and weight. A Tukey's post-hoc analysis was performed to test for significant differences between temperatures. There was no difference in the length of the individuals from different temperature treatments (Appendix B; Figure B2 A/B). Developmental temperature had a significant effect on wet weight (ANOVA p-value = 0.0223). Stickleback that were exposed to 12 °C during development and had a significantly higher wet weight compared to stickleback

exposed to 18 °C (p-value = .0459) or 24 °C (p-value = 0.0307) during development (Figure B.2 C/D). However, because some individuals within a treatment are from the same family, we cannot rule out the possibility that the observed effects of developmental temperature on adult wet weight are heritable transgenerational temperature effects of the parental and grand-parental environment (Shama & Wegner 2014; Shama *et al.* 2016), or that the analysis is affected by pseudo-replication at the family level in some treatments but not others (Appendix A; Table A.1).

We elected to examine gene expression in muscle tissue because previous work in a variety of species of fish suggests that both developmental temperature and adult thermal acclimation have substantial effects on muscle phenotype (Johnston 2006; Macqueen *et al.* 2008; Johnston *et al.* 2009; Finstad & Jonsson 2012; Salinas & Munch 2012; Scott & Johnston 2012; Schnurr *et al.* 2014). A muscle sample from the other side of the same individuals was used in a previous study that investigated the effects of developmental temperature and adult temperature acclimation on DNA methylation levels (Metzger & Schulte 2017; Chapter two of this thesis).

3.2.2 RNA isolation and sequencing

Total RNA was prepared from stickleback muscle tissue using TRIzol Reagent (Invitrogen Life Technologies). Approximately 20 mg of muscle tissue was homogenized in 1 mL of TRIzol in 1.5 mL Eppendorf® Safe-Lock micro centrifuge tubes containing approximately ten 1.0 mm ceria stabilized zirconium oxide beads (Next Advance) using a Bullet Blender24 (Next Advance). Total RNA was DNase treated using the Qiagen RNeasy DNase I on-column DNA digestion protocol. Total RNA was quantified using a QBit® RNA broad range assay kit (product # Q10210; ThermoFisher Scientific) and an Invitrogen™ Qubit® 2.0 Fluorometer.

RNA quality was assessed using an Agilent RNA 6000 Pico Kit (product # 5067-1514) and an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA integrity numbers (RIN) were between 7.9 - 9.1 (mean = 8.6 ± 0.4 SD). Preparation of cDNA libraries and 100 base-pair paired end sequencing was performed at the UBC Nucleic Acid Protein Service Unit (NAPS) and UBC Biodiversity Research Center's next generation sequencing facility. Briefly, mRNA was purified using BIO-O NEXTflex® Poly-A beads. Sequencing libraries were prepared using the BIO-O NEXTflex® Rapid RNA-Seq kit. Each sample was individually barcoded and samples from different treatments were evenly distributed across 3 sequencing lanes of an Illumina HiSeq 2000 flow cell (10 samples/lane, two samples from each treatment/lane). Mean sequenced library size was 39,737,041 reads (± 7,227,925 SD; Appendix B, Table B.1).

3.2.3 Sequence alignment and expression analysis

Reads were aligned to the stickleback genome (http://www.ensembl.org) using CLC genomics workbench v9.5. Average mapping efficiency of paired and broken reads was 88 %. Analysis of total read counts was performed in R v3.3.1 with *edgeR* v3.14.0 (Robinson *et al.* 2010; McCarthy *et al.* 2012b) based on the recommended guidelines in Lin *et al.* (2016). Genes with no reads were removed from the datasets. Counts were normalized using the relative log expression (RLE) method. However, temperature acclimation has generally been shown to induce the up-regulation of a large proportion of genes in fish (Gracey *et al.* 2004; Healy *et al.* 2017). Many methods for normalization of RNA-seq data (e.g. TMM and RLE) assume that the majority of genes in an RNA-seq dataset are not differentially expressed (Dillies *et al.* 2013). Thus, normalizing factors can become problematic when a large proportion expressed genes are differentially expressed between treatments, particularly when the direction of change is biased

in one direction (Dillies *et al.* 2013; Evans *et al.* 2017). Ideally, only those genes that are not differentially expressed should be used to calculate the normalization factors. Therefore, we applied the method describe in Healy *et al.* (2017), which utilizes a preliminary analysis of the dataset to identify and remove genes that are likely to be differentially expressed and then calculates standard RLE normalizing factors using the remaining dataset. To identify and remove putatively differentially expressed genes, two separate preliminary analyses were performed.

In the first preliminary analysis, putatively differentially expressed genes were identified without library normalization. Genes with low expression were filtered from the dataset. The minimum criterion for retaining a gene was at least 0.5 counts per million (~10 counts in the smallest library) in each of the six samples of each temperature. Tagwise dispersions were calculated using the robust method in edgeR. The data were then fit to a negative binomial generalized linear model using glmFit().

In the second preliminary analysis, sequencing libraries were normalized using the RLE method. Genes with low expression were filtered from the dataset using the same criteria as previously described. Tagwise dispersions were calculated using the robust method in edgeR. The data were then fit to a negative binomial generalized linear model using glmFit().

Genes that were identified as differentially expressed in each of these two preliminary analyses were then removed from datasets for the purposes of normalization. The majority of genes that remained following these steps are less likely to be differentially expressed in response to temperature stimuli and are thus suitable to calculate normalizing factors for the rest of the dataset. Normalizing factors using the RLE method were then calculated for the dataset that contained these remaining genes. These normalizing factors were then used in a final analysis of the data. Separate preliminary analyses and normalizing factors were calculated in

this way for each pairwise comparison of either a developmental or acclimation temperature treatment using stickleback that were held at 18 °C for the entire duration of the experiment as the control group.

For the final analysis of the data, differential expression was assessed using pairwise comparisons of the gene expression data for each developmental or acclimation treatment to stickleback that were held at 18 °C for the duration of the experiment. The minimum criterion for retaining a gene, following RLE normalization as described above, was for a gene to have at least 0.5 counts per million (CPM; ~10 counts in the smallest library) in each of the six samples of within a temperature treatment. If a gene had a read count less than 0.5 CPM in at least one sample within a treatment then it was discarded from the analysis. After normalization using this method and filtering of the dataset a total of 12,199 genes remained in the 5 °C acclimation dataset, 12,097 genes remained in the 25 °C acclimation dataset, 11,507 genes remained in the 12 °C development dataset, and 11,661 genes remained in the 24 °C development dataset for differential expression analysis. Tagwise dispersions were calculated using the robust method in edgeR. Differentially expressed (DE) genes were identified using the *glmFit*() function from *edgeR* to fit a negative binomial generalized linear model followed by a likelihood ratio test, *glmLRT*(). The resulting p-values were adjusted based on a false discovery rate (FDR) correction, and the threshold for significance of these adjusted p-values (q-value) was set at 0.05. Gene ontology (GO) pathway enrichment analyses were conducted using the *goseq* (v1.22.0) R package (Young *et al.* 2010), with FDR correction as previously described.

3.2.4 Differential methylation analysis

Differentially methylated cytosines (DMCs) associated with differentially expressed genes were identified by filtering previously identified DMCs (Metzger & Schulte 2017; Chapter two of this thesis) for those located within 5 kilobase pairs (kb) upstream or downstream of genes that were differentially expressed in stickleback from the same temperature treatment. Analysis of the genomic distribution of DMCs was conducted using the *annotateWithGeneParts*() function in the *genomation* v1.10 R package.

3.3 Results

3.3.1 Temperature dependent gene expression plasticity at different life stages

The expression levels of 10,140 genes were responsive to thermal acclimation (Figure 3.2). A total of 7,940 genes were differentially expressed in stickleback that were acclimated to 5 °C (Figure 3.2A/C) and 7,015 genes were differentially expressed in stickleback that were acclimated to 25 °C (Figure 3.2B/D). The majority of genes that responded to thermal acclimation changed by a $\log_2$ fold of less than two (i.e. an absolute fold change of less than four) for both cold (86 % of differentially expressed genes, Figure 3.2A) and warm (95 % of differentially expressed genes, Figure 3.2B) acclimated stickleback. In both cold and warm acclimation, substantially more genes were up-regulated than were down-regulated (cold-acclimated: 72% of all differentially expressed genes were up-regulated; warm-acclimated: 80% of all differentially expressed genes were up-regulated) (Figure 3.2C/D).

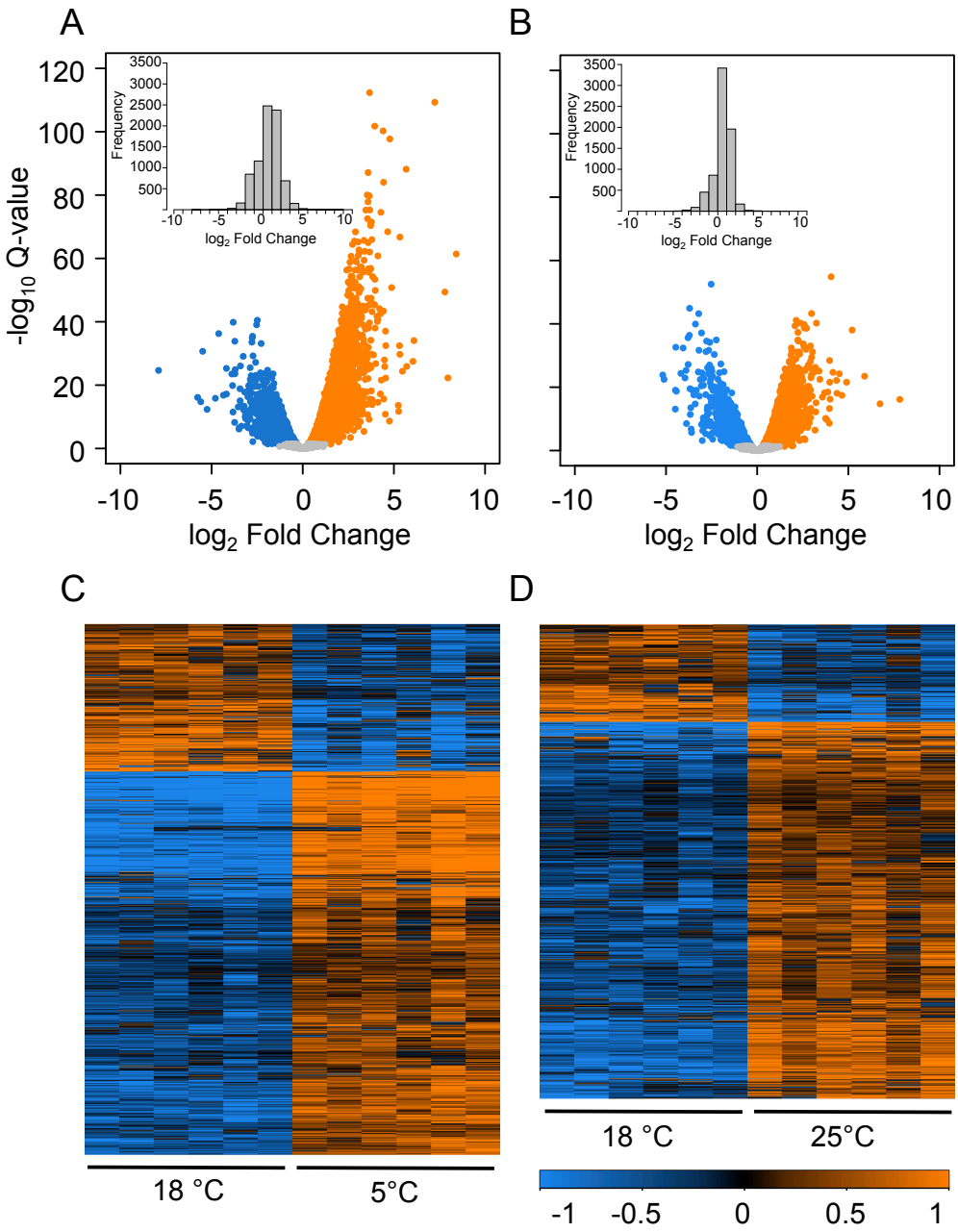

Figure 3.2: Differential expression in stickleback acclimated to either 5 °C (A/C) or 25 °C (B/D) relative to stickleback that were kept at 18 °C for the duration of the experiment. Panels A and B show the $-\log_{10}$ FDR corrected p-value (q-value) against $\log_2$ fold change for significantly ($q<0.05$) up-regulated (orange), down-regulated (blue) genes and non- differentially expressed genes (grey). Embedded plots are frequency histograms of $\log_2$ fold change (e.g. a bar above a $\log_2$ fold change of 2 represents the genes that range in a $\log_2$ fold change between 1 and 2). Panels C and D are heatmaps of differential expression in stickleback acclimated to 5 °C and 25 °C, respectively. Each row represents the expression value (log2 counts per million) for a single gene relative to the mean expression value for that gene across all individuals (orange representing higher expression and blue representing lower expression). Each column represents an individual stickleback. Columns 1-6 are stickleback that were kept at 18 °C for the duration of the experiment. Columns 7-12 are either stickleback that were kept at 18 °C for 8 months and then acclimated to 5 °C (C) or 25 °C (D) for four weeks.

Only 57 genes were differentially expressed in the muscle tissue of adult stickleback that were exposed to different temperatures during development (Figure 3.3). A total of 33 genes were differentially expressed in stickleback that had developed at 12 °C (Figure 3.3A/C) and 29 genes were differentially expressed in stickleback that had developed at 24 °C (Figure 3.3B/D). Similar to the patterns of differential expression in response to adult thermal acclimation, the majority of these genes had a $\log_2$ fold change less than two for stickleback that were exposed to colder (94 % of differentially expressed genes, Figure 3.3A) or warmer (90 % of differentially expressed genes, Figure 3.3B) temperatures during development. However, unlike the pattern that was observed in response to adult acclimation temperature, none of the genes affected by developmental temperature had a $\log_2$ fold change greater than five. As was the case for adult thermal acclimation, the majority of the differentially expressed genes in response to altered developmental temperature were up-regulated (cold-development: 64 % of all differentially expressed genes were up-regulated; warm-development: 83 % of all differentially expressed genes were up-regulated)(Figure 3.3C/D).

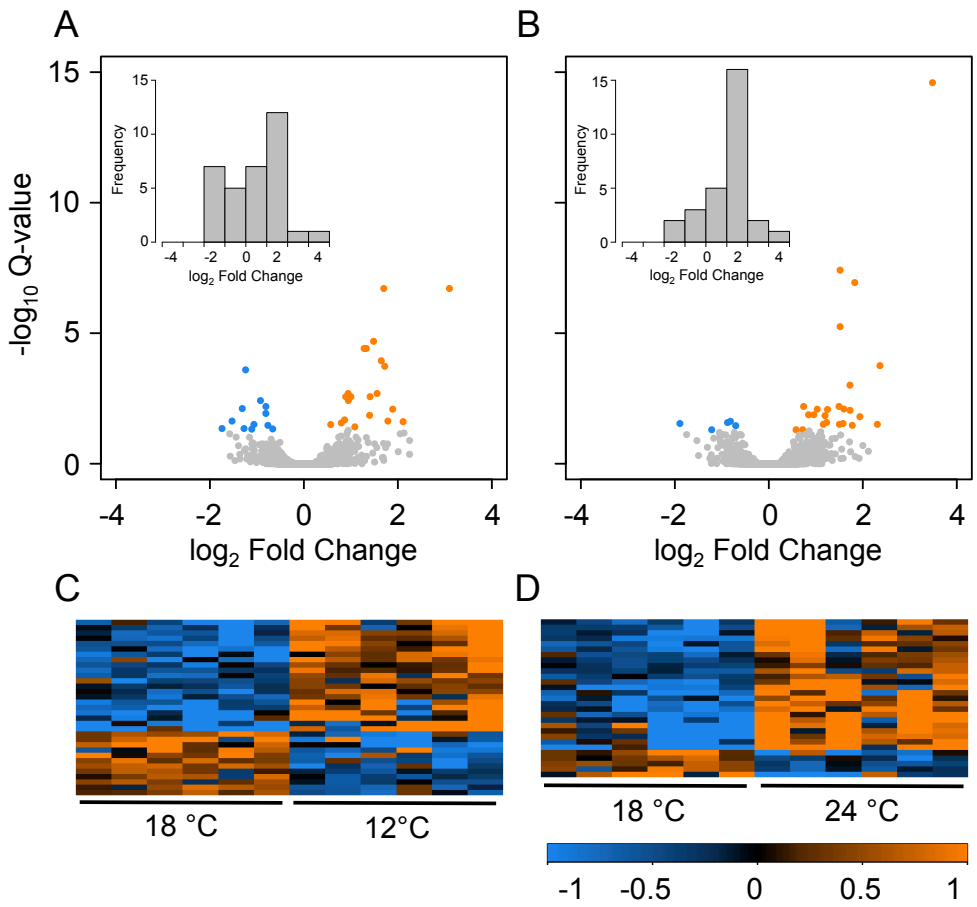

Figure 3.3: Differential expression in stickleback exposed to either 12 °C (A/C) or 24 °C (B/D) during development and then kept at 18 °C until 9 months of age relative to stickleback that were kept at 18 °C for the duration of the experiment. Panels A and B show the $-\log_{10}$ FDR corrected p-value (q-value) against $\log_2$ fold change for significantly ($q<0.05$) up-regulated (orange), down-regulated (blue) genes and non- differentially expressed genes (grey). Embedded plots are frequency histograms of $\log_2$ fold change (e.g. a bar above a $\log_2$ fold change of 2 represents the genes that range in a $\log_2$ fold change between 1 and 2). Panels C and D are heatmaps of differential expression in stickleback exposed to 12 °C and 24 °C during development, respectively. Each row represents the expression value (log2 counts per million) for a single gene relative to the mean expression value for that gene across all individuals (orange representing higher expression and blue representing lower expression). Each column represents an individual stickleback. Columns 1-6 are stickleback that were kept at 18 °C for the duration of the experiment. Columns 7-12 are either stickleback that were kept at 18 °C for 8 months and then acclimated to 5 °C (C) or 25 °C (D) for four weeks.

Of the 10,140 genes that were differentially expressed in response to adult thermal acclimation, 4,851 were differentially expressed in both cold- and warm-acclimated stickleback (Figure 3.4A), and the majority of these genes (4,235 genes) were differentially expressed in the same direction between acclimation temperatures (Figure 3.4A). In contrast, of the 57 genes

identified in stickleback from different developmental temperatures, only five were differentially expressed in stickleback from both warm and cold developmental treatment temperatures (Figure 3.4B). The expression of all five of these genes changed in the same direction in stickleback exposed to both cold and warm developmental temperatures.

To further assess the degree to which developmentally plastic responses to environmental temperature are consistent with adult thermal acclimation responses, we compared the list of genes that were differentially expressed between developmental treatments to those that were differentially expressed in adult stickleback acclimated to different temperatures. From this analysis, we identified 27 genes that were differentially expressed both in response to development at cold temperatures and in response to cold temperature acclimation (Figure 3.4C, Table 3.1). The direction of differential expression for all 27 genes was conserved across developmental and adult treatments. Similarly, we identified 18 genes that were differentially expressed in response to development at warm temperatures and in response to warm temperature acclimation (Figure 3.4C). The direction of differential expression for 17 of these genes was conserved between developmental and acclimation treatments (Table 3.1). Comparison of the differentially expressed genes from all four analyses identified four genes (*irs2b, klhl38b, gadd45ga,* and *slc3a2a*) that were differentially expressed in all treatments and each of these genes was up-regulated in each treatment (Table 3.1).

In addition to comparisons of gene expression of the 12 °C and 24 °C developed groups to the group developed at 18 °C, we also examined the list of expressed transcripts in the stickleback that developed at 12 °C and 24 °C to determine whether there were any novel genes that were expressed at one temperature but not the other, because these genes would not

necessarily be revealed by comparison to stickleback developed at 18 °C. However, there were no genes that fell into this category.

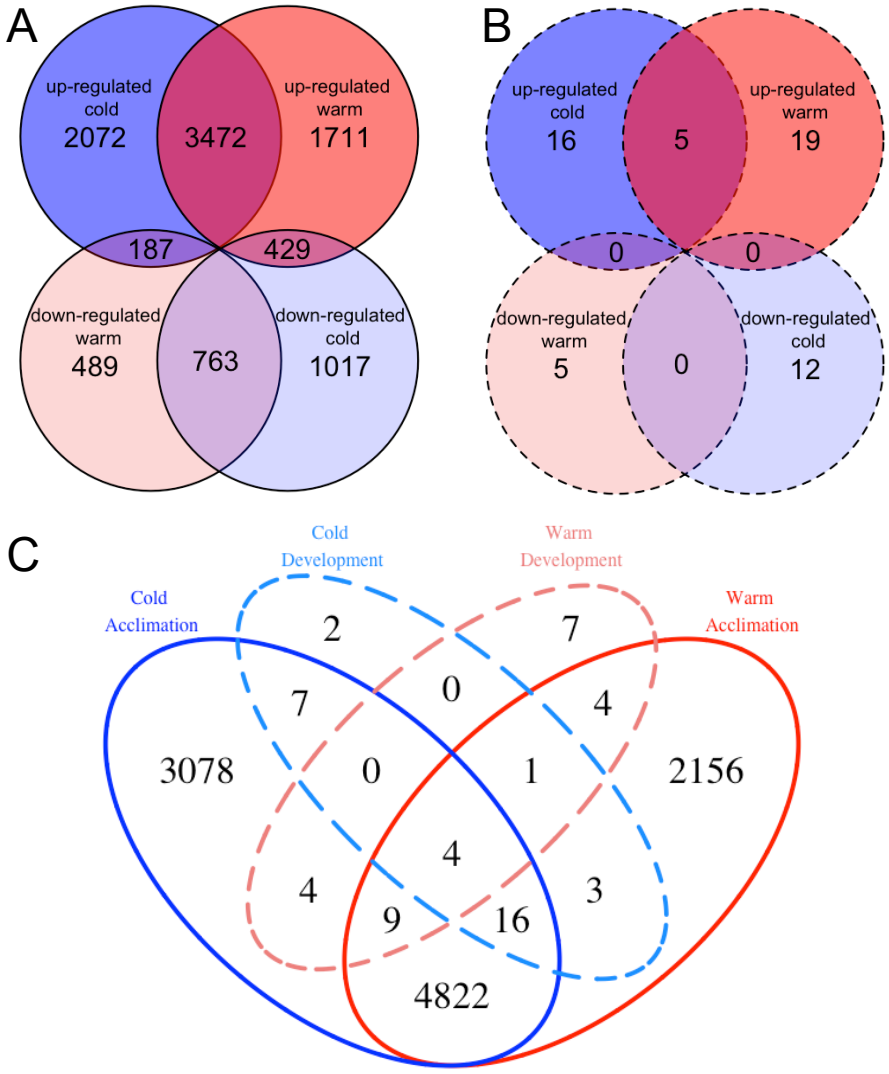

Figure 3.4: (A) Comparison of up- and down-regulated genes in stickleback muscle tissue from adult stickleback acclimated as adults to 5 °C (blue) or 25 °C (red) for four weeks. (B) Comparison of up- and down-regulated genes in stickleback muscle tissue from stickleback exposed to 12 °C (blue) or 24 °C (red) during development and then kept at 18 °C until 9 months of age. (C) Comparison of differentially expressed genes in stickleback muscle tissue. Cold acclimation = fish developed and reared at 18 °C for eight months and then acclimated to 5 °C for four weeks. Warm acclimation = fish developed and reared at 18 °C for eight months and then acclimated to 25 °C for four weeks. Cold development = fish developed at 12 °C until hatch, and then held at 18 °C for nine months. Warm development = fish developed at 24 °C until hatch, and then held at 18 °C for nine months. All differential expression was identified relative to fish held at 18 °C for the duration of the experiment.

Table 3.1: Genes that are differentially expressed in response to changes in both developmental temperatures and adult acclimation temperatures.

		Log_2 Fold Change			
Ensembl Gene ID	Gene Name	Cold Development	Cold Acclimation	Warm Development	Warm Acclimation
ENSGACG00000003564	irs2b	0.80	1.06	1.03	1.76
ENSGACG00000006793	gadd45ga	0.95	0.89	1.52	2.75
ENSGACG00000006167	klhl38b	1.08	2.60	1.25	1.15
ENSGACG00000019745	slc3a2a	1.48	1.28	1.23	2.02
ENSGACG00000013368	bcl2b	-1.31	-1.04	-	-
ENSGACG00000007797	vkorc1	-1.27	-2.56	-	-
ENSGACG00000014656	ifi35	-1.24	-1.00	-	-
ENSGACG00000008237	EVC	-1.11	-0.97	-	-
ENSGACG00000004133	pim1	-0.92	-1.33	-	-
ENSGACG00000003021	klf2b	-0.81	-1.71	-	-
ENSGACG00000019700	DDIT4L	-0.81	-3.73	-	-
ENSGACG00000012452	IL16	-0.77	-2.23	-	-
ENSGACG00000009575	id3	-0.66	-1.04	-	-
ENSGACG00000005010	tob1a	0.57	0.70	-	-
ENSGACG00000010010	ezrb	0.89	0.61	-	-
ENSGACG00000008895	sesn1	0.94	1.79	-	-
ENSGACG00000006997	alas1 (1 of 2)	1.00	1.66	-	-
ENSGACG00000015298	ENSGACG00000015298	1.28	-1.53	-	-
ENSGACG00000015297	METTL21C (2 of 2)	1.33	-3.44	-	-
ENSGACG00000013859	zc3h12a	1.40	0.80	-	-
ENSGACG00000005398	tfr1b	1.56	3.73	-	-
ENSGACG00000017927	cebpd	1.65	1.15	-	-
ENSGACG00000002379	ddit4	1.70	1.54	-	-
ENSGACG00000016373	tfr1a	1.72	2.60	-	-
ENSGACG00000010739	klf13	1.79	1.42	-	-
ENSGACG00000001466	fkbp5	1.89	3.16	-	-
ENSGACG00000001632	samhd1 (2 of 3)	2.11	2.72	-	-
ENSGACG00000000049	rasgef1ba	-	-	1.20	2.52
ENSGACG00000001607	trim63b	-	-	0.96	1.96
ENSGACG00000006161	fbxo32	-	-	1.52	2.15
ENSGACG00000006480	ddit3	-	-	0.58	0.42
ENSGACG00000006908	ENSGACG00000006908	-	-	1.94	-1.01
ENSGACG00000008429	ENSGACG00000008429	-	-	3.48	1.10
ENSGACG00000010788	nr4a1	-	-	-0.70	-1.97
ENSGACG00000010861	RASGRF1 (1 of 2)	-	-	-1.89	-3.88
ENSGACG00000011050	DUSP8	-	-	0.85	0.51
ENSGACG00000011743	HIVEP2 (1 of 2)	-	-	0.74	0.75
ENSGACG00000013469	pptc7a	-	-	1.50	1.57
ENSGACG00000014133	irs2a	-	-	0.72	0.88
ENSGACG00000015066	camk2n1a	-	-	1.49	2.36
ENSGACG00000016438	ALPK3 (2 of 2)	-	-	1.83	1.08

3.3.2 Identification of candidate biological processes affected by gene expression plasticity across timescales

Comparison of the most significantly enriched biological processes associated with genes that were differentially expressed in response to acclimation to low or high temperature revealed that acclimation to both low and high temperature induced changes in the expression of genes involved in a common set of biological processes (Table 3.2). Enriched biological processes for the up-regulated genes were generally associated with cell division, mRNA splicing, and protein degradation. Enriched biological processes for the down-regulated genes were generally associated with extracellular matrix organization and cell adhesion in both warm and cold-acclimated individuals.

There were no significantly enriched terms for genes that were uniquely up-regulated in cold-acclimated stickleback. Genes that were uniquely up-regulated in warm-acclimated stickleback were enriched for biological processes involved in protein translation and amino acid metabolism. Genes that were uniquely down-regulated in cold-acclimated stickleback were enriched for biological processes involved in muscle filament sliding, muscle contraction, oxidation-reduction, angiogenesis, and epidermis development. There were no significantly enriched processes associated with genes that were uniquely down-regulated in warm-acclimated stickleback.

Table 3.2: Significantly enriched biological process gene ontologies for genes that are up- or down-regulated in response to warm or cold thermal acclimation in adult stickleback muscle tissue.

	Cold Acclimation			Warm Acclimation		
	GO ID	GO term	Over represented p-value	GO ID	GO term	Over represented p-value
Up-regulated genes	GO:0000278	mitotic cell cycle	4.06E-21	GO:0010467	gene expression	1.41E-34
	GO:0010467	gene expression	1.53E-18	GO:0016032	viral process	4.78E-16
	GO:0000398	mRNA splicing, via spliceosome	1.08E-17	GO:0000278	mitotic cell cycle	2.47E-15
	GO:0008380	RNA splicing	3.51E-17	GO:0006364	rRNA processing	3.47E-15
	GO:0008033	tRNA processing	5.51E-13	GO:0008033	tRNA processing	4.40E-15
	GO:0006521	regulation of cellular amino acid metabolic process	1.03E-12	GO:0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	5.97E-15
	GO:0043687	post-translational protein modification	4.59E-10	GO:0051439	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	1.16E-14
	GO:0006364	rRNA processing	1.51E-09	GO:0051437	positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	2.24E-14
	GO:0018279	protein N-linked glycosylation via asparagine	1.57E-09	GO:0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	2.96E-14
	GO:0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	1.76E-09	GO:0043488	regulation of mRNA stability	5.12E-14
Down-regulated genes	GO:0030198	extracellular matrix organization	8.88E-28	GO:0030198	extracellular matrix organization	1.19E-32
	GO:0030574	collagen catabolic process	5.56E-17	GO:0030574	collagen catabolic process	6.14E-22
	GO:0007155	cell adhesion	9.80E-15	GO:0022617	extracellular matrix disassembly	1.30E-18
	GO:0030049	muscle filament sliding	2.11E-14	GO:0007155	cell adhesion	5.68E-15
	GO:0006936	muscle contraction	1.28E-13	GO:0030199	collagen fibril organization	4.14E-14
	GO:0022617	extracellular matrix disassembly	3.78E-13	GO:0007160	cell-matrix adhesion	7.29E-09
	GO:0001525	angiogenesis	1.95E-09	GO:0001501	skeletal system development	1.95E-08
	GO:0030199	collagen fibril organization	2.55E-09	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	1.95E-07
	GO:0001501	skeletal system development	7.58E-09	GO:0051056	regulation of small GTPase mediated signal transduction	3.40E-07
	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	2.87E-07	GO:0005980	glycogen catabolic process	1.28E-06

There was no significant enrichment of biological processes for genes differentially expressed between stickleback that experienced different temperatures during development. This is likely due to the relatively small number of genes affected by developmental temperature; however, many of these genes are known to be involved in the same processes that were enriched among genes differentially expressed in adult stickleback acclimated to different temperatures. For example, transcripts encoding genes for the DNA damage inducible transcript 4 (*ddit4/redd1*) and *ddit4*-like (*ddit4l/redd2*) were differentially expressed in stickleback reared at 12 °C and are thought to be involved in the attenuation of the mTORC1 protein synthesis which can result in muscle atrophy (Kelleher *et al.* 2013). In addition, several genes involved in muscle cell development, growth, aging and metabolism were also differentially expressed in stickleback that developed at 12 °C, including methyltransferase like 21C (*mettl21c*), CCAAT/enhancer binding protein delta (*cebpd)*, transferrin receptor 1a and 1b (*tfr1a, tfr1b*/*tfrc*), pim-1 proto-oncogene, serine/threonine kinase (*pim1*), B-cell CLL/lymphoma 2b (*bcl2b*)**,** FK506 binding protein 5 (*fkbp5*), Kruppel-like factor 2b and 13 (*klf2b*, *klf13*), transducer of ERBB2, 1 (*tob1*), and DNA-binding protein inhibitor ID-3 (*id3*).

A different set of genes that have been implicated in muscle development, growth and metabolism were differentially expressed in stickleback that developed at 24 °C including Ras protein specific guanine nucleotide releasing factor 1 (*rasgrf1*), alpha kinase 3 (*alpk3*), f-box protein 32 (*fbxo32*), dual specificity phosphatase 8 (*dusp8*), tripartite motif containing 63b (*trim63b*/*murf1*b), endothelial lipase G (*lipg*), nuclear receptor subfamily 4 group A member 1 (*nr4a1*/*nur77*), and plexin A2 (*plxna2*).

Comparison of genes that were differentially expressed by either warm or cold development identified five genes whose expression was affected by both developmental

temperatures (Figure 3.4B, 3.4C), including the solute carrier family 3 member 2a (*slc3a2a*), growth arrest and DNA-damage-inducible, gamma a (*gadd45ga*), insulin receptor substrate 2b (*irs2b*), kelch-like family member 38b (*klhl38b*), and one unannotated gene ENSGACG00000008429. All but the last of these genes were also affected by thermal acclimation.

3.3.3 Comparison to patterns of DNA methylation

Because the samples used in this experiment were derived from the same individuals used to demonstrate that both altered developmental temperature and altered adult acclimation temperature result in changes in DNA methylation patterns in threespine stickleback muscle tissue (Metzger & Schulte 2017; Chapter two of this thesis), it is possible to directly compare changes in DNA methylation patterns to changes in gene expression. In this analysis we compared the genes that we identified in this study as being differentially expressed to DMCs that were located near a gene (within 5 kb of either the transcription start site or the 3' end of the gene) identified in our previous study. Using this identification cutoff, none of the genes that were differentially expressed between developmental temperature treatments were associated with previously reported differential methylation, whereas 125 genes that responded to warm acclimation were associated with differentially methylated loci, and 199 genes that responded to cold acclimation were associated with differentially methylated loci. Of these DMCs, the majority were located in intergenic regions within 5 kb upstream or downstream of the differentially expressed genes (Figure 3.5), and only 5-6% (depending on the acclimation temperature) were located in promoter regions (within 2 kb upstream of the transcription start site). When considering the complete reduced representation bisulfite sequencing (RRBS)

dataset, approximately 9% of sequence data were from promoter regions. In the subset of the data analyzed here, which excludes intergenic sequences located more than 5kb upstream or downstream of a gene (thus effectively excluding much of the intergenic sequence), the proportion of promoter sequences in the background sequence data is higher. Thus, the fact that we observe a lower percent of DMCs in promoter regions suggests that there is no evidence for enrichment of differential methylation in the promoter regions of differentially expressed genes.

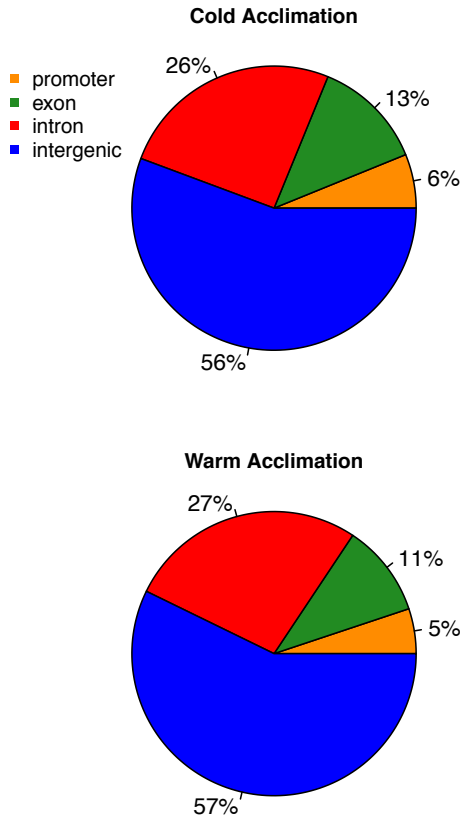

Figure 3.5: Genomic distribution of differentially methylated cytosines associated with differentially expressed genes (within 5 kilobase pairs) in stickleback muscle tissue from adult stickleback acclimated to 5 °C (cold acclimation) or 25 °C (warm acclimation).

We did not detect significant enrichment of gene ontologies among the differentially expressed genes associated with DMCs, but a number of these genes are involved in processes

that are likely affected by thermal acclimation based on the GO enrichment analysis of the DE genes, including processes such as proper formation of the sarcomere (eg. *sh3bgr* and *ttn.2*), mRNA splicing (*aqr*, *sf3b6,* and *pus10*), the ubiquitin proteasome pathway (eg. *psmd1, psmd13, ube2e2*), muscle cell growth and development (eg. *col12a1a* and *relb*), mitochondrial proliferation (eg. *mdh1*, *hpc2*/*elac2* and *ugp2b*), and myogenesis (eg. *foxk1i*).

3.4 Discussion

3.4.1 Conserved effects of cold and warm temperature on gene expression

In this study we present evidence of similarities in temperature-induced gene expression plasticity at different life stages on adult gene expression patterns. In both the developmental and adult acclimation treatments, colder temperatures resulted in the differential expression of more genes compared to warmer temperatures, and there were more differentially expressed genes that were up-regulated compared to down-regulated in all treatments. While the effects of adult thermal acclimation on gene expression were much more extensive than the effects of developmental temperature on gene expression in adult stickleback muscle, the majority of gene expression patterns that were affected by developmental temperature were also responsive to thermal acclimation, and these changes were generally in the same direction. Taken together these data suggest that there could be similar mechanisms that regulate plastic responses at these different life stages.

The effects of thermal acclimation on muscle gene expression have been examined in many fish species (eg. Gracey *et al.* 2004; Scott & Johnston 2012; Healy *et al.* 2016) including threespine stickleback (Morris *et al.* 2014b); however, studies that contrast the gene expression patterns of cold- or warm-acclimated fish are less common (eg. Ikeda *et al.* 2017). In this study

we characterized the transcriptional response to cold (5 °C) and warm (25 °C) temperature acclimation independently by comparing muscle gene expression patterns to those in individuals at a common control temperature (18 °C). By using this experimental design we are able to identify the common set of genes (Figure 3.4C) and biological processes (Table 3.2) that are differentially regulated in response to both warm and cold temperature acclimation, determine whether direction of regulation of these genes is similar at both temperatures (Figure 3.4B), identify genes that are uniquely regulated in one temperature but not the other (Figure 3.4A and 3.4C), and compare the overall magnitude of the transcriptomic response to warm and cold temperature acclimation (Figure 3.2). In contrast, experiments that compare differentially expressed between two temperature extremes could potentially lead to an oversimplification of patterns of thermal acclimation, if (as demonstrated here) both warm and cold acclimation lead to similar changes in gene expression. For example, a previous study of variation in thermal acclimation responses between marine and freshwater populations of stickleback compared gene expression patterns in stickleback acclimated to 7 °C compared to stickleback acclimated to 22 °C (Morris *et al.* 2014b). Many of the enriched biological processes that we identified for genes that were up-regulated in both cold- and warm-acclimated stickleback, such as gene expression and protein ubiquitination, were previously reported as being up-regulated in stickleback acclimated to warm temperatures (Morris *et al.* 2014b). The advantages of comparing thermal extremes to an intermediate temperature can be illustrated with an example – the gene encoding the peroxisome proliferator activated receptor alpha (*pparaa*). This gene has been previously associated with a genetically divergent region of the stickleback genome that is under positive selection between marine and freshwater stickleback populations (Jones *et al.* 2012a), and has been shown to be up-regulated in stickleback from a freshwater population acclimated to cold

conditions but not in a stickleback from a marine population acclimated to cold conditions (Morris *et al.* 2014b). Here, we detected up-regulation of *pparaa* transcripts in stickleback from a marine population in response to both warm and cold acclimation. Thus, if only stickleback acclimated at warm and cold temperatures are compared, patterns of differential expression can be missed.

Disruption in the timing or magnitude of gene expression during development can result in permanent changes to adult phenotypes (West-Eberhard 2005). These effects have been previously demonstrated in zebrafish where warm temperatures during development have been shown to impact muscle cell fiber composition, swimming performance, and metabolism (Scott & Johnston 2012). Interestingly, in zebrafish these large changes in muscle phenotype and function were not associated with large changes in the transcriptional program of muscle tissue in adults. The magnitude of the transcriptional response in zebrafish muscle was modest (26 DEGs with a q-value < 0.05) and similar to what we observed in stickleback (33 and 29 DEGs with a q-value < 0.05 in stickleback exposed to either cold or warm temperatures during development, respectively). Genes that were differentially expressed between stickleback that developed at different temperatures are know to be involved in metabolism and muscle cell development (eg. *cebpd, tfr1, fkbp5,* and *klf2* in stickleback that developed at 12 °C, *nr4a1* in stickleback that developed at 24 °C, and *slc3a2* in both developmental treatment temperatures). Among this relatively small set of genes there were two genes, *fkbp5* and *slc3a2,* that were also affected by developmental temperature in zebrafish, These data suggest that altered developmental temperatures induce changes in muscle phenotype that could impact the performance of adults, and that the effect of developmental temperature on the regulation of these genes may be conserved across distantly related fish species.

Plasticity is thought to influence evolutionary processes by revealing previously hidden sources of variation and by facilitating phenotypic variation and divergence among populations that inhabit different environments (Schlichting 2008; Schneider & Meyer 2017). Natural selection can then act on this variation to refine the plastic phenotype closer to the optimum (Levis & Pfennig 2016). For example, in a "plasticity-first" hypothesis, developmental plasticity can induce phenotypes that increase fitness in stressful environments (Levis & Pfennig 2016). However, this type of analysis assumes the presence of beneficial plasticity, whereas maladaptive or non-adaptive plasticity can also affect evolutionary trajectories (Ghalambor *et al.* 2015). The differential expression of genes in stickleback reared at colder or warmer temperatures is consistent with the potential effects of developmental temperature on metabolism and muscle cell fiber composition that could impact adult performance, but whether these changes are likely to be adaptive or maladaptive is unknown.

Examination of the genes differentially regulated in response to all four of our temperature treatments strongly suggests that changes in the regulation of muscle growth are a core phenomenon uniting phenotypic plasticity in response to temperature change at different life stages and in response to both low and high temperature. Although there have been relatively few studies that directly compare plastic responses at the molecular level between life stages, one study comparing rapid cold hardening and exposure to altered developmental temperatures in *Drosophila melanogaster* (Gerken *et al.* 2015) detected effects on similar functional classes of genes at different life stages, although the genes themselves differed. This commonality in responses across life stages and temperatures could potentially be interpreted in two different ways. First, this pattern could reflect a common underlying mechanism regulating plasticity. An alternative, but not mutually exclusive, interpretation of this pattern is that these plastic

responses at different life stages represent beneficial or adaptive responses to thermal change. For example, the persistent effects of developmental temperature on gene expression patterns could be adaptive by shifting gene expression levels closer to those observed in fish at lower and higher temperatures or by reducing the cost of thermal acclimation.

Although the phenotypic consequences of the gene expression patterns observed here are unknown, a previous study in zebrafish (Schaefer & Ryan 2006) found that acclimation to increased temperature increased whole-organism thermal tolerance, and increased developmental temperature had a similar, but much smaller, effect. Taken together, this suggests that changes in gene expression may have effects on a variety of traits at the whole-organism level.

3.4.2 Relationship to patterns of DNA methylation

Epigenetic mechanisms have been proposed to be important processes through which environmentally induced variation in phenotypic expression in one generation can impact subsequent generations and through which environmental conditions during development can have persistent phenotypic effects later in life. To explore the relationship between DNA methylation patterns and the effects of changes environmental temperature during development and in adults on gene expression, we compared the differentially expressed genes identified in this study to differentially methylated loci from the same stickleback reported in our previous study. Differentially expressed genes in stickleback from different developmental temperatures were not closely associated with any of the differentially methylated loci that were previously described, suggesting a potential role of DNA methylation on trans-acting factors that regulate gene expression. Alternatively, differentially methylated regions could be located on regulatory elements that are not within 5 kb of a gene, or the persistent effects of developmental

temperature on gene expression may not be regulated by DNA methylation and instead could be due to effects such as changes in histone acetylation or changes in miRNA activity. However, we cannot firmly rule out a potential role for changes in DNA methylation in regulating the changes in gene expression that we observed because important regulatory regions located near differentially expressed genes may not have been assessed in the reduced representation analysis of DNA methylation (RRBS). For example, only ~10% of the differentially expressed genes were represented within the RRBS dataset, highlighting a potential limitation when deducing functional relationships that are based on a correlation between a reduced representation approach and a more comprehensive technique such as RNA-seq.

Analysis of differentially expressed genes and differentially methylated loci in adult stickleback acclimated to warm and cold temperatures identified genes involved in several processes that are known to be differentially regulated in marine ectotherms in response to changes in environmental temperatures, including the ubiquitin-proteasome pathway, aerobic metabolism and mitochondrial proliferation, mRNA splicing, myogenesis, proper formation of the sarcomere, and muscle cell growth and development. These data suggest that modified DNA methylation levels are likely involved in many of the transcriptional responses of stickleback to variation in environmental temperatures. One potentially interesting candidate gene that was both differentially expressed and associated with at least one DMC in adult stickleback acclimated to different temperatures is phosphodiesterase 4B (*pde4ba*). Epigenetic mechanisms are thought to play an important role in an organism's capacity to adapt to environmental changes, particularly over shorter timescales, but there is little empirical evidence to support this hypothesis. The *pde4ba* gene has been previously associated with the genetic divergence of Baltic sea stickleback populations along a thermal gradient (Guo *et al.* 2015) and thus may play an adaptive role to

changes in environmental thermal regimes. It is therefore a strong candidate for subsequent analysis of the effects of DNA methylation on adaptive evolutionary processes.

Taken together, these data demonstrate that changes in DNA methylation patterns are likely implicated in short-term, potentially reversible transcriptional response of adults to changes in environmental temperature.

3.5 Conclusions

The data presented here demonstrate that the temperature experienced during early stages of development (before hatch) can have persistent effects on gene expression patterns in adult stickleback muscle tissue. In addition, we demonstrate that the majority of differentially expressed genes in stickleback from different developmental temperatures are also differentially expressed as part of the adult stickleback thermal acclimation response. This pattern suggests that developmental plasticity and phenotypic flexibility in gene expression in response to temperature change share some common underlying mechanisms and may have similar functional consequences. However, adult acclimation resulted in a much larger overall change in the transcriptome than did developmental temperature exposure. Some genes that were differentially expressed as a result of the adult acclimation treatments were also associated with previously identified temperature-dependent effects on DNA methylation patterns, suggesting a potential role for epigenetic mechanisms in regulating plastic responses during acclimation. Overall, these results emphasize both the similarities and differences between developmental plasticity and phenotypic flexibility in adults and highlight the relationships between plasticity acting across different time scales.

Chapter 4: The DNA methylation landscape of stickleback reveals patterns of sex chromosome evolution and effects of environmental salinity.

4.1 Introduction

Epigenetic variation has the potential to impact ecological and evolutionary processes, and thus affect species distributions and evolutionary trajectories (Bossdorf et al. 2008; Flores et al. 2013; Jablonka and Raz 2009; Varriale 2014; Franks and Hoffmann 2012). Currently, one of the best-studied mechanisms underlying epigenetic variation is DNA methylation, a heritable epigenetic modification in which a methyl group is added to position 5 of the pyrimidine ring on a cytosine (5mC), most commonly found on cytosine-phosphate-guanine (CpG) dinucleotides in vertebrates (Heard and Martienssen 2014). Changes in DNA methylation can have profound effects on chromatin structure, which can in turn alter gene expression (Klose and Bird 2006; Jaenisch and Bird 2003). The addition or removal of these methyl groups can be dynamically regulated in response to changes in the environment (Cooney *et al.* 2002; Kucharski *et al.* 2008; Boyko *et al.* 2010). Variation in DNA methylation levels have therefore been hypothesized to play a key role in mediating phenotypic responses to environmental change (Bossdorf *et al.* 2008; Flores *et al.* 2013; Hofmann 2017), and may represent a dynamic source of heritable variation that can respond to changes in the environment and influence phenotypic variation over multiple time-scales (Richards 2006).

In addition to its potential role in regulating gene expression in response to environmental change, DNA methylation is also critical in regulating gene expression in dosage compensation systems that have evolved to minimize the unequal expression of genes on heteromorphic sex

chromosomes (Graves 2016). In older XY sex chromosome systems, such as those found in most mammalian species, DNA methylation is involved in the global silencing of one of the two X chromosome in females (Graves 2016). In ZW sex chromosome systems (which have a female-specific W chromosome) such as those in birds and some reptiles and fishes, DNA methylation is involved in gene-specific dosage compensation via the activation or suppression of particular dosage sensitive genes (Graves 2016). While epigenetic silencing has been well established as a mechanism involved in dosage compensation of older heteromorphic sex chromosome systems, patterns of DNA methylation in young sex chromosome systems are less well understood, but have been hypothesized to play a key role sex chromosome evolution (Gorelick 2003).

The threespine stickleback (*Gasterosteus aculeatus*) has been extensively used to investigate the genetic basis of adaptive evolution to novel environments (Jones et al. 2012a; Jones et al. 2012b). Following the last glaciation, ancestral marine populations of stickleback colonized and adapted to newly available freshwater habitats in the north-temperate zone (Bell and Foster 1994). Adaptation to these novel environments drove the rapid parallel evolution of divergent phenotypes including changes in body shape, armor plate number, gene expression levels, and gene expression plasticity (Jones et al. 2012a; Gibbons et al. 2017; Morris et al. 2014; McCairns & Bernatchez 2010; Ishikawa et al. 2017). Several studies have used reduced representation approaches to characterize variation in DNA methylation patterns between stickleback that vary in their lateral plate morphology, and have suggested that variation in DNA methylation patterns may contribute to the phenotypic divergence observed between marine and freshwater populations (Smith *et al.* 2015; Trucchi *et al.* 2016; Artemov *et al.* 2017). Threespine stickleback also have a relatively young XY sex chromosome pair that has evolved since the species first arose ~13-16 Ma (Kawahara *et al.* 2009; Ross *et al.* 2009; Bell *et al.* 2009), and this

species has become a powerful model system to explore the evolution of heteromorphic sex chromosomes and dosage compensation mechanisms (Schultheiß *et al.* 2015; White *et al.* 2015). Thus, the threespine stickleback is an ideal model in which to investigate the complementary roles of DNA methylation in both environmental adaptation and the evolution of sex chromosome systems.

In this study we present the first high-resolution analysis of DNA methylation patterns in the stickleback genome using whole genome bisulfite sequencing (WGBS). This approach allowed us to characterize prominent features in the DNA methylation landscape of stickleback, including variation in DNA methylation patterns between males and females along the entire stickleback sex chromosome, which provides insight into the relationship between epigenetic mechanisms and sex chromosome evolution. By rearing putatively ancestral marine stickleback at both low and high salinity, we also describe the effects of environmental salinity on genomic DNA methylation patterns, and highlight potential salinity responsive genes that may be differentially regulated by DNA methylation.

4.2 Material and methods

4.2.1 Fish collection

All animal experimentation was conducted according to University of British Columbia approved animal care protocols (A10-0285 and A11-0372). Adult threespine stickleback (*G. aculeatus*) of the fully plated "marine" ecotype were collected at the beginning of their natural spawning season in May 2013 from Oyster Lagoon, British Columbia in Canada (GPS: 49.6121,-124.0314). Fish were separated into six 110-litre glass tanks (20 fish per tank) and acclimated to 21 ppt salt water (dechlorinated Vancouver municipal tap water supplemented with Instant

Ocean Sea Salt), 18 °C and 14:10 h light:dark photoperiod. These conditions mimic the natural environmental conditions at the collection location at the time of collection. Fish were fed daily to satiation with Hakari Bio-Pure frozen Mysis Shrimp and were acclimated to laboratory conditions for four weeks.

4.2.2 Fertilization and rearing procedure

To determine the impact of salinity on fertilization and hatching, eggs were collected from gravid female stickleback and immediately divided into six different petri dishes containing 5 ml of 2, 7, 14, 21, 28, or 35 ppt saltwater. Testes were collected from males displaying sexually mature characteristics and individually macerated in a 1.75 mL microcentrifuge tube containing 300 mL Ginzberg's fish Ringer's solution. Eggs from a single clutch were fertilized with sperm solution from a single male across all salinities (50 mL of sperm solution for each petri dish at each different salinity). Following fertilization, an additional 10mL of water at the appropriate salinity was added to each petri dish. This process was repeated ten times creating a total of ten different families, each fertilized at all salinities. Petri dishes were partially covered to prevent water loss via evaporation and to allow for surface gas exchange. Eggs were monitored twice daily during which time any unfertilized eggs were removed and 10 mL of water was changed with sterilized water of the appropriate salinity to prevent mold growth. Percent fertilization and percent hatch were recorded. Percent hatch is recorded as the proportion of fertilized embryos that hatched. The effect of salinity on fertilization and hatching success was analyzed using a logistic regression (Warton & Hui 2011) in the R v3.3.2 base stats package. Tukey post hoc analysis was performed using the glht() function in the *multicomp* v1.4-6 R package.

After all fish in a petri dish had hatched and the yolks had been absorbed (~15 days post fertilization), larvae were transferred to hanging net boxes (Aquaclear) in 110 L glass aquaria containing water at the fertilization salinity. Sponge filters were used for filtration and aeration. Each family was kept separate throughout the experiment. At one-month post hatch whole animals were snap frozen in liquid nitrogen.

4.2.3 Whole genome bisulfite sequencing (WGBS)

Genomic DNA was isolated from one-month old whole fish samples from the 2 ppt and the 21 ppt salinity treatments using a Qiagen DNeasy Blood and Tissue Kit following the manufacturer's recommended protocol for RNA-free genomic DNA using RNAase A. The sex of each sample was identified by PCR analysis using primers designed to *idh, gasm6*, and *stn190* following previously described methods (Toli *et al.* 2016). Genomic DNA samples from three males and three females from 2 ppt and 21 ppt (twelve samples total) were sent to the McGill University and Genome Quebec Innovation Center for DNA quality assessment, library preparation, bisulfite treatment, and 150 base pair paired-end sequencing using an Illumina HiSeqX. The 12 samples were split evenly across 3 sequencing lanes (4 samples/lane) such that one male and female sample from each of the salinity treatments were represented on each sequencing lane. Average sequencing library size was 102,011,555 reads (± 13,147,873 SD).

4.2.4 WGBS data analysis

Reads were mapped to a revised assembly of the stickleback genome (Glazer *et al.* 2015) obtained from the Dryad Digital Repository (http://datadryad.org/resource/doi:10.5061/dryad.q018v) and DNA methylation levels were

calculated using the bisulfite sequencing plugin v1.2 in CLC Genomics Workbench v10.0. Average mapping efficiency was 89.5 % (± 1% SD). DNA methylation data were exported from CLC and analyzed using in R v3.3.2 using *methylKit* package v3.5 (Akalin *et al.* 2012) following previously recommended guidelines for bisulfite sequence analysis (Ziller *et al.* 2015; Wreczycka *et al.* 2017). Sequenced CpG loci were filtered so that only sites with at least 10 reads in each of the 12 samples were retained. Sites that were in the 99.9th percentile of coverage were also removed from the analysis to account for potential PCR bias.

Hierarchical cluster analysis was conducted using Ward's method and was implemented using the clusterSamples() function. Pairwise comparisons between groups were performed using a logistic regression model with a correction for overdispersion using the calculateDiffMeth() function followed by a Chi-square test to identify significantly differentially methylated cytosines (DMCs) between groups. The p-values for DMCs were false discovery rate (FDR) corrected using the sliding linear model method (SLIM) with a maximum q-value threshold of 0.05 and a minimum change in percent methylation of 10%. For the comparison between males and females, salinity and family were included as covariates. For the comparison between salinity rearing treatments, sex and family were included as covariates. For comparisons between families, sex and salinity were included as covariates. To calculate mean methylation levels across 10 kilobase (kb) genomic regions, the tileMethylCounts() function in methylKit was used to calculate DNA methylation values across sequential 10 kb windows of the genome. All figures were generated in R.

To obtain nearest neighboring gene annotations, the gene coordinates in the annotation file (.gtf) provided by Glazer et al. (Glazer *et al.* 2015) in the dryad digital repository (which contains gene coordinates that correspond to the stickleback genome available in Ensembl) were

converted to the coordinates in the updated assembly using the R script convertCoordinate.R that is provided by the authors of the revised assembly. Distances to nearest neighboring genes were calculated using the annotatePeakInBatch() function in the R package *ChIPpeakAnno* v3.6.5.

CpG islands for the revised stickleback genome assembly were calculated using python scripts (https://github.com/lucasnell/TaJoCGI) that apply an algorithm based on the methods described by Takai and Jones (Takai & Jones 2002). The observed distribution of DMCs was compared to the distribution of CpGs across the genome using a Chi-square test.

4.2.5 Candidate gene analysis

Previous RNA-seq studies have identified many genes that respond to changes in salinity in stickleback (2, 771 in gill tissue (Gibbons *et al.* 2017) and 1,844 in kidney tissue (Wang *et al.* 2014)). To determine whether DNA methylation could be involved in the differential regulation of these candidate genes we compiled a list of salinity responsive genes from previous studies (4,615 candidate genes) and compared them to genes within 2 kb of DMCs in stickleback reared at different salinities.

Similarly, several studies have also characterized sex-biased gene expression patterns in stickleback. We therefore compiled a list of 2,282 genes that display sex-biased gene expression patterns in brain (1,255 genes (Metzger & Schulte 2016a)) and liver tissue (1,268 genes (Leder *et al.* 2010)), and compared these to genes within 2 kb of DMCs that were identified between male and female stickleback.

We also compared genes within 2 kb of DMCs between individuals reared a different salinities to genes associated with single nucleotide polymorphisms (SNPs) under positive

selection in threespine stickleback from freshwater environments compared to marine environments (Jones *et al.* 2012a).

4.3 Results and discussion

4.3.1 Characterization of the stickleback methylome

We performed WGBS on fish from a marine population of threespine stickleback reared from fertilization to the age of 1 month at a salinity of either 2 ppt or 21 ppt. These salinities represent the widest range that still allows good fertilization and hatching success in this population (Appendix C, Figure C.1). In this study we utilized a balanced design with WGBS performed on one male and one female from each of three families and each of the salinity rearing treatments. This design was chosen because genetic variation has been shown to have substantial effects on the divergence of DNA methylation patterns among individuals in both plants and animals (Gertz *et al.* 2011; McRae *et al.* 2014; Vidalis *et al.* 2016). Consistent with this observation, we detected strong effects of family on DNA methylation (Appendix C, Figure C.2 and C.3). However, family-level variation in DNA methylation levels could also be indicative of transgenerational environmental or maternal effects (Jablonka and Raz 2009).

CpG loci in the stickleback genome had an average methylation level of 70.3% (Appendix C, Figure C.4), which is consistent with whole genome assessments of methylation in other fish species (Feng *et al.* 2010; Zemach *et al.* 2010; Shao *et al.* 2014). However, there were several hypomethylated regions (<40% methylation) that are indicative of DNA methylation canyons or valleys (Xie *et al.* 2013; Jeong *et al.* 2014) with the most prominent of these located on chromosomes 4, 10, 11, 12, and 16 (Figure 4.1 and A4.4).

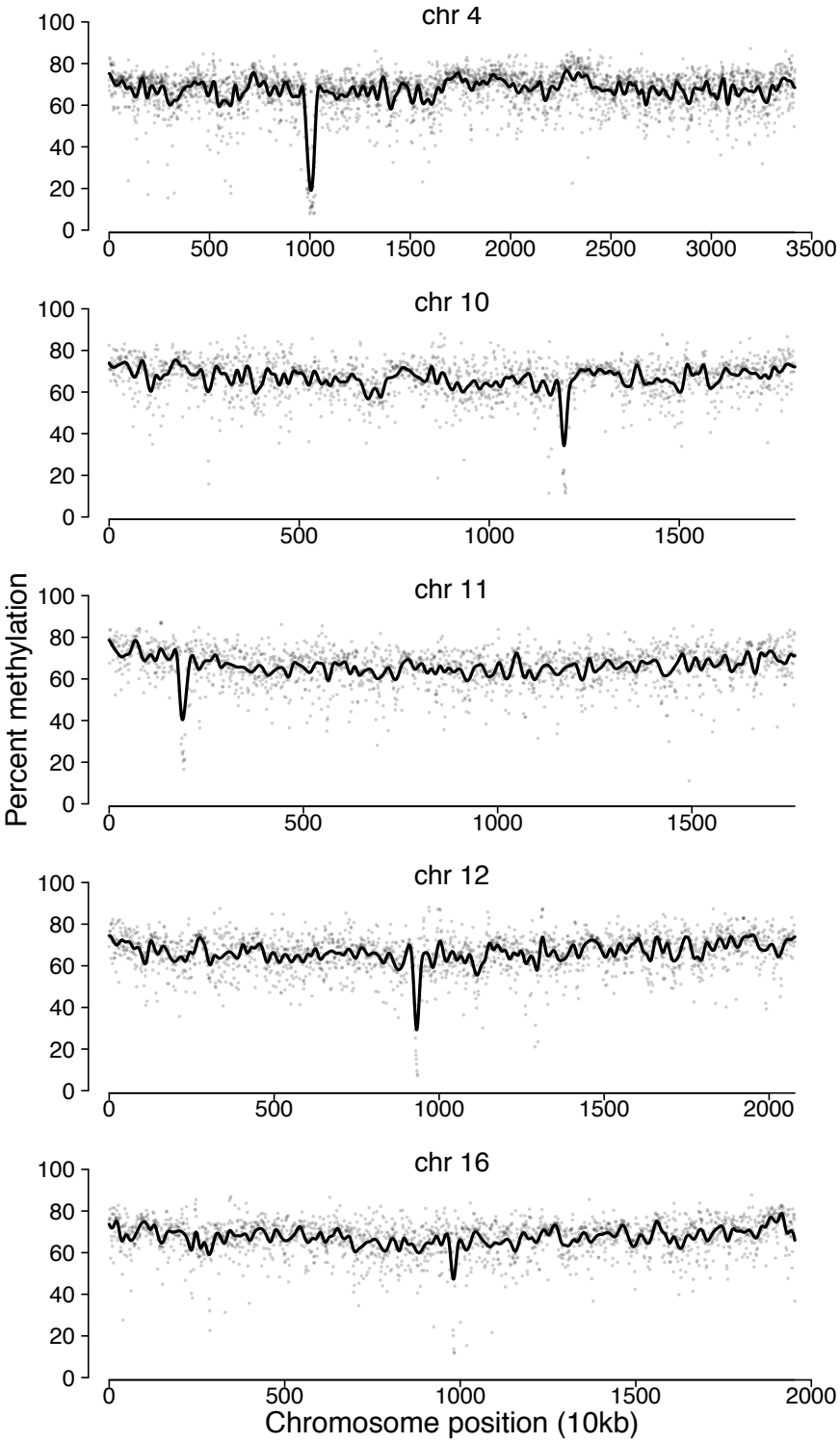

Figure 4.1: Mean CpG methylation level across chromosomes 4, 10, 11, 12, and 16. Each point represents the mean methylation level across all twelve individuals for a single 10 kb window. The solid line represents the smoothed spline fit to these data. Position along the x-axis represents the base position along the chromosome. The y-axis is the average DNA methylation level.

While the factors that determine the size of DNA methylation canyons remains unknown, larger hypomethylated canyons such as those described here in stickleback have been shown to be under strong transcriptional suppression due to the increased abundance of repressive histone H3 lysine 27 methylation that interacts with hypomethylated DNA (Nakamura et al., 2014). This mechanisms of transcriptional repression is thought to maintain these regions in a "poised" transcriptional state to allow rapid activation of gene transcription in these regions at specific times during embryonic development (Nakamura *et al.* 2014), but whether these hypomethylated canyons play a functional role in adults is unknown. However, genes that are essential for proper development typically dominate these regions (Xie *et al.* 2013; Jeong *et al.* 2014; Nakamura *et al.* 2014). Consistent with this pattern, genes located in the hypomethylated canyons in the stickleback genome include protocadherins on chromosome 4 and homeobox genes on chromosomes 10, 11, 12 and 16 (Appendix C, Table C.1), suggesting a conserved role of hypomethylated canyons across vertebrates.

4.3.2 Sex-biased DNA methylation patterns

We identified a total of 18,564 DMCs between males and females (Figure 4.2). Although relatively few studies have examined differential methylation patterns between males and females at the whole genome level in fishes, a study in tilapia detected a similar number of DMCs between males and females in muscle tissue (17,112 DMCs; Wan et al. 2016), whereas a study of sex-specific differential methylation in zebrafish brain detected only 914 DMCs (Chatterjee *et al.* 2016). These data suggest that the extent of sexually dimorphic methylation may be highly variable among teleosts, consistent with the wide range of sex-determining mechanisms in this group (Devlin & Nagahama 2002).

The distribution of DMCs across genomic features (e.g. promoters, exons, CpG islands) did not differ from the relative proportions of these features in the genome (Appendix C, Table C.2). No DMCs were identified between males and females in the mitochondrial genome. The majority of DMCs (90 %; 16,626 DMCs) between males and females showed a bias towards higher methylation in females relative to males suggesting that female stickleback genome is hypermethylated relative to male stickleback genome.

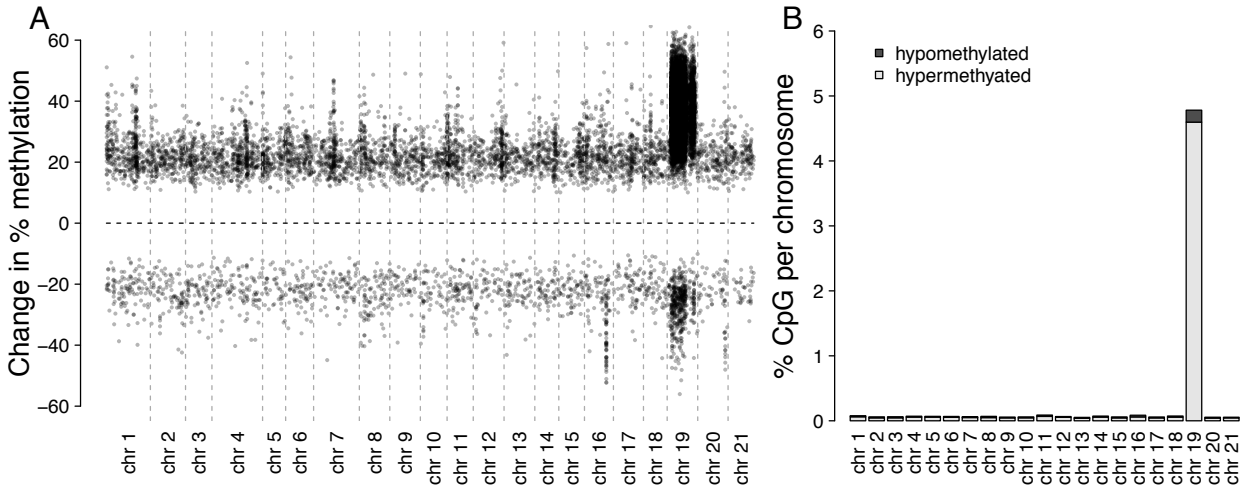

Figure 4.2: Differentially methylated CpG loci between male and female stickleback.
(A) Differentially methylated CpG (DMC) loci between male and female threespine stickleback. Each point represents an individual DMC. The y-axis indicates the percent difference in methylation between males and females. A positive value on the y-axis indicates a DMC that is hypermethylated in females relative to males. A negative value on the y-axis indicates a DMC that is hypomethylated in females relative to males. The x-axis indicates the position of the DMC in the stickleback genome. Chromosome boundaries are represented by vertical dashed lines. Only DMCs for which a change in methylation of > 10 % are presented. (B) The percentage of CpG loci on a given chromosome that were differentially methylated between male and female threespine stickleback. The light shading represents DMCs that are hypermethylated and the dark shading represents DMCs that are hypomethylated in female stickleback compared to males.

The most striking pattern in these data is the apparent hypermethylation of chr19 (the threespine stickleback sex chromosome) in females relative to males, which is where 65 % of the putative DMCs identified between males and females are located (12,112 DMCs). Chr19 also had the highest proportion of DMCs relative to the number of CpG sites on the chromosome (5

%) compared to the rest of the autosomes where ~0.07 % of CpG loci were differentially methylated between the sexes (Figure 4.2).

Three distinct regions (strata) have been characterized on chr19 based on the extent of divergence in these regions between the X and Y chromosome (Ross & Peichel 2008; White *et al.* 2015), and two of these strata no longer recombine between the X and Y: stratum two (the younger evolutionary stratum located between ~2.5 Mb and 12 Mb), and stratum one (the older evolutionary stratum located from ~12 Mb to the end of the chromosome). There is also a pseudoautosomal region (PAR) located in the first ~2.5 Mb of chr19 that is thought to still recombine between the X and Y chromosomes (White *et al.* 2015). To assess whether these evolutionary strata are also associated with sex-specific DNA methylation, we divided chr19 into 10 kb consecutive non-overlapping bins and calculated the frequency of CpG loci that were putatively identified as hypermethylated or hypomethylated in female stickleback relative to male stickleback (Figure 4.3). This analysis revealed clear patterns that correspond to the evolutionary strata of chr19 for loci that were hypermethylated in females relative to males, with the greatest apparent hypermethylation in stratum two and the least in the PAR (Figure 4.3B).

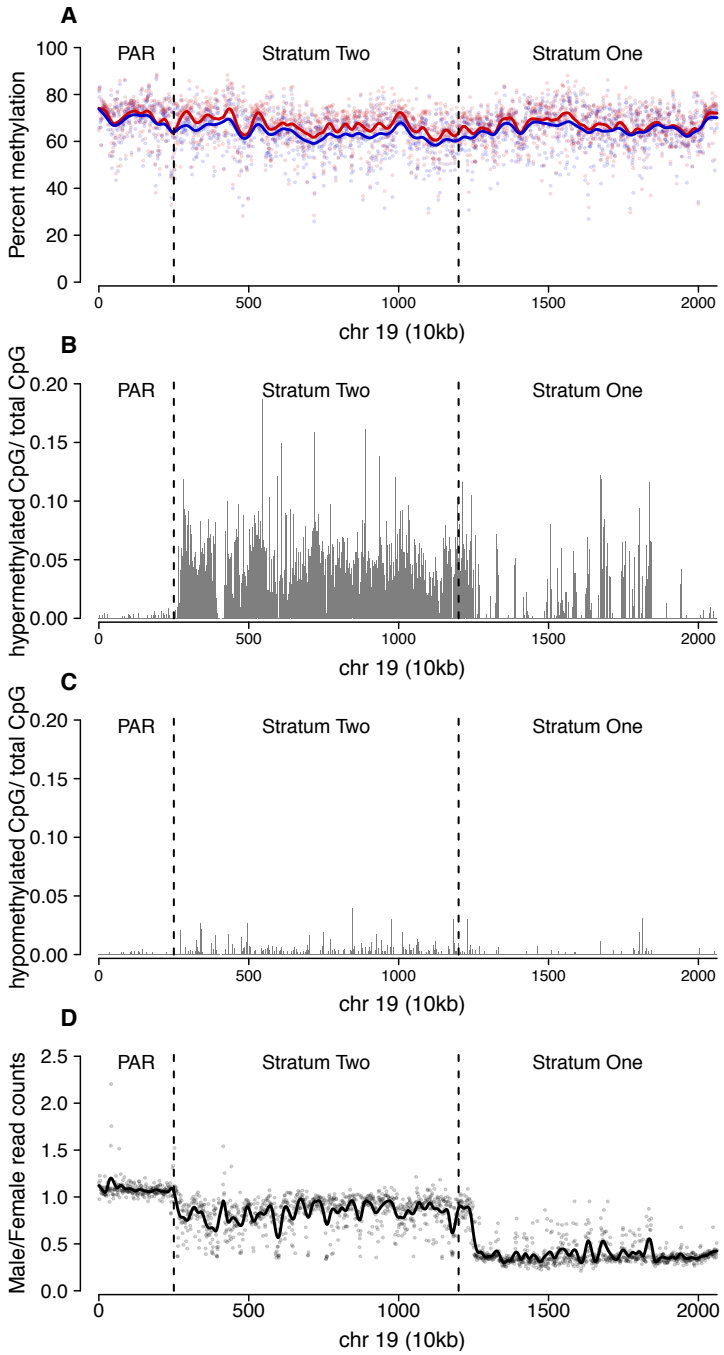

Figure 4.3: Differential methylation between sexes on chromosome 19 (chr19). (A) Mean DNA methylation levels for CpG loci along chromosome chr19. Each point represents the mean DNA methylation level in a 10 kb window for six individual stickleback that were either male (blue) or female (red). Solid lines represent the smooth spline fit for the DNA methylation levels in males (blue) and females (red). (B-C) Proportion of DMCs along chr 19 that are hypermethylated (B) or hypomethylated (C) in female stickleback compared to male stickleback. Values on the y-axis represent the total number of DMCs in a 10 kb window relative to the number of CpG loci in that same 10 kb window. (D) Ratio of mapped read counts for males relative to females along chr19. Each point represents the ratio of mean counts for a 10kb window in males compared to females. The solid black line is the smooth spline fit. Vertical dashed lines represent the boundaries between the three evolutionary strata on chr19: the pseudo autosomal region (PAR), stratum two, and stratum one.

4.3.3 Identifying differential methylation on the sex chromosome

One of the challenges for unambiguously determining levels of sex-specific DNA methylation on stickleback sex chromosomes is that there is currently no publically available sequence for the Y chromosome and the published sequence for chr19 is predominately derived from X chromosome sequence. Because DNA methylation is detected as sequence differences between bisulfite-treated DNA and the reference sequence at CpG sites, both divergence between the X and Y chromosome at CpG sites and differential methylation can result in the same patterns in the sequence data. Thus, the signal of differential methylation that we observe could be attributed to one of three possible mechanisms: 1) identical sequences but differential methylation between the X and Y chromosome, 2) differential methylation between X chromosomes in males and females, or 3) sequence divergence between the X and Y chromosomes resulting in alteration of CpG sites.

To address this issue, we again divided chr19 in to 10kb consecutive non-overlapping segments and compared the number of reads that mapped to chr19 in males and females for each 10kb segment. If chr19 reads map uniquely to the published X chromosome then we would expect half the number of reads to map to chr19 in males compared to females. Given that the PAR is known to recombine between the X and Y chromosomes, suggesting low levels of divergence in this region, we predicted that sequencing reads derived from both the X and Y chromosomes would map to the reference sequence, resulting in a ratio of one for the number of reads mapped in males and females in the PAR. The results from the read coverage analysis matched this prediction (Figure 4.3D).

For the younger, less diverged stratum (stratum two), we predicted a read count ratio between 0.5 and 1 because sequence similarity between the X and Y chromosome would result

in reads from both chromosomes mapping to the X chromosome reference sequence. The results from the read coverage analysis matched this prediction (Figure 4.3D). Thus, we cannot unambiguously determine whether differential methylation or X Y polymorphism is the cause of the apparent hypermethylation in females in stratum two.

Stratum one is thought to be the most divergent region of the sex chromosome, and thus we predicted that few reads from the Y chromosome would map to the chr19 reference, resulting in a ratio of 0.5 for the number of reads mapped in males and females. Again, the results from the read coverage analysis mostly matched this prediction (Figure 4.3D), although there were specific regions where the read count ratio was close to one. These regions in stratum one may correspond to regions that are thought to be under purifying selection to maintain dosage sensitive genes from being lost on the Y chromosome (White *et al.* 2015). We also detected apparent hypermethylation in females in these regions (Figure 4.3B and 4.3D). Thus, we cannot conclusively determine whether this apparent hypermethylation of chr19 in females is due to differential methylation or from the accumulation of TG polymorphisms on the Y that are being interpreted as unmethylated loci.

While we are unable to unambiguously determine the ultimate cause of the apparent DNA methylation differences between males and females on chr19, whether the patterns we observe are the result of sequence polymorphism between the X and Y chromosome that alters CpG sites, or are due to differential methylation of conserved sequences between males and females in chr19, the ultimate effect would be differences in methylation between the sex chromosomes. Thus, taken together, the patterns of putative differential methylation that we observe suggest that divergence in DNA methylation patterns between males and females on the

stickleback sex chromosome are closely associated with the known evolutionary history of this chromosome.

4.3.4 DNA methylation and sex chromosome evolution

Sex chromosome evolution from autosomes is thought to involve recombination suppression in sex determining regions, followed by the accumulation of deleterious mutations and the degeneration of the sex-specific (e.g. Y) chromosome (Graves 2016). Degradation of the sex-specific heteromorphic sex chromosome following recombination suppression has the potential to cause imbalances in gene expression. Many taxa with heteromorphic sex chromosome pairs have evolved dosage compensation mechanisms to resolve this effect, but the nature and extent of these dosage compensation mechanisms varies greatly among taxa (Graves 2016). DNA methylation has been proposed as a key mechanism responsible for regulating every step of the evolution of sex chromosomes from recombination suppression in the early stages of sex chromosome evolution to dosage compensation in more derived sex chromosome systems (Gorelick 2003); however, there has been little empirical evidence to test this hypothesis. Taxa with relatively "young" heteromorphic sex chromosomes, such as the threespine stickleback, provide an opportunity to investigate the potential role of DNA methylation in regulating sex chromosome recombination and the evolution of dosage compensation mechanisms. In the following section, we discuss the apparent differential methylation between males and females on chr19 in the context of the different stages of sex chromosome evolution in stickleback.

DNA methylation promotes the formation of heterochromatin (Melamed-Bessudo & Levy 2012; Mirouze *et al.* 2012; Yelina *et al.* 2015), and it is thought to play a role in suppressing recombination of sex chromosomes in plants (Zhang *et al.* 2008). In stickleback we

observed apparent hypermethylation of the younger evolutionary stratum in females (stratum two), and relatively less differential methylation between males and females along the older evolutionary stratum (stratum one) and the PAR. The apparent hypermethylation of stratum two on the X chromosome in females (hypomethylated in males) corresponds to the region hypothesized to have undergone the first chromosomal inversion during the evolution of the Y chromosome (Ross & Peichel 2008). The apparent differential methylation in stratum two between males and females (hypomethylated in males and hypermethylated in females) could have played a role in suppressing recombination between male and female sex chromosomes and in establishing the boundaries in which this inversion first occurred.

The next stage in sex chromosome evolution, following recombination suppression, is thought to be the accumulation of genetic variation and degradation in the non-recombining region(s). If methylated cytosines on the female X chromosome correspond to thymines on the male Y chromosome as previously discussed, then it is possible that the accelerated mutation rate of methylated cytosines, which can be deaminated to become thymines (Coulondre *et al.* 1978; Shen *et al.* 1994), could play an important role in the divergence between X and Y chromosomes. Alternatively, instead of being a direct result of C to T polymorphisms, the observed increase in hypermethylated loci in females could be closely linked to the accumulation of genetic polymorphisms. The frequency at which single nucleotide polymorphisms (SNPs) occur in genomes has been shown to be higher near methylated CpG loci (Qu *et al.* 2012). The CGCG motif has been identified as a candidate cis-element associated with this observation and is enriched in hypomethylated regions (Qu *et al.* 2012). Therefore, individuals with higher DNA methylation levels at particular loci would be predicted to have a higher degree of sequence divergence near those loci relative to individuals with lower methylation levels. The PAR had

the highest frequency of the CGCG motif (1.98 / 1kb) while stratum one and stratum two had a lower frequency of the CGCG motif (1.22 / 1kb and 1.18 / 1 kb respectively). These data suggest that DNA methylation may also be associated with the accumulation of genetic variation between the non-recombining regions of the male and female sex chromosomes.

We next explored whether the apparent differential methylation patterns between males and females on chr19 are consistent with the regulation of dosage sensitive genes. In stickleback, there are two conflicting hypotheses regarding the existence of a dosage compensation system. One hypothesis is that there is locally confined partial dosage compensation in stratum one in males that is also associated with a hypertranscription of genes in stratum one in females (Schultheiß *et al.* 2015). The second hypothesis suggests that dosage compensation has not evolved in the stickleback but that there is purifying selection to maintain dosage sensitive genes in stratum one of the Y chromosome (White *et al.* 2015). The differential methylation patterns between sexes along the X chromosome that we observe are not entirely consistent with either of these prevailing hypotheses. We observed apparent hypermethylation in stratum two in females. This might be expected to result in reduced transcription or partial silencing of genes in this region, which has not been observed in stickleback (Schultheiß *et al.* 2015; White *et al.* 2015). The less extensive and highly localized pattern of hypermethylation in stratum one that we observe is suggestive of gene-specific regulation, which is not consistent with a generalized hypertranscription of genes in stratum one in females (Schultheiß *et al.* 2015). Because the male to female coverage ratio is similar in these localized regions in stratum one, this localized pattern may be more consistent with the potential preservation of dosage sensitive genes in these regions (White *et al.* 2015); however, it is also possible that the differential methylation in these regions

is caused by the accumulation of C to T polymorphisms which is less consistent with purifying selection acting in these regions (White *et al.* 2015).

Taken together, the apparent differential methylation between male and female stickleback described in this study is consistent with the proposed role of DNA methylation in the evolution of sex chromosomes (Gorelick 2003). Thus differential DNA methylation could be playing a role in the evolution of this "young" heteromorphic sex chromosome system, either through influencing patterns of recombination or potentially through mediating the early stages of the development of dosage compensation.

4.3.5 DNA methylation and sex-biased gene expression

To determine whether the apparent variation in DNA methylation patterns between males and females could be influencing previously described sex-biased gene expression patterns in stickleback, we compared the list of genes near DMCs between males and females to previously identified genes that exhibit sex-biased gene expression patterns (Leder *et al.* 2010; Metzger & Schulte 2016a). Of the 2,282 genes that have been shown to exhibit sex biased gene expression patterns from these studies, 490 overlapped with genes near DMCs in our study of which 269 are on chr19 including genes located in the region considered to be tightly linked to sex determination in stickleback such as *sema4ba* and *idh2* (Peichel *et al.* 2004). This pattern is consistent with differential methylation between males and females playing a role in regulating sex-biased patterns of gene expression.

4.3.6 Effects of environmental salinity on DNA methylation

Variation in DNA methylation has also been suggested to be an important component of an organism's response to environmental change (Bossdorf *et al.* 2008; Flores *et al.* 2013; Hofmann 2017). Changes in environmental salinity are known to cause substantial changes in gene expression in many species of fish, including stickleback (Wang *et al.* 2014; Gibbons *et al.* 2017; Zhang *et al.* 2017). In order to explore whether changes in DNA methylation may be involved in environmental regulation of gene expression, we identified differentially methylated loci in stickleback reared at two salinities (2 and 21 ppt), and we compared genes near DMCs identified in fish reared at different salinities to genes that have been previously identified as salinity-responsive using RNA-seq (Wang *et al.* 2014; Gibbons *et al.* 2017).

1,259 CpG loci were differentially methylated between salinity treatments, and these DMCs were distributed across all chromosomes, with an average of 0.01 % of the CpG loci on each chromosome being differentially methylated (Figure 4.4). No DMCs were identified between individuals from low and high salinities in the mitochondrial genome. The distribution of DMCs across genomic features (e.g. promoters, exons, CpG islands) did not differ from the relative proportions of these features in the genome (Appendix C, Table C.2). The majority of DMCs (1,051) were hypomethylated in individuals from high salinity relative to low salinity. Analysis of the genes located close to these DMCs revealed several genes known to be involved in the response to salinity in fish. However, GO enrichment analysis did not detect significant enrichment for any GO categories following FDR correction. The ten GO terms with the lowest p-values are listed in tables A4.3-4.5.

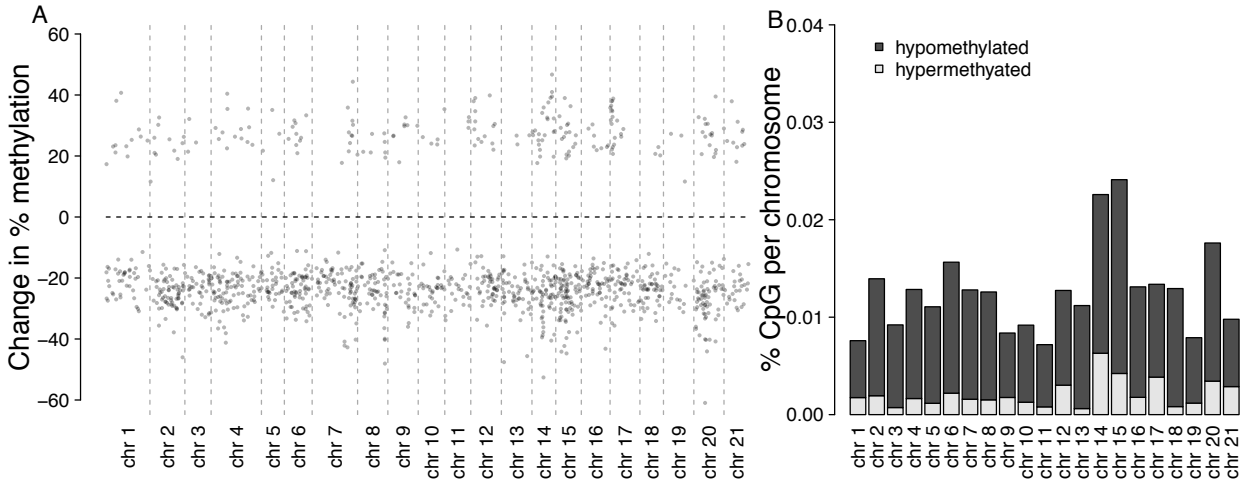

Figure 4.4: Differentially methylated CpG loci between stickleback reared a low and high salinity. (A) Differentially methylated CpG (DMC) loci between stickleback reared at a salinity of 2 ppt compared to 21 ppt. Each point represents an individual DMC. The y-axis indicates the percent difference in methylation between salinity rearing treatments. A positive value on the y-axis indicates a DMC this is hypermethylated in stickleback reared at 21 ppt relative 2 ppt. A negative value on the y-axis indicates a DMC that is hypomethylated in stickleback reared at 21 ppt relative to 2 ppt. The x-axis indicates the position of the DMC in the stickleback genome. Chromosome boundaries are represented by vertical dashed lines. Only DMCs for which a change in methylation of > 10 % are presented. (B) The percentage of CpG loci on a given chromosome that were detected as being differentially methylated between stickleback reared at 21 ppt and 2 ppt. The light shading represents DMCs that are hypermethylated and dark shading represents DMCs that are hypomethylated in stickleback reared at 21 ppt compared to 2 ppt respectively.

Comparison of genes located near DMCs in fish from reared at different salinities to previously identified as salinity-responsive using RNA-seq (Wang *et al.* 2014; Gibbons *et al.* 2017) identified 126 candidate genes with changes in both expression and methylation in response to salinity. Among the candidate genes that we identified are ion channels that are important for regulating cellular ion concentrations in hyper and hypo-osmotic conditions such as the calcium pump *atp2b4*, the sodium/chloride co-transporter *slc12a3*, and the sodium/potassium/2 chloride co-transporter *slc12a1*. Taken together, these data suggest that changes in DNA methylation could play a role in facilitating the transition between marine and freshwater environments.

In stickleback, a variety of genomic regions have been identified as having been subject to positive selection following colonization of freshwater habitats by ancestral marine fish (Jones

et al. 2012a). Because epigenetic variation has been suggested to be a driver of adaptive evolution (Flores *et al.* 2013), we screened our dataset of salinity responsive DMCs in marine fish to identify those associated with genes found in regions under positive selection in freshwater populations (Jones *et al.* 2012a). Very few of the DMCs identified in our study were near these genes, suggesting that salinity-responsive changes in DNA methylation are unlikely to have played a role in driving genetic divergence in these regions between marine and freshwater populations of stickleback.

4.4 Conclusions

In this study we used whole-genome bisulfite sequencing to identify novel DNA methylation features in the stickleback epigenome. Apparent hypermethylation of stratum two on the female X chromosome compared to levels in males suggests that DNA methylation could play an important role in the suppressing recombination between the X and Y chromosome, and potentially in regulating sex-biased gene expression patterns. We also detected significant changes in DNA methylation in response to rearing salinity, some of which were associated with genes known to be differentially regulated in response to changes in environmental salinity. This epigenetic change reflects a response to environmental salinity that could facilitate the accumulation of epigenetic variation between natural populations, and thus be implicated in long-term responses to environmental change.

Chapter 5: Maternal stress has divergent effects on gene expression patterns in the brains of male and female threespine stickleback

5.1 Introduction

Maternal exposure to stress has been shown to have persistent effects on the neurodevelopmental processes of offspring across a wide range of taxa, resulting in profound changes to the health and behavior of subsequent generations (Storm & Lima 2010; Bale 2015; Bell *et al.* 2016). For example, in humans, epidemiological studies suggest that prenatal exposure to maternal stress increases the prevalence of neurodevelopmental diseases such as schizophrenia and autism spectrum disorder in adult offspring (Babenko *et al.* 2015). In contrast, in a variety of ecological contexts the effects of maternal stress on offspring phenotypes have been suggested to be positive, conferring increased fitness in stressful environments (Sheriff & Love 2013). Whether maladaptive or adaptive, these effects are likely the result of a complex interaction between genes and the environment, mediated through a variety of epigenetic processes (Weaver *et al.* 2004; Murgatroyd *et al.* 2009).

In mammals, the effects of maternal stress on adult offspring have been repeatedly demonstrated to differ between males and females (Weinstock 2007; Jazin & Cahill 2010; McCarthy *et al.* 2012a). These sex-specific effects are thought to be regulated by complex maternal-fetal interactions via the placenta (Nugent & Bale 2015). Thus, it is unclear whether conclusions derived from studies in mammals can be broadly applied across non-placental taxa. Studies in birds have detected sexually dimorphic effects of maternal stress on offspring behavior (Holloway & Clayton 2001; Wade *et al.* 2004), and the regulation of the hypothalamic-

pituitary axis (HPA) (Schmidt *et al.* 2014), but the generality of these findings across oviparous vertebrates remains unknown. This is particularly important in the context of ongoing debates regarding the evolutionary implications of maternal effects in natural populations (Räsänen & Kruuk 2007; Badyaev 2008; Sheriff & Love 2013).

In this study we use threespine stickleback (*Gasterosteus aculeatus*) as a model to study the effects of maternal stress on the brain transcriptome of male and female offspring. There is compelling evidence from studies in this species that maternally derived stressors have profound and persistent effects on developmental, morphological and behavioral phenotypes of offspring (Bell *et al.* 2016) that can persist across generations (Shama *et al.* 2014, 2016; Shama & Wegner 2014; Shama 2015) making them an important model in which to study the adaptive potential of transgenerational plasticity, and an ideal candidate in which to explore the sexually dimorphic effects of maternal stress on the offspring of an oviparous vertebrate.

5.2 Materials and methods

5.2.1 Stress treatment and animal rearing

Adult threespine stickleback (*G. aculeatus*) of the fully plated "marine" ecotype were collected in May 2013 in Oyster lagoon (British Columbia, Canada, GPS: 49.6121,-124.0314) and acclimated to laboratory conditions for three weeks (see appendix C for details). Following the three-week acclimation period, fish were divided into two treatment groups (Unstressed and Stressed) with three replicate tanks per treatment. Fish in the stressed treatment were chased once daily with a fish net for 30 s before being captured and held out of water for a further 30 sec and then returned to their tank. This was intended to mimic similar predatory and handling stressors that have been used in investigate the effects of maternal stress on offspring behavior in

fish (Giesing *et al.* 2011; Sopinka *et al.* 2017). Timing of the stress treatment was randomized during each day to avoid conditioning. The stress treatment was applied for two weeks, and significantly increased plasma cortisol levels in mothers (ANOVA $p = 0.0014$; Appendix D, Figure D.1). Testes were then harvested from unstressed males and used to generate half-sib crosses, with the same male being used to fertilize the eggs of one stressed female and one unstressed female. A total of 6 pairs of half-sib crosses were produced.

After one year post hatch, and prior to reaching sexual maturity, brain tissue was dissected from the offspring and immediately frozen in liquid nitrogen and stored at -80°C until further use. Sex of the 1-year-old offspring was determined by PCR amplification of *Idh, Gasm6,* and *Stn190* as previously described (Toli *et al.* 2016). Table S1 summarizes the family information for the fish that were selected for RNA-seq. Because not all families had the required number of male and female offspring at one year post-hatch, we were unable to use a completely balanced design with male and female half sibs represented in both the unstressed and stressed treatments. However, we attempted to maximize the genetic diversity within the sample by limiting the use of full-sibs where possible (Appendix D, Table D.1).

5.2.2 RNA isolation and sequencing

Total RNA was isolated from stickleback whole brain tissue (3 fish per sex per stress treatment; 12 fish total) using TRIzol Reagent (Invitrogen Life Technologies) as previously described (Metzger *et al.* 2016) followed by DNase treatment (Qiagen Rneasy). RNA integrity numbers (RIN) were between 7.9 – 9.1 (mean = 8.6 ± 0.4 SD). Preparation of Illumina TruSeq cDNA libraries and 100 base-pair paired end sequencing was performed at the UBC Nucleic Acid Protein Service Unit (NAPS) and UBC Biodiversity Research Center's next generation

sequencing facility, and at the McGill University and Genome Quebec Innovation Center. Sequencing depth and sample multiplexing were devised to optimize statistical power given the total sequence generated (Ching *et al.* 2014; Wu *et al.* 2015). Mean library size was 19.8 million reads (Appendix D, Table D.2).

5.2.3 Sequence alignment and expression analysis

Reads were aligned to the stickleback genome (http://www.ensembl.org) using CLC genomics workbench v8.5 (Qiagen). Average mapping efficiency of paired and broken reads was 88.5 ± 0.9%. Analysis of total read counts was performed in R v3.2.2 with edgeR v3.12.0. Recommended RNA-seq analysis guidelines were followed (Lin *et al.* 2016) with the addition of RUVr from the RUVSeq protocol v1.4.0 (Risso *et al.* 2014) to account for batch effects. Genes with no reads were removed from the dataset. Counts were then normalized using the relative log expression (RLE) method (Anders *et al.* 2010). Following normalization, genes with low expression were filtered from the dataset. The minimum criterion for retaining a gene was at least 1 count per million (~10 counts in the smallest library) in each of the three samples of at least one sex by stress treatment group. A total of 16,477 genes were retained after normalization and filtering for subsequent expression analysis. Robust dispersions were calculated using the robust method in edgeR (Zhou *et al.* 2014). The data were then fit to a negative binomial generalized linear model using glmFit(). Likelihood ratio tests were run to assess effects of sex, maternal stress and the interaction of these main effects with factors calculated from RUVseq included in the model. The resulting p-values were adjusted based on FDR correction (Benjamini & Hochberg 1995), and the threshold for significance of these adjusted p-values was set at 0.05. Principal component analysis (PCA) was performed using the prcomp() function from the base

package in R on log 2 counts per million expression values. GO and KEGG pathway enrichment analyses were conducted using the goseq (v1.22.0) R package (Young *et al.* 2010), with FDR correction (see Appendix D for details). Cluster analysis of differentially expressed gene ontologies was performed using REVIGO (http://revigo.irb.hr), and the resulting source code was used to generate figures using the ggplot package in R.

5.3 Results

Analysis of RNA-seq data from the brains of one-year-old male and female threespine stickleback offspring from stressed and unstressed mothers revealed over 2,900 genes with either a significant main effect of sex (1,255 genes) or a significant interaction between maternal stress and sex (1,650 genes), but very few genes with a main effect of maternal stress (Figure 5.1A). Principal component (PC) analysis of all expressed genes distinguished four groups (Figure 5.1B). PC1 explained ~30% of the variation in the data and separated males and females that came from stressed mothers indicating that maternal stress has different effects on the brain transcriptome in male and female stickleback. PC2 explained ~11.5% of the variation in the data and separated males and females that came from unstressed mothers. Combined, PC1 and PC2 accounted for ~41% of the variation in the RNA-seq data.

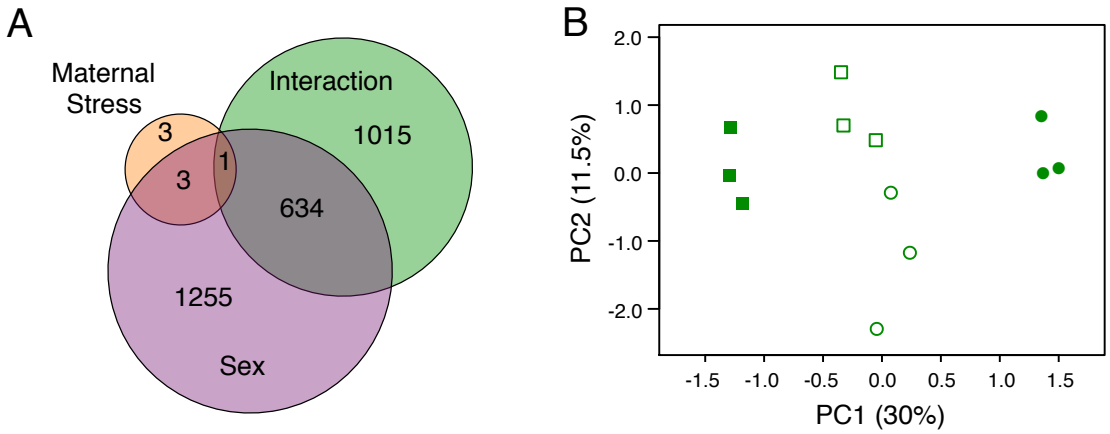

Figure 5.1: Differential expression and principal component analyses reveal sexually dimorphic effects of maternal stress in threespine stickleback offspring. (A) Venn diagram depicting the total number of differentially expressed genes for which a main effect of sex (purple), maternal stress (orange), or an interaction between sex and maternal stress (green) was detected. (B) Principal components 1 and 2 of expressed genes in the brain tissue of males (squares) and females (circles) from unstressed (open symbol) and stressed (closed symbol) mothers.

5.3.1 Sexual dimorphism in gene expression

Independent of the effects of maternal stress, there were differences in brain gene expression patterns between male and female threespine stickleback (Appendix D, Figure D.2). 614 genes were expressed at higher levels in females whereas 641 genes were expressed at higher levels in males (Appendix D, Figure D.2). Enrichment analysis (Gene-Ontology (GO)-enrichment, KEGG pathway enrichment) did not reveal any terms or pathways that were significantly enriched among the genes with a significant main effect of sex. However, GO enrichment analyses do not provide comprehensive categorization of the GO terms associated with a differentially expressed gene list (Primmer *et al.* 2013). Genes associated with metabolic processes were expressed at higher levels in females whereas genes associated with cytoskeletal organization, cell adhesion and developmental processes including nervous system development were expressed at higher levels in males (Appendix D, Figure D.3).

5.3.2 Sex-specific effects of maternal stress

The effects of maternal stress on brain gene expression were strikingly different in male and female offspring (Figure 5.2) and these effects were generally in opposing directions between the sexes such that genes that increased in expression in response to maternal stress in female offspring decreased in expression or did not change in response to maternal stress in male offspring and vice versa. This is in direct contrast to the small number of genes that showed a conserved effect of maternal stress in both males and females, with only three genes demonstrating a maternal stress effect without an effect of offspring sex or an interaction (Figure 5.1A).

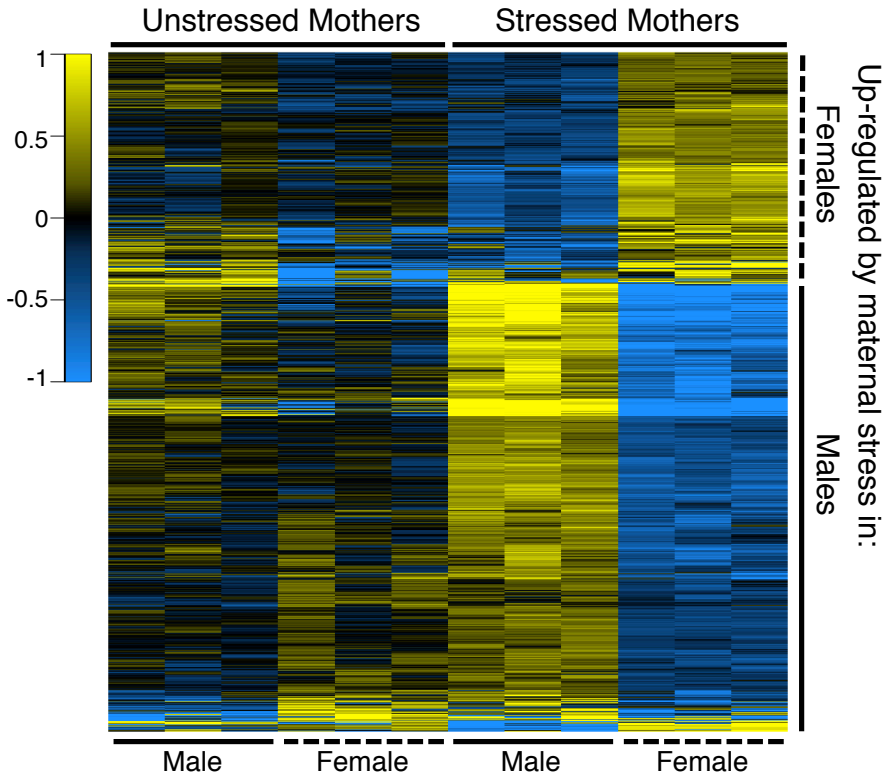

Figure 5.2: Heat map of all differentially expressed (DE) genes for which a significant interaction between sex and maternal stress was identified. Expression results are displayed a log2 counts per million normalized to the mean expression for all samples. Blue indicates lower expression and yellow indicates higher expression.

GO-enrichment analysis of genes with a significant interaction between maternal stress and offspring sex revealed several enriched GO categories that suggest differential expression of genes involved in protein translation, metabolism, and synapse organization and assembly (Appendix A, Table A5.6). Enrichment analysis of KEGG pathways identified those involved in protein synthesis, and several neurodegenerative diseases such as Huntington's, Parkinson's, and Alzheimer's disease, as well as pathways associated with synaptic function such as nicotine addiction and glutamatergic synapse (Table S7).

To further explore functional enrichment in the genes exhibiting significant interactions, we also performed separate GO enrichment analysis genes that were up-regulated by maternal stress in female offspring and genes that were up-regulated by maternal stress in male offspring. This analysis divided the previously identified enriched GO categories into two distinct groups (Figure 5.3). The majority of enriched GO categories for genes that were up-regulated by maternal stress in female offspring were involved in protein translation and regulation of metabolic processes, and this was reflected in both the biological process (Figure 5.3A) and cellular component (Figure 5.3B) ontologies. Enriched KEGG pathways up-regulated by maternal stress in female offspring were those associated with protein translation and diseases such as Parkinson's, Huntington's, and Alzheimer's disease, all of which have a metabolic component (Beal 1998; Schapira 1999; Appendix D, Table D.5). A different set of processes was identified as enriched for genes that were up-regulated by maternal stress in male offspring. The majority of enriched GO categories for genes up-regulated by maternal stress in males were involved in nervous system development and synapse formation, and again this was reflected in both the biological process (Figure 5.3A) and cellular component (Figure 5.3B) ontologies. Enriched KEGG pathways up-regulated by maternal stress in males were those associated with

synaptic function and organization including glutamatergic synapse, GABAergic synapse, long-term depression, and nicotine addiction (Appendix D, Table D.5).

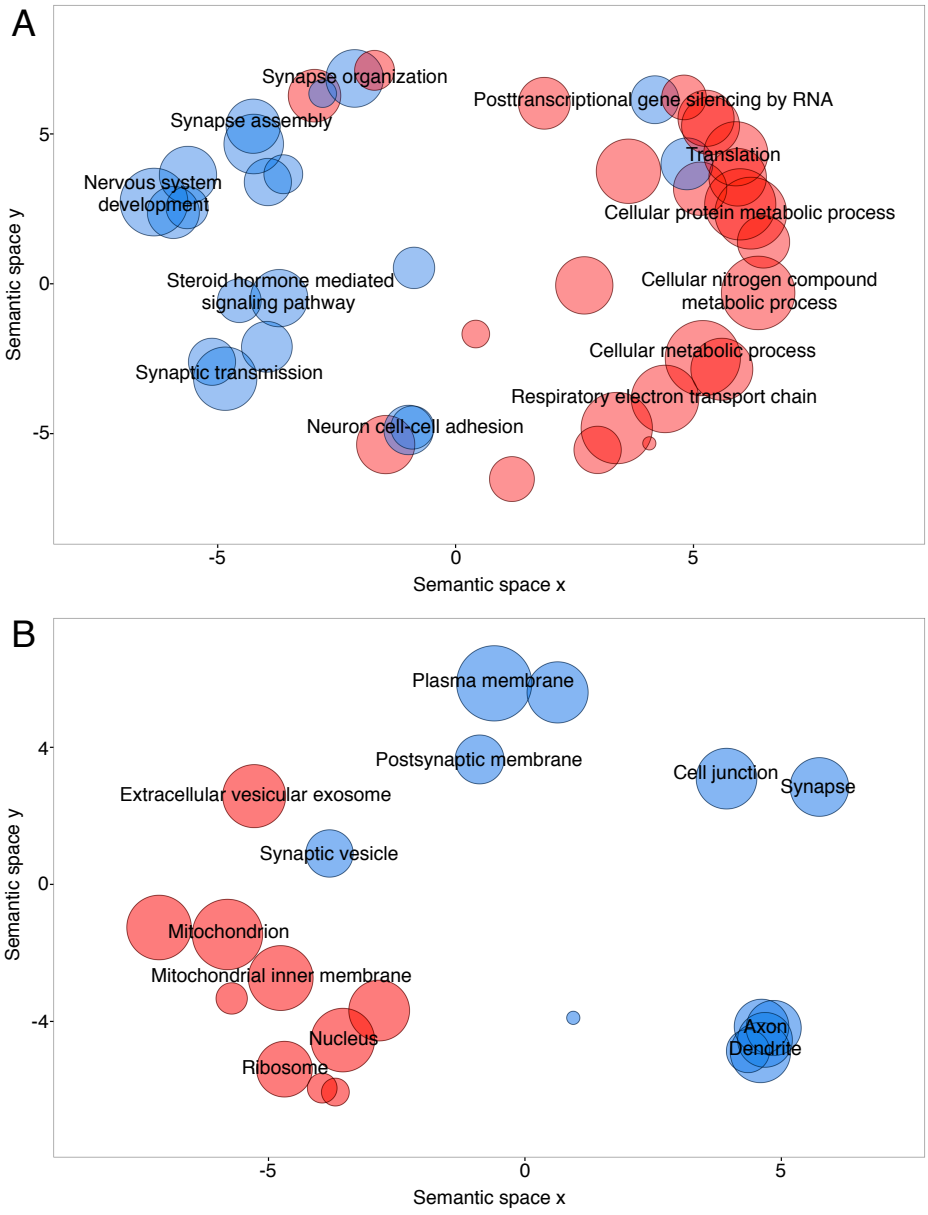

Figure 5.3: Cluster analysis of the significantly enriched gene ontology (GO) terms for biological process (A) and cellular component (B) over-represented among genes that increased in males (blue circles) or in females (red circles) from stressed mothers compared to unstressed mothers. Size of the circle is representative of the total number of DE genes in that GO category. Note that some names have been omitted from the figure due to semantic similarity.

5.3.3 Chromosomal location of differentially expressed genes

Of the 1,255 genes that exhibited a main effect of sex, 1170 could be localized to chromosomes, and of these, 585 were expressed at higher levels in females, and 585 were expressed at higher levels in males. A disproportionate number of these genes were located on the sex chromosome (chromosome 19; 441 genes, 38%), and 87% of these sex-chromosome localized genes (383 genes) were expressed at higher levels in females than in males (Figure 5.4A). In contrast, genes that were expressed at higher levels in males than in females were distributed fairly uniformly across the chromosomes. Because similar numbers of genes were expressed at higher levels in females and in males, genes with autosomal locations tended to be more highly expressed in males.

A strikingly different pattern of chromosomal location was observed for genes that differed in their response to maternal stress between male and female offspring (Figure 4B). In this case, 1,477 genes of the 1,650 genes with a significant interaction between offspring sex and maternal could be localized to chromosomes. Of these, 504 genes were expressed at higher levels in female offspring from stressed mothers, whereas 973 genes were expressed at higher levels in male offspring from stressed mothers. These genes were uniformly distributed across the 21 stickleback chromosomes in both sexes (Figure 4B), with each chromosome harboring 4.8 ± 1% of the genes that demonstrated a significant interaction between maternal stress and offspring sex.

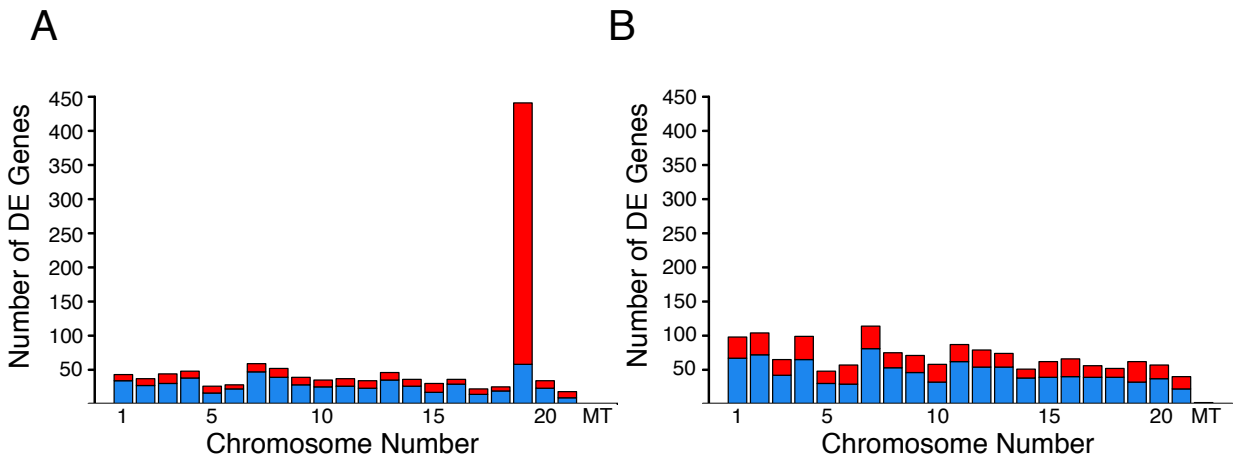

Figure 5.4: Histograms representing the total number of differentially expressed genes localized to each of the 21 stickleback chromosomes and the mitochondrial genome. (A) Genes for which a significant effect of offspring sex was detected. (B) Genes for which significant interaction between offspring sex and maternal stress was detected. Blue bars represent genes that are up-regulated in males (A) or are expressed at higher levels in males from stressed mothers compared to females from stressed mothers (B). Red bars represent genes that are up-regulated females (A) or are expressed at higher levels in females from stressed mothers compared to males from stressed mothers (B).

5.4 Discussion

The results presented here clearly demonstrate two distinct classes of sexual dimorphism in brain gene expression in threespine stickleback: 1) differences between males and females that are present under all conditions, and 2) differences in the response of the male and female brain to maternal stress. Sexual dimorphism in brain gene expression is known for a wide variety of taxa (Jazin & Cahill 2010), and has been previously observed in several fish species including threespine stickleback (Santos *et al.* 2008; Sreenivasan *et al.* 2008; Manousaki *et al.* 2014; Sharma *et al.* 2014; Schultheiß *et al.* 2015; Lu *et al.* 2015). In contrast, the critical importance of the interaction between maternal stress and offspring sex has been pointed out in mammals (McCarthy *et al.* 2012a), but has been poorly studied in other taxa and is often neglected in ecological and evolutionary studies. Our finding that maternal stress affects the brains of male and female stickleback offspring differently emphasizes the importance of taking sex into account when studying the effects of maternal stress on gene expression, neurodevelopment and

behavior and has important implications for the assessment of the ecological and evolutionary impacts of stress across generations.

5.4.1 Sex-specific effects of maternal stress

In this study we detected 1,650 genes that demonstrated a significant interaction between maternal stress and offspring sex, while only 6 genes demonstrated a significant effect of maternal stress with no interaction. This pattern is surprising because the power to detect differential expression is greater for the main effect of maternal stress (n=6 individuals per group) than it is for the interactive effects of maternal stress and offspring sex (n=3 individuals per group). Simulation studies suggest that six individuals per group should be sufficient to detect differential expression given the sequencing depth per sample used here (Wu *et al.* 2015), and thus we should be able to detect a general effect of maternal stress, if present. The lack of significant main effects of maternal stress is likely driven by the observation that genes up-regulated by maternal stress in male offspring were generally down-regulated in female offspring, and vice versa.

We had relatively low power to detect differences between males and females in the effects of maternal stress (i.e. the interaction between offspring sex and maternal stress), suggesting that the 1,650 genes for which we detected significant interactions may represent an underestimate of the sex-specific effects of maternal stress. Another key question is the extent of false positives within this dataset, as rates of false positives increase at low samples sizes (Wu *et al.* 2015). Two possible approaches to reduce this "false discovery cost" are to apply a very stringent FDR threshold, or to apply an effect-size filter and retain only genes above a certain fold-change cutoff as differentially expressed, which greatly reduces the probability of detecting

false positives. We applied each of these approaches (Appendix D, Figure D.4) and these analyses support the conclusion of sex-specific effects of maternal stress on brain gene expression patterns.

We also detected clear divergence in the pathways identified as enriched for genes up-regulated by maternal stress in male versus female offspring of stressed mothers. This pattern suggests that maternal stress has physiologically distinct effects on the brains of male and female offspring of stressed mothers. Enrichment analysis indicated that maternal stress up-regulates genes involved in mitochondrial respiration in females, while up-regulating genes involved in synaptic function in males, and these patterns were supported when very stringent effect size and FDR filters were applied to the data (see Appendix D for details). Although differences in gene expression do not necessarily result in changes in protein activity, the striking differences in expression patterns between the sexes and the sexual dimorphism in the biological processes identified and the enrichment of different biological processes between the sexes in response to maternal stress strongly suggests important differences in the physiological impacts of maternal stress between male and female offspring in threespine stickleback. In combination with similar patterns that have been observed in mammals (Weinstock 2007), these data suggest that sexually-dimorphic effects of maternal stress may be common across a wide range of taxa.

5.4.2 Sources of sexual dimorphism in brain gene expression

We also detected significant sexual dimorphism in brain gene expression independent of maternal stress. Although the specific functional consequences of the sexually dimorphic patterns of brain gene expression that we and others (Schultheiß *et al.* 2015; White *et al.* 2015) observe in threespine stickleback remain unknown, they may be either causes or consequences of

the many morphological and behavioral differences between male and female threespine stickleback (Bell & Foster 1994; Shaw *et al.* 2007; Kotrschal *et al.* 2012). For example, male stickleback perform several complex behaviors that females do not, such as nest building, courtship behaviors, and paternal care of offspring (Bell & Foster 1994). Associated with this sexual dimorphism in behavior, there is striking sexual dimorphism in brain size, with males having larger brains compared to females (Kotrschal *et al.* 2012; Samuk *et al.* 2014; Herczeg *et al.* 2015), and similar patterns have been observed in other fish species (Kolm *et al.* 2009; Herczeg *et al.* 2014). It has been proposed that the larger brain size of male stickleback may be due to the increased cognitive demand on males compared to females (Jacobs 1996; Kotrschal *et al.* 2012; Herczeg *et al.* 2015). In addition to differences in brain size and behavior between males and females, another possible cause of differences in brain gene expression between the sexes could be differences in the relative sizes of different brain regions. Such a pattern has been observed in a variety of species (Cooke *et al.* 1998), including stickleback (Park & Bell 2010).

In addition to differing in size and morphology between the sexes, the vertebrate brain also has substantial capacity for plasticity in many species (Kolb & Whishaw 1998; Kaslin *et al.* 2008), including stickleback (Park *et al.* 2012). Interestingly, male stickleback have been shown to have increased brain plasticity relative to female stickleback (Herczeg *et al.* 2015). The effects of maternal stress on brain gene expression represent a form of transgenerational plasticity. Here we observed increased expression of genes associated with neurodevelopment processes in males from stressed mothers but not in females from stressed mothers. This observation raises the intriguing possibility that the differential effects of maternal stress on the male and female brain could be due to underlying differences in brain plasticity between the sexes. Indeed, previous studies have detected a strong maternal effect on brain size and the relative sizes of brain regions

in stickleback (Noreikiene *et al.* 2015), and taken together with the known sexual dimorphism in brain plasticity in stickleback (Gonda *et al.* 2009; Park *et al.* 2012; Herczeg *et al.* 2015), could account for the differences in the impacts of maternal stress that we observe in brain gene expression between male and female offspring.

Another potential source of sexual dimorphism in gene expression is the biased genomic localization of these genes on divergent sex chromosomes (Rice 1984; Rinn & Snyder 2005; Ellegren & Parsch 2007; Leder *et al.* 2010; Chatterjee *et al.* 2016). Consistent with previous studies in threespine stickleback (Schultheiß *et al.* 2015) we observed that genes that were more highly expressed in females, independent of maternal stress, were heavily biased towards localization on the sex chromosome (Figure 5.4). In contrast, the genes that exhibited divergent expression patterns in the response to maternal stress between male and female offspring (i.e. those that showed a significant interaction between maternal stress and offspring sex) did not exhibit biases in genomic localization. This pattern strongly suggests that mechanisms acting in trans, such as regulation by sexually dimorphic genes localized on the sex chromosome, or via sexually dimorphic hormones, are likely to be responsible for the different responses to maternal stress observed in male and female brains.

5.4.3 Effects of maternal stress on stress-related genes

One of the clear conclusions emerging from previous studies in mammals is that stress experienced during early development, such as during prenatal development or during childhood, can induce changes in the hypothalamic-pituitary-adrenal stress response (Liu 1997; Francis *et al.* 1999), which can lead to the sensitization of adults to stress-related disorders (Oberlander *et al.* 2008). Similar effects have been observed in a variety of fish species, including threespine

stickleback (Mommer & Bell 2013). To explore the effects of maternal stress on the expression of genes associated with the glucocorticoid stress response, we examined the expression patterns of a suite of candidate genes known to be involved in this pathway.

Both of the isoforms of *nr3c1,* which encode glucocorticoid receptors, were up-regulated by maternal stress in male offspring and were down-regulated by maternal stress in female offspring. In addition, there was evidence for sexual dimorphism and sex differences in the response to maternal stress in the expression of several of the key regulators of the glucocorticoid receptor (Appendix D, Table D.4). Brain glucocorticoid receptors are important for the negative feedback regulation of the stress hormone axis (Liu 1997). If the changes in mRNA levels that we detect are predictive of protein levels, this implies that female stickleback from stressed mothers may show reduced ability to down regulate the stress-hormone axis compared to male offspring, although current evidence suggests that these effects are likely to be subtle, if present (Mommer & Bell 2013). However, taken together, these data suggest that maternal stress re-shapes the stress hormone axis, and that these effects differ between male and female stickleback.

5.4.4 Effects of maternal stress on epigenetic processes

Epigenetic mechanisms such as DNA methylation are known to be involved in modulating the effects of maternal stress on offspring phenotypes (Bale 2015) including changes in the expression of the glucocorticoid receptor (Palma-Gudiel *et al.* 2015). We identified multiple sex-specific effects of maternal stress on the expression of DNA-demethylases (e.g. TET) suggesting a potential role for epigenetic mechanisms influencing the dimorphic expression of genes in males and females from stressed mothers. Similarly, GO enrichment

analysis identified post-translational gene silencing by RNA (GO: 0035194, Figure 5.3A) as a process that is over-represented in the set of genes that are up-regulated by maternal stress in male, but not female, offspring. The overall up-regulation of genes involved in a variety of epigenetic processes by maternal stress in male offspring and not in female offspring suggests a potential difference in the mechanisms via which maternal stress is transduced between the sexes.

5.5 Conclusions

Our data demonstrate that the effects of maternal stress on brain gene expression are sex-specific in threespine stickleback. Maternal stress has the potential to result in adaptive alterations in offspring physiology and behavior, enabling mothers to prepare their offspring for the environment they will encounter as adults. Alternatively, this phenomenon can also be maladaptive when temporary stressors induce permanent developmental changes that are suboptimal for the environment or when they lead to the development of disease (Hales & Barker 2001; Ghalambor *et al.* 2007; Sheriff & Love 2013). Determining whether a specific offspring phenotype is the result of adaptive or maladaptive plasticity is a significant challenge in ecology and evolutionary biology. Our observation of sex-specific effects of maternal stress in stickleback thus has important implications for the analysis of the evolutionary significance of maternal effects on transgenerational plasticity.

Chapter 6: Conclusion

6.1 Overview

The three main objectives of this thesis were 1) to determine how DNA methylation and gene expression are regulated in response to changes in environmental conditions, 2) to investigate the persistent effects of developmental conditions on DNA methylation and gene expression levels, and 3) to assess the effects of sex on DNA methylation patterns. Addressing the first objective, I demonstrated that DNA methylation patterns in stickleback are modified in response to both temperature (chapter two) and salinity (chapter four). Addressing the second objective, I demonstrated that temperature (chapters two and three) salinity (chapter four) and maternal factors (chapter five) influence DNA methylation and gene expression patterns across a range of timescales within an organism's lifetime. Through this work I also found that maternal stress has divergent effects on gene expression patterns in the brain tissue between male and female offspring. Addressing the third objective, I identified genome wide differences in DNA methylation patterns between male and female stickleback, including apparent differential methylation on the stickleback sex chromosome that is associated with the known evolutionary history of this chromosome.

By addressing these objectives, this thesis provides insight into the inheritance of DNA methylation and gene expression patterns through mitotic cell division, provides evidence for a role of epigenetic mechanisms in modulating phenotypic responses to environmental stressors, and provides empirical evidence for a role of DNA methylation in the evolution of heteromorphic sex chromosomes. In this chapter I discuss how the studies described in this thesis have contributed to our understanding of the ecological and evolutionary role of DNA

methylation and discuss several potential future directions that have been inspired by these results.

6.2 Ecological epigenetics and threespine stickleback

One of the key questions in ecological epigenetic studies is to understand how the environment affects epigenetic variation. Several studies have demonstrated that variation in DNA methylation patterns can accumulate among individuals that inhabit different environments (Cervera *et al.* 2002; Riddle & Richards 2002; Keyte *et al.* 2006; Vaughn *et al.* 2007; Zhang *et al.* 2011). For example, a study of Kenyan house sparrows observed a negative correlation between DNA methylation diversity and genetic diversity but a positive correlation between epigenetic diversity and inbreeding among individuals from a rapidly expanding population (Liebl *et al.* 2013). Several studies have also demonstrated positive correlations between phenotypic diversity and variation in DNA methylation patterns (Lira-Medeiros *et al.* 2010; Lyko *et al.* 2010; Liebl *et al.* 2013) which could play a role the spread of invasive species or the colonization of new habitats (Liebl *et al.* 2013). However, our understanding of how environmental cues are involved in establishing or modulating this observed epigenetic variation remains largely unknown.

In this thesis I demonstrate that different environmental temperatures (chapter two) and salinities (chapter four) can have both persistent and plastic effects on DNA methylation patterns, suggesting that DNA methylation patterns are sensitive to environmental conditions and epigenetic variation between populations could be highly dependent on environmental differences. Thus, observed differences in DNA methylation patterns between natural populations may not necessarily be indicative of fixed population differences in DNA

methylation patterns. Therefore, differences in the environment conditions of populations should be considered when interpreting apparent differences in DNA methylation patterns between natural populations.

Many authors have suggested that in order to be evolutionarily relevant, epigenetic variation must be able to persist across multiple generations unaffected by subsequent environmental changes (Bossdorf *et al.* 2008; Jablonka & Raz 2009). Based on this idea of evolutionarily relevant epigenetic variation, one potential experiment that could be conducted in order to determine whether the effects of environmental temperature or salinity on DNA methylation patterns in stickleback are adaptive responses to environmental cues would be to compare DNA methylation patterns among wild populations that span a thermal or salinity gradient but that have been reared in controlled laboratory conditions for multiple generations. Differences in DNA methylation patterns that persist following acclimation to a common environment that are consistent the effects described in either chapter two or four would then be considered candidates for adaptive evolutionary changes.

However, this experimental design seems somewhat contradictory in that epigenetic variation must be both environmentally sensitive in one generation and fixed (i.e. environmentally independent) in the next. In this scenario, epigenetic mechanisms can act as a type of heritable "memory" about past environmental conditions. However, it is not clear why there should be a requirement for these epigenetic changes to stop being plastic in subsequent generations. It is possible for epigenetic states to persist in a population because the environmental condition that originally induced the epigenetic change has also persisted, or for epigenetic mechanisms to influence developmental trajectories but to still remain plastic in response to environmental cues in later stages of development. For example, the difference in

DNA methylation between stickleback from marine and freshwater populations when kept at their native salinity (Artemov *et al.* 2017) might be more representative of DNA methylation patterns in stickleback reared at two salinities and not evolved difference in DNA methylation patterns between populations. Differences in DNA methylation patterns between marine and freshwater populations could also be due to environmental effects on epigenetic drift. Epigenetic drift is an age-dependent change in DNA methylation patterns over time due to the accumulation of errors in maintaining DNA methylation states through successive rounds of cell division (Fraga *et al.* 2005). Epigenetic drift has been shown to be affected by both genetic and environmental factors (Shah *et al.* 2014), thus epigenetic variation between populations may not be due to the active regulation of DNA methylation patterns but on the passive accumulation of DNA methylation variation over the lifetime of the individual. Environmentally dependent and persistent DNA methylation patterns could still be considered adaptive, or maladaptive, and could influence evolutionary trajectories by manipulation the expression of different phenotypes in different environments. Therefore, instead of searching for fixed epigenetic differences between populations that are no longer responsive to environmental cues, selection might act on the plasticity of epigenetic processes. Thus, a more productive approach to understanding the adaptive, or maladaptive consequences of epigenetic variation would be for future studies to investigate differences in the plasticity of the reaction norms of epigenetic processes (e.g. DNA methylation levels) in response to environmental cues.

6.3 Sex specific effects on DNA methylation and gene expression

Another important result that emerged from the data in this thesis is that sex has a large effect on responses to environmental stress and on DNA methylation patterns in the stickleback

genome. These effects are clearly demonstrated in the whole genome DNA methylation patterns described in chapter four and in the effects of maternal stress on gene expression patterns in the brain tissue of offspring in chapter five.

6.3.1 Differential methylation on the stickleback sex chromosome

The major finding from chapter four is that the majority of DMCs identified between males and females were located on chr19, which is the threespine stickleback sex chromosome. Furthermore, the differential methylation pattern patterns on chr19 were closely associated with the known evolutionary strata of the stickleback sex chromosome.

These results appear to be consistent with the theory proposed by Gorelick (2003) who suggested a role for DNA methylation in accelerating the divergence of heteromorphic sex chromosomes by accelerating the effects of Muller's ratchet on chromosome degradation. Muller's ratchet is a process that describes chromosomal degradation resulting from suppressed recombination between chromosome pairs followed by the irreversible accumulation of deleterious mutations (Muller 1964; Felsenstein 1974). High levels of DNA methylation are associated with the formation of heterochromatin, and heterochromatin suppresses chromosome recombination. Thus, differences in the DNA methylation levels of chr19 between males and females could have been responsible for suppressing recombination. Methylated cytosines are hypermutable and deaminate to become thymines at a faster rate than unmethylated cytosines (Duncan & Miller 1980; Bulmer 1986; Britten *et al.* 1988; Sved & Bird 1990). Therefore, differences in DNA methylation levels between males and females could also result in differences in the rate at which genetic polymorphisms accumulated on one sex chromosome but not the other. Data from chapter 4 strongly implicate variation in DNA methylation patterns

between males and females in the evolution of heteromorphic sex chromosomes, and future analysis of DNA methylation levels on the Y chromosome will provide further insight into the role of DNA methylation in sex chromosome evolution.

6.3.2 Divergent effects of maternal stress in male and female stickleback

Maternal exposure to stress has been shown to have persistent effects on the neurodevelopmental processes of their offspring across a wide range of taxa resulting in profound changes to the health and behavior of subsequent generations (Storm & Lima 2010; Bale 2015; Bell *et al.* 2016). These effects are thought to be the result of a complex interaction between genes and the environment, mediated through a range of epigenetic processes (Darnaudéry & Maccari 2008; Bale 2011; Mychasiuk *et al.* 2011; Zucchi *et al.* 2013). Unlike mammalian systems where complex maternal-fetal interactions via the placenta are thought to regulate the sexually dimorphic effects of maternal stress (Nugent & Bale 2015; Bronson & Bale 2016), oviparous embryos develop outside the body, separated from the further influence of maternal conditioning. Despite differences in the developmental environment of mammalian and teleost embryos, several studies have observed effects of maternal stress on offspring behavior and the regulation of the hypothalamic-pituitary-interrenal (HPI) axis in teleost fishes (Mommer & Bell 2013; Bell *et al.* 2016; Sopinka *et al.* 2017). The results from chapter five add to this growing body of research by demonstrating that the persistent effects of maternal stress are different depending on whether the offspring is male or female. For example, the divergent effects of maternal stress on gene expression patterns between males and females may result in differences in neural development and metabolic processes. The importance of considering sexually dimorphic effects of maternal stress on neurodevelopmental processes has been

repeatedly highlighted and discussed in the mammalian literature (McCarthy *et al.* 2012a; Cahill & Aswad 2015). The apparent similarity in the sexually dimorphic effects of maternal stress in placental and oviparous organisms suggests that there may be more similarities in the mechanisms that regulate maternal effects among placental and oviparous organisms than previously thought.

One of the unresolved questions in studies addressing maternal stress effects on offspring phenotypes is what mechanism or mechanisms are responsible for transferring information about the maternal environment to the offspring. In this thesis I measured circulating levels of cortisol, a glucocorticoid hormone involved in the HPI stress response, as a proxy for determining whether the stress treatment applied in this study was adequate to elicit a stress response. Previous studies in stickleback have shown that similar stress protocols resulting in elevated levels of circulating cortisol can also result in elevated cortisol levels in the eggs (Giesing *et al.* 2011). While glucocorticoids, such as cortisol, are known to regulate many developmental processes, it is unclear what effects maternally contributed cortisol has on developmental processes because maternal cortisol is rapidly metabolized in the embryo shortly after fertilization (Paitz *et al.* 2016). However, maternal stress can also affect other biological processes. For example, maternal stress has been shown to affect the nutritional content of eggs from stressed song sparrows and these changes in egg nutrient content have been shown to influence developmental processes of their offspring (Schmidt *et al.* 2012, 2014, 2015). Epigenetic processes have also been hypothesized to mediate the effects of maternal stress on offspring phenotypes (Bale 2015). While I did not measure epigenetics processes directly in chapter five, one of the significantly enriched biological processes that was identified as differentially expressed was the regulation of micro RNA expression. Micro RNAs are thought

to be one of the mechanisms that could play a role in epigenetic changes (Chuang & Jones 2007; Sætrom *et al.* 2007). It is possible that maternal stress could influence the composition of maternally derived micro RNAs that are deposited into the developing egg and these micro RNAs could influence developmental processes.

The results described in chapter 5 are particularly important in the context of ongoing debates regarding the evolutionary implications of maternal effects in natural populations (Räsänen & Kruuk 2007; Badyaev 2008; Sheriff & Love 2013). The environmental/maternal-matching hypothesis (Gluckman & Hanson 2004; Monaghan 2008; Love & Williams 2008; Sheriff & Love 2013; Sheriff *et al.* 2017) describes the adaptive potential of the effects of maternal stress on offspring phenotypes when the maternal environment is predictive of the environment experienced by the offspring. In the context of this hypothesis, divergent effects of maternal stress in males and females could suggest that the optimal phenotype in a stressful environment could differ depending on the sex of the offspring. In contrast, divergent effects in males and females could also suggest that the effects of maternal programming may be adaptive for one sex and maladaptive for the other in a stressful environment. However, both scenarios suggest that generalized effects of maternal stress on offspring performance should take the sex of the offspring into account. Whether maternal stress effects are adaptive or maladaptive will require additional studies that consider the fitness consequences of these effects in different environmental stress scenarios.

Stickleback exhibit paternal care of their offspring in which the male stickleback protects the developing embryos in a nest. The paternal duties of a stickleback include fanning the eggs to provide sufficiently oxygenated water to the clutch as well as defending the clutch from predators (Van Iersel 1953). Studies of adult offspring from predator-exposed fathers have

observed a decrease in offspring condition and offspring activity (Stein & Bell 2014; McGhee & Bell 2014; Bell *et al.* 2016). Interestingly reduced parental care in mammals (generally maternal care) is associated with the epigenetic modification of gene regulatory networks (Weaver *et al.* 2004). Whether the underlying mechanisms impacted by variation in paternal care in stickleback are similar to those impacted by altered maternal care in mammalian systems remains unknown.

6.4 Methodological considerations

The current gold-standard for characterizing DNA methylation levels at high resolution is the application of bisulfite sequencing, which can be applied to single candidate loci, subsets of the whole genome (e.g. RRBS), or as WGBS. Bisulfite sequencing of candidate loci is cost-effective and straightforward, but is critically dependent on the appropriate choice of candidate gene. This can be difficult in the absence of prior information suggesting that a genomic region is likely to be differentially methylated. In chapters two and four of this thesis I identify several candidate loci that are differentially methylated in adults from different developmental temperatures, in adults acclimated to different temperatures, and in stickleback reared at different salinities. In chapter five I describe the effects of maternal stress on gene expression patterns and many of the genes identified are known be regulated by DNA methylation (e.g. *nr3c1*). Taken together, these data chapters highlight several candidate loci that could be studied using a cost effective candidate approach that could further investigate environmentally dependent relationships between DNA methylation and gene expression.

In contrast, WGBS provides complete information about the methylation status of every cytosine in the genome, but this technique remains prohibitively expensive for many studies. RRBS is thought to provide cost effective alternative that captures a subset of the methylated

regions of the genome at a more reasonable cost. Similarly, the recently developed non-bisulfite-dependent method of EpiRADseq (Schield *et al.* 2016) represents another approach to increase the tractability of high-resolution epigenomic techniques. However, based on results described in chapters two and three, it is difficult to determine the extent to which the effects of developmental temperature on adult gene expression patterns may be associated with changes to DNA methylation patterns when using a reduced representation approach such as RRBS or EpiRADseq. RRBS covers a very small subset of the CpG loci in the genome making it very difficult to correlate changes in DNA methylation in specific regions of interest (ie. regions near specific genes), or to datasets with a small number of differentially expressed genes. Instead, RRBS may be particularly useful for situations where changes in transcript abundance for a large number of genes are observed, such as in the adult thermal acclimation response, so that the odds of obtaining DNA methylation information near differentially expressed genes are better. Even under these conditions, RRBS does not provide a comprehensive analysis of the DNA methylation state of differentially expressed genes, but it can provide evidence of a role for DNA methylation in the regulation of genes involved in key biological processes.

One key challenge for epigenomic studies of DNA methylation is that these studies examine correlations between changes in methylation patterns and phenotypic changes, which does not directly establish causation. To demonstrate that changes DNA methylation levels are causing the observed changes in phenotype requires direct functional assays such as mutagenesis of the putatively important methylated sites. New technologies such as the CRISPR/Cas9 endonuclease system (Hsu *et al.* 2014) should allow these causal links to be established. This technique has been proposed to allow targeted genetic manipulation of essentially any species and has already been successfully applied in a marine fish species (Aluru *et al.* 2015), and recent

technological advances (eg CRISP-Cas SunTag-directed DNMT3A; Huang *et al.* 2017b) have modified the CRISPR/Cas9 system such that is now possible to modify the DNA methylation level of specific target regions of the genome.

6.5 Future epigenomic research in fishes

One of the biggest unresolved issues in epigenomic research is determining if, and understanding how, epigenetic marks such as DNA methylation are transferred from one generation to the next. Studies in mammalian systems suggest that DNA methylation patterns are largely erased and replaced during early development, thus the efficacy of transgenerational epigenetic inheritance in vertebrates has been in doubt. Earlier in this chapter I argued that ecologically and evolutionarily relevant epigenetic changes do not necessary have to be heritable from one generation to the next to be important for adaptive evolution and that the environment may play a large role in establishing epigenetic patterns in a population. However, my intent was not to suggest that heritable epigenetic variation does not exist, or that heritable epigenetic variation is not an important form of heritable variation. Epidemiological studies in humans, for example, clearly show that maternal condition can have transgenerational effects on DNA methylation patterns that are associated with increased susceptibility of developing chronic diseases (Babenko *et al.* 2015), yet how these epigenetic effects are maintained during development is not well understood. Results from chapter five of this thesis suggest that many of these maternal effects may be acting through conserved pathways in mammals and fish, yet fish have many unique attributes compared to other model systems that make them a powerful system in which to investigate the mechanisms of transgenerational epigenetic inheritance.

In mammals, an environmentally induced phenotype must be maintained at least to the F3 generation to convincingly demonstrate a heritable epigenetic effect. This is because exposure of the gestating female (F0) simultaneously exposes the developing embryo (F1) and the developing germ line of the embryo (F2). In contrast, in fishes only the mother (FO) and her eggs (F1) are exposed. Thus, in fishes it is possible to conclusively demonstrate an epigenetic effect by the F2 generation. In addition, because many fishes develop externally, it is relatively straightforward to manipulate the environment during development, especially in comparison to the challenges of these approaches in mammals. Many species also have large clutch sizes, which allows for full-sib comparisons by using a split-clutch design in which multiple members of the same family are exposed to different treatments. Similarly, a split clutch design can be used to generate multiple half-sib families, which could help to disentangle paternal and maternal effects.

Fishes are particularly well suited for future research on the role of DNA methylation in sex-determination and the evolution of sex chromosomes. Differential methylation has been shown to be involved in the sex-specific regulation of key genes involved in ESD mechanisms (Navarro-Martín *et al.* 2011; Chen *et al.* 2014; Shao *et al.* 2014; Wen *et al.* 2014), establishing a foundation in which to build on our understanding of the role of DNA methylation in the evolution of ESD processes. The data presented in chapter 4 describe an apparent association between sex-specific methylation patterns and the evolution of heteromorphic sex chromosomes. While the X and Y chromosomes in stickleback are at a relatively early stage of differentiation, sex chromosome systems in other fish species are independently derived and are thought to be at various stages of differentiation (Mank *et al.* 2006; Wright *et al.* 2016), providing a model framework to investigate the theories described by Gorelick (2003) and the roles of DNA

methylation as the underlying mechanism regulating the evolution of sex determination mechanisms.

Although fish and mammals share many common features of their methylation patterns, which are distinct from the patterns in other major taxonomic groups such as the invertebrates, fungi and plants (Zemach *et al.* 2010), there are also fundamental differences in methylation patterns and mechanisms between fish and mammals (Goll & Halpern 2011) that are interesting for future research. For example, studies in zebrafish suggest that fish and mammals may differ in the extent to which methylation patterns are erased early in development, with current studies suggesting that fish retain a higher proportion of methylated sites (Jiang *et al.* 2013; Potok *et al.* 2013). Whether other species of fish have similar developmental methylation levels and what the mechanisms are that either retain or re-establish epigenetic patterns is unknown.

Fish genomes also contain additional DNA methyltransferase genes not present in the genomes of other organisms. As in mammals, the major proteins involved in DNA methylation in fishes are the DNA methyltransferases (DNMTs). The zebrafish genome contains 8 DNA methyltransferase orthologs: one *dnmt1* ortholog, one *dnmt2* ortholog, two *dnmt3a* orthologs, and four *dnmt3b* orthologs, although they lack an ortholog of the mammalian *dnmt3L* isoform (Wu *et al.* 2011). The teleosts are known to have undergone a whole-genome duplication relative to mammals (Jaillon *et al.* 2004), thus zebrafish might be expected to have two copies of each mammalian Dnmt family member. This suggests that the duplicates of *dnmt1* and *dnmt2* were lost during the rediploidization following the whole genome-duplication event, and that *dnmt3b* has undergone an additional round or rounds of duplication in zebrafish following the teleost whole-genome duplication. Whether these genes facilitate a unique functional role of DNA methylation, and the evolution of this gene family in fish remains to be studied. For example, the

lack of a *dnmt3l* gene in zebrafish is consistent with the reduced importance of imprinting in fishes. For example, in contrast to mammals, homozygous diploid clones are viable in fishes demonstrating that imprinting must be fundamentally different in fishes compared to mammals (Streisinger *et al.* 1981). *DNMT3L* is also absent from amphibian and bird genomes, suggesting that this gene may have arisen in mammals along with the evolution of X-inactivation (Yokomine *et al.* 2006).

6.6 Conclusions

The threespine stickleback is an effective model system for studying the mechanisms of natural selection and the evolution of adaptive phenotypes. In this thesis I have demonstrated a relationship between DNA methylation and several key ecological and evolutionary processes, which builds on the rich body of research in stickleback and establishes a new base from which to continue to examine and develop the ecological and evolutionary of role(s) of DNA methylation.

Because there was very little empirical data that demonstrates a relationship between environmental conditions and epigenetic processes in stickleback, I started exploring this relationship by investigating plasticity of DNA methylation patterns and gene expression at multiple timescales in response to changes in temperature, salinity, and maternal stress. From these studies, we now know that DNA methylation patterns are dynamically regulated in response to several key environmental factors that are thought to drive directional selection and divergence of many phenotypic traits in stickleback. I also show that the dynamic regulation of DNA methylation patterns is controlled by both developmental processes and in adult acclimation responses, which suggests that manipulating DNA methylation patterns is a key

component of developmental and adult phenotypic responses to environmental change. These results also suggest that this dynamic regulation of DNA methylation could contribute to much of the observed epigenetic variation observed between distinct populations of stickleback and should be considered in the interpretations of environmental epigenetic datasets.

I have also demonstrated that DNA methylation patterns along the sex chromosome in stickleback are different between males and females and that these differences correspond to the known evolutionary history of the stickleback sex chromosome. To my knowledge, this is the first time that an epigenetic mechanism has been empirically tested and correlated with sex chromosome evolution in vertebrates. This result not only provides the foundation for future experimental work on the role of DNA methylation in sex chromosome evolution, but also to addresses fundamental questions about the relationship between DNA methylation and genetic divergence using sex chromosomes as a model.

Furthermore, I have demonstrated that the effects of maternal stress differ between male and female offspring in stickleback. This result is particularly important because it suggests that the adaptive potential of transgenerational environmental effects might depend on the sex of the offspring. These data also suggest that the effects of maternal stress gene expression in offspring likely impact similar cellular mechanisms in both stickleback and mammalian systems suggesting that future research in this area may have broader implications beyond the ecology and evolution of stickleback.

Taken together, the results of my research clearly demonstrate the high degree of plasticity in DNA methylation and gene expression in response to environmental change across multiple time-scales, and highlight potential role of variation between sexes in these epigenetic processes.

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Appendices

Appendix A Supporting information for chapter two

Table A.1: Sample sizes from each family in each developmental treatment

	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6
12 °C Development	2	0	1	2	1	0
18 °C Development	1	1	0	3	0	1
24 °C Development	2	1	1	2	0	0

Table A.2: Genomic mean and median percent methylation

Treatment	Mean genomic percent DNA methylation	Standard deviation	Median genomic percent DNA methylation	Standard deviation
12 °C development, 18 °C acclimation	73.66%	2.21%	82.62%	3.68%
24 °C development, 18 °C acclimation	72.31%	0.89%	80.30%	0.74%
18 °C development, 18 °C acclimation	72.03%	2.20%	80.98%	3.32%
18 °C development, 5 °C acclimation	73.62%	1.24%	83.38%	2.07%
18 °C development, 25 °C acclimation	73.96%	1.48%	84.12%	3.56%

n=6

Table A.3: Kolmogorov-Smirnov test statistics

	p-value	D statistic
12 °C Development, 18 °C acclimation	2.2 E-16	0.02336
24 °C Development, 18 °C acclimation	2.2 E-16	0.042084
18 °C Development, 5 °C acclimation	2.2 E-16	0.05126
18 °C Development, 25 °C acclimation	2.2 E-16	0.081229

Table A.4: Difference in percent DNA methylation level for DMRs common to all treatments

	DMR position		12 °C Development		24 °C Devlopment		5 °C Acclimation		25 °C Acclimation	
chr	start	end	qvalue	DiffMeth	qvalue	DiffMeth	qvalue	DiffMeth	qvalue	DiffMeth
groupI	9551801	9551900	3.52E-02	21	1.29E-02	23	9.78E-04	26	1.03E-02	24
groupI	24585101	24585200	1.23E-05	-16	6.34E-05	-14	3.24E-05	-15	9.61E-04	-13
groupI	27994101	27994200	7.90E-05	20	4.49E-03	17	2.48E-08	28	6.72E-03	17
groupII	18989201	18989300	8.13E-06	15	4.91E-08	18	3.00E-16	24	3.31E-04	16
groupII	21905101	21905200	1.44E-02	10	5.17E-03	11	4.30E-06	15	5.37E-03	11
groupIII	879601	879700	5.51E-09	23	3.10E-06	19	4.03E-03	12	1.15E-15	31
groupIII	9626701	9626800	2.23E-67	32	3.89E-07	12	1.18E-18	19	2.31E-30	25
groupIV	132901	133000	1.57E-03	14	3.36E-05	16	7.14E-03	11	9.56E-03	13
groupIV	2314301	2314400	2.43E-15	-10	8.36E-17	-10	9.82E-30	12	3.58E-34	-15
groupIV	2758901	2759000	3.15E-02	14	3.08E-02	15	3.91E-03	18	2.05E-03	19
groupIV	11319801	11319900	4.00E-04	-14	3.95E-03	-11	4.07E-04	-13	4.39E-06	-17
groupIV	12200201	12200300	1.07E-03	17	4.32E-03	16	6.87E-06	22	8.46E-04	19
groupIV	29800001	29800100	1.03E-06	15	5.09E-08	18	7.18E-19	26	6.79E-13	28
groupIX	353601	353700	9.20E-06	-13	4.96E-04	-11	4.24E-14	-22	4.86E-11	-18
groupIX	8238301	8238400	7.43E-03	-15	2.42E-04	-19	2.11E-03	-15	6.51E-04	-17
groupIX	17934101	17934200	4.70E-02	13	6.68E-03	16	5.86E-03	15	2.00E-02	16
groupV	9288901	9289000	3.64E-04	-14	1.34E-02	-11	6.73E-10	-25	1.27E-02	-10
groupVI	1530101	1530200	4.31E-08	26	5.66E-03	16	1.97E-11	27	8.73E-03	14
groupVI	4132101	4132200	1.01E-37	13	1.80E-12	-12	1.11E-52	-27	3.86E-37	13
groupVII	5901801	5901900	4.96E-11	-20	3.75E-18	-27	3.17E-51	-55	9.13E-06	-12
groupVII	15623001	15623100	4.60E-06	21	9.36E-03	14	3.56E-04	17	6.62E-04	17
groupVIII	351301	351400	3.04E-02	12	1.23E-03	18	1.08E-08	27	1.33E-05	27
groupX	97101	97200	9.08E-10	36	9.73E-05	22	2.46E-15	37	9.17E-04	23
groupX	2872701	2872800	4.20E-06	-16	2.29E-04	-12	6.29E-05	-13	8.74E-04	-11
groupXI	2274501	2274600	8.61E-05	20	1.22E-02	15	6.30E-07	24	2.93E-06	24
groupXII	7504001	7504100	7.04E-04	14	7.12E-04	14	3.86E-06	18	1.42E-02	11
groupXIII	8471801	8471900	9.75E-06	32	1.38E-04	30	4.85E-09	37	1.28E-03	24
groupXIV	3410601	3410700	2.65E-02	-14	4.41E-02	-13	3.38E-02	-13	2.62E-02	-13
groupXIV	3943001	3943100	3.40E-02	-14	1.56E-02	-14	4.76E-05	-23	8.94E-03	-12
groupXIV	10441601	10441700	2.17E-02	-14	1.60E-04	-20	7.08E-07	-25	7.12E-03	-16
groupXIX	8793401	8793500	8.41E-08	22	2.15E-06	17	1.08E-09	23	1.44E-03	14
groupXIX	10454801	10454900	3.29E-03	-13	1.49E-07	-21	2.58E-10	-23	5.99E-03	-12
groupXIX	15351001	15351100	2.35E-03	15	6.51E-03	13	1.44E-04	18	2.20E-03	15
groupXV	1970801	1970900	1.34E-04	-21	5.07E-06	-25	9.26E-04	-17	1.54E-03	-16
groupXV	4546301	4546400	4.46E-187	36	1.83E-100	27	1.56E-52	19	4.13E-109	29
groupXVI	8645601	8645700	5.14E-03	23	2.09E-02	19	5.51E-03	20	3.47E-02	17
groupXVI	17985101	17985200	2.93E-06	32	4.05E-04	26	6.06E-08	38	1.18E-07	37
groupXVI	17985201	17985300	8.29E-05	23	1.01E-02	16	4.03E-06	26	1.54E-08	31
groupXVII	5773201	5773300	8.78E-03	-14	1.04E-02	-12	9.66E-11	-27	4.55E-02	-12
groupXVII	11445901	11446000	8.57E-04	16	1.13E-03	16	2.00E-05	20	7.49E-03	14
groupXVIII	7284901	7285000	3.63E-02	16	4.98E-02	13	3.09E-04	23	3.65E-05	24
groupXVIII	15625401	15625500	1.20E-12	26	3.85E-06	18	2.37E-04	17	1.06E-04	18
groupXXI	5934501	5934600	5.07E-06	-28	1.44E-05	-30	5.01E-06	-27	1.62E-04	-24
groupXXI	8811701	8811800	7.07E-04	12	2.27E-03	10	1.32E-04	13	1.05E-12	30
scaffold_1331	2901	3000	3.73E-05	-20	9.70E-05	-19	2.79E-07	-22	3.76E-07	-25
scaffold_1498	5201	5300	1.62E-07	11	4.63E-06	10	1.50E-13	16	7.16E-11	15
scaffold_167	70001	70100	4.98E-12	-25	2.32E-03	-15	3.08E-13	-24	3.31E-10	-24
scaffold_235	12101	12200	4.14E-03	17	8.66E-03	17	1.11E-05	23	1.07E-03	19
scaffold_515	5601	5700	5.90E-16	16	2.25E-13	13	5.99E-45	22	6.37E-35	18
scaffold_990	401	500	2.99E-03	22	4.79E-02	13	6.34E-08	32	2.42E-03	19

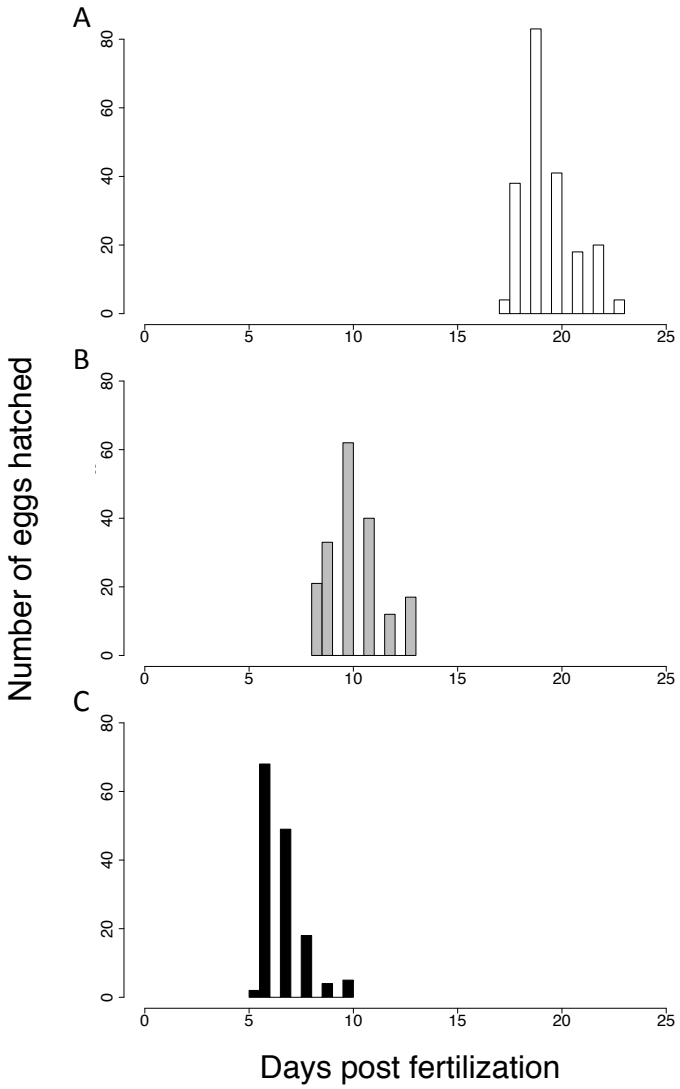

Figure A.1: The effects of developmental temperature on time to hatch of threespine stickleback embryos. Histograms represent the number of threespine stickleback embryos that hatched on a particular day post fertilization when reared at 12 °C (A; white), 18 °C (B; grey), or 24 °C (C; black) . Day 0 represents the day of fertilizaiton.

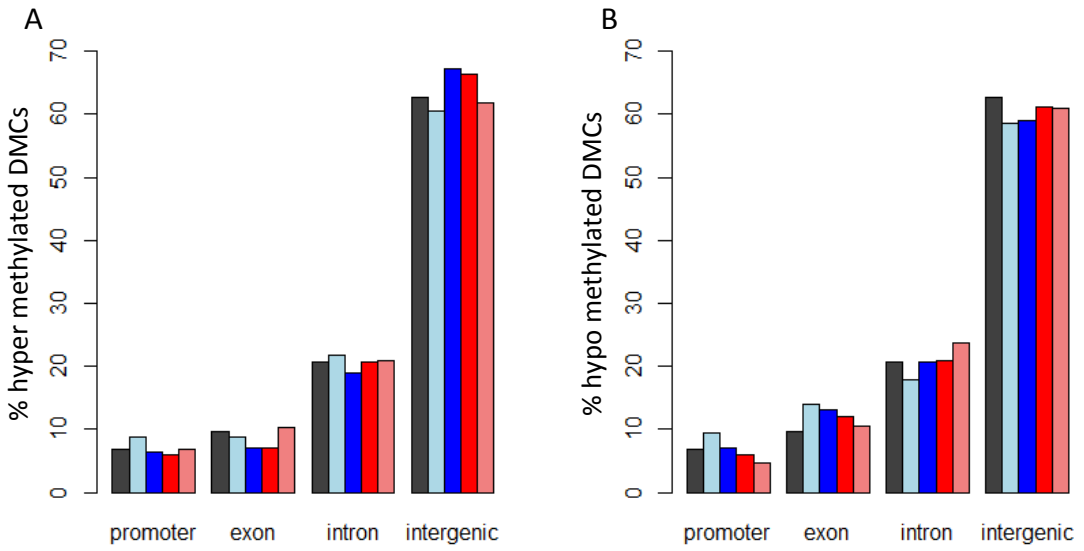

Figure A.2: Genomic distributions of (A) significantly hypermethylated and (B) significantly hypomethylated cytosines for each treatment. Different colors correspond to the different treatments with 12 °C development as light blue, 5 °C acclimation as dark blue, 25 °C acclimation as red, 24 °C development as pink. The background distribution of the sequenced CpG loci is in grey and is replicated in panels A and B.

Appendix B Supporting information for chapter three

Table B.1: RNA-sequencing library sizes

Sample ID	Developmental Temperature (°C)	Acclimation Temperature (°C)	Sequencing library size (# of reads)
TSS1	12	18	31366410
TSS2	12	18	36206008
TSS3	12	18	41908012
TSS4	12	18	32977256
TSS5	12	18	35373834
TSS6	12	18	44243698
TSS7	18	18	32459910
TSS8	18	18	41973418
TSS9	18	18	37587956
TSS10	18	18	40668456
TSS11	18	18	45922122
TSS12	18	18	36567744
TSS13	24	18	39881784
TSS14	24	18	40313224
TSS15	24	18	41374452
TSS16	24	18	37452680
TSS17	24	18	36030292
TSS18	24	18	31786428
TSS19	18	5	46315050
TSS20	18	5	41645694
TSS21	18	5	28716160
TSS22	18	5	36478166
TSS23	18	5	35062444
TSS24	18	5	42850618
TSS25	18	25	49687968
TSS26	18	25	43084226
TSS27	18	25	35147542
TSS28	18	25	33675164
TSS29	18	25	34663342
TSS30	18	25	40017684
TSS31	18	18	57013628
TSS32	18	18	63133950

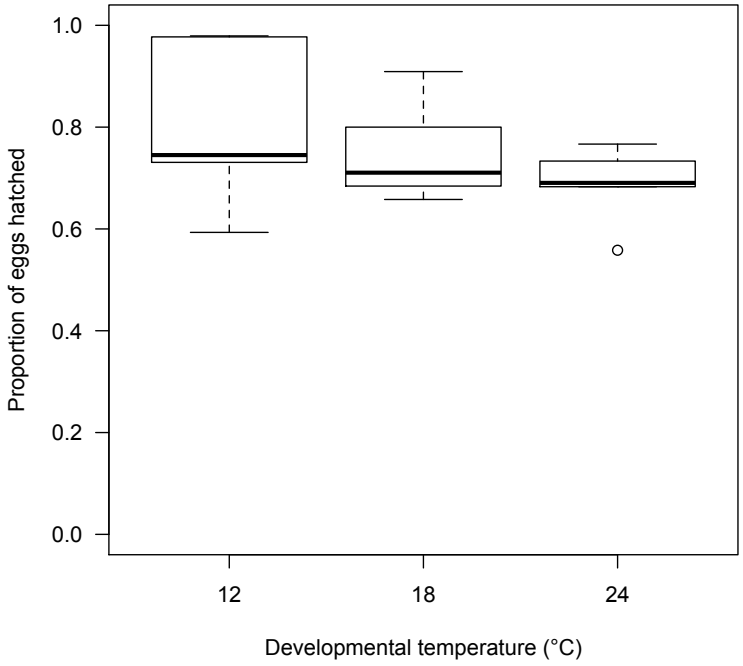

Figure B.1: Hatching success of threespine stickleback reared at 12, 18, and 24 °C. Hatching success is presented as the proportion of fertilized eggs that hatched per family (n=6 families/temperature). The lines in each box plot indicates the median proportion of hatched individuals for each temperature, the box defines the interquartile range (IQR), and the whiskers represent the maximum and minimum values, excluding values greater than 1.5x IQR (which are shown as individual points). There were no significant differences in hatching success among the temperature treatments (one-way ANOVA $p>0.05$).

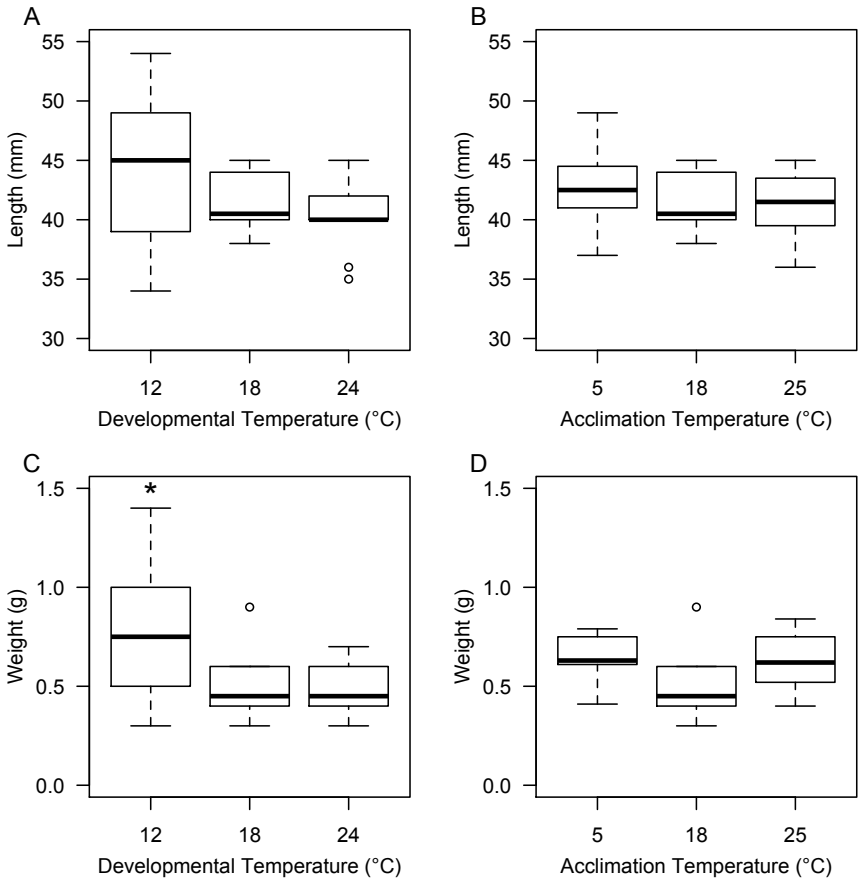

Figure B.2: Length (A/B) and weight (C/D) of nine-month-old threespine stickleback exposed to different temperatures during development until hatch and then kept at 18 °C until nine months old (A/B) or developed at 18 °C to eight months old and then acclimated to different temperatures for four weeks as adults (B/D). The lines in each box plot indicate the median value for each group, the box defines the interquartile range (IQR), and the whiskers represent the maximum and minimum values, excluding values greater than 1.5x IQR (which are shown as individual points). A significant effect of developmental temperature on wet weight was detected by a one-way ANOVA analysis ($p < 0.05$). An asterisk (*) indicates a significant difference in mean value from the other two treatments based on a Tukey's post hoc analysis ($p < 0.05$).

Appendix C Supporting information for chapter four

C.1 Effects of environmental salinity on fertilization and hatching success

Salinity had a significant effect on both fertilization ($p < 2.2 \times 10^{-16}$) and hatching success ($p < 2.2 \times 10^{-16}$). Percent fertilization was highest at 2 ppt, intermediate at salinities from 7 ppt to 21 ppt, and lowest at 28 and 35 ppt (Figure S1). Similarly, the percent of fertilized embryos that hatched was highest at salinities from 2 to 14 ppt, intermediate at 21 ppt, and lowest at 28 and 35 ppt. These data indicate that fertilization and hatching success the "fully plated" marine stickleback ecotype used in this study are optimal at low and brackish salinities and that higher salinities closer to full strength sea water represent a stressful reproductive environment and early developmental environment. Therefore, for subsequent studies of the epigenome we selected the salinities of 2 ppt and 21 ppt as representative of salinity extremes that do not impose excessive mortality in this population.

C.2 Characterizing family effects on DNA methylation patterns in stickleback

Principle component analysis of genome-wide DNA methylation patterns in stickleback clearly separated the samples into three distinct groups based on family, and the first three principle components accounted for 31.73 % of the variation in the dataset (PC1 = 11.03 %, PC2 = 10.65 %, PC3 = 10.05 %; Figure S2). Hierarchical cluster analysis also revealed three major groupings corresponding to the three separate families used in this study. Within each stickleback family individuals clustered either by sex (two of three families) or by environmental salinity (one family, Figure S3). Together, these

data indicate that family has a larger effect on inter-individual variation in DNA methylation levels than either sex or environmental salinity.

C.3 Supporting figures and tables for chapter four

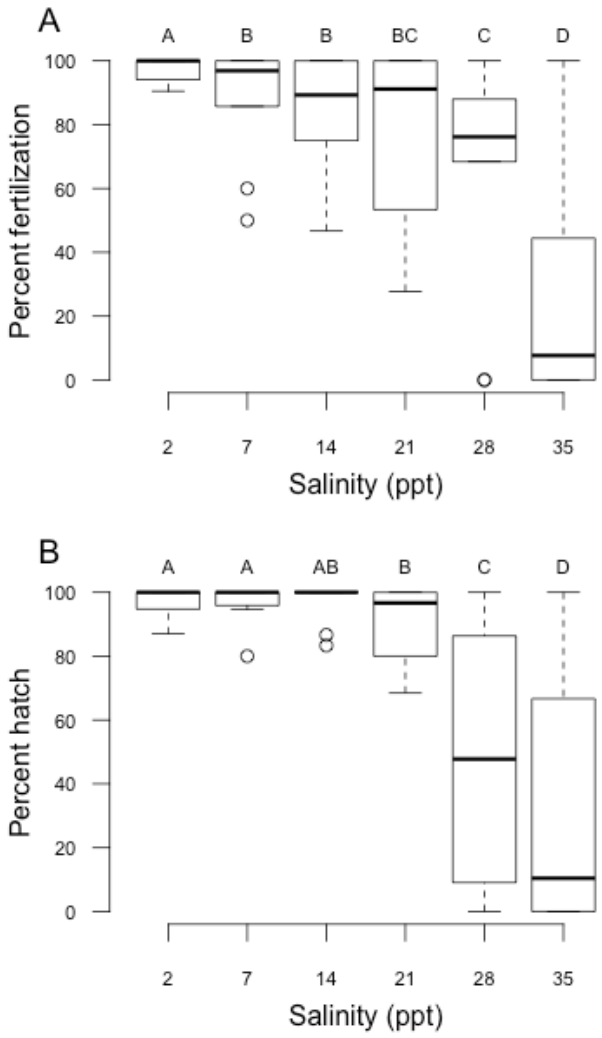

Figure C.1: Percent fertilization and hatching at different salinities. A) Percent of embryos that were successfully fertilized and B) percent of fertilized embryos that successfully hatched at salinities 2, 7, 14, 21, 28, or 35 ppt. Different letters indicate a significant difference between two groups detected using a Tukey test ($q \leq 0.05$).

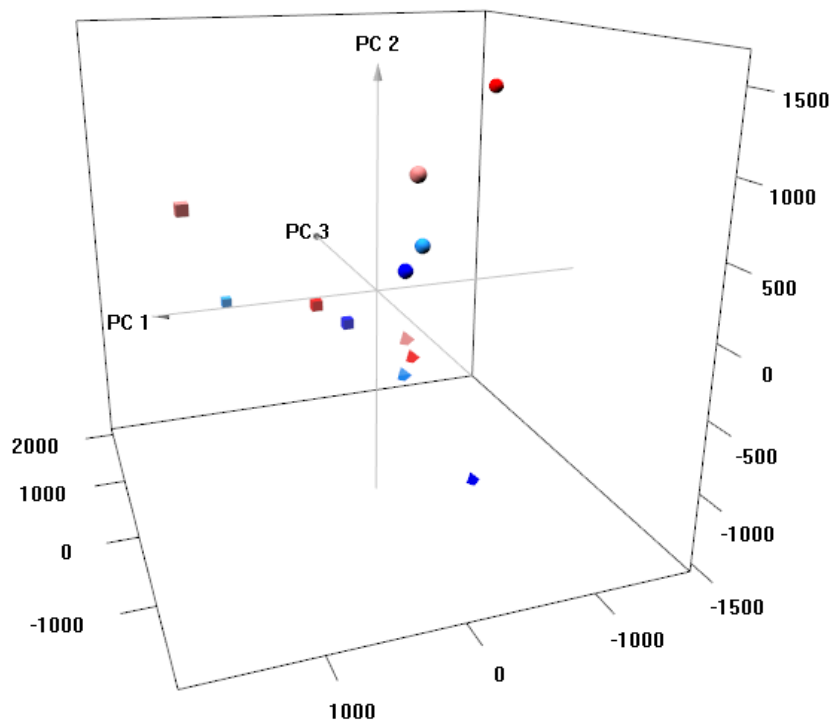

Figure C.2: Principle component analysis of DNA methylation levels. 3D plot of the first three principal components (PC) of the variation in DNA methylation levels among all twelve individuals. PC1 = 11.03 %, PC2 = 10.65 %, and PC3 = 10.05 % of the variation in the data. Shapes represent families. Colors represent rearing salinity and sex. Dark red = females reared at 21 ppt, light red = females reared at 2 ppt, dark blue = males reared at 21 ppt, light blue = males reared at 2 ppt.

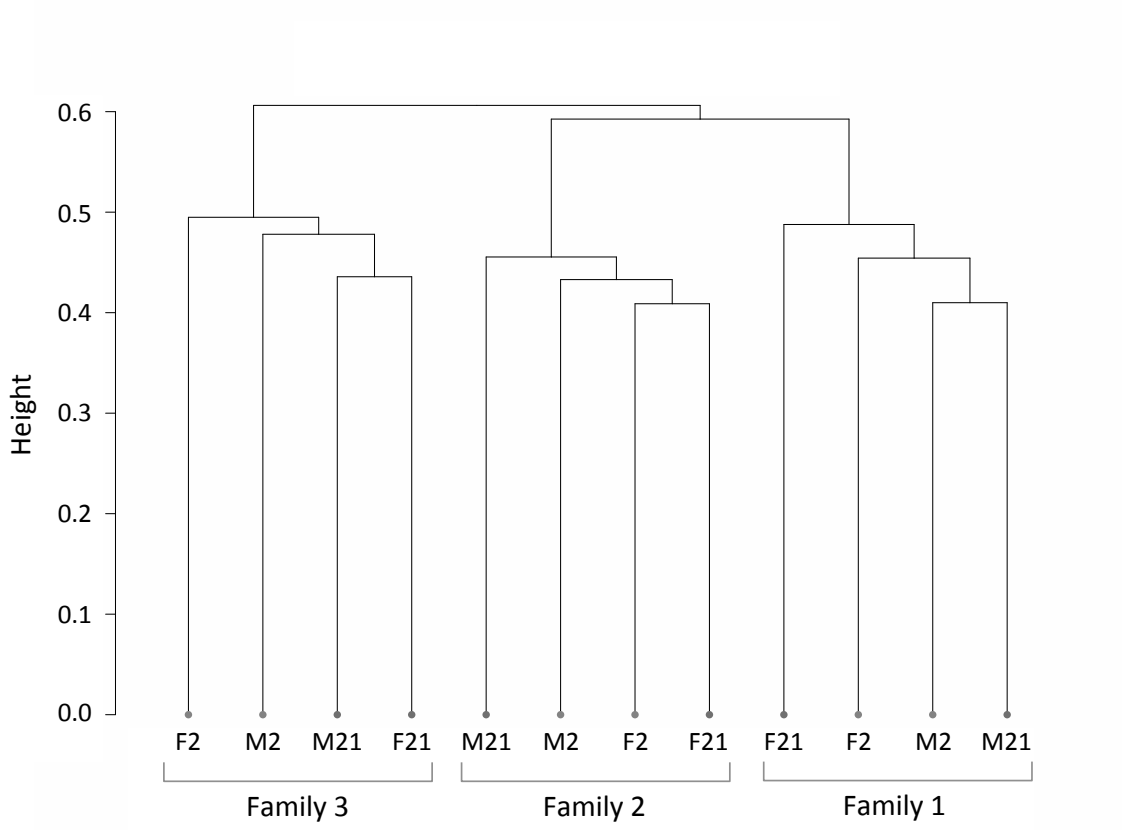

Figure C.3: Cluster analysis of whole genome bisulfite sequencing data of twelve individual threespine stickleback. Each family consists of two females (F) and two males (M) reared at an environmental salinity of either 2 ppt or 21 ppt.

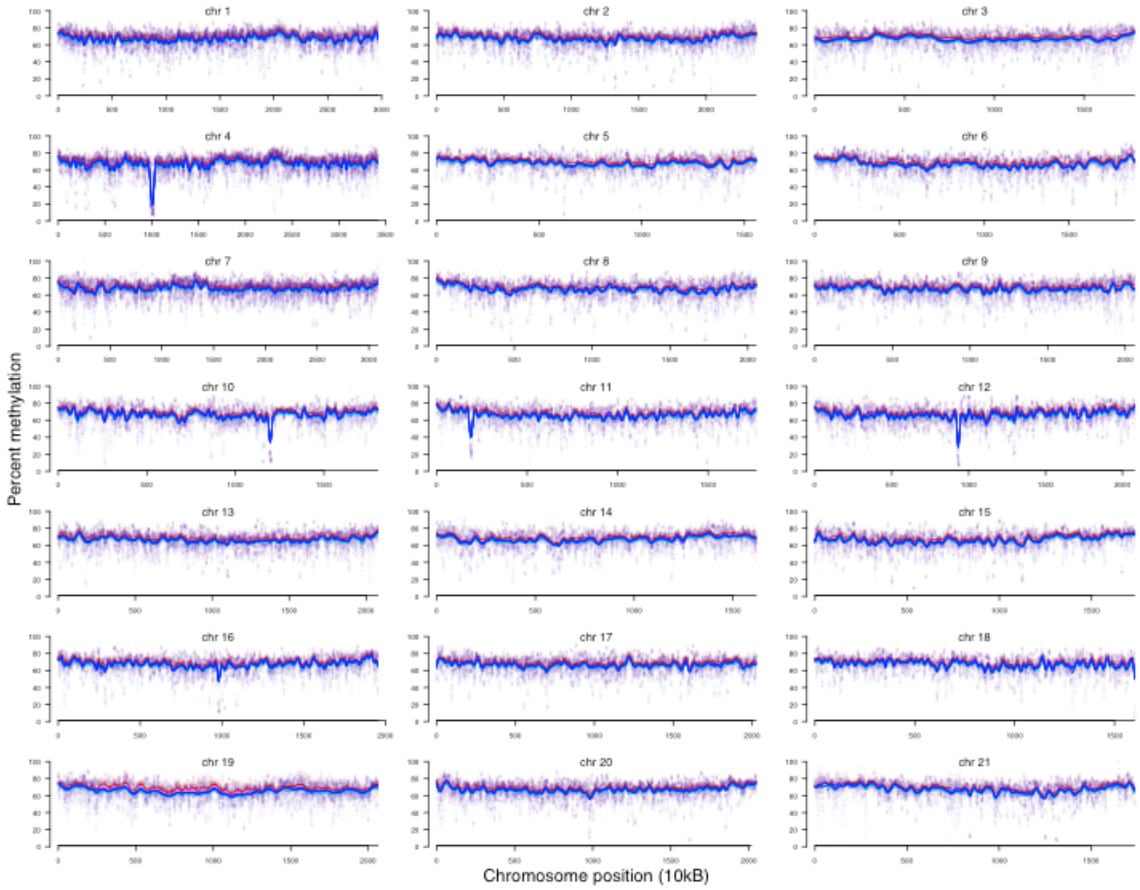

Figure C.4: Chromosomal DNA methylation levels for each individual. Each point represents the mean DNA methylation level in a 10 kb window for an individual stickleback that were either male stickleback reared at 2 ppt (light blue) or 21 ppt (dark blue) or female stickleback reared at 2 ppt (light red) or 21 ppt (dark red). Solid lines represent the smooth spline fit for each individual.

Table C.1: DNA hypomethylated canyon gene annotations

Ensembl Gene ID	Chromosome	Gene Name	Ensembl Gene ID	Chromosome	Gene Name
ENSGACG00000017891	chr4	trpc7	ENSGACG00000005622	chr11	si:dkeyp-101e12.1
ENSGACG00000017895	chr4	not annotated	ENSGACG00000005623	chr11	not annotated
ENSGACG00000017896	chr4	diaph7	ENSGACG00000005626	chr11	not annotated
ENSGACG00000017901	chr4	pcdhgc5 (1 of many)	ENSGACG00000005628	chr11	hoxb4a
ENSGACG00000017905	chr4	pcdhgc5 (1 of many)	ENSGACG00000005631	chr11	hoxa3
ENSGACG00000017907	chr4	not annotated	ENSGACG00000005633	chr11	hoxb2a
ENSGACG00000017910	chr4	not annotated	ENSGACG00000005635	chr11	not annotated
ENSGACG00000017911	chr4	not annotated	ENSGACG00000021289	chr11	not annotated
ENSGACG00000017912	chr4	not annotated	ENSGACG00000022266	chr11	not annotated
ENSGACG00000017913	chr4	pcdh2g17	ENSGACG00000009377	chr12	calcoco1a
ENSGACG00000017914	chr4	pcdh2g12	ENSGACG00000009389	chr12	hoxc13a
ENSGACG00000017915	chr4	pcdh2ac	ENSGACG00000009391	chr12	hoxc12a
ENSGACG00000017917	chr4	not annotated	ENSGACG00000009392	chr12	hoxc11a
ENSGACG00000017919	chr4	not annotated	ENSGACG00000009394	chr12	hoxc10a
ENSGACG00000017920	chr4	si:ch73-233f7.1	ENSGACG00000009396	chr12	hoxc9a
ENSGACG00000017921	chr4	fgf18a	ENSGACG00000009401	chr12	hoxc8a
ENSGACG00000017925	chr4	fbxw11a	ENSGACG00000009405	chr12	hoxc6a
ENSGACG00000017928	chr4	etf1a	ENSGACG00000009416	chr12	hoxc5a
ENSGACG00000022802	chr4	vault	ENSGACG00000009421	chr12	hoxc4a
ENSGACG00000007085	chr10	not annotated	ENSGACG00000009429	chr12	not annotated
ENSGACG00000007090	chr10	not annotated	ENSGACG00000009430	chr12	not annotated
ENSGACG00000007094	chr10	hoxb3a	ENSGACG00000009431	chr12	not annotated
ENSGACG00000007100	chr10	hoxa4	ENSGACG00000009433	chr12	cbx5
ENSGACG00000007108	chr10	hoxa5a	ENSGACG00000009435	chr12	hnrnpa1b
ENSGACG00000007112	chr10	hoxa7	ENSGACG00000009442	chr12	nfe2
ENSGACG00000007123	chr10	hoxa9a	ENSGACG00000021244	chr12	not annotated
ENSGACG00000007128	chr10	not annotated	ENSGACG00000021427	chr12	not annotated
ENSGACG00000007132	chr10	not annotated	ENSGACG00000004548	chr16	hoxd3a
ENSGACG00000007134	chr10	hoxa13a	ENSGACG00000004551	chr16	hoxd4a
ENSGACG00000007148	chr10	evx1	ENSGACG00000004556	chr16	hoxd9a
ENSGACG00000007155	chr10	hibadha	ENSGACG00000004564	chr16	hoxd10a
ENSGACG00000022170	chr10	not annotated	ENSGACG00000004569	chr16	hoxd11a
			ENSGACG00000004574	chr16	hoxd12a
			ENSGACG00000004579	chr16	not annotated
			ENSGACG00000004584	chr16	lnpa
			ENSGACG00000021403	chr16	not annotated

Table C.2: Differentially methylated cytosine distributions

DMC Analysis	Promoter	Exon	Intron	Intergenic	CpGi	CpGi shores	CpGi other
Background Distrubtions for All CpG Loci	12.33	5.75	26.36	55.56	3.34	26.43	70.23
Sex	13.69	5.12	23.94	57.25	2.41	23.57	74.01
Salinity	16.92	6.75	25.81	50.52	3.18	29.94	66.88
Hypomethylated in Females	18.27	6.04	25.44	50.26	3.87	31.11	65.02
Hypermethylated in Females	13.15	5.01	23.77	58.07	2.24	22.69	75.06
Hypomethylated at High salinity	16.94	6.57	25.40	51.09	3.43	28.16	68.41
Hypermethylatd at High Salinity	16.83	7.69	27.88	47.60	1.92	38.94	59.13

Table C.3: Top ten enriched biological process gene ontology categories

DMC analysis	category	over represented pvalue	under represented pvalue	numDEInCat	numInCat	term	ontology
Sex	GO:0007411	1.20E-06*	0.999999299	123	610	axon guidance	BP
Sex	GO:0030036	2.97E-05	0.999987721	38	146	actin cytoskeleton organization	BP
Sex	GO:0030574	3.97E-05	0.999987447	24	77	collagen catabolic process	BP
Sex	GO:0061337	4.24281E-05	0.999983448	33	122	cardiac conduction	BP
Sex	GO:0030198	8.31041E-05	0.99995392	69	330	extracellular matrix organization	BP
Sex	GO:0022617	9.54257E-05	0.999960232	34	132	extracellular matrix disassembly	BP
Sex	GO:0034765	0.000211102	0.999911756	31	121	regulation of ion transmembrane transport	BP
Sex	GO:0051056	0.000313878	0.999851784	38	162	regulation of small GTPase mediated signal transduction	BP
Sex	GO:0030199	0.000536729	0.999840788	16	50	collagen fibril organization	BP
Sex	GO:0007268	0.00069141	0.999553526	90	486	chemical synaptic transmission	BP
Salinity	GO:0051924	1.98E-05	0.999997917	8	37	regulation of calcium ion transport	BP
Salinity	GO:0007156	7.17E-05	0.999982454	14	128	homophilic cell adhesion via plasma membrane adhesion molecules	BP
Salinity	GO:0033280	0.000214507	0.999984769	5	18	response to vitamin D	BP
Salinity	GO:0072006	0.000325122	0.999994625	3	5	nephron development	BP
Salinity	GO:0061444	0.001054532	1	2	2	endocardial cushion cell development	BP
Salinity	GO:0006812	0.001587505	0.999810676	5	27	cation transport	BP
Salinity	GO:0010976	0.001640774	0.999564522	10	101	positive regulation of neuron projection development	BP
Salinity	GO:0007096	0.001692083	0.999930411	3	8	regulation of exit from mitosis	BP
Salinity	GO:0034765	0.001958714	0.999425251	11	121	regulation of ion transmembrane transport	BP
Salinity	GO:0051056	0.002457297	0.999165564	13	162	regulation of small GTPase mediated signal transduction	BP

*p-value < 0.05 after FDR correction

Table C.4: Top ten enriched cellular component gene ontology categories

DMC analysis	category	over represented pvalue	under represented pvalue	numDEInCat	numInCat	term	ontology
Sex	GO:0016020	1.07E-06*	0.999999226	345	2056	membrane	CC
Sex	GO:0045211	6.89E-06*	0.999996657	62	267	postsynaptic membrane	CC
Sex	GO:0043195	8.1882E-05	0.999971094	26	90	terminal bouton	CC
Sex	GO:0031095	0.000151272	0.99998868	7	11	platelet dense tubular network membrane	CC
Sex	GO:0042734	0.00032697	0.999877656	24	87	presynaptic membrane	CC
Sex	GO:0016529	0.000370163	0.999900351	15	44	sarcoplasmic reticulum	CC
Sex	GO:0008021	0.000442366	0.999811842	29	115	synaptic vesicle	CC
Sex	GO:0030054	0.000533742	0.999650229	100	546	cell junction	CC
Sex	GO:0030424	0.000552069	0.999686132	58	284	axon	CC
Sex	GO:0005903	0.000741204	0.999751522	18	61	brush border	CC
Salinity	GO:0005911	0.000646235	0.999782529	16	195	cell-cell junction	CC
Salinity	GO:0005886	0.00243756	0.998240739	141	3505	plasma membrane	CC
Salinity	GO:0005891	0.002512918	0.999570773	6	43	voltage-gated calcium channel complex	CC
Salinity	GO:0005958	0.003095305	0.999965854	2	3	DNA-dependent protein kinase-DNA ligase 4 complex	CC
Salinity	GO:0060187	0.003095305	0.999965854	2	3	cell pole	CC
Salinity	GO:1990423	0.003095305	0.999965854	2	3	RZZ complex	CC
Salinity	GO:0043235	0.003447861	0.998841329	12	149	receptor complex	CC
Salinity	GO:1902711	0.004251369	0.999530043	4	21	GABA-A receptor complex	CC
Salinity	GO:0030424	0.005489377	0.997564702	18	284	axon	CC
Salinity	GO:0070852	0.006032377	0.99955649	3	12	cell body fiber	CC

*p-value < 0.05 after FDR correction

Table C.5: Top ten enriched molecular function gene ontology categories

DMC analysis	category	over represented pvalue	under represented pvalue	numDEInCat	numInCat	term	ontology
Sex	GO:0005509	4.58E-06*	0.999997239	124	632	calcium ion binding	MF
Sex	GO:0004252	5.50E-05	0.999979802	29	103	serine-type endopeptidase activity	MF
Sex	GO:0000146	7.74151E-05	0.999986659	12	27	microfilament motor activity	MF
Sex	GO:0005089	8.1882E-05	0.999971094	26	90	Rho guanyl-nucleotide exchange factor activity	MF
Sex	GO:0005516	0.000103789	0.999948231	50	221	calmodulin binding	MF
Sex	GO:0003779	0.000106332	0.999937966	79	393	actin binding	MF
Sex	GO:0030020	0.000123928	0.999994691	6	8	extracellular matrix structural constituent conferring tensile strength	MF
Sex	GO:0005220	0.00031733	1	4	4	inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity	MF
Sex	GO:0038191	0.000346476	0.999948396	9	19	neuropilin binding	MF
Sex	GO:0005524	0.000359602	0.99972823	243	1492	ATP binding	MF
Salinity	GO:0005261	0.000367817	0.999969914	5	20	cation channel activity	MF
Salinity	GO:0005509	0.000800742	0.999592781	36	632	calcium ion binding	MF
Salinity	GO:0005096	0.001254622	0.999502823	19	268	GTPase activator activity	MF
Salinity	GO:0004222	0.002704885	0.999228699	10	108	metalloendopeptidase activity	MF
Salinity	GO:0008381	0.003095305	0.999965854	2	3	mechanically-gated ion channel activity	MF
Salinity	GO:0030594	0.003095305	0.999965854	2	3	neurotransmitter receptor activity	MF
Salinity	GO:0005230	0.0035311	0.999632183	4	20	extracellular ligand-gated ion channel activity	MF
Salinity	GO:0046872	0.004488283	0.997071819	64	1420	metal ion binding	MF
Salinity	GO:0004890	0.005064548	0.999407937	4	22	GABA-A receptor activity	MF
Salinity	GO:0019992	0.006032377	0.99955649	3	12	diacylglycerol binding	MF

Appendix D Supporting information for chapter five

D.1 Fish collection and acclimation

Adult threespine stickleback (*G. aculeatus*) used in this study were collected in May 2013 from Oyster lagoon (British Columbia, Canada, GPS: 49.6121,-124.0314) and transported to UBC. This population contains almost entirely fish of the fully plated "marine" ecotype (Barrett *et al.* 2008), and all fish used in this experiment were fully plated. Captured fish were transported to UBC where they were separated into six 110-litre glass tanks (20 fish/tank) and acclimated to 20 ppt salt water (instant ocean), 18°C and 14:10h light:dark photoperiod. Fish were fed daily to satiation with Hakari Bio-Pure frozen Mysis Shrimp. Fish were acclimated to these conditions for three weeks prior to the initiation of the experimental stress treatment.

D.2 Experimental stress treatment, crossing design and rearing

Following the three-week acclimation period to laboratory conditions, three tanks were designated as the unstressed treatment (tank #'s 1-3) and three tanks were designated as the stressed treatment (tank #'s 4-6). The four sides of the tanks containing the unstressed group were wrapped in black plastic to reduce visual disturbances. The top of the tanks as well as a small window on the front of each tank were left uncovered to allow for continued health monitoring of the fish during the treatment. Fish in the stressed treatment were chased once daily with an 8 in x 6 in blue aquarium fish net for 30sec before being captured and held out of water for a further 30 sec and then returned to their tank.

After two weeks, gravid females were harvested from both the stressed and unstressed treatments. As not all females reached reproductive readiness at the same time, eggs were

harvested over the course of 4 days. The daily stress treatment continued throughout. Six unstressed males were crossed to six unstressed females and six stressed females to generate a total of twelve families (6 half-sib pairs). Gravid females were removed from the experimental tanks and eggs were collected by gently applying pressure to the abdomen with a thumb and forefinger. Testes were dissected from unstressed males and macerated in a 1.75 mL microcentrifuge tube containing 100 uL Ginzberg's fish ringers solution. Eggs collected from females were arranged as a monolayer in petri dishes containing 5mL of 20ppt seawater. 50 µL of the sperm solution was applied directly on the egg mass and left for 30 min to allow fertilization to occur. Following fertilization, an additional 10mL of 20 ppt saltwater was added to each petri dish. Petri dishes were partially covered to prevent water loss from evaporation and allow for surface gas exchange. Eggs were monitored twice daily during which time any unfertilized eggs were removed and 10 mL of water was changed to prevent mold growth. After all fish in a petri dish had hatched and the yolks had been absorbed (~15 days post fertilization), larvae were transferred to 110 L glass aquaria equipped with hanging box filters (Aquaclear) and sponge filters for filtration and aeration. Larval stickleback were fed live brine shrimp nauplii twice daily ad libitum until they were large enough to feed on frozen Mysis shrimp. Each family was reared in a separate tank. Although density varied because family size varied, as the juvenile stickleback grew towards adulthood families were culled to a maximum final density of ~1 fish per 5 L. After one year post hatch, and prior to reaching sexual maturity, whole brain tissue was dissected and immediately frozen in liquid nitrogen and stored at -80°C until further use. Family information for the fish used for RNA-seq is provided in Table D.1 (Appendix D).

D.3 DNA Isolation and Sex Identification

Genomic DNA was isolated from fin clips using the Qiagen DNeasy Blood & Tissue mini spin columns (Product # 69504). The sex of each fish was determined by PCR (Toli *et al.* 2016). PCR amplification was conducted using PTC-200 a Peltier Thermal Cycler (MJ Research) in 10 µl reactions containing 0.5 µM of each primer, 5 ng DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 0.25 units Taq polymerase (Fermentas) with the following thermal cycling conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min with a final extension of 72 °C for 10 min. PCR products were separated by gel electrophoresis on a 2% agarose gel containing SYBR safe DNA gel Stain (product # S33102; ThermoFisher Scientific).

D.4 Plasma cortisol

To verify that the maternal stress treatment induced an increase in the circulating cortisol levels, two gravid female fish were randomly selected from each of the three control and three experimental tanks (n = 6 for each treatment) and rapidly sacrificed with a cranial blow. Blood was collected into heparinized capillary tubes by caudal severance. Plasma was separated by centrifugation, frozen in liquid nitrogen and stored at -80°C until use. Steroids were extracted with diethyl ether, and cortisol levels were assessed using an enzyme-linked immunosorbent assay (ELISA) (product# 402710; Neogen) according to the manufacturer's instructions.

D.5 RNA isolation

Total RNA was isolated from stickleback brain tissue using TRIzol Reagent (Invitrogen Life Technologies) following the manufacturer's recommended protocol. Briefly, tissue was

homogenized in 1 mL of TRIzol containing approximately 10, 1.0 mm ceria stabilized zirconium oxide beads (Next Advance, NY, USA) using a Bullet Blender24 (Next Advance, NY, USA). Total RNA was DNase treated using the Qiagen RNeasy (product # 74104) DNase I (product # 79254) on column DNA digestion protocol. Total RNA was quantified using the QBit® RNA broad range assay kit (product # Q10210; ThermoFisher Scientific) and an Invitrogen™ Qubit® 2.0 Flurometer. RNA quality was assessed using the Agilent RNA 6000 Pico Kit (product # 5067-1514) and an Agilent 2100 Bioanalyzer (Agilent Technologies).

D.6 Gene Ontology and pathway analysis

Orthologous Ensembl gene IDs for stickleback and human and the corresponding human UniProt accession numbers were obtained from the Ensembl biomart database and identifiers for Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) IDs were obtained using the UniProt online mapping tool (http://www.uniprot.org/uploadlists/) to generate a database of stickleback Ensembl gene IDs linked to orthologous human GO and KEGG gene IDs. The keggLink() function in the KEGGREST v1.6.4 R package was then used to obtain KEGG pathway identifiers for the KEGG gene IDs obtained from the UniProt database. A total of 15,646 genes were assigned GO identifiers and 3,213 genes were assigned KEGG pathway IDs using this method. GO and KEGG pathway enrichment analyses was conducted using the goseq (v1.22.0) R package (Young *et al.* 2010). The goseq package contains gene length information for stickleback ("gasAcu1) based on Ensembl gene IDs (“ensGene"). Results from the enrichment analysis were corrected for the false discovery rate (FDR) of multiple comparisons (Benjamini & Hochberg 1995) using a q value cutoff of <0.05.

D.7 Differential expression between males and females

1,255 genes exhibited a significant effect of sex, independent of maternal stress (at an FDR $q<0.05$). Figure D.2 (Appendix D) provides a heatmap summarizing the expression patterns of these genes.

D.8 Effects of increased stringency thresholds on detection of sexually-dimorphic effects of maternal stress

To determine the robustness of the detected sexually dimorphic response to maternal stress we analyzed the RNA-seq data using two different approaches to reduce the false discovery cost. When we applied cutoff of $q<0.01$ (increasing the stringency of the false discovery rate criterion), we detected 821 genes with a significant interaction between the effects of sex and maternal stress (compared with 1650 with a cutoff of $q<0.05$). Figure D.4A (Appendix D) provides a heatmap summarizing the patterns of expression for these genes. When we applied a fold-change cutoff of >2.0 we detected 675 genes with a significant interaction between the effects of sex and maternal stress. Figure D.4B provides a heatmap summarizing the patterns of expression for these genes. If both criteria are applied simultaneously 472 genes are detected with a significant interaction between the effects of sex and maternal stress.

GO-enrichment analysis of these datasets detected different enriched processes in males and females, as was observed in the analyses presented in the main text. Enrichment results for genes with a fold-change greater than two are summarized in Table D.3.

D.9 Candidate genes involved in the stress response and epigenetic regulation

Genes with known roles in the regulation of the glucocorticoid receptor expression and activity exhibited significant interactions between maternal stress and offspring sex are listed in Table D.4. In addition, the early growth response gene *egr,* which is known to regulate the transcription of the glucocorticoid receptor (Szyf *et al.* 2005) demonstrated a significant effect of sex, with higher expression in males compared to females and there was a trend for an interaction between maternal stress and sex ($p = 0.053$)

With respect to genes with known roles in epigenetic processes, we identified several members of the ten-eleven translocation (TET) family of enzymes, which are involved in active DNA demethylation (Li & Zhang 2014). A significant interaction between maternal stress and offspring stress was identified for two members of the TET family of DNA-demethylases, *tet2* and *tet3,* which were up-regulated in male offspring of stressed mothers, but down-regulated in female offspring. These sex-specific effects of maternal stress on the expression of DNA-demethylases suggest a potential role for epigenetic mechanisms influencing the dimorphic expression of genes in males and females from stressed mothers.

In contrast, we did not identify a significant interaction between maternal stress and offspring sex for any of the DNA (Cytosine-5-)-Methyltransferase 3 Alpha (*dmnt3a*) isoforms that are involved in regulating de novo methylation patterns in the genome (Li & Zhang 2014). However, *dnmt3aa* and *dmnt3ab* were both differentially expressed between the sexes and for *dnmt3aa* there was a trend towards an interaction between offspring sex and maternal stress ($p = 0.083$; FDR corrected).

A variety of other genes whose products are known to associate with methylated DNA also exhibited sex-specific effects of maternal stress, including zinc finger and BTB domain

containing 7A (*zbtb7a),* methyl-CpG binding domain protein 5 (*mbd5*), and myocyte enhancer factor 2C (*mef2c*), which are also up-regulated in male, but not female, offspring of stressed mothers. *Zbtb7a* (also referred to as FBI-1) has been shown to interact with the methyl binding domain containing protein *mbd3* to recruit DNA methylation machinery to silence the promoters of target genes (Choi *et al.* 2013). *mbd5* is another member of the MBD protein family that plays an important role regulating expression of genes involved in neural development (Talkowski *et al.* 2011, 2012) and growth (Du *et al.* 2012) but cannot bind methylated DNA (Laget *et al.* 2010). *mbd5* is thought bind heterochromatin by interacting with *mef2c* (Gigek *et al.* 2016).

Genes involved in a variety of other epigenetic mechanisms also exhibited sex-specific effects of maternal stress including bromodomain adjacent to zinc finger domain 2A (*baz2a*), which is involved in gene silencing by recruiting enhancer of zeste 2 polycomb repressive complex 2 subunit (*ezh2*) which has known histone methyltransferase activity (Gu *et al.* 2015).

The GO category "translational gene silencing by RNA" (GO: 0035194) was also significantly enriched among genes that demonstrated a significant interaction between offspring sex and maternal stress. The genes associated with this term include several members of the argonaute (*ago*) and trinucleotide repeat containing 6 (*tnrc*) gene family including *ago2*, *ago3*, *ago4*, *tnrc6a*, *tnrc6b*, *tnrc6c*, and *tnrc6c2.* These genes have been shown to interact with each other and regulate micro RNA (miRNA) silencing activity (Liu *et al.* 2004; Rehwinkel *et al.* 2005; Baillat & Shiekhattar 2009).

D.10 Supporting tables and figures for chapter five

Table D.1: Fish used for RNA-seq

Offspring Fish #	Offspring Sex	Maternal Stress Treatment	Mother #	Mother Tank #	Father #	Father Tank #
UM1	Male	Unstressed	1	1	1	1
UM2	Male	Unstressed	2	1	2	1
UM3	Male	Unstressed	1	1	1	1
UF1	Female	Unstressed	3	2	3	2
UF2	Female	Unstressed	4	3	4	3
UF3	Female	Unstressed	5	1	5	1
SM1	Male	Stressed	6	4	1	1
SM2	Male	Stressed	7	5	4	3
SM3	Male	Stressed	8	4	5	1
SF1	Female	Stressed	9	5	2	1
SF2	Female	Stressed	10	6	6	2
SF3	Female	Stressed	9	5	2	1

Table D.2: RNA quality and library sizes

Sample Name	RNA Integrity Number (RIN)	RNA-seq Library Size
UM1	8.5	11383120
UM2	7.9	11891132
UM3	8.9	22702505
UF1	8.9	21626954
UF2	7.9	18841249
UF3	9.1	31112715
SM1	8.1	18149183
SM2	8.2	16236161
SM3	8.7	18104469
SF1	8.5	15446367
SF2	9.0	24948538
SF3	9.0	27018611

Table D.3: GO enrichment analysis of genes with fold-change >2.0 with a significant interaction between maternal stress and offspring sex

Enriched GO terms of genes expressed at higher levels in males from stressed mothers		Enriched GO terms of genes expressed at higher levels in females from stressed mothers	
Biological Process			
GO:0007268	Synaptic transmission	GO:0044281	Small molecule metabolic process
GO:0043401	Steroid hormone mediate signaling pathway	GO:0044237	Cellular metabolic process
GO:0043484	Regulation of RNA splicing	GO:0022904	Respiratory electron transport chain
		GO:0006414	Translational elongation
		GO:0006415	Translational termination
		GO 0016259	Selenocysteine metabolic process
		GO:0019083	Viral transcription
		GO:0001887	Selenium compound metabolic process
Cellular Component			
GO:0005887	Integral component of plasma membrane	GO:0005743	Mitochondrial inner membrane
GO:0030425	Dendrite		
GO:0045211	Post-synaptic membrane		
Molecular function			
GO:0003700	Transcription factor activity, sequence-specific DNA binding		
GO:0003707	Steroid hormone receptor activity		

Table D.4: Candidate genes with known roles in the regulation of glucocorticoid receptor expression and activity

Gene symbol	Gene name	Protein function
crebbpb	CREB binding protein b	histone acetyltransferase (Weaver *et al.* 2007; McGowan *et al.* 2009; Hellstrom *et al.* 2012)
ncoa1	Nuclear receptor coactivator 1	steroid receptor coactivator protein; (Heitzer *et al.* 2007)
ncoa3	Nuclear receptor coactivator 3	steroid receptor coactivator protein (Heitzer *et al.* 2007)
nrip1b	Nuclear receptor interacting protein 1b	nuclear receptor interacting protein (Windahl *et al.* 1999)
rela	v-rel avian reticuloendotheliosis viral oncogene homolog A	*NF-κB* subunit (Wissink *et al.* 1997)
pou2f1	POU class 2 homeobox 1	POU domain factor with roles in neuroendocrine function (Préfontaine *et al.* 1998; Andersen & Rosenfeld 2001)
pou2f2	POU class 2 homeobox 2	POU domain factor with roles in neuroendocrine function (Préfontaine *et al.* 1998; Andersen & Rosenfeld 2001)

Table D.5: Significantly enriched KEGG pathways

	Enriched KEGG Pathway ID (FDR q < 0.05)	KEGG ID	pvalue	Number of DE Genes With Annotation	Number of Genes With Annotation
All DE Genes	Ribosome	hsa03010	1.81E-08	22	67
	Huntington's disease	hsa05016	2.72E-08	30	90
	Oxidative phosphorylation	hsa00190	3.00E-08	22	65
	Parkinson's disease	hsa05012	2.13E-07	22	71
	Alzheimer's disease	hsa05010	4.37E-07	29	89
	Non-alcoholic fatty liver disease	hsa04932	1.25E-06	19	61
	Nicotine addiction	hsa05033	2.17E-05	14	33
	Glutamatergic synapse	hsa04724	0.00012917	23	72
	Morphine addiction	hsa05032	0.00038931	14	48
	Retrograde endocannabinoid signaling	hsa04723	0.00080708	19	65
DE genes up-regulated in Females	Oxidative phosphorylation	hsa00190	5.66E-08	22	65
	Parkinson's disease	hsa05012	1.23E-06	21	71
	Huntington's disease	hsa05016	6.70E-06	21	90
	Ribosome	hsa03010	1.24E-05	22	67
	Alzheimer's disease	hsa05010	3.39E-05	19	89
	Non-alcoholic fatty liver disease	hsa04932	6.18E-05	16	61
DE genes up-regulated in Males	Nicotine addiction	hsa05033	1.94E-07	14	33
	Morphine addiction	hsa05032	6.79E-07	14	48
	Glutamatergic synapse	hsa04724	2.28E-06	22	72
	Retrograde endocannabinoid signaling	hsa04723	3.86E-06	19	65
	Neuroactive ligand-receptor interaction	hsa04080	2.24E-05	19	120
	Cocaine addiction	hsa05030	0.0001104	10	34
	Cell adhesion molecules	hsa04514	0.00015629	11	41
	GABAergic synapse	hsa04727	0.00017636	12	50
	Rap1 signaling pathway	hsa04015	0.00027316	18	94
	cAMP signaling pathway	hsa04024	0.00094925	19	105
	Transcriptional misregulation in cancer	hsa05202	0.00166575	10	52
	Long-term potentiation	hsa04720	0.00171489	12	43
	MAPK signaling pathway	hsa04010	0.00173098	18	127
	Long-term depression	hsa04730	0.00238347	10	38
	Calcium signaling pathway	hsa04020	0.00246922	18	89

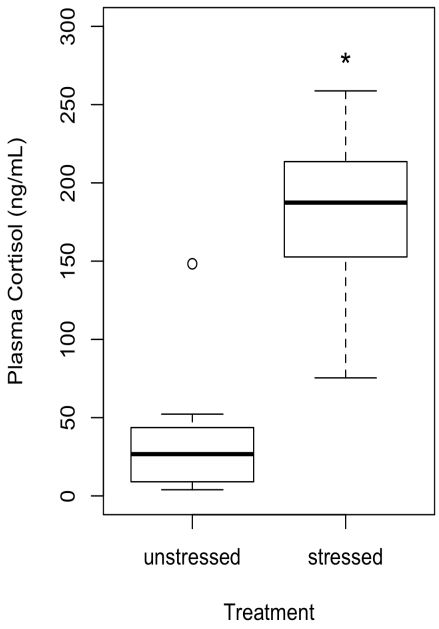

Figure D.1: Plasma cortisol levels of adult stickleback mothers from the unstressed and stressed treatments. Data are presented as box and whisker plots generated using default settings in R. Asterisk indicates significant difference in mean plasma cortisol levels between stressed and unstressed mothers detected by one-way ANOVA in R ($p = 0.0014$). $n = 6$ fish per treatment group.

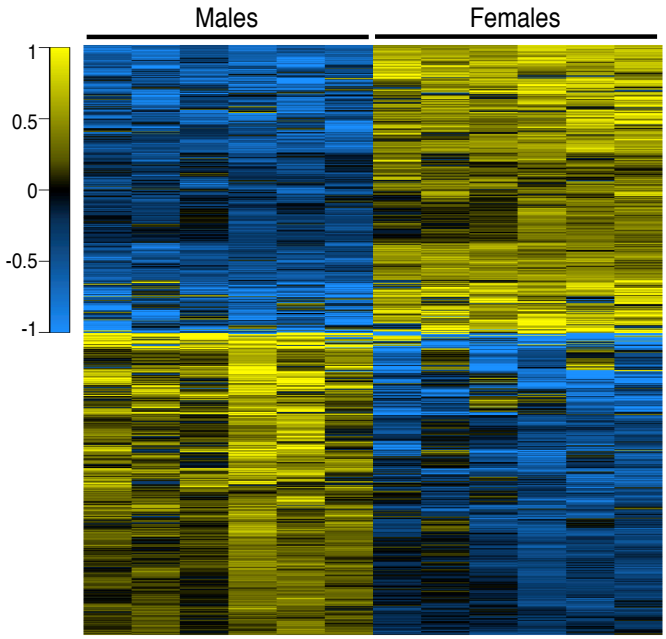

Figure D.2: Heat map of all differentially expressed (DE) genes for which a significant effect of offspring sex was identified. Expression results are displayed as log2 counts per million normalized to the mean expression for all samples. Blue indicates lower expression and yellow indicates higher expression.

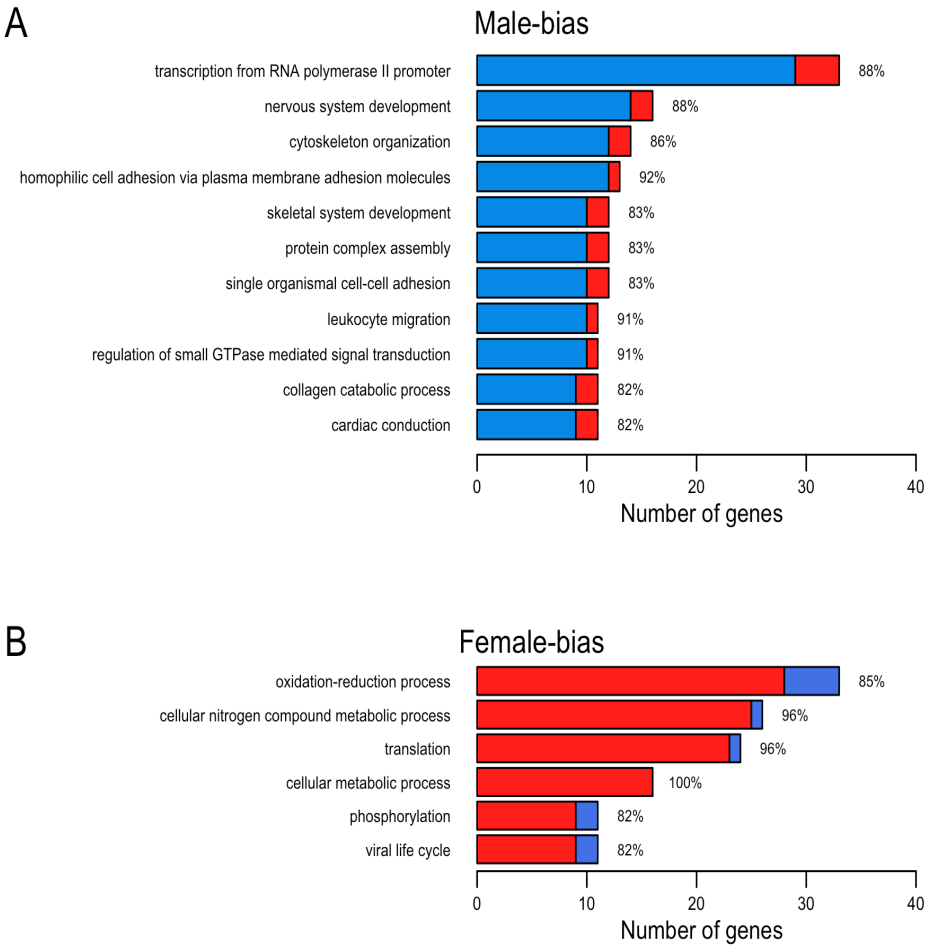

Figure D.3: Summary of gene ontology (GO) categories for genes that exhibited sexually dimorphic expression patterns independent of maternal stress. GO categories represent those that contained at least ten genes that were differentially expressed between males and females and where 80% of the genes in that GO category exhibit a bias of higher expression in males (A) or higher expression in females (B). Blue bars represent the number of genes expressed at higher levels in males while red bars represent genes expressed at higher levels in females. The percent of genes within a category that exhibit a given bias in expression is also shown.

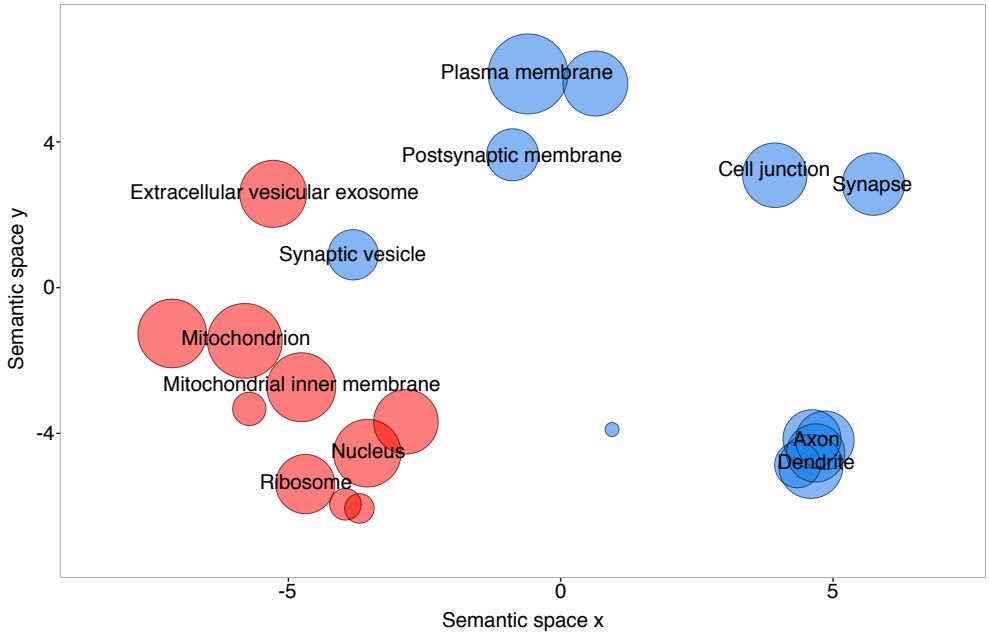

Figure D.4. Cluster analysis of the significantly enriched cellular component gene ontology (GO) terms over-represented among genes that increased in males (blue circles) or increased in females (red circles) from stressed mothers compared to unstressed mothers. Size of the circle is representative of the total number of DE genes in that GO category. Note that some names have been omitted from the figure due to semantic similarity.

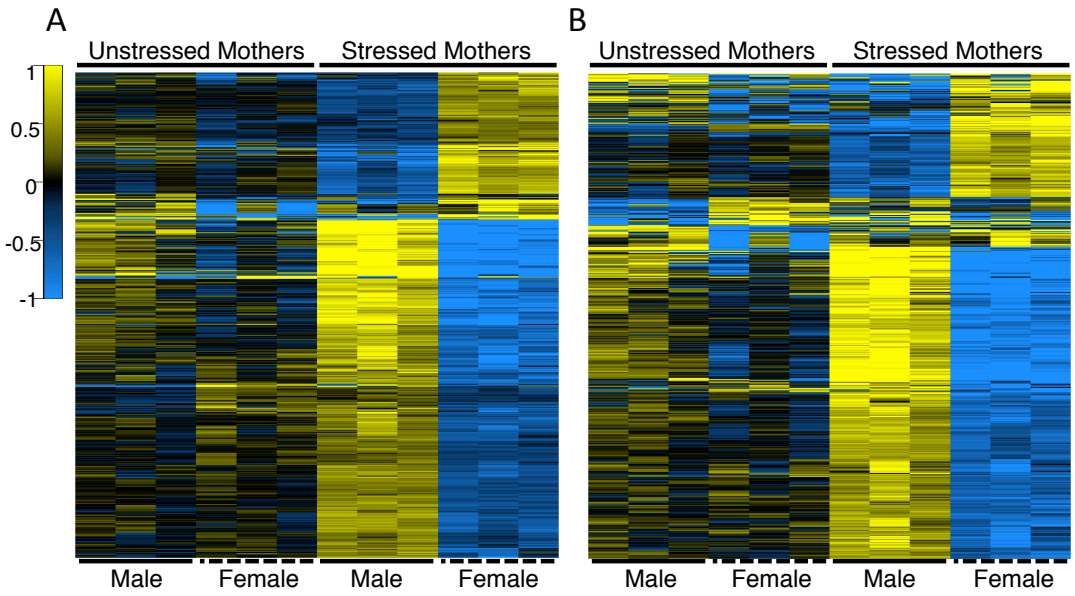

Figure D.5: Heatmap displaying the expression patterns of genes with a significant interaction between sex and maternal stress with A) FDR q<0.01 or B) fold change >2.0. Expression results are displayed as log2 counts per million normalized to the mean expression for all samples. Blue indicates lower expression and yellow indicates higher expression.