University of South Florida

DIGITAL COMMONS
@ UNIVERSITY OF SOUTH FLORIDA

Digital Commons @ University of South Florida

USF Tampa Graduate Theses and Dissertations

USF Graduate Theses and Dissertations

June 2017

Epigenetic Response to Challenging Environmental Conditions

Marta Robertson
University of South Florida, marta.h.robertson@gmail.com

Follow this and additional works at: https://digitalcommons.usf.edu/etd

Part of the Biology Commons, Ecology and Evolutionary Biology Commons, and the Plant Sciences Commons

Scholar Commons Citation
Robertson, Marta, "Epigenetic Response to Challenging Environmental Conditions" (2017). *USF Tampa Graduate Theses and Dissertations.*
https://digitalcommons.usf.edu/etd/6939

This Dissertation is brought to you for free and open access by the USF Graduate Theses and Dissertations at Digital Commons @ University of South Florida. It has been accepted for inclusion in USF Tampa Graduate Theses and Dissertations by an authorized administrator of Digital Commons @ University of South Florida. For more information, please contact digitalcommons@usf.edu.

Epigenetic Response to Challenging Environmental Conditions

by

Marta Robertson

A dissertation submitted in partial fulfillment
of the requirements of the degree of
Doctor of Philosophy in Biology
with a concentration in Ecology and Evolution
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Major Professor: Christina L. Richards, Ph.D.
Gordon Fox, Ph.D.
Kathleen Scott, Ph.D.
Aaron W. Schrey, Ph.D.

Date of Approval:
April 14, 2017

Keywords: plant epigenetics, stress, response to the environment, DNA methylation

Copyright © 2017, Marta Robertson

Dedication

This dissertation is dedicated to all of the people who have helped me in my pursuits. My parents, who taught me to work hard and question all things; my sister, my role model; and my grandparents, whose library ignited my education. This endeavor would not have been possible without the pervasive influence of my undergraduate research advisor, turned lifelong mentor and friend, who introduced me to epigenetics and the philosophy of biology.

Acknowledgments

Special thanks to my research advisor, Christina Richards, who has provided unwavering support. I also thank my committee members, Gordon Fox, Kathleen Scott, and Aaron Schrey, for their insight and guidance. Lastly, thank you to all of my fellow lab members, who have been available for needed brainstorming and encouragement.

Financial support was provided by the Student Government travel grant, Department of Biology travel grant, and the University of South Florida Tharp Summer Fellowship.

Table of Contents

List of Tables i

List of Figures ii

Abstract iv

Chapter One: Non-genetic inheritance in evolutionary theory—the importance of plant studies 1
- Authors 1
- Abstract 1
- Introduction 2
- Evidence for Epigenetic Inheritance 4
  - Epigenetic mechanisms impact ecologically important traits 7
  - Epigenetic variation can be environmentally induced 7
  - Epigenetic state is independent of genetic sequence 8
- Objects to Evolutionary Epigenetics 9
  - How should epigenetics be defined? 9
  - Soft inheritance 11
  - Stability of epigenetic variation 12
  - Epigenetic reset 14
  - Population genetics models 16
- Future Directions 17
- Acknowledgements 19
- References 19

Chapter Two: Genetic and epigenetic variation in *Spartina alterniflora* following the *Deepwater Horizon* oil spill 28
- Authors 28
- Abstract 28
- Introduction 29
- Materials and Methods 32
  - Sample collection 32
  - AFLP genotyping 33
  - MS-AFLP genotyping 34
  - Data analysis 35
- Results 37

Genetic diversity and structure 37
Epigenetic diversity and structure 38
Discussion 39
Genetic and epigenetic response to pollution 40
Acknowledgements 43
References 44
Tables and Figures 54

Chapter Three: Conservation of methylation response to seasonal variation 62
Authors 62
Abstract 62
Introduction 63
Materials and Methods 65
Study sites and species 65
DNA extractions and library prep 67
Data pre-processing and epigenetic analysis 67
Results 69
Discussion 70
Future directions 73
Acknowledgements 74
References 74
Tables and Figures 81

Chapter Four: Effects of CMT3 expression on phenotype under stress 91
Authors 91
Abstract 91
Introduction 92
Materials and Methods 95
Plant material 95
Experimental conditions: maternal generation 95
Experimental conditions: progeny 96
Analyses 96
Results 97
Main effect of CMT3 on maternal survival and fitness 97
Effect of biotic and abiotic stress on maternal survival and fitness 98
Effect of maternal environment on progeny survival and growth 98
Discussion 99
Effects of stress but not genotype 100
Transgenerational interactions between parent environment and CMT3 expression 101
Acknowledgements 103
References 103
Tables and Figures 108

List of Tables

Table 2.1: GPS coordinates of seven study sites. 54

Table 2.2: Mean AFLP haplotype and MS-AFLP epigenotype diversity (*h*) and percent polymorphic loci by site (%P), based on 71 AFLP and 39 MS-AFLP loci. 55

Table 2.3: Summary of hierarchical AMOVA for AFLP and MS-AFLP data sets among site type (Φ_{RT}), among populations within site type (Φ_{PR}) and within populations (Φ_{PT}). 56

Table 2.4: Pairwise Φ_{PT} comparisons of variation among study sites. 57

Table 3.1: Adapter and primer sequences used in library preparation. 81

Table 3.2: Structure of genetic variation in each species explained by individual, season, and year. 82

Table 3.3: Structure of epigenetic variation in each species explained by individual, season, and year. 83

Table 4.1: Results of linear model for growth traits in *A. thaliana* maternal plants. 108

Table 4.2: Results of linear model for growth traits in *A. thaliana*, second generation. 109

List of Figures

Figure 2.1: Map of seven study sites and their relative locations in the Gulf coast, with site specific oil intensity following the *DWH* oil spill, according to NRDA databases, and the results of Bayesian clustering. 58

Figure 2.2: Examples of (a) non-contaminated (GIN1) and (b) contaminated sites (GIO1) in the Gulf Coast following the DWH oil spill. 59

Figure 2.3: Frequencies of genetic loci significantly correlated to oil contamination across seven populations in locus-by-locus analysis. 60

Figure 2.4: Frequencies of epigenetic loci significantly correlated to oil contamination across seven sites in locus-by-locus analysis. 61

Figure 3.1: Number of sequenced cytosines per sample for *S. alterniflora* shown in dark grey. 84

Figure 3.2: Number of sequenced cytosines per sample for *I. imbricata* shown in dark grey. 85

Figure 3.3: Number of sequenced cytosines per sample for *O. axillaris* shown in dark grey. 86

Figure 3.4: Methylation rate by context for *S. alterniflora* by season. 87

Figure 3.5: Methylation rate by context for *I. imbricata* by season. 88

Figure 3.6: Methylation rate by context for *O. axillaris* by season. 89

Figure 3.7: Proportion of methylation by context for *S. alterniflora, I. imbricata,* and *O. axillaris* by season. 90

Figure 4.1: Time to (a) germination and bolting (b) without treatment and (c) under salt stress. 110

Figure 4.2: Reaction norms for number of fruits, flowers, branches and leaves in the rosette at bolting; final height; and rosette diameter at five weeks (means ± s.e.) averaged over each genotype of *A. thaliana* in each treatment in the maternal generation. 111

Figure 4.3: Time to germination in seeds from (a) maternal lines without treatment and (b) maternal lines under salt stress. 112

Figure 4.4: Time to bolting in seeds from (a) maternal lines without treatment and (b) maternal lines under salt stress. 113

Figure 4.5: Time to flowering in seeds from (a) maternal lines without treatment and (b) maternal lines under salt stress. 114

Figure 4.6: Reaction norms for number of leaves in the rosette at bolting and rosette diameter at five weeks (means ± s.e.) averaged over each genotype of *A. thaliana* in each treatment in the second generation. 115

Abstract

The discovery of epigenetic mechanisms has ignited speculation into their role in ecological and evolutionary processes. In particular, the contribution of epigenetic variation to adaptation or phenotypic plasticity that is distinct from genetic variation would be an important addition to existing evolutionary mechanisms. Although the research of epigenetic mechanisms from an ecological and evolutionary (or eco-evolutionary) perspective has been growing, it is still unclear how epigenetic variation might function in natural populations and settings and to what extent it might serve to mediate population response to changing environmental conditions over time. Over the course of my dissertation, I explored the importance of DNA methylation in population response to a variety of environmental conditions.

In the first chapter of my dissertation, I reviewed existing literature on the relationship between DNA methylation and environmental response. I argued that given the weight of current evidence, DNA methylation, in addition to other epigenetic mechanisms, needs to be included the evolutionary synthesis. Additionally, I identified a number of outstanding questions and outlined research directions that would help elucidate the role of epigenetic mechanisms in evolution.

In my second chapter, I studied the genetic and epigenetic composition of populations of *Spartina alterniflora* that were impacted by the *Deepwater Horizon* oil spill in 2010. Current evolutionary theory predicts that following a severe environmental stressor, populations may

experience a bottleneck effect, in which one or only a few genotypes survive to reproduce in subsequent generations. However, it is unclear whether these patterns are reflected in epigenetic variation as well, because novel environmental perturbations may serve to induce epigenetic variation rather than diminish it. We found a significant genetic signature of oil exposure in exposed populations, but did not see a similar effect in the epigenetic composition of exposed populations. These data suggest that epigenetic modifications, such as DNA methylation, may not always increase in number during stressful episodes, but may instead follow genetic variation. These results provide valuable information for the development of nascent population epigenetic theory, and may help parameterize expectations about conditions that provoke epigenetic variation, particularly when genetic variation may be limited.

In addition to strong, unpredictable stressors, populations also respond via phenotypic changes over time through developmental stages and life histories that coincide with seasonal, regular environmental cues. Epigenetic mechanisms influence these regulatory and developmental changes that occur within an individual over time. In my third chapter, I examined the epigenetic response to seasonality in multiple coastal plant species. We found a weak signature of single methylation polymorphisms that was associated with seasonal environmental change within the studied species, as well as global patterns of methylation that were consistent across species. The results of this study indicate the possibility of conservation of methylation patterns across phylogenetic histories.

In my fourth chapter, I explored in detail how the ability to maintain methylation might affect stress response. We compared individuals of the model plant *Arabidopsis thaliana* that were deficient in maintenance methylation machinery to control genotypes under both abiotic

and biotic stresses, and then studied the growth of their offspring in the absence of stress. We found inherited phenotypic signatures of parental stress in the offspring generation and interactive effects of parental stress and genotype. This study not only reinforces the correlations that we observed in our field studies, but adds to the growing body of literature highlighting the importance of DNA methylation both in immediate environmental response and as a mechanism for heritability.

Overall, this dissertation demonstrates that DNA methylation is highly abundant in natural populations and may be part of the response to various stressors at a number of time scales. The integration of DNA methylation in the evolutionary synthesis will aid in the explanation of phenomena such as phenotypic plasticity or adaptation, and will be an important contribution to the existing body of evolutionary mechanisms.

Chapter One:
Non-genetic inheritance in evolutionary theory- the importance of plant studies[1]

Authors

Marta Robertson, Christina Richards

Abstract

Emerging evidence points to a causal role for epigenetic variation in evolution, but evolutionary biologists have been reluctant to incorporate epigenetics into modern evolutionary theory. Part of this ambivalence comes from the assumption that epigenetic inheritance is only relevant to the evolution of plants, which is perpetuated by a comparative lack of evolutionary studies in animals. However, although most of the evidence for epigenetic inheritance comes from plants, plants and animals share many homologous epigenetic mechanisms, and plants provide a more tractable system for investigating the causal role of epigenetic mechanisms underlying phenotypic variation and its relationship with fitness. The insights from studies of epigenetic inheritance in plants may be applicable across a broad range of taxa once we establish commonalities and differences in epigenetic machinery. In this paper we present evidence for a role of epigenetic mechanisms in the evolutionary process and discuss common objections to

[1] This chapter has been previously published, open access, in Non-Genetic Inheritance (Robertson, M., and Richards, C. "Non-genetic inheritance in evolutionary theory- the importance of plant studies" Non-Genetic Inheritance (2015) 2: 3-11.) and is reproduced under the Creative Commons license.

incorporating epigenetics into evolutionary theory. This review is not exhaustive, but is meant to demonstrate that epigenetic inheritance can be incorporated into current evolutionary theory without overhauling its foundations.

Introduction

When Darwin described the mechanism of evolutionary change, effectively establishing the current dominant paradigm of evolutionary theory, he could not define the unit of heredity. For the next half-century, related discussions focused on various hypotheses of the unit of heredity, including inheritance of acquired characteristics. The topic was debated until Weismann's doctrine was popularized in the late 1890s (Buss 1983, Weissman 2011). Weismann's doctrine argued that only genes and the germline are responsible for heredity, dismissing an important role for the soma in evolutionary change. Later, this doctrine was incorporated into the Modern Synthesis and was central to the development of population genetics (Bonduriansky and Day 2009, Weissman 2011), which established a quantitative way to explain gradual phenotypic change in the context of Mendel's laws of inheritance. In the Modern Synthesis (MS), evolutionary change is often equated with genetic divergence, and phenotypic variation is solely dependent on the emergence of random sequence mutations in the germ line (Jablonka and Lamb 2008). Under this framework, evolution is described as changing allele frequencies over time (Pigliucci 2007).

This is a convenient but limited definition of evolutionary change because it does not include current findings in development, plasticity, or non-genetic inheritance (Müller 2007), it neglects the creative roles of the environment and disease in adaptation, and it neglects to

consider taxa which do not have a clearly separated soma and germ line, such as bacteria, plants, and many animals (see Buss 1983 for a more complete list and discussion). With a focus on population genetics, the MS has become a “theory of genes” (Pigliucci 2007), but several evolutionary biologists are exposing the limitations of a gene-centric definition of evolution (Jablonka and Lamb 2008, Laland et al. 2014, Noble et al. 2014, Pigliucci 2007, Travisano and Shaw 2014), including its inability to fully describe morphological change and evolutionary process. For example, it is not possible to explain all of heritability using only genetic variation, and quantitative genetics models often fail to predict offspring phenotype from parental genotype and environmental experience alone (Greisemer 2011). Genetics also explains only a portion of complex traits, such as behavior and disease, despite recent advances in sequencing and genotype-to-phenotype mapping (Slatkin 2009). Additionally, in contrast to the expectations at the foundation of the MS, epigenetic phenomena, such as genomic imprinting, cause parent-of-origin effects and violate Mendel’s laws of segregation and independent assortment. Furthermore, the definition of a gene itself has become unclear, as gene regulatory networks and alternative splicing have blurred the functional boundaries of a gene. New definitions acknowledge complex gene interactions, placing an emphasis on final functional products (Gerstein et al. 2007). These findings weaken the gene- centric association of changing allele frequencies with evolutionary change.

As gene frequencies become a dissatisfying and incomplete explanation of evolutionary change, more inclusive units of heredity must be identified and it is becoming clear that changes must be made to accommodate emerging evidence from developmental and molecular biology (Greisemer 2002). In particular, rapidly growing evidence makes a strong case that epigenetic

mechanisms contribute to complex, heritable traits, and thus evolution. Epigenetic mechanisms, such as DNA methylation, have been shown to be more variable than DNA base pair sequences, responsive to the environment, and heritable (Cortijo et al. 2014, Johannes et al. 2009, Latzel et al. 2012, Schmitz et al. 2013, Verhoeven et al. 2010a). Importantly, epigenetic mechanisms can have a phenotypic effect independent of genotype (Cortijo et al. 2014, Cubas et al. 1999, Herrera and Bazaga 2013, Zhang et al. 2013). Epigenetic mechanisms also provide promising explanations for various aspects of human health and disease including cancers, infertility, obesity, diabetes, cardiovascular disease and several neurological disorders (e.g. Alzheimer's, schizophrenia, autism; Liebers et al. 2014, Slatkin 2009). In this paper, we present evidence that epigenetic mechanisms can contribute to phenotype and inheritance, and discuss many of the objections evolutionary biologists have to incorporating epigenetic mechanisms into evolutionary theory. We focus on plants as tractable, easily- manipulated models of epigenetic evolutionary change. Plant systems can be used to experimentally explore the evolutionarily conserved epigenetic mechanisms, and insights can be applied more generally across taxa to describe how epigenetic mechanisms may be inherited with consequences for phenotype and evolution. However, clarification of the similarities and differences between plant and animal epigenetic processes is needed before we can use current plant epigenetics studies to develop a broad understanding of the implications for evolution.

Evidence for Epigenetic Inheritance

Most of the current evidence for a role of epigenetic mechanisms in evolution comes from plants, with limited examples in animals. This is partly due to the fact that several resources

are available for plants that are not available for animals, such as epigenetic recombinant inbred lines (epiRILs), which can be used to measure effects of DNA methylation in constant genetic backgrounds (Cortijo et al. 2014, Johannes et al. 2009, Latzel et al. 2012, Zhang et al. 2013). Inheritance of induced epigenetic changes may also be easier to detect in plants than in animals, since plants do not sequester their germline and the environment may play a more obvious role in shaping their epigenomes. Additionally, some plant species harbor higher loads of transposable elements than animals (Fedoroff 2012) and hybridize more frequently, providing greater opportunity for epigenetic regulation to come into play, and increasing the likelihood of detecting epigenetic variation in plant systems rather than animal systems (reviewed in Richards et al. 2012b). However, plants and animals share homologous epigenetic mechanisms, and many insights from studies of epigenetic inheritance in plants are applicable across diverse taxa (Fedoroff 2012, Feschotte 2008). Because experiments on humans and some animals are limited in number, the research community can benefit by using plant systems to explore the evolutionarily conserved epigenetic mechanisms that are involved in response to environment, and how those mechanisms may be inherited with consequences for phenotype and evolution.

For example, DNA methylation often suppresses the activity of transposable elements, and can spread to neighboring regions of the genome (Furner et al. 2011, Lippman et al. 2004, Schmitz et al. 2011). Most insertions of transposable elements (TEs) are thought to be neutral or deleterious (reviewed in Brookfield 2005, Slotkin et al. 2012), but in both plants and animals some TEs provide novel regulatory function, promote alternative splicing or develop into transcription factor binding sites (Feschotte 2008, Kidwell and Lisch 2000, Slotkin et al. 2012). In the human genome, transposable elements are often found in the promoter regions of genes,

affecting gene expression. The presence and control of transposable elements may have contributed to diversification of flowering plants (Joly-Lopez et al. 2012) and primates (Feschotte 2008).

Another commonality between plants and animals is that methylation of cytosines is controlled by methyltransferases, which either maintain DNA methylation at a specific locus or establish methylation *de novo*. Homologs of many methyltransferases are found in plants and animals; however, plants also use an RNA directed DNA methylation pathway (RdDM) that is not found in animals to methylate *de novo*. This pathway may contribute to environmentally induced and functional epigenetic variation in plants (Law and Jacobsen 2010, Schmitz et al. 2011, 2013) that is not seen in animals. The functional patterns of DNA methylation that result from these mechanisms are still not well understood in either plants or animals, and this has led to disagreement about the possibility of epigenetic inheritance (Daxinger and Whitelaw 2012, Ptashne 2013). For example, some authors argue that most epigenetic mechanisms are non-specific and unstable, minimizing the possibility of true independent epigenetic inheritance (Grossniklaus et al. 2013, Ptashne 2013). However, several studies, discussed above and below, provide empirical support for epigenetic inheritance. Detailed studies of the mechanistic basis of transgenerational methylation transmission in both plants and animals will be required to resolve the ongoing debates about the molecular mechanisms behind epigenetic inheritance.

Several studies in plant systems are building convincing evidence that epigenetic mechanisms can be environmentally induced and can affect ecologically relevant phenotypes (reviewed in Kilvitis et al. 2014, Richards et al. 2012b). Importantly, this epigenetic variation is not completely reducible to genetic variation; in many of these examples epigenetic variation is

at least partly independent of genetic variation. Although no single study has found the smoking gun, several independent studies have assembled compelling evidence in support of epigenetics in evolution and many of these studies demonstrate that epigenetic variation is present in populations, can cause phenotypic variation, and can be stably inherited (see below, also see (Jablonka and Raz 2009) for examples of epigenetic inheritance).

Epigenetic mechanisms impact ecologically important traits

Several studies have demonstrated an epigenetic impact on functional traits (Alonso et al. 2014, Cortijo et al. 2014, Cubas et al. 1999). One of the earliest and best examples of epigenetic potential in evolution is in the pioneering study of the epi-allele at the *L-CYC* gene in *Linaria vulgaris* (Cubas et al. 1999). In this species, a peloric mutant exhibits radial symmetry in the flower, whereas the wild type exhibits bilateral symmetry in the flower. The L-CYC gene controls floral symmetry in *L. vulgaris*; however, both variants of *L. vulgaris* share the same genetic sequence at the *Lcyc* locus (Cubas et al. 1999). Radial symmetry is associated with hypermethylation and transcriptional silencing at the *Lcyc* locus. This epigenotype and the associated phenotype are stably heritable. This study shows that heritable DNA methylation can contribute to ecologically relevant traits, suggesting that it may play a substantial role in evolution.

Epigenetic variation can be environmentally induced

Environmental influence on epigenetic variation has been demonstrated in a number of species, including *Viola elatior* (Schulz et al. 2014), S*olanum lycopersicum* (González et al.

2013) and *A. thaliana* (Dowen et al. 2012). In an apomictic dandelion, *Taraxacum officinale* (Verhoeven et al. 2010a, 2010b, and 2012), seeds are produced via parthenogenesis, resulting in clonal offspring and little genetic variation within populations. When exposed to salt stress, nutrient stress, salicylic acid and jasmonic acid, *T. officinale* showed no genetic polymorphism, but did show increased variation in DNA methylation. Furthermore, offspring of stressed parents that were raised in a common unstressed environment maintained DNA methylation changes that were induced in the parents. The environmentally induced changes in DNA methylation in this system were heritable from parent to offspring. This study supports the idea that epigenetic mechanisms are susceptible to environmental stressors and may facilitate rapid evolution.

Epigenetic state is independent of genetic sequence

Though epigenetic variation can change independently from genetic variation, the correlation between epigenetic and genetic variation is graded and is difficult to tease apart (Richards 2006, Richards 2008). However, a few studies have taken advantage of systems with clonal reproduction or little genetic variation to demonstrate independent effects of epigenetic variation (Bossdorf et al. 2010, , Latzel et al. 2012, Verhoeven et al. 2014, Zhang et al. 2013). Richards et al. (2012a) showed that Japanese knotweed has little genetic variation in invasive populations along the east coast of the United States, but maintained differential epigenetic patterns in a broad range of habitats. By propagating individuals in a common garden, they were able to conclude that epigenetic state persisted through clonal reproduction rather than just being reset or necessarily induced by the environment. However, this study is not able to determine

local adaptation due to differential epigenetic state since they did not characterize the relationship between these patterns and fitness in situ.

Objections to Evolutionary Epigenetics

Despite growing evidence of heritable epigenetic phenomena, many evolutionary biologists are reluctant to include the concept in evolutionary theory (see Laland et al. 2014). Many of the challenges evolutionary biologists raise against epigenetic inheritance focus on the relative importance of the phenomenon (Greisemer 2011). For example, is epigenetic inheritance relevant in many taxa, and can a theory that incorporates both genetic and epigenetic mechanisms of heredity explain more of the organismal phenotype than genetics alone? Below, we address some of the common objections evolutionary biologists make which are aimed at dismissing the relative importance of epigenetic inheritance.

How should epigenetics be defined?

The Greek root “epi” means “upon, near to, or in addition,” and so epigenetics can be considered the study of mechanisms literally found on the DNA sequence (Greisemer 2011). Waddington (1942) first coined the term “epigenetics” during his studies to describe developmental effects not caused by genetic mutation (Jablonka and Lamb 2002). More recently, epigenetics has been used to describe developmental cellular processes and molecular controls that can be inherited across meiosis (Cubas et al. 1999, Herrera et al. 2013). The newest definitions of epigenetics include mechanisms that affect gene expression and are stable across meiotic and mitotic divisions (Kilvitis et al. 2014, Jablonka and Raz 2009), such as DNA

methylation, histone modifications, and small interfering RNA. However, these definitions and uses of the term 'epigenetics' have been met with some disagreement from authors who claim that a much more narrow definition of epigenetics is necessary to discriminate effects that are grounded in sequence-based changes (Bossdorf et al. 2008, Ptashne 2007, 2013). For example, although generally labeled 'epigenetic' and tightly associated with DNA methylation, histone modifications have not been shown to be heritable (Ptashne 2013). Furthermore, these broad definitions could theoretically include transcription factors and other regulatory elements that affect gene expression without change in DNA sequence, which are not usually considered epigenetic.

While there is some disagreement and ambiguity about what constitutes an epigenetic effect, for this review we will leave our definition of epigenetics intentionally broad (i.e. including mechanisms that affect gene expression and can be stable across meiotic and mitotic divisions), and define epigenetic inheritance as heritable modifications that can change gene expression without changes to the DNA sequence (Jablonka and Raz 2009, Kilvitis et al. 2014). Despite the debate on the definition of epigenetics, most research in epigenetic inheritance has focused on DNA methylation, and most authors agree that DNA methylation is both epigenetic and can be heritable (Feng et al. 2010, Grossniklaus et al. 2013, Ptashne 2013). We wish to emphasize the importance of a system of inheritance other than DNA sequence, and will focus on examples of DNA methylation here.

Soft inheritance

Similar to the disagreement about the definition of "epigenetics", some evolutionary biologists object to the concept of "soft inheritance". Epigenetic mechanisms are often discussed in concert with soft inheritance, traditionally defined as a mechanism by which the environment molds favorable alleles (in contrast to "hard inheritance," in which genetic changes arise randomly and are stable regardless of environment; (Richards 2006)). During the formation of the Modern Synthesis, soft inheritance was used to describe many theories of inheritance, including evolution by inheritance of acquired characteristics and use and disuse of organs. At the time, there was little evidence to suggest that the environment had any effect on genotype or evolution, and the improbability of soft inheritance was used to solidify the Modern Synthesis, which depended on population genetic theory, and thus hard inheritance (Wilkins 2011).

Some critics argue that epigenetic inheritance is a revival of soft inheritance and Lamarckism. However, in its current usage, soft inheritance should not be equated with neo-Lamarckism. Instead, the modern rendition of soft inheritance refers to environmentally induced epigenetic variation, both in somatic and germ lines (Richards 2006). When differentially methylated regions are passed to offspring in the absence of that same stimulus, they can be considered inherited epigenetic states (Jablonka and Raz 2009). Epigenetic variation is not necessarily adaptive, but if functional, it is subject to many of the same evolutionary processes as DNA sequence polymorphisms (i.e. natural selection, drift, and mutation; (Jablonka and Raz 2009)). This places epigenetic inheritance well within Darwin's paradigm, but epigenetic inheritance does not fit explicitly within the Modern Synthesis as it was constructed in the 1940s.

However, the extent by which epigenetic mechanisms serve to differentiate populations still needs to be determined.

Stability of epigenetic variation

A common argument against a substantial role for epigenetic variation in evolutionary change is the supposed instability of epigenetic state. Because epigenetic state can be labile and DNA methylation mutation rates are higher than mutation rates in DNA sequence base pairs, many opponents to epigenetic inheritance claim that epigenetic variation cannot have lasting effects on organismal evolution. They also argue that natural selection cannot work if parent epigenotype is not correlated to offspring epigenotype. However, the stability of epigenetic marks is context dependent: some marks are more persistent than others and more work is needed to understand the importance of genomic context dependency in epigenetics (eg. Johannes et al. 2009, Verhoeven et al. 2010a). Further, even when the state is labile, extensive modeling and ample empirical evidence find that epigenetic variation can have substantial effects on the evolution of populations. For example, a study of the methylation variation in five species of Darwin's finches found a greater number of differentially methylated regions than genetic mutations among the species. This study also found that the amount of epigenetic variation among species increases with phylogenetic difference whereas the amount of genetic variation does not (Skinner et al. 2014). Although these data were collected in the field and therefore cannot control for environmentally induced differences among the species, they indicate that epigenetic mechanisms reflect evolutionary relationships and may contribute to long- term changes in populations.

Although epigenetic marks are often characterized as labile and transient, there are many examples across all taxa that show epigenetic variation is stable (Jablonka and Raz 2009). Strong evidence for the stability of epigenetic variation comes from several studies on *Arabidopsis thaliana* epigenetic recombinant inbred lines (epiRILs). EpiRILs are inbred lines that contain little genetic variation, but high epigenetic variation (Cortijo et al. 2014, Johannes et al. 2009, Latzel et al. 2012, Zhang et al. 2013). These lines show heritability of epigenetic differences over at least eight generations (Johannes et al. 2009). Furthermore, significant heritable phenotypic differences among epiRILs under stress highlight the potential importance of methylation variation in adaptation (Latzel et al. 2012, Zhang et al. 2013). Significant selection coefficients were found for biomass, root:shoot mass, and plant height in response to nutrient stress and drought (Zhang et al. 2013). Finally, further studies with these epiRILs have identified epigenetic quantitative trait loci (QTL^{epi}), which account for 90% of the heritability of flowering time and 60% of the heritability of primary root length (Cortijo et al. 2014). These findings offer strong support that epigenetic variation is stable, can affect phenotype independently of genotype, and is subject to natural selection.

Other proponents of epigenetic inheritance contend that even if epigenetic variation induced in one generation is prone to reversion in the next, evolutionary dynamics can still be affected in the long term. Importantly, when epigenetic variation is induced by environmental stressors, it is likely to be induced in many individuals in the population simultaneously (Rapp and Wendel 2005). This is in contrast to genetic mutations, which arise in single individuals and must be propagated throughout the population over generations. Even if epigenetic state is only

stable for very few generations, their population-level effect allows for stronger and more rapid influence on evolutionary trajectories than genetic mutations.

Epigenetic reset

One of the biggest objections to epigenetic inheritance is evidence of the resetting of epigenetic variation during gametogenesis, which questions the validity of epigenetic mechanisms as a unit of heredity (see Ptashne 2013). Both plants and animals tend to reprogram at least some epigenetic variation during gametogenesis, although it is unclear to what extent or by which mechanisms this occurs (Badyaev 2014), and emerging evidence shows that many epigenetic states are not reprogrammed during fertilization (Wei et al. 2014). Objections to epigenetic inheritance claim that DNA methylation is erased and resulting DNA methylation in offspring is environmentally influenced. Any correlation between parent and offspring DNA methylation is due to a shared environment, and does not represent a transfer of information from the parent to offspring, but rather from environment to developing organism (Daxinger and Whitelaw 2012). However, evidence suggests that plants and animals, despite their divergent reproductive strategies, share mechanisms to copy DNA methylation information from parent to offspring (Feng et al. 2010). In plants, it has been demonstrated that the endosperm is widely demethylated, creating a feedback loop to reinforce methylation of transposable elements in the developing embryo. Small interfering RNA (siRNA) are produced as transposable elements are demethylated, and are passed from the endosperm to the egg cell, directing methylation in the developing zygote (see Feng et al. 2010).

Mammals may similarly pass information about methylation states from parent to offspring (Daxinger and Whitelaw 2012, Feng et al. 2010). For example, in mice, expression of the A^{vy} allele of the agouti locus is correlated with the amount of DNA methylation of a transposable element upstream of the genic region. Low methylation of the transposable element is correlated with the yellow-agouti phenotype; yellow coat color and diseases such as obesity, diabetes, and tumors. High methylation of the transposable element is associated with the pseudo-agouti phenotype; brown coat color and lessened disease risk (Daxinger and Whitelaw 2012). Female mice with the yellow- agouti phenotype give birth to offspring with the yellow-agouti phenotype at a higher rate than pseudo-agouti females. This suggests that epigenetic state is maintained from parent to offspring at the agouti locus in pseudo- agouti mice. To rule out maternal effects, fertilized embryos from the yellow-agouti females were transferred to pseudo-agouti females. The offspring maintained a high rate of yellow-agouti phenotype, suggesting that the (DNA methylation associated) pseudo-agouti phenotype normally seen in pseudo-agouti mothers is maintained via transmission of epigenetic state rather than maternal effects during fetal development (Morgan et al. 1999). Daxinger and Whitelaw (2012) have proposed that this is due to elements in the cytoplasm, such as siRNA. Other studies suggest that the mammalian placenta may mediate methylation states in a similar fashion to the endosperm of plants (see Feng et al. 2010 for a more detailed discussion).

As mentioned earlier, many studies have demonstrated transmission of DNA methylation changes from parent to offspring in plants. In *T. officinale*, 74%-92% of polymorphic DNA methylation induced in parents is transmitted faithfully to offspring (Verhoeven et al. 2010a). Similarly, high transmission of epigenetic state from parents to pollen granules has also been

demonstrated in the field in *Helleborus* (Herrera et al. 2013). This study reported that 75% of the DNA methylation found in parents was found in the gamete, and that the parental epigenetic population structure persisted in the pollen. However, large variation in transmission of epigenetic state exists, suggesting that transmissibility itself is a variable trait. This is consistent with the idea that variations in gametic epigenotype are guided by traces of the parent epigenotype and different genotypes have different epigenetic potential.

Population genetics models

A major hurdle for the study of epigenetic inheritance is incorporating epigenetic mechanisms into current population genetic and quantitative genetic theory (Richards et al. 2010). Many models of epigenetic inheritance modify current population genetics models with added parameters for highly labile epigenetic states (Day and Bonduriansky 2011, Goeghegan and Spencer 2012, Klironomos et al. 2013). These models show that epigenetic inheritance is adaptive in quickly changing environments, which has also been demonstrated in several other modeling efforts (Lachmann and Jablonka 1996, Pál 1998, Pál and Miklos 1999, Tal et al. 2010). Although few of these models have been tested directly, the assumption of labile epigenetic state is confirmed by studies of epimutation rate, shown to be orders of magnitude higher than genetic mutation rate. In mutation accumulation lines of *A. thaliana*, the epimutation rate is 4.46×10^{-4} DNA methylation polymorphisms (Becker et al. 2011, Schmitz et al. 2011) compared to a rate of 7×10^{-9} in SNPs (Ossowski et al. 2010) in the same mutation accumulation lines. As a consequence, there is tremendous epigenetic variation within species (Schmitz et al. 2013,

Vaughn et al. 2007), and measures of epigenetic variation are often greater than genetic variation (Richards et al. 2012a, 2012b). Further, while some models make oversimplifying assumptions, Day and Bonduriansky (2011) present a model that allows for the fact that some genetic contexts are more likely to acquire methyl groups than others, and the change in the epigenetic component may be dependent on genotype.

Some authors argue that epigenetic state must be stable over at least 3 generations to discount parental and grandparental effects (Grossniklaus et al. 2013), and any modeling efforts must take this multi-generational approach (Furrow and Feldman 2014). Furrow (2014) models a multi-generational effect of epigenetic variation, and finds that as epimutation rates increase, the response to selection is slowed. However, this model fails to include the effect of changing environmental conditions, which theorists have identified as a likely scenario in which epigenetic variation will have a large effect on adaptation, and which will affect epimutation rate and the strength of selection (Lachmann and Jablonka 1996). Klironomos et al. (2013) show that epigenetic variation may be used to increase adaptive phenotypic variation before genetic variation is codified, and can decouple fitness effects from changes in the genome during selective events, increasing evolutionary potential. These effects may be mediated through environmentally induced epigenetic variation and unstable epigenetic variation. However, these findings have not been tested experimentally.

Future Directions

Although a large body of evidence supports the possibility that epigenetic mechanisms are a unit of heredity, controversy still exists over the potential role for epigenetics in evolution

(Laland et al. 2014). Researchers interested in advocating for the importance of epigenetics should take precautions to design experiments that can address questions about long-term fitness effects due to epigenotype. Few studies have investigated adaptive and evolutionary potential for epigenetic variation by demonstrating effects on phenotype and fitness due to inherited epigenetic state. Many studies which show correlation of phenotypic variation and epigenetic patterns in different environments fail to discern whether these patterns are inherited or environmentally induced in each generation (Herrera and Bazaga 2011, Lira-Medeiros et al. 2010). Future studies will need to measure phenotype and fitness in a reciprocal transplant or common garden design to draw stronger conclusions about adaptive epigenetic variation and avoid these conceptual pitfalls (Bossdorf et al. 2008). These manipulative approaches are much more feasible in plant systems and can provide information applicable to understanding epigenetic dynamics in a wide range of human health related phenomena.

Secondly, epigenetic experimental evolution studies will help evaluate the stability of epigenetic variation and its potential contribution to evolutionary divergence. Several studies show that epigenetic variation is re-patterned during plant hybridization and polyploidization events (Hegarty et al. 2011, Paun et al. 2010, Salmon et al. 2005, Verhoeven et al. 2010b; reviewed in Richards et al. 2012b), which suggests that epigenetic mechanisms can be important in long-term evolution. However, experimental studies have focused mainly on effects in a single generation, despite the fact that epigenetic stability has been demonstrated for 8 generations (Johannes et al. 2009). Characterizing long-term behavior of epigenetic variation through these processes, and in response to environmental challenges will lead to more robust models of its importance in an evolutionary context.

Acknowledgements

Thank you to Mariano Alvarez, Christy Foust, Holly Kilvitis, and Nancy Sheridan for helpful advice and feedback on earlier versions of this manuscript.

References

Alonso C., Pérez R., Bazaga P., Medrano M., & Herrera C.M. (2014) Individual variation in size and fecundity is correlated with differences in global DNA cytosine methylation in the perennial herb Helleborus foetidus (Ranunculaceae). American Journal of Botany, 101:1309-1313.

Badyaev A.V. (2014) Epigenetic resolution of the 'curse of complexity' in adaptive evolution of complex traits. The Journal of Physiology, 592:2251-2260.

Becker C., Hagmann J., Müller J., Koenig D., Stegle O., Borgwardt K., & Weigel D. (2011) Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. Nature, 480:245-249.

Bonduriansky R. & Day T. (2009) Nongenetic Inheritance and Its Evolutionary Implications. Annual Review of Ecology, Evolution, and Systematics, 40:103-125.

Bossdorf O., Arcuri D., Richards C.L., & Pigliucci M. (2010) Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana, Evolutionary Ecology, 24(3):541-553. doi: 10.1007/s10682-010-9372-7

Bossdorf O., Richards C.L., & Pigliucci M.(2008) Epigenetics for ecologists, Ecology Letters, 11:106-115.

Brookfield J.F. (2005) The ecology of the genome—mobile DNA elements and their hosts. Nature Reviews Genetics, 6:128-136.

Buss L.W. (1983) Evolution, development, and the units of selection. Proceedings of the National Academy of Sciences, 80:1387-1391.

Cortijo S., Wardenaar R., Colomé-Tatché M., Gilly A., Etcheverry M., Labadie K., . . . Roudier F. (2014) Mapping the epigenetic basis of complex traits. Science, 343:1145-1148.

Cubas P., Vincent C., & Coen E. (1999) An epigenetic mutation responsible for natural variation in floral symmetry. Nature, 401:57-161.

Daxinger L. & Whitelaw E. (2012) Understanding transgenerational epigenetic inheritance via the gametes in mammals. Nature Reviews Genetics, 13:153-162.

Day T. & Bonduriansky R. (2011) A unified approach to the evolutionary consequences of genetic and nongenetic inheritance. The American Naturalist, 178:E18-E36.

Dowen R.H., Pelizzola M., Schmitz R.J., Lister R., Dowen J.M., Nery J.R., . . . Ecker J.R. (2012) Widespread dynamic DNA methylation in response to biotic stress, Proceedings of the National Academy of Sciences, 109:E2183-E2191.

Fedoroff N.V. (2012) Transposable elements, epigenetics, and genome evolution. Science, 338:758-767.

Feng S., Jacobsen S.E., & Reik W.(2010) Epigenetic reprogramming in plant and animal development. Science, 330:622-627.

Feschotte C. (2008) Transposable elements and the evolution of regulatory networks. Nature Reviews Genetics, 9:397-405.

Furner I.J. & Matzke M. (2011) Methylation and demethylation of the Arabidopsis genome. Current Opinion in Plant Biology, 14:137-141.

Furrow R.E. & Feldman M.W. (2014) Genetic variation and the evolution of epigenetic regulation. Evolution, 68:673-683.

Geoghegan J.L. & Spencer H.G. (2012) Population-epigenetic models of selection. Theoretical population biology, 81:232-242

Gerstein, M. B., Bruce, C., Rozowsky, J. S., Zheng, D., Du, J., Korbel, J. O., . . . & Snyder, M. (2007) What is a gene, post-ENCODE? History and updated definition. Genome Research, 17:669-681.

González, R. M., Ricardi, M. M., & Iusem, N. D. (2013) Epigenetic marks in an adaptive water stress-responsive gene in tomato roots under normal and drought conditions. Epigenetics, 8:864-872.

Griesemer J. (2002) What is "epi" about epigenetics?. Annals of the New York Academy of Sciences, 981:97-110.

Griesemer J. (2011) The relative significance of epigenetic inheritance in evolution: Some philosophical considerations. Transformations of Lamarckism, 331-344.

Grossniklaus U., Kelly W.G., Ferguson-Smith A.C., Pembrey M., & Lindquist S. (2013) Transgenerational epigenetic inheritance: how important is it?. Nature Reviews Genetics, 14:228-235.

Hegarty M.J., Batstone T., Barker G.L., Edwards K.J., Abbott R.J., & Hiscock S.J. (2011) Nonadditive changes to cytosine methylation as a consequence of hybridization and genome duplication in Senecio (Asteraceae), Molecular Ecology, 20:105-113.

Herrera C.M. & Bazaga P. (2011) Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory, Molecular Ecology, 20:1675-1688.

Herrera C.M. & Bazaga P. (2013) Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous Ilex aquifolium (Aquifoliaceae) trees. Botanical Journal of the Linnean Society, 171:441-452.

Herrera C.M., Medrano M., & Bazaga P. (2013) Epigenetic differentiation persists after male gametogenesis in natural populations of the perennial herb Helleborus foetidus (Ranunculaceae). PloS one, 8:e70730.

Jablonka E. & Lamb M.J. (2002) The changing concept of epigenetics. Annals of the New York Academy of Sciences, 981:82-96.

Jablonka E. & Lamb M.J. (2008) Soft inheritance: challenging the modern synthesis. Genetics and Molecular Biology, 31:389-395.

Jablonka E. & Raz G. (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution, The Quarterly Review of Biology, 84:131-176.

Johannes, F., Porcher, E., Teixeira, F. K., Saliba-Colombani, V., Simon, M., Agier, N., ... & Bouchez, D. (2009) Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS Genet, 5:e1000530.

Joly-Lopez Z., Forczek E., Hoen D.R., Juretic N., & Bureau T.E. (2012) A gene family derived from transposable elements during early angiosperm evolution has reproductive fitness benefits in Arabidopsis thaliana. PLoS genetics, 8:e1002931.

Kidwell M.G. & Lisch D.R. (2000) Transposable elements and host genome evolution, Trends in Ecology & Evolution, 15:95-99.

Kilvitis, H. J., Alvarez, M., Foust, C. M., Schrey, A. W., Robertson, M., & Richards, C. L. (2014). Ecological epigenetics. In Ecological Genomics (pp. 191-210). Springer Netherlands.

Klironomos F.D., Berg J., & Collins S. (2013) How epigenetic mutations can affect genetic evolution: model and mechanism. Bioessays, 35:571-578.

Lachmann M. & Jablonka E. (1996) The inheritance of phenotypes: an adaptation to fluctuating environments. Journal of Theoretical Biology, 181:1-9.

Laland K., Uller T., Feldman M., Sterelny K., Müller G.B., Moczek A., . . . Hoekstra H.E. (2014) Does evolutionary theory need a rethink?. Nature, 514:161.

Latzel V., Zhang Y., Moritz K.K., Fischer M., & Bossdorf O. (2012) Epigenetic variation in plant responses to defence hormones. Annals of Botany, 110:1423-1428.

Law J.A. & Jacobsen S.E. (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nature Reviews Genetics, 11:204-220.

Liebers R., Rassoulzadegan M., & Lyko F. (2014) Epigenetic regulation by heritable RNA. PLoS genetics, 10:e1004296.

Lippman Z., Gendrel A.-V., Black M., Vaughn M.W., Dedhia N., McCombie W.R., . . . Kasschau K.D. (2004) Role of transposable elements in heterochromatin and epigenetic control. Nature, 430:471-476.

Lira-Medeiros C.F., Parisod C., Fernandes R.A., Mata C.S., Cardoso M.A., & Ferreira P.C. (2010) Epigenetic variation in mangrove plants occurring in contrasting natural environment. PLoS One, 5:e10326.

Morgan H.D., Sutherland H.G., Martin D.I., & Whitelaw E. (1999) Epigenetic inheritance at the agouti locus in the mouse. Nature Genetics, 23:314-318.

Müller G.B. (2007) Evo–devo: extending the evolutionary synthesis. Nature Reviews Genetics, 8:943-949.

Noble D., Jablonka E., Joyner M.J., Müller G.B., & Omholt S.W. (2014) Evolution evolves: physiology returns to centre stage. The Journal of Physiology, 592:2237-2244.

Ossowski S., Schneeberger K., Lucas-Lledo J.I., Warthmann N., Clark R.M., Shaw R.G., . . . Lynch M. (2010) The rate and molecular spectrum of spontaneous mutations in Arabidopsis thaliana, Science, 327:92-94.

Pal C. (1998) Plasticity, memory and the adaptive landscape of the genotype. Proceedings of the Royal Society of London. Series B: Biological Sciences, 265:1319-1323.

Pál C. & Miklós I. (1999) Epigenetic inheritance, genetic assimilation and speciation. Journal of Theoretical Biology, 200:19-37.

Paun O., Bateman R.M., Fay M.F., Hedren M., Civeyrel L., & Chase M.W. (2010) Stable epigenetic effects impact adaptation in allopolyploid orchids (Dactylorhiza: Orchidaceae), Mol Biol Evol, 27:2465-2473.

Pigliucci M. (2007) Do we need an extended evolutionary synthesis?. Evolution, 61:2743-2749.

Ptashne M. (2007) On the use of the word ‘epigenetic’. Current Biology, 17:R233-R236.

Ptashne M. (2013) Epigenetics: core misconcept. Proceedings of the National Academy of Sciences, 110:7101-7103.

Rapp R.A. & Wendel J.F. (2005) Epigenetics and plant evolution. New Phytologist, 168:81-91.

Richards C.L., Bossdorf O., & Pigliucci M. (2010) What role does heritable epigenetic variation play in phenotypic evolution?. BioScience, 60:232-237.

Richards C.L., Schrey A.W., & Pigliucci M. (2012a) Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. Ecology Letters, 15:1016-1025.

Richards, C. L., Verhoeven, K. J., & Bossdorf, O. (2012). Evolutionary significance of epigenetic variation. In Plant Genome Diversity Volume 1 (pp. 257-274). Springer Vienna.

Richards E.J. (2006) Inherited epigenetic variation—revisiting soft inheritance. Nature Reviews Genetics, 7:395-401.

Richards E.J. (2008) Population epigenetics. Current Opinion in Genetics and Development, 18:221-226.

Salmon A., Ainouche M.L., & Wendel J.F. (2005) Genetic and epigenetic consequences of recent hybridization and polyploidy in Spartina (Poaceae). Molecular Ecology, 14:1163-1175.

Schmitz R.J., Schultz M.D., Lewsey M.G., O'Malley R.C., Urich M.A., Libiger O., . . . Ecker J.R. (2011) Transgenerational epigenetic instability is a source of novel methylation variants. Science, 334:369-373.

Schmitz R.J., Schultz M.D., Urich M.A., Nery J.R., Pelizzola M., Libiger O., . . . Schork N.J. (2013) Patterns of population epigenomic diversity. Nature, 495:193-198.

Schulz B., Eckstein R.L., & Durka W. (2014) Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. Molecular Ecology, 23:3523-3537.

Skinner M.K., Gurerrero-Bosagna C., Haque M.M., Nilsson E.E., Koop J.A., Knutie S.A., & Clayton D.H. (2014) Epigenetics and the Evolution of Darwin's Finches. Genome Biology and Evolution, 6:1972-1989.

Slatkin M. (2009) Epigenetic inheritance and the missing heritability problem. Genetics, 182:845-850.

Slotkin, R. K., Nuthikattu, S., & Jiang, N. (2012). The impact of transposable elements on gene and genome evolution. In Plant Genome Diversity Volume 1 (pp. 35-58). Springer Vienna.

Tal O., Kisdi E., & Jablonka E. (2010) Epigenetic contribution to covariance between relatives. Genetics, 184:1037-1050.

Travisano M. & Shaw R.G. (2013) Lost in the map. Evolution, 67:305-314.

Vaughn M.W., Tanurdžić M., Lippman Z., Jiang H., Carrasquillo R., Rabinowicz P.D., . . . Bulski A. (2007) Epigenetic natural variation in Arabidopsis thaliana. PLoS biology, 5:e174.

Verhoeven K.J., Jansen J.J., van Dijk P.J., & Biere A. (2010a) Stress- induced DNA methylation changes and their heritability in asexual dandelions. New Phytologist, 185:1108-1118.

Verhoeven K.J., Van Dijk P.J., & Biere A. (2010b) Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. Molecular Ecology, 19:315-324.

Verhoeven K.J. & van Gurp T.P. (2012) Transgenerational effects of stress exposure on offspring phenotypes in apomictic dandelion. PloS one, 7:e38605.

Verhoeven K.J. & Preite V. (2014) Epigenetic variation in asexually reproducing organisms. Evolution, 68:644-655.

Waddington C.H. (1942) Canalization of development and the inheritance of acquired characters. Nature, 150:563-565.

Wei Y., Schatten H., & Sun Q.-Y. (2014) Environmental epigenetic inheritance through gametes and implications for human reproduction. Human reproduction update, dmu061.

Weissman C. (2011) Germinal selection: a Weismannian solution to Lamarckian problematics. Transformations of Lamarckism: From Subtle Fluids to Molecular Biology, 57.

Wilkins A. (2011) Why did the modern synthesis give short shrift to 'soft inheritance'. Transformations of Lamarckism: From Subtle Fluids to Molecular Biology, 127.

Zhang Y.Y., Fischer M., Colot V., & Bossdorf O. (2013) Epigenetic variation creates potential for evolution of plant phenotypic plasticity. New Phytologist, 197:314-322.

Chapter Two:
Genetic and epigenetic variation in *Spartina alterniflora* following the *Deepwater Horizon* oil spill[2]

Authors

Marta Robertson, Aaron Schrey, Ashley Shayter, Christina J Moss, Christina Richards

Abstract

Catastrophic events offer unique opportunities to study rapid population response to stress in natural settings. In concert with genetic variation, epigenetic mechanisms like DNA methylation may offer a mechanism of rapid response to organisms facing severe environmental challenges, and contribute to the high resilience of species like *Spartina alterniflora*, a foundation salt marsh grass which shows resilience to strong environmental disturbance. In 2010, the *Deepwater Horizon* oil spill devastated large portions of the coastline along the Gulf of Mexico. Following the spill, we simultaneously examined the genetic and epigenetic structure of recovering populations of *S. alterniflora* to oil exposure. We quantified genetic and DNA methylation variation using AFLP and MS-AFLP to test the hypothesis that response to oil exposure in *S. alterniflora* resulted in genetically and epigenetically based population

---

[2] This chapter has been previously published, open access, in Evolutionary Applications (Robertson, M., Schrey, A., Shayter, A., Moss, C.J., and Richards, C. "Genetic and epigenetic variation in *Spartina alterniflora* following the *Deepwater Horizon* oil spill" Evolutionary Applications (2017) 00:1–10. https://doi.org/10.1111/eva.12482.) and is reproduced under the Creative Commons license.

differentiation. We found high genetic and epigenetic variation within and among sites, and found significant genetic differentiation between contaminated and uncontaminated sites, which may reflect non-random mortality in response to oil exposure. Additionally, despite a lack of genome wide patterns in DNA methylation between contaminated and uncontaminated sites, we found five MS-AFLP loci (12% of polymorphic MS-AFLP loci) that were correlated with oil exposure. Overall, our findings support genetically based differentiation correlated to exposure to the oil spill in this system, but also suggest a potential role for epigenetic mechanisms in population differentiation.

Introduction

Ecological theory predicts that adaptation to local conditions can result when populations harbor heritable phenotypic variation for traits that increase tolerance to local conditions. Classic population genetics studies demonstrate that natural selection in different microhabitats can result in associations of genotypes, or alleles of candidate genes, with habitat type (e.g., Hamrick & Allard 1972, Salzman 1985, Schmidt & Rand 1999, Schmidt et al. 2008). In concert with other evolutionary mechanisms, disturbance events may also create population genetic structure, by diminishing standing genetic diversity through mortality (Orr & Betancourt 2001, Hermisson & Pennings 2005). These classic predictions are intuitive and often supported empirically (e.g. Clausen et al. 1948). However, in some cases, data across a diversity of taxa show either no association of genetic differences with habitat (e.g. Richards et al. 2004, Foust et al. 2016; examples in Schmidt et al. 2008) or that low levels of molecular diversity are not associated with decreased phenotypic variation (Dlugosch & Parker 2008, Richards et al. 2008). The disconnect

between empirical findings and ecological theory suggests the possibility of additional, under-explored molecular mechanisms, such as epigenetic modifications, that mediate the relationship between phenotype and environment.

The recent application of molecular techniques to ecological questions has revealed that epigenetic regulatory mechanisms, such as DNA methylation, may respond dynamically and independently to sudden changes in the environment (e.g. Gugger et al. 2016, Trucchi et al. 2016). Although there are several epigenetic mechanisms that can alter gene expression (e.g. chromatin remodeling, histone modifications, small interfering RNAs), DNA methylation of cytosines is the most widely studied (Schrey et al. 2013, Verhoeven et al. 2016) and can have important ecological effects. For example, studies in *Taraxacum officinale* show that when DNA methylation machinery is disrupted, flowering time differences among populations of these plants are removed (Wilschut et al. 2016). Additionally, natural populations typically harbor high amounts of epigenetic variation (Paun et al. 2010, Richards et al. 2012, Keller et al. 2016), which can be structured by local environmental conditions along with genetic variation. Variation in DNA methylation is correlated with habitat-type in mangroves (Lira-Medeiros et al. 2010) and knotweed (Richards et al. 2012), herbivory in viola (Herrera & Bazaga 2010), and climate in natural accessions of *Arabidopsis thaliana* (Keller et al. 2016). This association between DNA methylation and plant ecology may reflect the modulation of gene expression (Zilberman et al. 2007, Bewick et al. 2012) or recombination rates (Mirouze et al. 2012), the release of transposable elements (Dowen et al. 2012), or other regulatory processes in response to environmental conditions in addition to covariance with genetic structure. In some cases, epigenetic variation can be restructured during periods of environmental stress and these changes can persist after the stress is relieved (Verhoeven et al. 2010, Dowen et al. 2012 but see Wibowo

et al. 2016). These findings suggest that epigenetic mechanisms may allow for rapid modification of phenotype in response to immediate and acute stressors (Rapp & Wendel 2005).

In this study, we simultaneously examined genetic and epigenetic patterns in populations of *S. alterniflora* along the Gulf coast that were exposed to heavy oiling following the *Deepwater Horizon* (*DWH*) oil spill ("heavy" sensu Lin et al. 2016, Nixon et al. 2016). In 2010, 4.9 million barrels of oil spilled into the Gulf of Mexico over a period of three months, with devastating effects on coastal ecology and salt marsh ecosystems (Lin & Mendelssohn 2012, Silliman et al. 2012, Whitehead et al. 2012, Lin et al. 2016). As the dominant plant on the leading edge of salt marshes, many *S. alterniflora* populations across the northern Gulf of Mexico were negatively impacted by the *DWH* oil spill. Despite large die-off of above-ground biomass and reduced carbon-fixation and transpiration in heavily oiled populations, *S. alterniflora* showed high resilience to the hydrocarbon exposure (Lin & Mendelssohn 2012, Lin et al. 2016), and above-ground biomass and live stem density levels recovered to the same level as uncontaminated reference marshes within 18 months (Lin et al. 2016). However, while these and other studies support that *S. alterniflora* is resilient to hydrocarbon stress, the extent of intraspecific variation in resilience is uncertain, and it remains unknown if there was differential mortality among *S. alterniflora* genotypes in natural populations exposed to the *DWH* oil spill. We measured genetic and epigenetic variation using AFLP and MS-AFLP to test the hypothesis that oil exposure in *S. alterniflora* resulted in genetic and epigenetic signatures of population differentiation. As in previous studies of *S. alterniflora* (Richards et al. 2004, Travis et al. 2004, Edwards et al. 2005, Hughes & Lotterhos 2014, Foust et al. 2016), we expected to see high levels of genetic and epigenetic variation. However, we anticipated that moderate, non-random differential mortality in response to oil exposure would result in genetic differentiation of oil-exposed populations

from unexposed populations. Further, we anticipated a concurrent but stronger epigenetic signature of oil exposure, given its reflection of gene expression and physiological response to environmental stimuli (Verhoeven et al. 2010, Dowen et al. 2012, Xie et al. 2015).

Materials and Methods

Sample collection

Spartina alterniflora is a clonal halophyte, native to the east coast of the United States and invasive in coastlines around the world (Pennings & Bertness 2001, Ayres et al. 2004, Ainouche et al. 2009). *Spartina alterniflora* displays diverse phenotypes in response to the natural environmental gradients in marshes, producing less above-ground biomass in response to increasingly saline soil (Richards et al. 2005). Populations of *S. alterniflora* display high genetic diversity (Richards et al. 2004, Travis et al. 2004, Edwards et al. 2005, Hughes & Lotterhos 2014, Foust et al. 2016) and substantial resilience to both natural variation in the salt marsh (Pennings & Bertness 2001) as well as anthropogenic stressors, such as crude oil (Lin & Mendelssohn 2012, Lin et al. 2016).

We collected leaf tissue from *S. alterniflora* stems at approximately ten-meter intervals along the shoreline from three oil-contaminated and four uncontaminated reference sites along the Gulf Coast in August 2010, while oil was still standing on the soil surface at contaminated sites (Table 2.1; Figure 2.1). Oil contamination was defined by visually-confirmed presence of crude oil in the sediment and complete above ground die-back of *S. alterniflora* in populations on the leading edge of the marsh. The only visible live tissue was the regrowth of stems from rhizomes through the wrack of dead above-ground *S. alterniflora* (Figure 2.2) from which we collected leaf tissue. Contamination levels were later confirmed through Natural Resource

Damage Assessment databases (2014; Figure 2.1). Shoreline Cleanup Assessment Technique categories delineate oil contamination into five categories from “no oil observed” to “heavy oiling” (Nixon et al. 2016, Zengel et al. 2016). Our three contaminated sites fit the description of heavily oiled marshes whereas the four uncontaminated sites had no visible oiling or impacted vegetation at the time samples were collected, and were not annotated as contaminated in the oil assessment databases. Samples were collected from the middle of the so-called "tall plant" zone near the leading edge of the marsh (sensu "low-salt habitat" in Foust et al. 2016). From each plant, we collected the 3rd fully expanded leaf to standardize age and minimize developmental bias in sampling. The contaminated sites were: Grand Isle, LA site 1 (GIO1) (n = 6), Grand Isle, LA site 2 (GIO2) (n = 7), and Bay St. Louis, MS (MSO) (n = 8). Nearby uncontaminated reference sites were: GIN1 (n=9), GIN2 (n=10), and MSN (n=8). Because the minimum number of populations required to detect differences between two groups at the level of alpha = 0.05 is suggested to be n = 7 (Fitzpatrick 2009), we also sampled one additional reference site, Aransas, TX (AR) (n = 10), which was not affected by the *DWH* oil spill (Table 2.1). Sites in Mississippi and Louisiana were separated by a minimum of 10 km and maximum of 35 km, and AR was 775 km from Mississippi. Tissue samples were snap frozen in liquid nitrogen and stored at -80˚C.

AFLP genotyping

We used AFLP to assess genetic variation between the field sites using a standard protocol described in Richards et al. (2012). Briefly, we isolated DNA in duplicate from leaf tissue with the Qiagen DNeasy Plant Mini kit according to the manufacturer’s recommended protocol (Qiagen, Valencia, CA), and conducted the entire protocol on duplicate reactions to ensure the consistent scoring of fragments, and control for the potential error rate of AFLP

markers. For selective PCR, we used fluorescently labeled primers *Eco*RI+AGC (6-FAM) and +ACG (HEX) and unlabeled *Mse*I+CAC primers. We sent selective PCR products to the DNA Facility at Iowa State University, IA, USA, where they were electrophoresed on an ABI 3130XL. We scored resulting fragments in duplicate as “1” for present and “0” for absent using PeakScanner (ThermoFisher Scientific), and excluded markers that were not supported in duplicate.

MS-AFLP epigenotyping

We used MS-AFLP to assess genome-wide DNA methylation on the same duplicate DNA extractions used in the AFLP protocol (Reyna-Lopez et al. 1997). We used *MspI* and *HpaII* restriction enzymes, which have different sensitivities to cytosine methylation of the same CCGG sequence (Reyna-Lopez et al. 1997, Salmon et al. 2008). DNA extracts were digested with both *EcoRI*/*MspI* and *EcoRI*/*HpaII* enzyme combinations independently for each individual, and selective PCR was run with fluorescently labeled primers *EcoRI*+AGC (6-FAM) and +ACG (HEX) and unlabeled primers *HpaII*/*MspI* +TCAC and *HpaII*/*MspI*+TCAT. We sent selective PCR products to the DNA Facility at Iowa State University, IA, USA, where they were analyzed on an ABI3130XL. We visualized the resulting electropherograms using PeakScanner and scored fragments as “1” when present and “0” when absent.

Together, *MspI* and *HpaII* produce four types of evaluative variation (Salmon et al. 2008). *MspI* does not cut when the external cytosines are fully or hemi-methylated and *HpaII* does not cut when either the internal or external cytosines are methylated on both strands. Likewise, cleaving by both enzymes is blocked when both cytosines are methylated. The resulting fragments can be classified as either: Type I when the corresponding sequence

restriction site is non-methylated and fragments occur in both digests, Type II when fragments are absent in *EcoRI* + *HpaII* digests but present in *MspI*, Type III when fragments are absent in *EcoRI* + *MspI* digests only, or Type IV when no fragments occur in either digest. We treated Type IV variation as missing data, because the methylation state cannot be specified (Salmon et al. 2008). Although some advocate for discriminating between Type II and Type III methylation as these types are expected to capture methylation in CG versus CHG contexts (Medrano et al. 2014, Schulz et al. 2014), Types II and III variation cannot simply be interpreted as CG versus CHG methylation since apparent CHG methylation can be caused by the nesting of internal restriction sites within MS-AFLP fragments that exhibit differential CG methylation (Fulnecek & Kovarik 2014). Therefore, we combined type II and III variation to represent the presence of DNA methylation in any context. Throughout this manuscript, we use "locus" to indicate a specific fragment size in the AFLP and MS-AFLP results. We use "haplotype" to indicate the binary variable positions (dominant genotypes) for each individual's collection of AFLP loci, and "epigenotype" to indicate the collection of binary variable positions of MS-AFLP loci.

Data analysis

To identify the number of different genetic groups represented in our collection independent of sampling location in our populations, we performed Bayesian clustering of the genetic data only using Structure v.2.3.4 (Pritchard et al. 2000, Falush et al. 2003, Falush et al. 2007, Hubisz et al 2009). Our previous work has shown population structure within native *S. alterniflora* populations (Richards et al. 2004, Foust et al. 2016). Although we designed our sampling to avoid subpopulation structure in this study by only sampling near the leading edge of the marsh (i.e. the low-salt habitat, sensu Foust et al. 2016), we tested for the possibility of

finding more populations than expected. We tested ten populations (k=1 - 10) with ten independent runs at each k. We performed analyses with 50,000 burn-in sweeps and 1,000,000 post burn-in sweeps, assuming admixture and without including sample location, or any geographic information as priors in the analysis. We estimated the number of clusters represented by the data using Evanno's Delta K (Evanno et al. 2005).

We used GenALex version 6.41 (Peakall & Smouse 2012) to estimate the haplotype and epigenotype diversity (*h*-AFLP and *h*-MS-AFLP). We also used GenALex to calculate estimates of genetic differentiation over all AFLP and MS-AFLP loci with a hierarchical AMOVA, nesting study sites within oil exposure to compare genetic variation among oil-contaminated and uncontaminated sites (Φ_{RT}), among sites within contamination level (Φ_{PR}), and within sites (Φ_{PT}). We also used GenALex to conduct a locus-by-locus AMOVA to characterize genetic and epigenetic differentiation at each locus, using the same hierarchical design. Finally, we performed pairwise AMOVA comparisons to determine which populations were differentiated. For all AMOVA analyses, we used 9999 permutations to estimate statistical significance and adjusted for multiple comparisons using the sequential Bonferroni method whenever multiple tests were performed.

In addition to AMOVA, we tested for the effect of oil on AFLP and MS-AFLP multi-locus marker profiles via permutational multivariate analysis of variance (perMANOVA), which allows for comparison of nested terms within hierarchical experimental design. Using the Adonis function within the Vegan package of R (Oksanen et al. 2007), we derived p-values based on 9999 permutations within populations using the following formula: Adonis (AFLP genetic distance matrix~ oil exposure, strata = population, permutations= 10,000). In each analysis, variation in marker profiles was represented by the Euclidean distance matrices as calculated

from the binary AFLP and MS-AFLP methylation data (with interpolation of missing values) generated by GenALex 6.41. We also used the RDA function within the Vegan package of R (Oksanen et al. 2007) to conduct a partial redundancy analysis of the relationship between contamination level (presence or absence) and MS-AFLP, while removing the effects of AFLP. We used the following formula: RDA [x~y + z] where x = the Euclidean epigenetic distance matrix generated by GenALex, y = site condition (presence or absence of oil) and z = the Euclidean genetic distance matrix generated by GenALex. To create the site condition matrix, we used zero to indicate uncontaminated sites and one to indicate contaminated sites. This strategy makes the assumption that differences between contaminated and uncontaminated populations will be essentially the same magnitude regardless of individual population differences.

Results

Genetic diversity and structure

A power analysis indicated that we could detect an effect of oil contamination among seven groups using our sample sizes (Fitzpatrick 2009), and previous work reports population differentiation in hierarchical analyses is detectable with as few as five individuals per population (Nelson & Anderson 2013). We found 71 polymorphic loci, which is well above the minimum of 30 markers reported in previous work to be required to detect significant patterns of differentiation (Nelson & Anderson 2013). Of these loci, six were present or absent in only one sample. We ran these analyses with and without including these single-variable loci, and found no substantial differences in the results. The data presented here are based on the complete set of 71 polymorphic loci. Although a modest data set, our markers identified that genetic diversity was high (*h*-AFLP ranged from 0.103 to 0.206), and 55 of 56 individuals displayed a unique

genotype. There was no difference in genotype diversity between contaminated and uncontaminated sites (*P* = 0.262). Bayesian clustering identified 2 genetic groups (ΔK= 1517.81); however these groups did not clearly reflect either differentiation by oil-contamination or geographic separation (Figure 2.1).

Hierarchical AMOVA revealed significant variation between contaminated and unaffected populations (explaining 6% of the genetic variance), and among populations within site type (explaining 16% of the genetic variance; Table 2.3), as well as most (66%) pairwise comparisons between sites (Table 2.4), indicating the presence of population structure between contamination types and among populations. These results were supported by perMANOVA, which showed a significant effect of oil contamination on multi-locus genetic marker profiles (F = 0.092, *P* = 0.017). Locus-by-locus AMOVA revealed 17 loci that varied significantly between oil-contaminated and unaffected sites (Figure 2.3).

Epigenetic diversity and structure

We found 39 polymorphic epigenetic loci from 71 observed. Of these loci, seven were present or absent in only one sample. We ran these analyses with and without including these single-variable loci, and found no substantial differences in the results. The data presented here are based on the complete set of 39 polymorphic loci. Epigenotype diversity was high (*h*-MSAFLP ranged from 0.089 to 0.222), and each individual displayed a unique epigenotype. Like the estimates for genetic patterns, there was no difference in epigenotype diversity between affected and unaffected sites (*P* = 0.993), and as in our previous studies of *Spartina alterniflora* (Foust et al. 2016), *h*-MS-AFLP tended to be lower than *h*-AFLP (Table 2.2).

Hierarchical AMOVA failed to detect a significant effect of oil contamination on epigenetic differentiation, but among populations within site type explained 7% of the epigenetic variance (Table 2.3), and 38% of the pairwise comparisons between sites were significant (Table 2.4). The lack of an effect of oil contamination on overall epigenetic variation was supported by perMANOVA (F = 0.373, $P = 0.815$) and redundancy analysis (F = 6.7269, $P = 0.22$). Locus-by-locus AMOVA revealed five loci were significantly differentiated between oil-contaminated and unaffected sites (Figure 2.4).

Discussion

Following the *Deepwater Horizon* oil spill in 2010, we sampled contaminated and uncontaminated populations of *S. alterniflora* along the coast of the Gulf of Mexico in populations that had experienced heavy oiling and complete above ground die-back. Despite reports of full recovery of above ground biomass and stem density in heavily oiled populations after 18 months (Lin et al 2016), our hierarchical AMOVA returned evidence of genetic differentiation among oil- contaminated and non-contaminated populations. However, we did not find evidence of decreased genetic diversity in contaminated populations, as nearly all individuals displayed a unique genotype in both contaminated and non-contaminated sites. These findings are consistent with other genetic surveys of *S. alterniflora* (Richards et al. 2004, Hughes & Lotterhos 2014, Foust et al. 2016), which also show high levels of genetic variation. With our small sample size ($n < 10$ at most sites), it is possible that we were unable to capture a change in genetic diversity among populations if one occurred in response to the oil spill.

In addition, we found no evidence of epigenetic differentiation over all loci between oil-contaminated and uncontaminated populations, but five loci showed epigenetic differentiation

due to oil exposure in the locus-by-locus analysis. Further study is required to determine if these loci are indicative of a regulatory response acting in concert with a few, but important epigenetic loci. However, redundancy analysis shows that overall patterns of methylation were not significantly correlated with oil exposure when controlling for the effects of genetic variation, which suggests that patterns of DNA methylation are explained almost entirely by genetic effects. Although we did expect to find epigenetic differentiation due to oil presence, it is possible that oil either did not induce any epigenetic changes between the population types, or that any existing epigenetic signature was too labile or too weak to be detected given the high epigenetic variation between individuals at our sites. Alternatively, our MS-AFLP may provide too few, anonymous markers to quantify epigenetic differentiation, and our small sample size may not have sufficient power to detect effects of rare epigenetic alleles or weak signatures of epigenetic change among the genetically differentiated populations. Many previous studies of epigenetic variation have taken advantage of low genetic diversity in natural systems to more clearly delineate population epigenetic effects (e.g. Gao et al. 2010, Richards et al. 2012). However, *S. alterniflora* is an out-crossing, wind-pollinated grass with extremely high genetic diversity (Richards et al 2004, Hughes & Lotterhos 2014, Foust et al. 2016). These high levels of genetic polymorphism make it more difficult to partition epigenetic structure due to increased statistical noise and genetic-dependent effects, particularly using anonymous genetic markers like AFLP (but see e.g. Foust et al. 2016).

Genetic and epigenetic response to pollution

Human-mediated environmental impacts have been well-documented as potential evolutionary drivers of population differentiation. A classic example is the rapid phenotypic

change experienced by the peppered moth as a result of coal pollution (Kettlewell 1958), which was recently explained by the activity of transposable elements that alter its development (van't Hof et al. 2016). Several studies also describe molecular differentiation in marine organisms across the eastern coast of the United States in response to aquatic pollution (Williams & Oleksiak 2008, Chapman et al. 2011, Whitehead et al. 2012). For example, populations of Atlantic killifish (*Fundulus heteroclitus*) in severely polluted habitats show broad genetic differentiation, including an allelic variant of cytochrome CYP1A (Williams & Oleksiak 2008, 2011), which is correlated with changes in gill morphology. Populations of the related Gulf killifish (*F. grandis*) in the Gulf of Mexico also showed differential expression of CYP1A among affected and unaffected populations following the *Deepwater Horizon* oil spill (Whitehead et al. 2012). Together, these studies highlight the role of anthropogenic stress in selection, adaptation, and divergence (Lande 1998, Hoffman & Sgro 2011). Despite previous literature suggesting that *S. alterniflora* is robust to heavy oil exposure, we found a signature of genetic differentiation between oil-exposed and unexposed populations. These results suggest at least some mortality in oil-exposed populations, consistent with findings of initial losses in live belowground biomass (Lin et al 2016). By examining the genetic and epigenetic composition of marshes after the *DWH* oil spill, our study adds to the growing number of ecological and evolutionary genomics studies describing population-level response to pollution.

Populations in coastal habitats, and salt marshes in particular, have long been models for phenotypic differentiation across natural environmental gradients (Schmidt & Rand 1999, Schmidt et al. 2008), and we expected to detect population level differentiation of DNA methylation in response to oil contamination as well (Richards et al. 2012, Foust et al. 2016). The idea that epigenetic mechanisms can contribute to population differentiation as a source of

heritable phenotypic variation has been challenged in recent literature (Laland et al. 2014, Wibowo et al. 2016). However, DNA methylation has been posited as a mechanism of phenotypic plasticity as well as a marker of stress response, and a number of studies have found a relationship between epigenetic variation and environment in support of this hypothesis (Jablonka & Raz 2009, Herman & Sultan 2016, Verhoeven et al. 2016). Environmental stressors can induce variation in DNA methylation and in some cases, these environmentally-induced methylation patterns can be inherited (Herrera & Bazaga 2010, Verhoeven et al. 2010, Herrera & Bazaga 2011), suggesting the potential for a signature of environmental response that is partially distinct from genetic variation.

Although we found high levels of epigenetic variation among individuals within and among populations, we failed to detect epigenetic differentiation in response to oil contamination. Our previous work showed a weak correlation between environmental conditions and epigenetic variation in *S. alterniflora* in a Georgia salt marsh (Foust et al. 2016). However, these data were collected from relatively protected habitat, and populations from this area are unlikely to have been exposed to a stress as severe as the *DWH* oil spill, which resulted in total above-ground die back, and reduction by approximately 84-95% of belowground biomass of the leading 5-10 meters of *S. alterniflora* in heavily oiled Gulf of Mexico marshes (Silliman et al. 2012, Lin et al. 2016). This impact may be far beyond what is normally experienced by *S. alterniflora* including natural disturbance events (Pennings & Bertness 2001).

Epigenetic mechanisms of response, such as DNA methylation, are expected to be evolutionarily favorable when the periodicity of a stressor is short (Lachmann & Jablonka 1996), such as cyclic patterns of rainfall, nutrient flows, and salinity that cause the zonation patterns observed among salt marsh plants (Pennings & Bertness 2001). In contrast, the *Deepwater*

Horizon oil spill may have acted as a single, discrete event that changed the make-up of the extensive genetic variation present in *S. alterniflora* rather than inducing a plastic or regulatory response that could be captured by assaying DNA methylation.

As studies of epigenetic variation in natural populations move away from quantifying the amount of standing genetic and epigenetic variation in natural populations to describing the role of that variation and the relative contribution of genetic and epigenetic variation to population differentiation, more precise sampling techniques and analyses will be needed. In future studies, a reduced-representation bisulphite sequencing approach would allow the direct comparison of genetic and epigenetic data sets, and at a much finer scale, with substantially increased statistical power to detect epigenetic differences between populations (Schrey et al. 2013, Robertson & Richards 2015, Trucchi et al. 2016, van Gurp et al. 2016). In addition, sequencing-based methods provide an increased ability to disentangle the relationship of methylation variation and gene function when fragments overlap with the promoters or coding regions of genes. By increasing the number of loci surveyed, future studies may better identify the environmental conditions under which genetic or epigenetic variation is associated with environmental cues (Robertson & Richards 2015).

Acknowledgements

We thank the Office of Undergraduate Research at the University of South Florida for financial support. We thank B DeLoach McCall and the S Pennings' lab for help locating and accessing field sites. We thank the Louisiana University Marine Consortium, especially A Kolker, for advice on oiled site locations in Louisiana, Superintendent J Malbrough for providing boat access and captain M Wike for navigating the field sites and trouble shooting issues related to

collections. We also thank C Strobel at Aransas NWR, J Dingee at Brazoria NWR, P Walther at Anahuac NWR, R Gosnell at Sabine NWR, T Augustine at Grand Isle State Park, A Rupp at the Mississippi Coastal Preserves Program, F Coleman and R Hughes at FSU Marine Lab for providing access to field sites. This work was partially supported by funding from a University of South Florida New Researcher Award and the National Science Foundation (U.S.A.) through DEB-1419960 (to CLR).

References

Aïnouche, M. L., Fortune, P., Salmon, A., Parisod, C., Grandbastien, M.-A., Fukunaga, K., . . . Misset, M.-T. (2009). Hybridization, polyploidy and invasion: lessons from Spartina (Poaceae). Biological Invasions, 11, 1159-1173.

Ayres, D. R., Smith, D. L., Zaremba, K., Klohr, S., & Strong, D. R. (2004). Spread of exotic cordgrasses and hybrids (Spartina sp.) in the tidal marshes of San Francisco Bay, California, USA. Biological Invasions, 6, 221-231.

Bewick, A. J., Ji, L., Niederhuth, C. E., Willing, E.-M., Hofmeister, B. T., Shi, X., . . . Hartwig, B. (2016). On the origin and evolutionary consequences of gene body DNA methylation. Proceedings of the National Academy of Sciences, 113, 9111-9116.

Chapman, R. W., Mancia, A., Beal, M., Veloso, A., Rathburn, C., Blair, A., . . . Sokolova, I. M. (2011). The transcriptomic responses of the eastern oyster, Crassostrea virginica, to environmental conditions. Molecular Ecology, 20, 1431-1449.

Clausen, J., Keck, D. D., & Hiesey, W. M. (1948). Experimental studies on the nature of species. III. Environresponses of climatic races of Achillea. Experimental studies on the nature of species. III. Environresponses of climatic races of Achillea.(Publ. No. 581).

Dlugosch, K. M., & Parker, I. (2008). Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. Molecular Ecology, 17, 431-449.

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., . . . Ecker, J. R. (2012). Widespread dynamic DNA methylation in response to biotic stress. Proceedings of the National Academy of Sciences, 109, E2183-E2191.

Edwards, K. R., Travis, S. E., & Proffitt, C. E. (2005). Genetic effects of a large-scaleSpartina alterniflora (smooth cordgrass) dieback and recovery in the northern Gulf of Mexico. Estuaries and Coasts, 28, 204-214.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology, 14, 2611-2620.

Falush, D., Stephens, M., & Pritchard, J. K. (2003). Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics, 164, 1567-1587.

Falush, D., Stephens, M., & Pritchard, J. K. (2007). Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes, 7, 574-578.

Fitzpatrick, B. M. (2009). Power and sample size for nested analysis of molecular variance. Molecular Ecology, 18, 3961-3966.

Foust, C., Preite, V., Schrey, A. W., Alvarez, M., Robertson, M., Verhoeven, K., & Richards, C. (2016). Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. Molecular Ecology, 25, 1639-1652.

Fulneček, J., & Kovařík, A. (2014). How to interpret methylation sensitive amplified polymorphism (MSAP) profiles? BMC Genetics, 15(1), 1.

Gao, L., Geng, Y., Li, B., Chen, J., & Yang, J. (2010). Genome-wide DNA methylation alterations of Alternanthera philoxeroides in natural and manipulated habitats: implications for epigenetic regulation of rapid responses to environmental fluctuation and phenotypic variation. Plant, Cell & Environment, 33, 1820-1827.

Gugger, P. F., Fitz-Gibbon, S., Pellegrini, M., & Sork, V. L. (2016). Species-wide patterns of DNA methylation variation in Quercus lobata and its association with climate gradients. Molecular Ecology, 25, 1665-1680.

Hamrick, J., & Allard, R. W. (1972). Microgeographical variation in allozyme frequencies in Avena barbata. Proceedings of the National Academy of Sciences, 69(8), 2100-2104.

Herman, J. J., & Sultan, S. E. (2016). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. Proc. R. Soc. B., 283, 20160988, doi: 10.1098/rspb. 2016.0988.

Hermisson, J., & Pennings, P. S. (2005). Soft sweeps. Genetics, 169, 2335-2352.

Herrera, C. M., & Bazaga, P. (2010). Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet Viola cazorlensis. New Phytologist, 187, 867-876. doi: 10.1111/j.1469-8137.2010.03298.x

Herrera, C. M., & Bazaga, P. (2011). Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory. Molecular Ecology, 20, 1675-1688. doi: 10.1111/j.1365-294X.2011.05026.x

Hoffmann, A. A., & Sgrò, C. M. (2011). Climate change and evolutionary adaptation. Nature, 470(7335), 479-485.

Hubisz, M. J., Falush, D., Stephens, M., & Pritchard, J. K. (2009). Inferring weak population structure with the assistance of sample group information. Molecular ecology resources, 9, 1322-1332.

Hughes, A. R., & Lotterhos, K. E. (2014). Genotypic diversity at multiple spatial scales in the foundation marsh species, Spartina alterniflora. Marine Ecology Progress Series, 497, 105-117.

Jablonka, E., & Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. The Quarterly review of biology, 84, 131-176.

Keller, T. E., Lasky, J. R., & Yi, S. V. (2016). The multivariate association between genome-wide DNA methylation and climate across the range of Arabidopsis thaliana. Molecular Ecology, 25, 1823-1837.

Kettlewell, H. D. (1958). A survey of the frequencies of Biston betularia (L.)(Lep.) and its melanic forms in Great Britain. Heredity, 12, 51-72.

Lachmann, M., & Jablonka, E. (1996). The inheritance of phenotypes: an adaptation to fluctuating environments. Journal of theoretical biology, 181, 1-9.

Laland, K., Uller, T., Feldman, M., Sterelny, K., Müller, G. B., Moczek, A., . . . Hoekstra, H. E. (2014). Does evolutionary theory need a rethink? Nature, 514(7521), 161.

Lande, R. (1998). Anthropogenic, ecological and genetic factors in extinction and conservation. Researches on population ecology, 40, 259-269.

Lin, Q., & Mendelssohn, I. A. (2012). Impacts and recovery of the Deepwater Horizon oil spill on vegetation structure and function of coastal salt marshes in the Northern Gulf of Mexico. Environmental Science & Technology, 46, 3737-3743.

Lin, Q., Mendelssohn, I. A., Graham, S. A., Hou, A., Fleeger, J. W., & Deis, D. R. (2016). Response of salt marshes to oiling from the Deepwater Horizon spill: Implications for plant growth, soil surface-erosion, and shoreline stability. Science of the Total Environment, 557, 369-377.

Lira-Medeiros, C. F., Parisod, C., Fernandes, R. A., Mata, C. S., Cardoso, M. A., & Ferreira, P. C. (2010). Epigenetic variation in mangrove plants occurring in contrasting natural environment. PloS one, 5, e10326. doi: 10.1371/journal.pone.0010326

Medrano, M., Herrera, C. M., & Bazaga, P. (2014). Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. Molecular Ecology, 23, 4926-4938.

Mirouze, M., Lieberman-Lazarovich, M., Aversano, R., Bucher, E., Nicolet, J., Reinders, J., & Paszkowski, J. (2012). Loss of DNA methylation affects the recombination landscape in Arabidopsis. Proceedings of the National Academy of Sciences, 109, 5880-5885.

Nelson, M. F., & Anderson, N. O. (2013). How many marker loci are necessary? Analysis of dominant marker data sets using two popular population genetic algorithms. Ecology and Evolution, 3, 3455-3470.

Nixon, Z., Zengel, S., Baker, M., Steinhoff, M., Fricano, G., Rouhani, S., & Michel, J. (2016). Shoreline oiling from the Deepwater Horizon oil spill. Marine pollution bulletin, 107, 170-178.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. (2007). The vegan package. Community ecology package, 10.

Orr, H. A., & Betancourt, A. J. (2001). Haldane's sieve and adaptation from the standing genetic variation. Genetics, 157, 875-884.

Paun, O., Bateman, R. M., Fay, M. F., Hedren, M., Civeyrel, L., & Chase, M. W. (2010). Stable epigenetic effects impact adaptation in allopolyploid orchids (Dactylorhiza: Orchidaceae). Mol Biol Evol, 27, 2465-2473. doi: 10.1093/molbev/msq150

Peakall, R., & Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics, 28, 2537-2539.

Pennings, S. C., & Bertness, M. D. (2001). Salt marsh communities. In: Marine Community Ecology, 289-316.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. Genetics, 155, 945-959.

Rapp, R. A., & Wendel, J. F. (2005). Epigenetics and plant evolution. New Phytologist, 168, 81-91. doi: 10.1111/j.1469-8137.2005.01491.x

Reyna-Lopez, G., Simpson, J., & Ruiz-Herrera, J. (1997). Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. Molecular and General Genetics MGG, 253, 703-710.

Richards, C. L., Hamrick, J., Donovan, L. A., & Mauricio, R. (2004). Unexpectedly high clonal diversity of two salt marsh perennials across a severe environmental gradient. Ecology Letters, 7, 1155-1162.

Richards, C. L., Pennings, S. C., & Donovan, L. A. (2005). Habitat range and phenotypic variation in salt marsh plants. Plant Ecology, 176, 263-273.

Richards, C. L., Schrey, A. W., & Pigliucci, M. (2012). Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. Ecology Letters, 15, 1016-1025.

Richards, C. L., Walls, R. L., Bailey, J. P., Parameswaran, R., George, T., & Pigliucci, M. (2008). Plasticity in salt tolerance traits allows for invasion of novel habitat by Japanese knotweed sl (Fallopia japonica and F.× bohemica, Polygonaceae). American Journal of Botany, 95, 931-942.

Robertson, M., & Richards, C. (2015). Opportunities and challenges of next-generation sequencing applications in ecological epigenetics. Molecular Ecology, 24, 3799-3801.

Salmon, A., Clotault, J., Jenczewski, E., Chable, V., & Manzanares-Dauleux, M. J. (2008). Brassica oleracea displays a high level of DNA methylation polymorphism. Plant Science, 174, 61-70. doi: 10.1016/j.plantsci.2007.09.012

Salzman, A. G. (1985). Habitat selection in a clonal plant. Science, 228(4699), 603-604.

Schmidt, P. S., & Rand, D. M. (1999). Intertidal microhabitat and selection at MPI: interlocus contrasts in the northern acorn barnacle, Semibalanus balanoides. Evolution, 53, 135-146.

Schmidt, P. S., Serrão, E. A., Pearson, G. A., Riginos, C., Rawson, P. D., Hilbish, T. J., . . . Wethey, D. S. (2008). Ecological genetics in the North Atlantic: environmental gradients and adaptation at specific loci. Ecology, 89(sp11), 91-107.

Schrey, A. W., Alvarez, M., Foust, C. M., Kilvitis, H. J., Lee, J. D., Liebl, A. L., . . . Robertson, M. (2013). Ecological epigenetics: beyond MS-AFLP. Integrative and comparative biology, 53, 340-350.

Schulz, B., Eckstein, R. L., & Durka, W. (2014). Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. Molecular Ecology, 23, 3523-3537. doi: 10.1111/ mec.12835

Silliman, B. R., van de Koppel, J., McCoy, M. W., Diller, J., Kasozi, G. N., Earl, K., . . . Zimmerman, A. R. (2012). Degradation and resilience in Louisiana salt marshes after the BP–Deepwater Horizon oil spill. Proceedings of the National Academy of Sciences, 109, 11234-11239.

Travis, S. E., Proffitt, C. E., & Ritland, K. (2004). Population structure and inbreeding vary with successional stage in created Spartina alterniflora marshes. Ecological Applications, 14, 1189-1202.

Trucchi, E., Mazzarella, A. B., Gilfillan, G. D., Romero, M. L., Schönswetter, P., & Paun, O. (2016). BsRADseq: screening DNA methylation in natural populations of non-model species. Molecular Ecology, 25, 1697-1713.

van Gurp, T. P., Wagemaker, N. C., Wouters, B., Vergeer, P., Ouborg, J. N., & Verhoeven, K. J. (2016). epiGBS: reference-free reduced representation bisulfite sequencing. Nature Methods, 13, 322-324.

van't Hof, A. E., Campagne, P., Rigden, D. J., Yung, C. J., Lingley, J., Quail, M. A., . . . Saccheri, I. J. (2016). The industrial melanism mutation in British peppered moths is a transposable element. Nature, 534(7605), 102-105.

Verhoeven, K. J., Jansen, J. J., van Dijk, P. J., & Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. New Phytologist, 185, 1108-1118. doi: 10.1111/j.1469-8137.2009.03121.x

Verhoeven, K. J., Van Dijk, P. J., & Biere, A. (2010). Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. Molecular Ecology, 19, 315-324.

Whitehead, A., Dubansky, B., Bodinier, C., Garcia, T. I., Miles, S., Pilley, C., . . . Walter, R. B. (2012). Genomic and physiological footprint of the Deepwater Horizon oil spill on resident marsh fishes. Proceedings of the National Academy of Sciences, 109, 20298-20302.

Wibowo, A., Becker, C., Marconi, G., Durr, J., Price, J., Hagmann, J., . . . Becker, J. (2016). Hyperosmotic stress memory in Arabidopsis is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. eLife, 5, e13546.

Williams, L. M., & Oleksiak, M. F. (2008). Signatures of selection in natural populations adapted to chronic pollution. BMC Evolutionary Biology, 8, 282.

Williams, L. M., & Oleksiak, M. F. (2011). Evolutionary and functional analyses of cytochrome P4501A promoter polymorphisms in natural populations. Molecular Ecology, 20, 5236-5247.

Wilschut, R. A., Oplaat, C., Snoek, L. B., Kirschner, J., & Verhoeven, K. J. (2016). Natural epigenetic variation contributes to heritable flowering divergence in a widespread asexual dandelion lineage. Molecular Ecology, 25, 1759-1768.

Xie, H. J., Li, A. H., Liu, A. D., Dai, W. M., He, J. Y., Lin, S., Duan, H., Liu, L. L., Chen, S. G., Song, X. L., Valverde, B. E. & Qiang, S. (2015). ICE1 demethylation drives the range expansion of a plant invader through cold tolerance divergence. Molecular Ecology, 24, 835–850.

Zengel, S., Weaver, J., Pennings, S. C., Silliman, B., Deis, D. R., Montague, C. L., . . . Zimmerman, A. R. (2016). Five years of Deepwater Horizon oil spill effects on marsh periwinkles Littoraria irrorata. Marine Ecology Progress Series.

Zilberman, D., Gehring, M., Tran, R. K., Ballinger, T., & Henikoff, S. (2007). Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nature Genetics, 39, 61-69.

Tables and Figures

Table 2.1. GPS coordinates of seven study sites.

		Coordinates	
Population	N	Longitude	Latitude
Oil Contaminated:			
GIO 1	6	29°26'42.8"N	89°55'45.7"W
GIO 2	7	29°26'11.2"N	89°54'35.9"W
MSO	8	30°15'29.1"N	89°24'45.6"W
Unaffected:			
GIN 1	9	29°10'09.2"N	90°09'05.7"W
GIN 2	8	29°10'49.4"N	90°06'31.6"W
MSN	8	30°20'21.1"N	89°21'15.3"W
AR	10	28°13'00.3"N	96°59'16.8"W

Table 2.2. Mean AFLP haplotype and MS-AFLP epigenotype diversity (*h*) and percent polymorphic loci by site (%P), based on 71 AFLP and 39 MS-AFLP loci.

	AFLP		MS-AFLP	
Population	h- (SE)	% P	h- (SE)	% P
Oil Contaminated:				
GIO 1	0.216 (0.031)	40.85%	0.179 (0.037)	41.03%
GIO 2	0.246 (0.035)	42.25%	0.185 (0.037)	43.59%
MSO	0.216 (0.028)	50.70%	0.161 (0.030)	41.03%
Unaffected:				
GIN 1	0.246 (0.027)	57.75%	0.226 (0.031)	66.67%
GIN 2	0.190 (0.022)	57.75%	0.152 (0.031)	46.15%
MSN	0.138 (0.020)	50.70%	0.204 (0.037)	48.72%
AR	0.103 (0.021)	28.17%	0.132 (0.033)	33.33%

Table 2.3. Summary of hierarchical AMOVA for AFLP and MS-AFLP data sets among site type (Φ_{RT}), among populations within site type (Φ_{PR}) and within populations (Φ_{PT}). Φ-statistics were calculated using 9999 permutations. d.f., degrees of freedom; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, NS = non-significant following sequential Bonferroni correction.

	Genetic			Epigenetic		
	Φ-statistics	% variation	d.f.	Φ-statistics	% variation	d.f.
Among site type	0.056 *	6	1	0.017 NS	1	1
Among populations within site type	0.168 ***	16	5	0.076 ***	7	5
Within subpopulations	0.215 ***	78	49	0.071 ***	92	49

Table 2.4. Pairwise Φ_{PT} comparisons of variation among study sites. Epigenetic comparisons are shown above the diagonal, genetic below. Statistical significance after sequential Bonferroni correction denoted by bolded numbers.

	Unaffected Sites				Oil-Contaminated Sites		
	GIN1	GIN2	MSN	AR	GIO1	GIO2	MSO
GIN1		0.017	0.033	**0.100**	0.000	0.057	0.052
GIN2	**0.179**		0.018	**0.182**	0.000	0.000	**0.111**
MSN	**0.201**	**0.144**		**0.133**	0.011	0.032	0.054
AR	**0.189**	**0.082**	**0.161**		**0.139**	**0.149**	**0.165**
GIO1	0.029	0.195	**0.165**	**0.215**		0.000	0.077
GIO2	0.123	**0.388**	**0.410**	**0.472**	0.163		**0.115**
MSO	0.045	**0.202**	**0.199**	**0.235**	0.068	0.125	

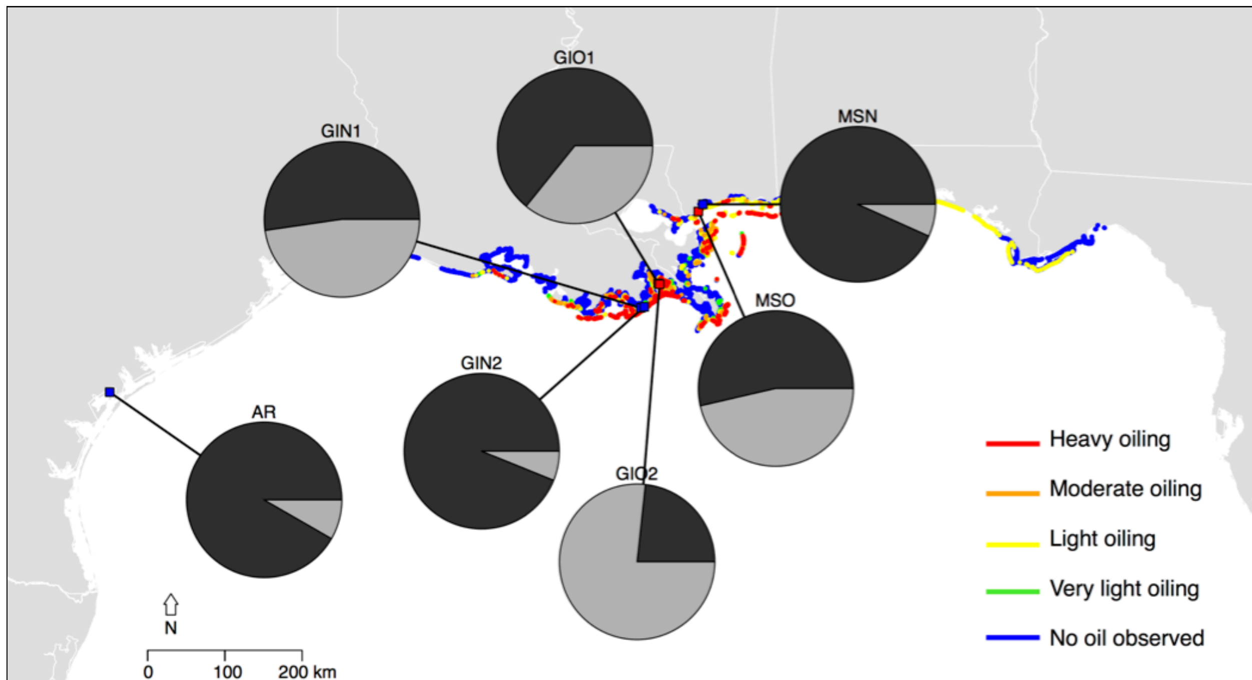


Figure 2.1. Map of seven study sites and their relative locations in the Gulf coast, with site specific oil intensity following the *DWH* oil spill, according to NRDA databases, and the results of Bayesian clustering. Population assignment to two groups is indicated by the shaded portion of the circle for each species. Group 1 = dark grey, group 2 = light grey.

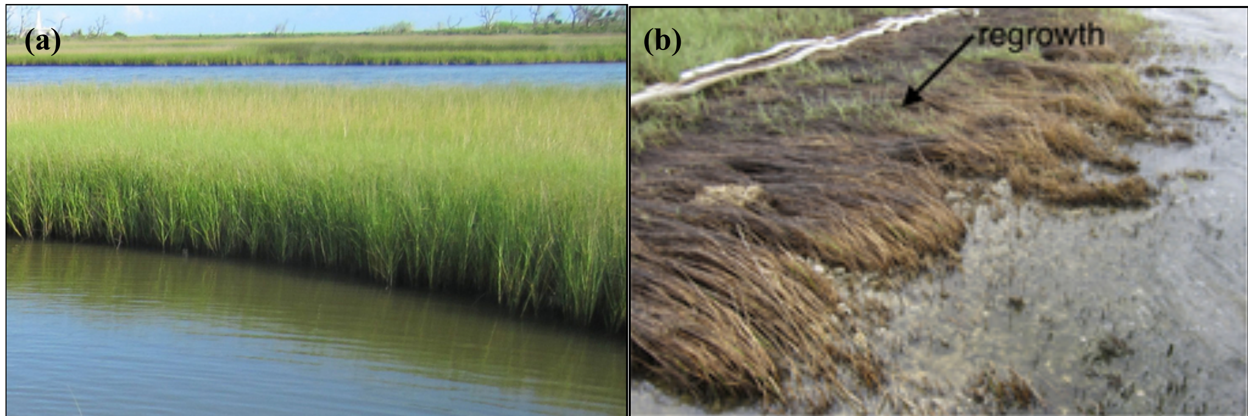


Figure 2.2. Examples of (a) non-contaminated (GIN1) and (b) contaminated sites (GIO1) in the Gulf Coast following the DWH oil spill. Oil was present on the soil surface at the time of sampling, and plants experienced substantial die-back. New growth sampled for this study (arrow) can be seen emerging from ramets under the soil surface through the dead wrack above ground.

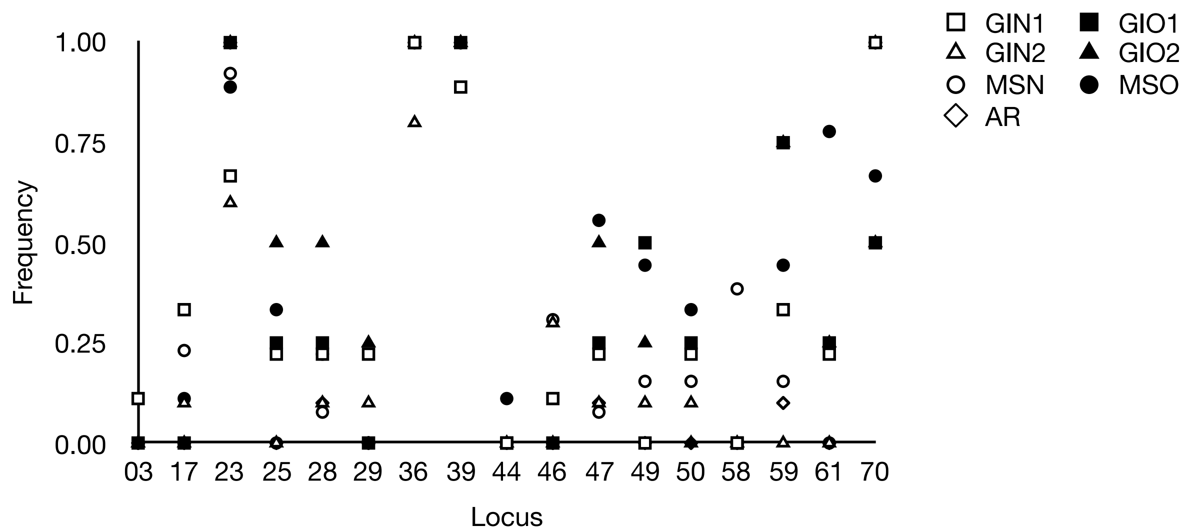


Figure 2.3. Frequencies of genetic loci significantly correlated to oil contamination across seven populations in locus-by-locus analysis. Contaminated sites are shown in open shapes and uncontaminated sites in closed shapes.

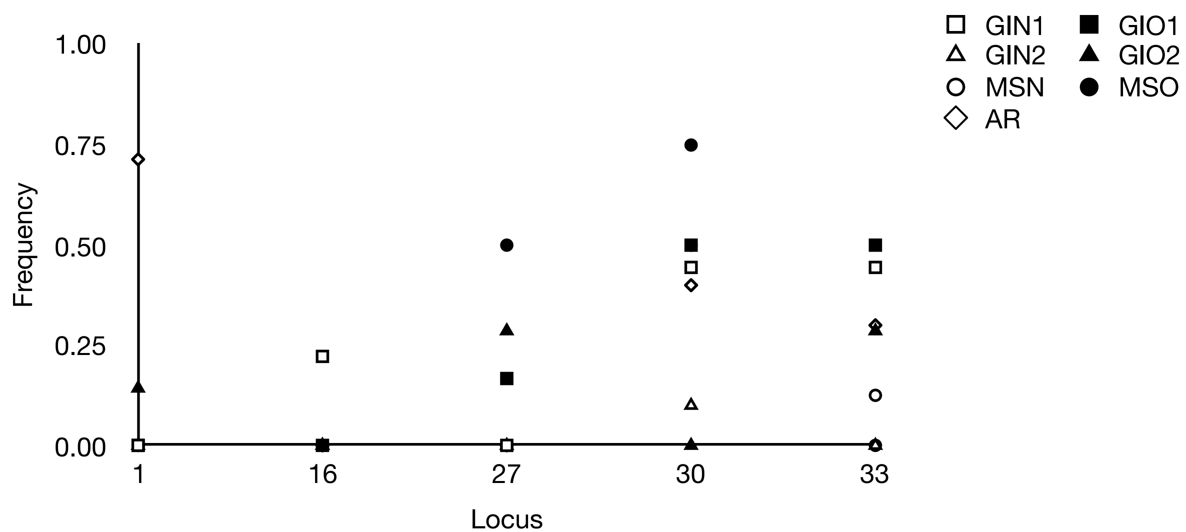


Figure 2.4. Frequencies of epigenetic loci significantly correlated to oil contamination across seven sites in locus-by-locus analysis. Contaminated sites are shown in open shapes and uncontaminated sites in closed shapes.

Chapter Three:
Conservation of methylation response to seasonal variation

Authors

Marta Robertson, Niccy Aitken, Adrienne Nicotra, Christina Richards

Abstract

Coastal ecosystems are subject to varied and periodic environmental stressors, reflecting environmental heterogeneity at several time scales. For example, daily tides or seasonal changes in rain and temperature may affect the prevalence and severity of ecological stressors, such as the abundance of herbivores, pathogens, or salt content in the soil. Organisms in these kinds of ecosystems employ several mechanisms to tolerate and adapt to the these conditions. Epigenetic mechanisms, such as DNA methylation, are potentially responsive to these dynamic changes in environmental stressors, and may provide a mechanism of plasticity under environmental change. We sampled eight individuals from three dominant species in different coastal ecosystems in the spring and fall over two years and found weak but significant variation in methylation between time points within individuals. Importantly, all three species showed similar patterns of temporal variation, suggesting conserved responses of DNA methylation across plant lineages.

Introduction

In the wild, organisms must regularly respond to dynamic environmental cues that are generated not only by spatial heterogeneity, but also by temporal variation in biotic and abiotic factors. These cues may manifest as stochastic cues or cyclical diurnal or seasonal cues, which can affect water and nutrient availability, pathogen abundance, light availability, and competitive interactions, among others (Nicotra et al. 2010). Additionally, plants also respond to internal developmental cues as they age and move between life history stages (Feng et al. 2010, Gutierrez-Marcos and Dickinson 2012). These exogenous and endogenous temporal cycles require dynamic regulation of biological processes by organisms in order to maintain homeostasis and persist across environments that are heterogenous in space and time. This is especially true in perennial plants, which respond to stochastic environmental variation and cyclical environmental variation as they control their own development from vegetative and reproductive growth to senescence (Rios et al. 2014, Shim et al. 2014).

Temporal variation affects a number of molecular processes, including gene expression, protein abundance, and regulatory variation. In particular, emerging work suggests that DNA methylation patterns may be highly variable across an organism's lifetime, varying both with environmental inputs (Dubin et al. 2105) and developmental stage (Rios et al. 2014). DNA methylation tends to occur on cytosines in specific motifs of the genome and is usually found in CG (in plants and animals), CHG, or CHH (in plants) contexts, where H is C, T or A. DNA methylation can regulate the movement of transposable elements and gene expression (Bewick et al. 2016), and may act as a link between environmental cues and organism phenotype (Herman et al. 2014). There have been several studies examining the response of DNA methylation to

seasonality in plants, but they have primarily been limited to model systems or controlled settings (e.g. Shen et al. 2014, Bratzel and Turck 2015, Keller et al. 2016). For example, the response of flowering time to seasonality is well-studied in the model system *Arabidopsis thaliana*. In this species, flowering is induced by the down-regulation of FLC in response to a cold period, and the expression of FLC is controlled by methylation (Shindo et al. 2005, Simpson and Dean 2002, Burn et al. 1993). Genome-wide CHH methylation can also increase with increasing temperature (Dubin et al. 2015) and patterns of methylation are variable with longer-term climatic variables (Keller at al. 2016). However, despite the evidence that methylation variation over time may be both extensive and correlated with seasonal variation, little work has been done to catalog epigenetic change over time in natural populations.

Coastal ecosystems are ideal systems in which to study temporal effects on epigenetic variation. Coastal systems are productive and dynamic environments that are subject to severe salt and nutrient gradients, periodic disturbance events, and dramatic fluctuations in seasonal cues. For example, tidal and rainfall influences create wide salinity and nutrient gradients across the salt marsh, which structures variation within and between species (Callaway et al. 1990, Pennings and Bertness 2001). Additionally, seasonal variation in biotic stressors, such as herbivory and pathogen prevalence, may interact with existing abiotic stressors to exacerbate stressful conditions (Silliman and Newell 2003, Mordecai 2011, Wieski and Pennings 2014). These predictable natural fluctuations create a useful model to disentangle natural standing variation from seasonal variation over time.

Although species have evolved different strategies to cope with this environmental heterogeneity, they may share some common mechanisms of response, like common DNA

methylation machinery, to maintain homeostasis in dynamic environments. For example, the foundation grass species *Spartina alterniflora* displays phenotypic variation along natural environmental gradients in the salt marsh, which is partially correlated with DNA methylation variation (Foust et al. 2016). Much of the enzymatic DNA methylation machinery is conserved across plant species (Feng and Jacobsen 2011), such as maintenance methyltransferases (CMT3, DRM2) and *de novo* methyltransferases (CMT2). Although different species vary greatly in genome-wide methylation pattern and the extent of methylation present (Niederhuth et al. 2016), they may share common patterns of response to environmental stimuli, such as an increase of CHH methylation mediated by CMT2 in response to heat (Shen et al. 2014, Dubin et al. 2015). Thus, variation in global methylation or variation in the relative proportions of context-specific methylation across the genome may be broadly indicative of regulatory shifts across plant species. In this study, we examined the methylation variation present in a dominant plant species from each of three coastal ecosystem types. We used a reduced representation bisulfite sequencing approach, epigenotyping-by-sequencing (epiGBS), to measure cytosine methylation in the spring and fall over the course of two years in eight replicates of each species. We expected that methylation patterns and targets would correlate with season, and that broad-scale patterns would be detectable across all species, despite different habitat types.

Materials and Methods

Study sites and species

Our sampling scheme allows for the broad study of DNA methylation changes over time, as we have repeatedly sampled eight individuals from three perennial plant species in the spring

and autumn over two years (N=96). In the spring, plants were beginning to establish new growth and in the fall had begun to senesce. Our sampling design allows us to decipher DNA methylation associated with the development of a plant over seasons and the aging of a perennial plant from year to year. We sampled a dominant plant species in each of three coastal ecosystem types: a clonal grass, *S. alterniflora* (Poaceae) from the salt marsh (Chassahowitzka National Wildlife Refuge, FL, USA) and two asters, *Iva imbricata* (Asteraceae) from the beach (Ft. Desoto State Park, FL, USA), and *Olearia axillaris* (Asteraceae) from the coastal foredune ridge (Jervis Bay National Park, Australia). All three species have been recognized as key components in shoreline stabilization and coastal restoration efforts (Colosi and McCormick 1978, Hinchliffe and Conran 2004, Lin et al. 2016), and all three species have a rich ecological history. *Spartina alterniflora* is salt tolerant and spreads vegetatively via rhizomes, although it does produce seed. It is a native foundation salt marsh grass species on the east coast of the United States, but is also highly invasive worldwide. *Iva imbricata* is also salt tolerant and has a woody stem and a thick cuticle (Colosi and McCormick 1978). Its seeds are wind and water dispersed and it is native to southeastern United States. *Olearia axillaris* is woody and salt tolerant. Its seeds are wind dispersed and it is native to the southern coast of Australia and Tasmania (Hinchliffe and Conran 2004). We controlled for genetic variation over time by repeatedly sampling the same individuals, with the exception of *S. alterniflora*, in which the aboveground biomass dies back regularly. In this case, we used a 0.25 m^2 quadrat to find a ramet from approximately the same location at each sampling point.

DNA extractions and library prep

We isolated DNA from eight samples across four time points for each species using the Qiagen DNeasy plant mini kit according to the manufacturer's protocol. We prepared epiGBS libraries *sensu* VanGurp et al. (2016). Briefly, isolated DNA was fragmented with PstI, which is sensitive to CHG methylation and biases resulting libraries toward coding regions (van Gurp et al. 2016). Adapters with variable barcodes were ligated to either end of the resulting fragments (Table 3.1), which were then treated with sodium bisulfite to detect cytosine methylation. Adapters had methylated cytosines to ensure their sequence fidelity through the subsequent bisulfite treatment. We used the Zymo EZ Lightning methylation kit to bisulfite treat and clean the DNA. Libraries were then amplified with the KAPA Uracil Hotstart Ready Mix with the following PCR conditions: an initial denaturation step at 98C for 1 min followed by 24 cycles of 98C for 30s, 60C for 45s, and 72C for 45s, with a final extension of 72C for 5 min. We sequenced paired-end reads on Illumina HiSeq 5000 at the Australian National University.

Data pre-processing and epigenetic analysis

We used the pipeline published by VanGurp et al. (2016) to assemble the de novo reference sequence, trim adapter sequences, and call SNP variation and methylation (https://github.com/thomasvangurp/epiGBS). We estimated the genetic variation (SNP) and epigenetic variation (percent methylation per locus) among our samples that could be explained by season and year with a perMANOVA using the vegan package in R (Oksanen et al. 2016).

We used the command line tool MACAU (Lea et al. 2015) to identify differentially methylated positions (DMPs) between season and year, and then applied a 1% FDR correction

using the R package qvalue (Storey et al. 2015). MACAU tests for locus-by-locus associations with predictors of interest using a binomial mixed model, which is appropriate for count data generated by bisulfite sequencing, and controls for effects of population structure on DNA methylation variation with a genetic relatedness matrix (Lea et al. 2015). Thus, we were able to assess changes in DNA methylation after controlling for the effects of genotype and relatedness of our samples.

Although population level epigenetic sequencing is growing in popularity, statistical analyses are still being developed for these data, particularly for complex, ecologically relevant designs. We modeled the main effects of season and year as well as the interaction between the two, and assessed significance for the two main effects. However, we were unable to test the significance of the interaction effect specifically due to constraints in the implementation of MACAU. Additionally, although a repeated measures test would be appropriate for our data, the MACAU framework does not allow for the inclusion of random effects, and instead models the effect of individual through the genetic relatedness matrix.

To prepare our data for use in MACAU, we restricted our dataset to common loci, defined as loci with reads in more than 90% of samples of our dataset. We then imputed over remaining missing data using the R package Amelia (Honaker et al. 2011). We generated a genetic relatedness matrix using GEMMA (Zhou and Stevens 2012) to control for population structure and we identified differentially methylated regions (DMRs) using the jackknife function from the Bootstrap package in R (Leisch 2007). Other methods for DMR identification rely on a sliding window approach, in which a DMR is defined as a cluster of DMPs, usually occurring within 200 basepairs (Schmitz et al. 2013, Keller et al. 2016). However, most of our

fragments are 100-200 basepairs long and we do not have reference genomes, so the sliding window approach is not feasible for our dataset. Alternatively, a jackknife approach allows us to identify fragments that have more DMPs than expected by chance.

Finally, we explored the change in methylation pattern over time by calculating the proportion of methylation in each context over time per sample and the measured methylation rate of each context per sample. We defined proportion of methylation as the fraction of methylated positions present in each context out of the total methylation measured over all contexts. We defined methylation rate as the percentage of methylated positions present in each context out of the total number of loci of each context.

Results

We analyzed 62,806 fragments from *S. alterniflora*, 31,384 from *I. imbricata,* and 75,828 from *O. axillaris.* Due to stochasticity in the sequencing process, four samples of *S. alterniflora* had few reads and little overlap with other samples, and were omitted from the subsequent analysis.

As expected, our perMANOVA showed high amounts of genetic variation between individuals in *I. imbricata* and *O. axillaris* (Table 3.2). However, we did not find significant effect of individual in *S. alterniflora,* indicating that there is a much variation within as between our purported individuals. However, individual still explained a high amount of variance in the perMANOVA (R^2, Table 3.2), suggesting genetic variation is still the most important driver of epigenetic variation. In locus-by-locus-modeling (MACAU), genetic variance is controlled through a genetic relatedness matrix. Epigenetic variation was high between individuals in all

three species (Table 3.3) and was also partially structured by year in *S. alterniflora* and season in *O. axillaris,* although these effects explained little of the variance in each case (Table 3.3, R^2).

After filtering reads with little overlap between samples and imputing missing data, we identified 31,641 common loci in *S. alterniflora* (Figure 3.1), 55,597 common loci in *I. imbricata* (Figure 3.2)*,* and 55,285 common loci in *O. axillaris* (Figure 3.3)*.* MACAU identified 8 DMPs (0.025%) between spring and fall for *S. alterniflora,* 50 (0.09%) for *I. imbricata*, and 124 (0.22%) for *O. axillaris*. MACAU also identified 4 DMPs (0.013%) between 2012 and 2013 for *S. alterniflora*, 81 (0.15%) for *I. imbricata*, and 24 (0.043%) for *O. axillaris*. Of the identified DMPs, 2 were CG, 2 were CHG, and 8 were CHH contexts in *S. alterniflora;* 88 were CG, 11 were CHG, and 32 were CHH in *I. imbricata*; and 100 were CG, 14 were CHG, and 34 were CHH in *O. axillaris*. We were unable to detect any DMRs between seasons or years in any of the specie

All species showed a higher rate of CG methylation compared to non-CG methylation across all collection points (Figure 3.4-6), but no significant effect of season or year on methylation rates ($P > 0.05$). Although CG sites were most consistently methylated, CHH methylation accounted for a larger portion of measured methylation across all species (Figure 3.7). However, again, these proportions did not change significantly over time.

Discussion

Natural plant populations harbor high amounts of epigenetic variation, and organisms may use epigenetic mechanisms to modulate phenotype in response to seasonal change (Herman and Sultan 2016). Although stressors such as herbivory (Herrera and Bazaga 2011), salinity (Lira-

Medeiros et al. 2010, Verhoeven et al. 2010), pathogen attack (Dowen et al. 2012), and nutrient availability (Lira-Medeiros et al. 2010, Verhoeven et al. 2010) have all been shown to affect DNA methylation patterns in plants, the effect of natural environmental fluctuations on epigenetic variation is not well characterized. In this study, we measured standing DNA methylation variation in three coastal plant species at four time points over two years.

We used a novel reduced representation sequencing technique, epiGBS, to examine DNA methylation variation in natural populations of non model species. EpiGBS is particularly advantageous because it biases library construction to coding regions (VanGurp et al. 2016), increasing the likelihood of finding methylation variation with phenotypic effects. EpiGBS also uses a double-barcoded, paired end read system to multiplex a large number of individuals in a single sequencing lane, reducing the cost of sequencing and allowing the study of sample sizes appropriate for ecological studies. Finally, bisulfite sequencing approaches are typically prohibitively expensive for non model species because they require the use of a non-bisulfite treated control sequence to parse methylation information (Robertson and Richards 2015). Instead, epiGBS employs a bioinformatic approach, using the double barcodes and Watson and Crick strands, to create a reference sequence from the bisulfite treated DNA (VanGurp et al. 2016), which is then assembled *de novo* and used to identify methylation and genetic polymorphisms. The use of epiGBS in this study provided a rich dataset to study the temporal epigenomic variation of three species over two years.

Despite the large dataset provided by the use of epiGBS, we did not find a genome-wide effect of season on methylation rate or methylation proportion by context. However, we did identify DMPs between measured seasons and years. In the coastal environments where these

plants are found, water availability, temperature, and salinity vary with season, and these differentially methylated loci may reflect specific regulation in response to these cues. The lack of genome-wide methylation pattern suggests that these plants are not using broad-scale repatterning of methylation to maintain homeostasis in response to seasonal environmental cues. However, they may be using methylation as part of a targeted response. Interestingly, we found the fewest DMPs in *S. alterniflora*, which also had the fewest common loci among samples. Constructed libraries may be more inconsistent among samples of *S. alterniflora* than for the other species because *S. alterniflora* has a large genome (1763.9 Mbp; Bedre et al. 2016) and amplified fragments may have less likelihood of matching across all samples.

Our use of three species also allows for comparative studies of DNA methylation in response to seasonal variation. Early studies in comparative epigenomics of plant species have identified methylation machinery that has been conserved across taxa, and have identified types of methylation that are associated with that machinery (Bewick et al. 2016). For example, DNA methylation can be found in three contexts: CG, CHG, and CHH, where H is any nucleotide except G. When present, CG and CHG methylation are both maintained by methyltranferases during DNA replication, whereas CHH methylation is placed *de novo*, suggesting a role in response to external perturbation. Global patterns of methylation context are consistent across many species. For example, a pattern of decreased CG methylation at start and stop sites, with enriched CG methylation in the gene body, is associated with increased gene expression (Niederhuth and Schmitz 2017), whereas non-CG methylation is associated with the suppression of transposable element activity and is typically enriched in the pericentromere (Mirouze et al. 2012).

However, these comparative studies have also found a high degree of variation in the extent of methylation present within and among species studied so far. The methylation rates we have measured in this study are consistent with those reported by Niederhuth et al. (2016) in a study of whole genome bisulfite sequences for 34 angiosperm species. They report methylation rates of approximately 60% CG, 30% CHG, and 5% CHH methylation in *Oryza sativa*, the closest relative to *S. alterniflora* in their study. Although our methylation rates are generally lower than those reported by Niederhuth et al. (2016), the relative pattern of methylation rates that we measured are consistent, where CG sites show the highest methylation rate and CHG and CHH methylation are similarly low. The discrepancy between our data set and Niederhuth et al. (2016) may be explained by the use of PstI in epiGBS. This enzyme is sensitive to CHG methylation, and biases toward coding regions (VanGurp et al. 2016), so the resulting libraries are biased samples of the genome. However, at a broad scale, our data is consistent with data reported by Niederhuth et al. (2016) and our use of multiple individuals measured at multiple time points accounts for some of the variation that may otherwise be introduced by a biased sampling of the genome.

Future directions

We know of two other studies that use bisulfite sequencing to measure epigenetic variation in natural plant populations (Platt et al. 2015, Gugger et al. 2016), but those studies employed RRBS at a great cost to sample size. The techniques presented here are an important first step in deciphering the role of epigenetic variation in natural systems. Our findings suggest that seasonal variation has some effect on DNA methylation, but does not shape methylation

patterns across the genome. Plants may be responding to environmental cues in a targeted way, but further work in additional species will need to be done to determine the consistency of the patterns found here.

Acknowledgments

I would like to thank Mariano Alvarez, Laura Lotero, Janny Peters, and Sandy Voors, for help in the field, and Thomas VanGurp and Neils Wagemaker for assistance with epiGBS. Sampling at Chassahowitzka National Wildlife Refuge was possible due to the generous assistance from Jody Pack and Bob Quarles, and sampling at Fort DeSoto State Park through generosity of everyone at Topwater Kayak Outpost . This work was partially supported by funding by the Australian Government through the Australian Research Council's Discovery Projects funding scheme (project number DP120100945; to AN).

References

Alvarez, M., Schrey, A. W., & Richards, C. L. (2015). Ten years of transcriptomics in wild populations: what have we learned about their ecology and evolution?. Molecular Ecology, 24(4), 710-725.

Bedre, R., Mangu, V. R., Srivastava, S., Sanchez, L. E., & Baisakh, N. (2016). Transcriptome analysis of smooth cordgrass (Spartina alterniflora Loisel), a monocot halophyte, reveals candidate genes involved in its adaptation to salinity. BMC genomics, 17(1), 657.

Bewick, A. J., Ji, L., Niederhuth, C. E., Willing, E.-M., Hofmeister, B. T., Shi, X., . . . Hartwig, B. (2016). On the origin and evolutionary consequences of gene body DNA methylation. Proceedings of the National Academy of Sciences, 113(32), 9111-9116.

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana. Evolutionary Ecology, 24(3), 541-553. doi: 10.1007/s10682-010-9372-7

Bratzel, F., & Turck, F. (2015). Molecular memories in the regulation of seasonal flowering: from competence to cessation. Genome biology, 16(1), 192.

Burn, J. E., Bagnall, D. J., Metzger, J. D., Dennis, E. S., & Peacock, W. J. (1993). DNA methylation, vernalization, and the initiation of flowering. Proceedings of the National Academy of Sciences, 90(1), 287-291.

Colosi, J., & McCormick, J. (1978). Population Structure of Iva imbricata in Five Coastal Dune Habitats. Bulletin of the Torrey Botanical Club, 105(3), 175-186. doi:10.2307/2484112

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., . . . Ecker, J. R. (2012). Widespread dynamic DNA methylation in response to biotic stress. Proceedings of the National Academy of Sciences, 109(32), E2183-E2191.

Dubin, M. J., Zhang, P., Meng, D., Remigereau, M. S., Osborne, E. J., Casale, F. P., ... & Jagoda, J. (2015). DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. eLife:4, e05255.

Feng, S., & Jacobsen, S. E. (2011). Epigenetic modifications in plants: an evolutionary perspective. Current Opinion in Plant Biology, 14(2), 179–186. http://doi.org/10.1016/j.pbi.2010.12.002

Feng, S., Jacobsen, S. E., & Reik, W. (2010). Epigenetic reprogramming in plant and animal development. Science, 330(6004), 622-627.

Foust, C., Preite, V., Schrey, A. W., Alvarez, M., Robertson, M., Verhoeven, K., & Richards, C. (2016). Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. Molecular Ecology, 25(8), 1639-1652.

Franks, S. & Weis, A. (2008). A change in climate causes rapid evolution of multiple life‐history traits and their interactions in an annual plant. Journal of Evolutionary Biology 21(5): 1321-1334.

Gutierrez-Marcos, J. F. & Dickinson, H. G. (2012). Epigenetic reprogramming in plant reproductive lineages. Plant and Cell Physiology, 53(5), 817-823.

Herman, J. J., Spencer, H. G., Donohue, K., & Sultan, S. E. (2014). How stable 'should' epigenetic modifications be? Insights from adaptive plasticity and bet hedging. Evolution, 68(3), 632-643.

Herman, J. J. & Sultan , S. E. (2016). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. Paper presented at the Proc. R. Soc. B.

Herrera, C. M. & Bazaga, P. (2011). Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory. Molecular Ecology 20(8): 1675-1688.

Hinchliffe, J. & Conran, J. G. (2005). Life-class stages in coast daisy-bush (Olearia axillaris: Asteraceae) as a possible means of monitoring coastal dunes. Australian Journal of Botany 53(2): 133-139.

Honaker, J., King, G., & Blackwell, M. (2011). Amelia II: A program for missing data. Journal of statistical software, 45(7), 1-47.

Keller, T. E., Lasky, J. R., & Yi, S. V. (2016). The multivariate association between genome‐wide DNA methylation and climate across the range of Arabidopsis thaliana. Molecular Ecology, 25, 1823-1837.

Lea, A. J., Tung, J., & Zhou, X. (2015). A flexible, efficient binomial mixed model for identifying differential DNA methylation in bisulfite sequencing data. PLoS Genet, 11(11), e1005650.

Leisch, F. (2007). "bootstrap: Functions for the Book “An Introduction to the Bootstrap." R package version 1: 21.

Lin, Q., Mendelssohn, I. A., Graham, S. A., Hou, A., Fleeger, J. W., & Deis, D. R. (2016). Response of salt marshes to oiling from the Deepwater Horizon spill: Implications for plant growth, soil surface-erosion, and shoreline stability. Science of the Total Environment, 557, 369-377.

Lira-Medeiros, C. F., Parisod, C., Fernandes, R. A., Mata, C. S., Cardoso, M. A., & Ferreira, P. C. (2010). Epigenetic variation in mangrove plants occurring in contrasting natural environment. PloS one, 5, e10326. doi: 10.1371/journal.pone.0010326

Mirouze, M., Lieberman-Lazarovich, M., Aversano, R., Bucher, E., Nicolet, J., Reinders, J., & Paszkowski, J. (2012). Loss of DNA methylation affects the recombination landscape in Arabidopsis. Proceedings of the National Academy of Sciences, 109, 5880-5885.

Mordecai, E. A. (2011). Pathogen impacts on plant communities: unifying theory, concepts, and empirical work. Ecological Monographs, 81(3), 429-441.

Nicotra, A. B., Atkin, O. K., Bonser, S. P., Davidson, A. M., Finnegan, E. J., Mathesius, U., ... & van Kleunen, M. (2010). Plant phenotypic plasticity in a changing climate. Trends in plant science, 15(12), 684-692.

Niederhuth, C. E., Bewick, A. J., Ji, L., Alabady, M. S., Do Kim, K., Li, Q., ... & Egesi, C. (2016). Widespread natural variation of DNA methylation within angiosperms. Genome Biology, 17(1), 194.

Niederhuth, C. E. & Schmitz, R. J. (2017). Putting DNA methylation in context: from genomes to gene expression in plants. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1860(1), 149-156.

Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, . . .,and H. Wagner (2016). vegan: Community Ecology Package. R package version 2.4-1. https://CRAN.R-project.org/package=vegan

Pennings, S. C. & Bertness, M. D. (2001). Salt marsh communities. Marine Community Ecology: 289-316.

Platt, A., Gugger, P. F., Pellegrini, M., & Sork, V. L. (2015). Genome−wide signature of local adaptation linked to variable CpG methylation in oak populations. Molecular ecology, 24(15), 3823-3830.

Ríos, G., Leida, C., Conejero, A., & Badenes, M. L. (2014). Epigenetic regulation of bud dormancy events in perennial plants. Frontiers in Plant Science, 5, 247. http://doi.org/10.3389/fpls.2014.00247

Robertson, M. & Richards C. (2015). Opportunities and challenges of next-generation sequencing applications in ecological epigenetics. Molecular Ecology, 24(15), 3799-3801.

Schmitz, R. J., Schultz M.D., Urich M.A., Nery J.R., Pelizzola M., Libiger O., . . . Schork N.J. (2013). Patterns of population epigenomic diversity. Nature, 495(7440), 193-198.

Schrey, A. W., Alvarez, M., Foust, C. M., Kilvitis, H. J., Lee, J. D., Liebl, A. L., . . . Robertson, M. (2013). Ecological epigenetics: beyond MS-AFLP. Integrative and Comparative Biology, 53, 340-350.

Silliman, B. R. & Newell S. Y. (2003). Fungal farming in a snail. Proceedings of the National Academy of Sciences, 100(26), 15643-15648.

Shen, X., De Jonge, J., Forsberg, S. K., Pettersson, M. E., Sheng, Z., Hennig, L., & Carlborg, Ö. (2014). Natural CMT2 variation is associated with genome-wide methylation changes and temperature seasonality. PLoS Genet, 10(12), e1004842.

Shim, D., Ko, J. H., Kim, W. C., Wang, Q., Keathley, D. E., & Han, K. H. (2014). A molecular framework for seasonal growth-dormancy regulation in perennial plants. Horticulture research, 1.

Shindo, C., Aranzana, M. J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M., & Dean, C. (2005). Role of FRIGIDA and FLOWERING LOCUS C in determining variation in flowering time of Arabidopsis. Plant physiology, 138(2), 1163-1173.

Simpson, G. G., & Dean, C. (2002). Arabidopsis, the Rosetta stone of flowering time?. Science, 296(5566), 285-289.

Smith, H., Casal, J. J., & Jackson, G. M. (1990). Reflection signals and the perception by phytochrome of the proximity of neighbouring vegetation. Plant, Cell & Environment, 13(1), 73-78.

Storey, J. D., Bass, A. J., Dabney, A., & Robinson, D. (2015). qvalue: Q-value estimation for false discovery rate control. R package version 2.4.2. http://github.com/jdstorey/qvalue

Tatra, G. S., Miranda, J., Chinnappa, C. C., & Reid, D. M. (2000). Effect of light quality and 5‐azacytidine on genomic methylation and stem elongation in two ecotypes of Stellaria longipes. Physiologia Plantarum, 109(3), 313-321.

van Gurp, T. P., Wagemaker, N. C., Wouters, B., Vergeer, P., Ouborg, J. N., & Verhoeven, K. J. (2016). epiGBS: reference-free reduced representation bisulfite sequencing. Nature Methods, 13, 322-324.

Verhoeven, K. J., Jansen, J. J., van Dijk, P. J., & Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. New Phytologist, 185, 1108-1118. doi: 10.1111/j.1469-8137.2009.03121.x

Więski, K., & Pennings, S. (2014). Latitudinal variation in resistance and tolerance to herbivory of a salt marsh shrub. Ecography, 37(8), 763-769.

Zhou, X. & Stephens, M. (2012). Genome-wide efficient mixed-model analysis for association studies. Nature Genetics, 44(7): 821-824.

Tables and Figures

Table 3.1. Adapter and primer sequences used in library preparation. xxxx and yyyy indicate location of variable nucleotide sequences, used to identify individuals multiplexed in one sequencing lane. We used twelve barcodes in the forward adapter and eight barcodes in the reverse adapter, for a total of 96 uniquely barcoded individuals per sequencing lane. Cytosines in the adapter sequences are methylated to maintain binding fidelity after bisulfite treatment.

	Sequence
Forward adapter	5’ - ACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxTGCA - 3’
	3’ - TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGAyyyy - 5’
Reverse adapter	5’ - xxxxAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG - 3’
	3’ - ACGTyyyyTCTAGCCTTCTCGCCAAGTCGTCCTTACGGCTC - 5’
Primer A	5’ - AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA CGCTCTTCCGATCT - 3’
Primer B	5’ - CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTG AACCGCTCTTCCGATCT - 3’

Table 3.2. Structure of genetic variation in each species explained by individual, season, and year. Adjusted R^2 and F statistics are reported from a perMANOVA using the adonis function in the vegan package for R; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Species	Source	df	R^2	F	
S. alterniflora	Individual	7	0.2433	1.098	
	Season	1	0.0427	1.350	
	Year	1	0.0155	0.490	
I. imbricata	Individual	7	0.3889	2.165	***
	Season	1	0.0231	0.899	
	Year	1	0.0234	0.913	
O. axillaris	Individual	7	0.2381	1.069	***
	Season	1	0.0316	0.994	
	Year	1	0.0307	0.966	

Table 3.3. Structure of epigenetic variation in each species explained by individual, season, and year. Adjusted R^2 and F statistics are reported from a perMANOVA using the adonis function in the vegan package for R;* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Species	Source	df	R^2	F	
S. alterniflora	Individual	7	0.3257	1.412	**
	Season	1	0.0407	1.236	
	Year	1	0.0436	1.323	*
I. imbricata	Individual	7	0.6964	8.025	***
	Season	1	0.0151	1.214	
	Year	1	0.0164	1.321	
O. axillaris	Individual	7	0.6916	8.003	***
	Season	1	0.0216	1.750	*
	Year	1	0.0146	1.187	

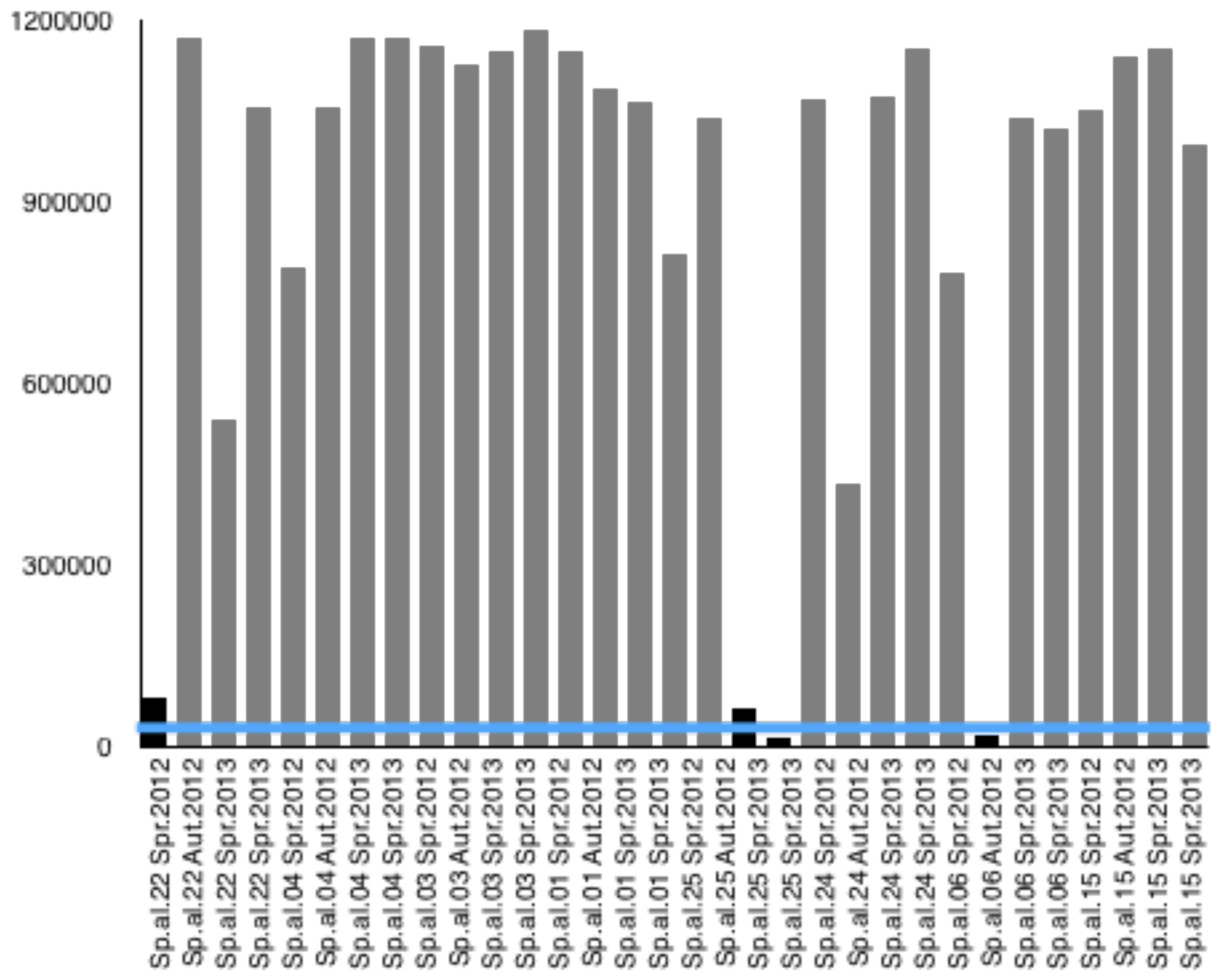


Figure 3.1. Number of sequenced cytosines per sample for *S. alterniflora* shown in dark grey. The number of common loci for locus-by-locus analysis is indicated by the blue line. Four samples with poor read coverage were excluded from analysis, shown in black.

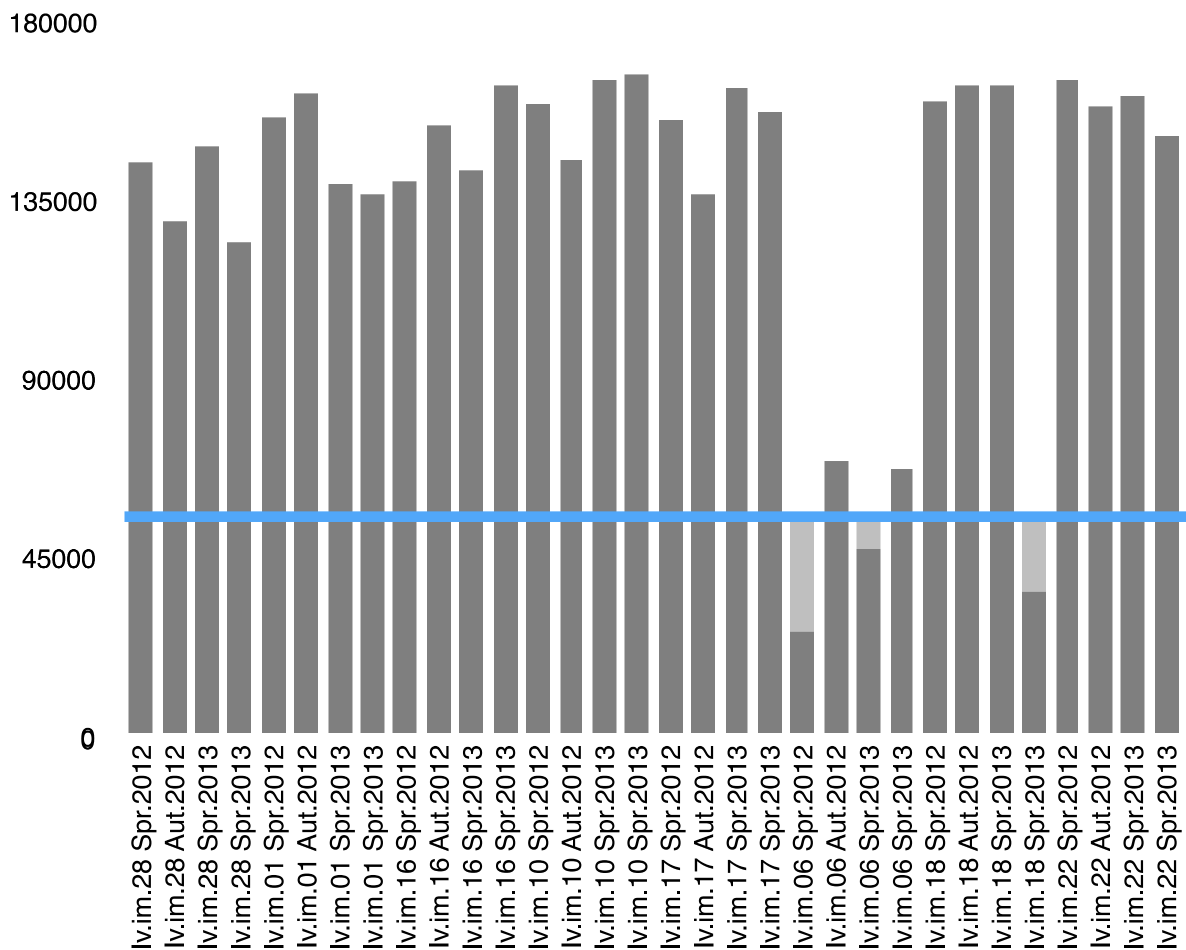


Figure 3.2. Number of sequenced cytosines per sample for *I. imbricata* shown in dark grey. Imputed data shown in light grey. The number of common loci for locus-by-locus analysis is indicated by the blue line.

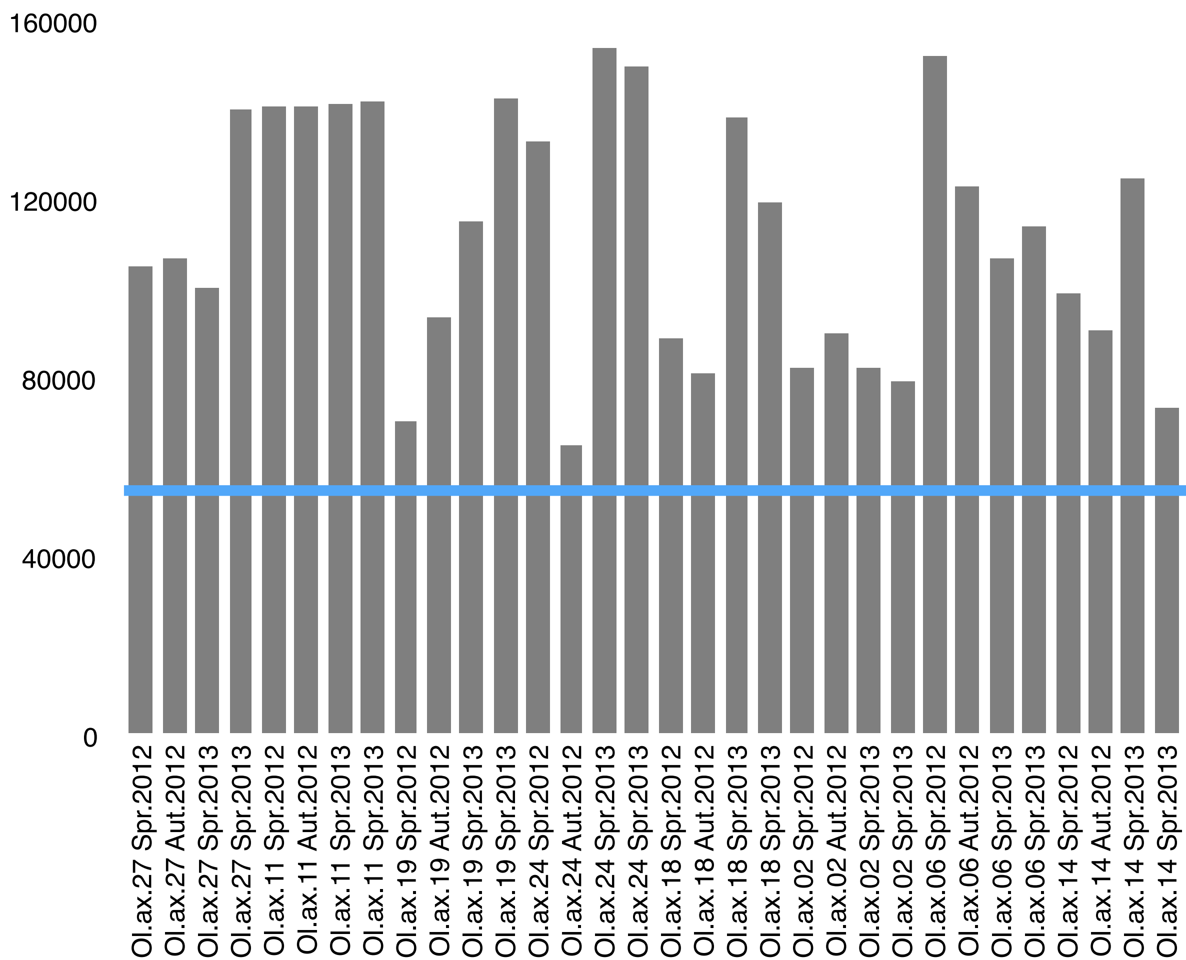


Figure 3.3. Number of sequenced cytosines per sample for *O. axillaris* shown in dark grey. The number of common loci for locus-by-locus analysis is indicated by the blue line.

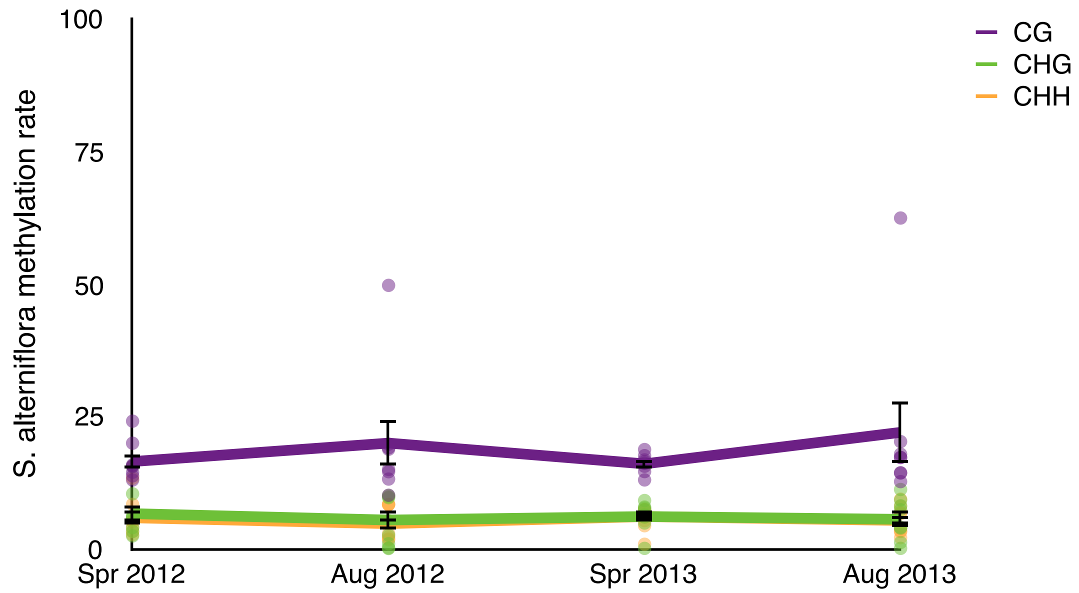


Figure 3.4. Methylation rate by context for *S. alterniflora* by season. Individual measures are shown in the background and species means ± s.e. are represented by the sold lines.

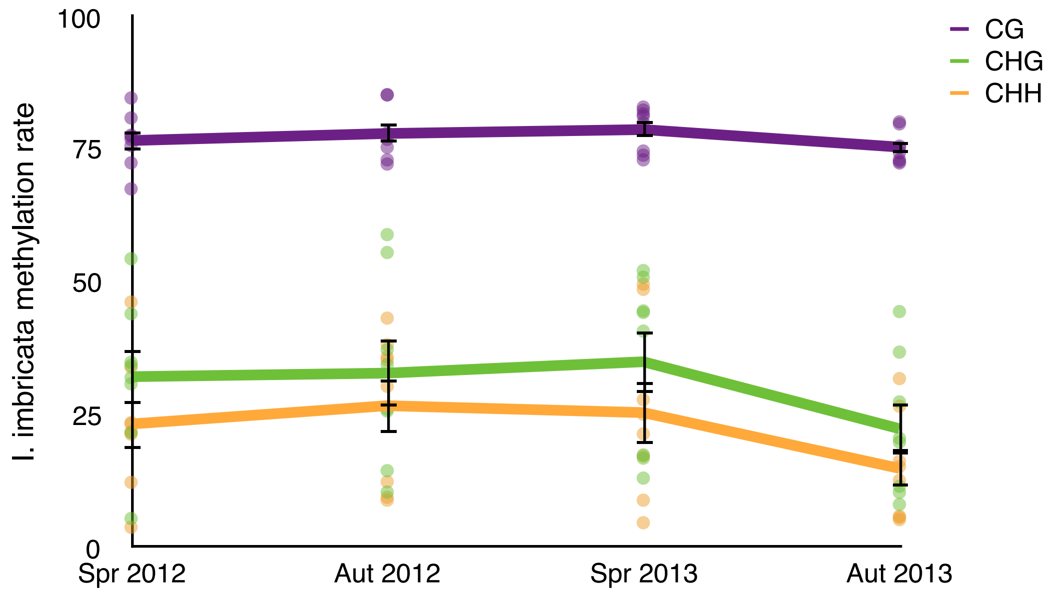


Figure 3.5. Methylation rate by context for *I. imbricata* by season. Individual measures are shown in the background and species means ± s.e. are represented by the sold lines. CG methylation is shown in purple, CHG in green, and CHH in orange.

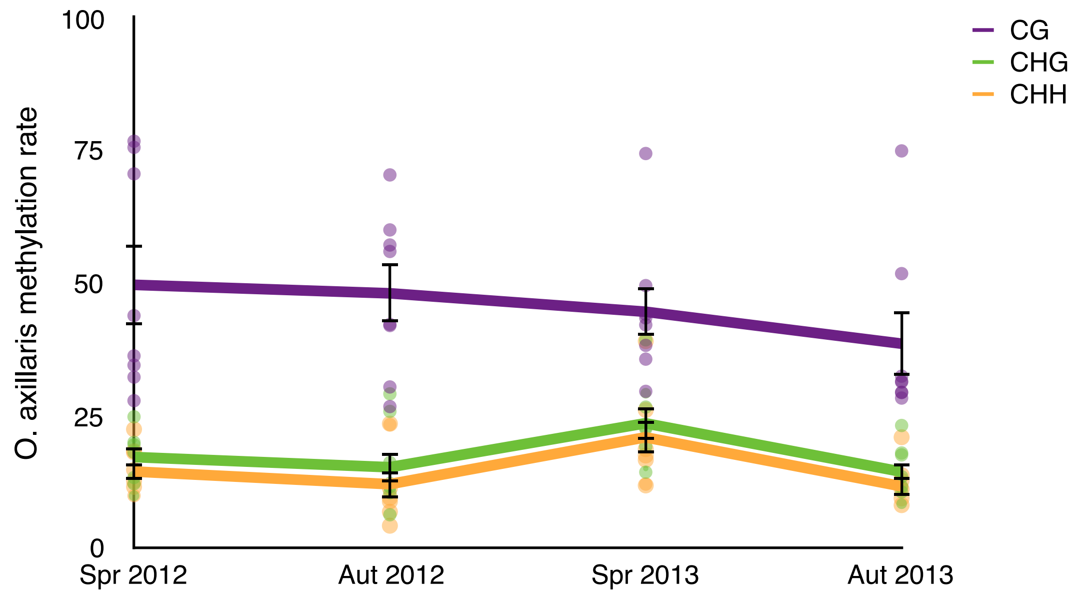


Figure 3.6. Methylation rate by context for *O. axillaris* by season. Individual measures are shown in the background and species means ± s.e. are represented by the sold lines. CG methylation is shown in purple, CHG in green, and CHH in orange.

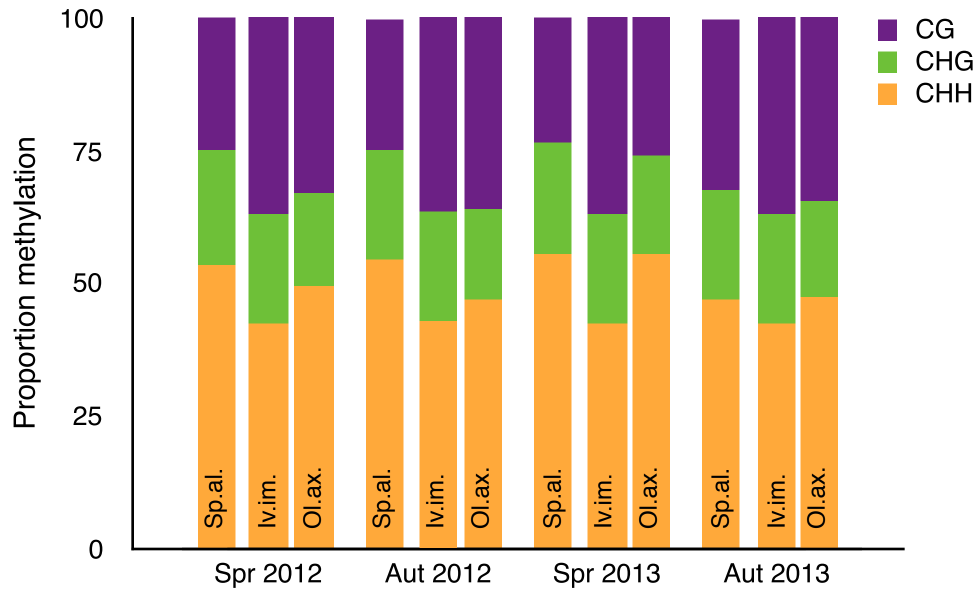


Figure 3.7. Proportion of methylation by context for *S. alterniflora, I. imbricata,* and *O. axillaris* by season. CG methylation is shown in purple, CHG in green, and CHH in orange.

Chapter Four:
Effects of CMT3 expression on phenotype under stress

Authors

Marta Robertson, Angela Roles, Josh Banta, Christina Richards

Abstract

Genome activity can be modulated in response to stressful conditions through dynamic regulatory and structural changes. DNA methylation is a well-studied epigenetic mark which may be environmentally responsive and may alter organismal phenotype both within and between generations. We studied the effects of environmental stress on the growth and survival of three genotypes of *Arabidopsis thaliana* which differed in their ability to maintain existing DNA methylation patterns. We compared phenotypes of CMT3 over expression lines and CMT3 knockouts to the wild type, Col-0, under two ecologically-relevant stresses, NaCl and salicylic acid, and then grew their offspring in a common unstressed environment. We found significant effects of one of the ecological stressors, NaCl, on growth and survival of the maternal plants, but no effect of salicylic acid. Additionally, we found that the offspring, although not exposed to stressors themselves, showed effects of parental stress in their growth and survival, regardless of stressor type. Furthermore, offspring from CMT3 over expression lines and CMT knockouts showed different effects of maternal stress than the wild type genotype. In concert with a

growing body of literature, these results suggest that DNA methylation may mediate transgenerational effects of stress in *A. thaliana*.

Introduction

Plants have evolved multiple strategies to cope with environmental heterogeneity, including phenotypically plastic responses to environmental fluctuations. In variable environments, it may be advantageous to pass information about phenotypic response to offspring (Herman and Sultan 2011), particularly when parental environments are accurate predictors of offspring environment (Lachmann and Jablonka 1996). Trans-generational priming or induced responses to ecological interactions have been documented in model and natural systems (Zas et al. 2013, Alsdurf et al. 2016, Herman and Sultan 2016). These responses manifest as inherited changes in gene expression and maternal effects (Holeski et al. 2012), where change in the phenotype of progeny depends on signals from the previous generation or in the inheritance of a phenotype induced in the previous generation.

Although the molecular mechanisms by which organisms regulate their responses to environmental cues remain poorly understood, DNA methylation has recently emerged as one possible mechanism for the regulation of phenotype in response to environmental heterogeneity (Foust et al. 2016, Herman and Sultan 2016). DNA methylation is the most commonly studied epigenetic mechanism (Schrey et al. 2013) and can affect gene expression (Saze 2008, Dowen et al. 2012, Nätt et al. 2012). DNA methylation tends to occur on cytosines in specific motifs of the genome and is usually found in CG (in plants and animals), CHG, or CHH (in plants) contexts, where H is C, T or A. Methylation of cytosines is controlled by methyltransferases such as

MET1 and CMT3, which either maintain DNA methylation at specific loci (MET1, CMT3) or establish methylation *de novo* (DRM2, CMT2; Niederhuth and Schmitz 2017). Importantly, DNA methylation is responsive to environmental stimuli (reviewed in Quadrana and Colot 2016). Biotic stimuli, such as herbivory (Herrera and Bazaga 2011) and pathogen attack (Dowen et al. 2012), as well as abiotic stressors such as salinity (Lira-Medeiros et al. 2010, Verhoeven et al. 2010), seasonal cues (Bastow et al. 2004), and nutrient availability (Bossdorf et al. 2010, Lira-Medeiros et al. 2010, Verhoeven et al. 2010) have all been shown to affect DNA methylation patterns in plants. In some cases, DNA methylation has been shown to be stably inherited across both mitotic and meiotic division (Verhoeven et al. 2010, Zhang et al. 2012), with consequences for environmental response. For example, Deficient in DNA Methylation 1 (DDM1) maintains DNA methylation across the genome. Epigenetic recombinant inbred lines (epiRILs) result from the crossing of DDM1 mutants with the wild type, Col-0. After several generations of inbreeding, the epiRILs are nearly genetically identical to Col-0, but have extensive DNA methylation variation among lines (Johannes et al. 2009). When subjected to drought and nutrient stress, these lines show heritable phenotypic effects mediated by differences in DNA methylation (Zhang et al. 2012), making DNA methylation a potential mechanism of transgenerational plasticity in isogenic lines. However, the mechanisms by which DNA methylation patterns interact with and respond to environment, both within and across generations, are still not well understood.

One potentially important maintenance methyltransferase in plants is chromomethyltransferase3 (CMT3). Phylogenetic evidence suggests that CMT3 function is central to the shaping of the epigenome over evolutionary history and has conserved methylation patterns in plants over evolutionary time (Bewick et al. 2016). CMT3 maintains cytosine

methylation by copying methylation from one strand to another in hemi-methylated contexts and preferentially in CHG sequences (Stroud et al. 2014), which tends to silence the expression of transposable elements (Kato et al. 2003). However, CMT3 also maintains gene body methylation in CG contexts through a positive feedback loop with methylation of H3K9 (Bewick et al. 2016). The pattern of gene body methylation, where CG methylation is depleted at transcriptional start and stop sites and enriched in the gene body, tends to be associated with gene expression (Bewick et al. 2016, Zilberman 2007, Nordborg et al. 2016), and plants with defective CMT3 have shown widespread DNA demethylation in addition to broad transcriptional changes under stress (Dowen et al. 2012). However, it is unclear whether natural variation in expression of CMT3 affects the distribution and lability of DNA methylation across the genome, and whether its effects on methylation distribution are dependent on environmental context.

As a major methyltransferase, CMT3 plays a crucial role in regulating the distribution of methylation across CG and CHG contexts in the genome. As such, variation in the expression of CMT3 may generate variation in phenotype, either through the downstream modulation of gene expression or through the release of transposable elements. Because plants modulate environmental response through the regulation of gene expression, variation in the expression of DNA methyltransferases may interfere with this response. Furthermore, since DNA methylation can be heritable (Zhang et al. 2012), the interaction of the expression of methyltransferases and environmental context in parental generations may alter the degree to which downstream phenotypic variation persists across generations. In this study, we used CMT mutant and over-expression lines to examine the effect of CMT3 on phenotype under stress in maternal plants and their progeny. We hypothesized that individuals deficient in CMT3 would be less capable of tuning

transcriptional response and therefore would be less tolerant of stressful conditions, show decreased fitness, and have less fit progeny. Conversely, we hypothesized that individuals with high expression of CMT3 would be more capable of modulating transcriptional response and therefore robust to environmental challenge.

Materials and Methods

Plant material

To study the effect of CMT3 on phenotype, we compared a mutation accumulation line with increased expression of CMT3 (identified and confirmed via qPCR and microarray, Roles unpublished) and a homozygous T-DNA CMT3 knockout (CS16392; Alonso et al. 2003) to the parent line, Col-0. Both CMT3 mutants have a background genotype of Col-0 and should show little genetic divergence from the parent line. We exposed these genotypes of *A. thaliana* to hyperosmotic (NaCl) and salicylic acid (SA) stress. SA is a plant hormone involved in the pathogen defense pathway, and its experimental application mimics the effect of infection and stimulates the defense response (Verhoeven et al. 2010). Both NaCl and SA have heritable effects on plant phenotype (Jiang et al. 2014, Latzel et al. 2012) and DNA methylation patterns (Jiang et al. 2014, Latzel et al. 2012), but the effects have not been tested on genotypes with upregulated and down regulated expression of CMT3.

Experimental conditions: maternal generation

After dark stratification at 4ºC for four days, we sewed seeds in MetroMix 360 potting soil (SunGro) and grew them in a Percival environmental chamber on a 16/8H light/dark cycle at

22ºC during the day and 18ºC at night. We exposed 20 replicates of each genotype to one of four treatments: 0.5 mM salicylic acid (SA), 150 mM NaCl, both SA and NaCl, and a non-treatment control (N=240). We started SA and NaCl treatments four weeks after planting and administered treatments every other day. We applied SA by spraying rosettes with solution until the leaves were wet and applied NaCl solution to the soil in lieu of water. We recorded time to germination, time to bolting, and time to flowering. We also measured leaves in the rosette at bolting time as an indicator of resource allocation and rosette diameter five weeks after planting. Ten weeks after planting, we measured plant height and counted the number of branches, fruits, and flowers as a measure of reproduction and collected seeds. Collected seeds were stored at 4ºC.

Experimental conditions: progeny

To study the effect of maternal genotype and environment on offspring phenotype, we grew twelve replicate offspring from two randomly selected mothers of each genotype from each treatment (N=288). Growing conditions were the same for offspring as parents, but offspring were grown in a common environment without NaCl or SA treatments. Again, we recorded time to germination, time to bolting, and time to flowering. We also counted leaves in the rosette at bolting time and measured the diameter of the rosette five weeks after planting.

Analyses

We analyzed the effect of genotype on time to germination and the interactive effect of genotype and treatment on time to bolting and time to flowering in a Kaplan Meier survival model with right censored data points. We chose to analyze germination time, bolting time, and

flowering time with a survival function because these are time-to-event data, where we have followed the progress of individuals over time. Because some individuals died or did not progress past a certain developmental stage, our data are also right censored. We also explored the risk of occurrence of an event (germination, bolting, or flowering) by genotype and treatment with a Cox proportional hazards test. We used the coxph function in the survival package in R (Therneau 2015) with main effects of genotype and treatment. Finally, we explored the timing of those events with an accelerated failure time model using the survreg function in the survival package of R (Therneau 2015) with main effects of genotype and treatment and a log logistic distribution.

We modeled both the main and interactive effects of genotype and treatment on the remaining phenotypic traits using a linear model with a negative binomial distribution for count data and a gaussian distribution for rosette diameter and height.

Finally, we analyzed the interactive effect of maternal genotype and environment on progeny survival and phenotype. Models were the same as in the parent generation, except offspring traits were modeled by genotype and maternal environment.

Results

Main effect of CMT3 on maternal survival and fitness

In the first generation, of 240 seeds planted, seven did not germinate, an additional seven did not bolt, and one more did not flower. We found no differences in survival curves or event timing for germination, bolting, or flowering among genotypes ($P > 0.05$ in all cases; Figure 4.1a). We also found no differences in phenotype among genotypes (Table 4.1).

Effect of biotic and abiotic stress on maternal survival and fitness

After controlling for germination date, we found that individuals exposed to NaCl, but not SA, were less likely to bolt ($\chi^2 = 16.3048$, $P < 0.001$) and less likely to flower ($\chi^2 = 4.9538$, $P = 0.026$). Similarly, our accelerated failure time model found that NaCl treatment, but not SA, tends to decrease time to bolting ($\chi^2 = 13.5726$, $P < 0.001$) and time to flowering ($\chi^2 = 7.7706$, $P < 0.01$) after germination (Figure 4.1b-c). Replicates under NaCl treatment had stunted growth, which was reflected in fewer fruits ($\chi^2 = 18.8348$, $P < 0.001$), fewer flowers ($\chi^2 = 30.3100$, $P < 0.001$), fewer branches ($\chi^2 = 38.823$, $P < 0.001$), shorter height ($F = 14.6262$, $P < 0.001$), and fewer leaves in the rosette at bolting ($\chi^2 = 11.2196$, $P < 0.001$) than replicates in control treatment (Figure 4.2). However, we found no effect of SA treatment on these same traits. Additionally, we found no differences in the interaction effect of NaCl or SA treatment with genotype.

Effect of maternal environment on progeny survival and growth

In the second generation, of 288 seeds planted, 17 did not germinate and two more did not bolt. Of the seeds that did not germinate, 12 were of the same maternal line, a CMT3 knockout subjected to NaCl treatments. We found a strong effect of genotype and maternal environment on survival curves for germination time ($\chi^2 = 35.5$, $P < 0.001$; Figure 4.3), bolting time ($\chi^2 = 19.5$, $P = 0.05$; Figure 4.4) and flowering time ($\chi^2 = 21.2$, $P = 0.03$; Figure 4.5). After controlling for germination date, our Cox proportional hazard model found an effect of maternal genotype on chance of germination ($\chi^2 = 12.3332$, $P < 0.01$), chance of bolting ($\chi^2 = 9.5597$, $P <$

0.01), and chance of flowering (χ^2 = 10.3363, $P < 0.01$). In these cases, the CMT3 knockout was less likely to germinate (χ^2 = 6.77 $P < 0.01$) or flower (χ^2 = 4.41, $P < 0.05$). Progeny from maternal lines subject to NaCl treatment were less likely to germinate (χ^2 = 10.1686, $P < 0.01$) or bolt (χ^2 = 3.9280, $P < 0.05$). We also found interactive effects of genotype and maternal NaCl treatment (χ^2 = 16.4354, $P < 0.001$), interactive effects of maternal SA treatment and maternal NaCl treatment (χ^2 = 15.6126, $P < 0.001$), and a three way interaction of genotype, maternal SA treatment, and maternal NaCl treatment (χ^2 = 20.0501, $P < 0.001$) on chance of germination. Similarly, our accelerated failure time model found that CMT3 knockout lines were slow to bolt (χ^2 = 11.3519, $P < 0.01$) and slow to flower (χ^2 = 12.2614, $P < 0.01$) and found an interactive effect of maternal NaCl treatment and genotype on time to bolting (χ^2 = 7.3273, $P < 0.05$) and time to flowering (χ^2 = 7.3288, $P < 0.05$) in the offspring. Additionally, we found an effect of maternal genotype (χ^2 = 13.9443, $P < 0.001$) and maternal NaCl treatment (χ^2 = 3.8817, $P <$ 0.05) on the number of leaves at bolting and maternal NaCl treatment on rosette diameter (F = 24.6849, $P < 0.001$; Table 4.2). Post-hoc analysis revealed both CMT3 over expression lines and CMT3 knockout lines had fewer leaves in the rosette at bolting than Col-0 when the maternal plants were exposed to NaCl (Figure 4.6).

Discussion

We grew three lines of *A. thaliana* with differing expression of CMT3, but the same background genotype, under a biotic and an abiotic stress. Although we did not observe significant effects of CMT3 expression on phenotype in the parent generation, we did observe strong effects of NaCl stress. We observed strong effects of NaCl stress, but not CMT3

expression, on survival and growth of *A. thaliana*. However, we found strong effects of parent environment on progeny survival and growth, which differed by genotype. Our findings suggest that CMT3 contributes to heritable methylation variation under environmental stress with important effects on fitness.

Effects of stress but not genotype

As expected, we found significant phenotypic differentiation in response to NaCl treatment in the parental generation. Plants were plastic in response to NaCl treatment in leaves on rosette at bolting, final height, and numbers of fruits, flowers, and branches. The control genotype, Col-0, was less plastic in response to NaCl treatment than either of the CMT3 expression lines, although this effect was not statistically significant (Figure 4.2). However, we did not find an effect of our biotic stress, salicylic acid, on phenotype in the parent generation. Although other studies have found that *A. thaliana* is more resistant to disease when global methylation is depressed (Dowen et al. 2012), we found no effect of SA on plant phenotype.

Previous studies also indicate that lines exposed to NaCl accumulate DNA methylation polymorphisms at a higher rate than lines in control conditions (Jiang et al. 2015). We expected that CMT3 knockout lines would be less able to buffer against this kind of mutagenesis and that this response would be exaggerated in our *cmt3* knockout lines. However, we did not observe a phenotypic effect due to CMT3 alone or a CMT3 expression by environment interaction, indicating that CMT3 neither affects phenotype nor mediates the observed phenotypic plasticity in the parental generation. This may be due to redundancy in methylation targets by other maintenance methyltransferases, such as MET1, which may have buffered against the loss of

methylation at CMT3 target sites. Alternatively, CMT3 may affect phenotype through the release of transposons rather than the modulation of gene expression, which may not manifest until subsequent generations.

Transgenerational interactions between parent environment and CMT3 expression

Evidence is gathering that plants have a generational stress memory, mediated by DNA methylation (Wibowo et al. 2016, Herman and Sultan 2016). Hyperosmotic stress in particular has been shown to induce changes in methylation patterns that persist over generations (Jiang et al. 2015, Wibowo et al. 2016). CMT3 is responsible for the maintenance of most CHG methylation in pollen (Hsieh et al. 2016), suggesting potential for a strong effect of CMT3 expression on offspring phenotype and methylation pattern. We expected that progeny from lines subject to stress would show inhibited survival and growth in comparison to progeny from unstressed parents, and that CMT3 expression and stress might interact to govern progeny phenotypes. Our results are consistent with these hypotheses, as we found that progeny from maternal lines subject to NaCl treatment were late to bolt and flower, and that this effect was exaggerated by the loss of CMT3. Furthermore, although we found no effect of SA in the parent generation, we found that progeny from CMT3 knockout lines exposed to SA were also inhibited in germination. In addition, we found that progeny from maternal lines subject to NaCl treatment produced fewer leaves in their rosette, although this effect was less extreme in individuals without CMT3. These results suggest that offspring from CMT3 mutant lines subject to NaCl stress may have difficulty transitioning from vegetative growth to reproductive growth, as bolting is delayed and rosette production continues. This may be due to variation in gene

expression resulting from changes in CMT3 expression during development, or from the release of transposons in the parental generation that generate random but potentially deleterious phenotypic responses to environmental cues in offspring generations.

In addition, we found that one of the CMT3 knockout maternal lines subject to NaCl failed to produce viable seed. McClintock suggested that environmental stress can cause "genomic shocks" that result large mutations in genome sequence and activate transposons (McClintock 1983). Since CMT3 is responsible for silencing transposons, it is possible that the maternal line that produced non-viable seed may have developed a lethal mutation in the germ line due to increased transposon movement. This may suggest that maintenance of DNA methylation machinery in stressful environments can increase inclusive fitness by buffering against deleterious mutations. However, changes in DNA methylation can be heritable for at least eight generations (Johannes et al. 2009), and our experiment has only examined the effects of stress and maternal genotype on the next generation. Further studies would be beneficial to examine the longevity of stress memory in these plants, as well as the rate of transposon-induced mutation.

Our study adds to the growing literature on epigenetically mediated environmental response by examining the role of the methyltransferase, CMT3, in stress response and the effects on progeny. In nature, plants are often phenotypically plastic in response to changing environmental cues. One of the ways plants might incorporate cues from their environment and alter regulation of gene expression is through epigenetic mechanisms, which are environmentally labile (Herman et al. 2014) and may be heritable in some cases (Verhoeven et al. 2010, Wibowo et al. 2016). The loss of epigenetic mechanisms may inhibit an organism's ability to respond to

its environment, leading to a decrease in fitness. Further work will need to be done to determine the evolutionary effects of methylation under environmental challenge, particularly in natural populations.

Acknowledgements

Thank you to Maria Nikolopoulos and Sandy Voors for help with data collection. This work was partially supported by funding from the National Science Foundation (U.S.A.) through DEB-1419960 and IOS-1556820 (to CLR).

References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., . . . Gadrinab, C. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science, 301(5633), 653-657.

Alsdurf, J., Anderson, C., & Siemens, D. H. (2016). Epigenetics of drought-induced trans-generational plasticity: consequences for range limit development. AoB Plants, 8, plv146.

Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A., & Dean, C. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. Nature, 427(6970), 164-167.

Bewick, A. J., Ji, L., Niederhuth, C. E., Willing, E.-M., Hofmeister, B. T., Shi, X., . . . Hartwig, B. (2016). On the origin and evolutionary consequences of gene body DNA methylation. Proceedings of the National Academy of Sciences, 113(32), 9111-9116.

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana. Evolutionary Ecology, 24(3), 541-553. doi: 10.1007/ s10682-010-9372-7

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., . . . Ecker, J. R. (2012). Widespread dynamic DNA methylation in response to biotic stress. Proceedings of the National Academy of Sciences, 109(32), E2183-E2191.

Foust, C., Preite, V., Schrey, A. W., Alvarez, M., Robertson, M., Verhoeven, K., & Richards, C. (2016). Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. Molecular Ecology, 25(8), 1639-1652.

Herman, J. J., Spencer, H. G., Donohue, K., & Sultan, S. E. (2014). How stable 'should' epigenetic modifications be? Insights from adaptive plasticity and bet hedging. Evolution, 68(3), 632-643.

Herman, J. J., & Sultan, S. E. (2011). Adaptive transgenerational plasticity in plants: case studies, mechanisms, and implications for natural populations. Frontiers in plant science, 2, 102.

Herman, J. J., & Sultan, S. E. (2016). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. Paper presented at the Proc. R. Soc. B.

Herrera, C. M., & Bazaga, P. (2011). Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory. Molecular Ecology, 20(8), 1675-1688. doi: 10.1111/j.1365-294X.2011.05026.x

Holeski, L. M., Jander, G., & Agrawal, A. A. (2012). Transgenerational defense induction and epigenetic inheritance in plants. Trends in ecology & evolution, 27(11), 618-626.

Jiang, C., Mithani, A., Belfield, E. J., Mott, R., Hurst, L. D., & Harberd, N. P. (2014). Environmentally responsive genome-wide accumulation of de novo Arabidopsis thaliana mutations and epimutations. Genome research. doi: 10.1101/gr.177659.114

Johannes, F., Porcher, E., Teixeira, F. K., Saliba-Colombani, V., Simon, M., Agier, N., . . . Colot, V. (2009). Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS Genet, 5(6), e1000530. doi: 10.1371/journal.pgen.1000530

Kato, M., Miura, A., Bender, J., Jacobsen, S. E., & Kakutani, T. (2003). Role of CG and non-CG methylation in immobilization of transposons in Arabidopsis. Current Biology, 13(5), 421-426.

Lachmann, M., & Jablonka, E. (1996). The inheritance of phenotypes: an adaptation to fluctuating environments. Journal of theoretical biology, 181(1), 1-9.

Latzel, V., Zhang, Y., Moritz, K. K., Fischer, M., & Bossdorf, O. (2012). Epigenetic variation in plant responses to defense hormones. Annals of botany, 110(7), 1423-1428.

Lira-Medeiros, C. F., Parisod, C., Fernandes, R. A., Mata, C. S., Cardoso, M. A., & Ferreira, P. C. (2010). Epigenetic variation in mangrove plants occurring in contrasting natural environment. PloS one, 5(4), e10326. doi: 10.1371/journal.pone.0010326

McClintock, B. (1983). Nobel Lecture: The Significance of Responses of the Genome to Challenge, Nobel Foundation, Stockholm.

Mirouze, M., Lieberman-Lazarovich, M., Aversano, R., Bucher, E., Nicolet, J., Reinders, J., & Paszkowski, J. (2012). Loss of DNA methylation affects the recombination landscape in Arabidopsis. Proceedings of the National Academy of Sciences, 109(15), 5880-5885.

Nätt, D., Rubin, C.-J., Wright, D., Johnsson, M., Beltéky, J., Andersson, L., & Jensen, P. (2012). Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. BMC genomics, 13(1), 59.

Quadrana, L., & Colot, V. (2016). Plant Transgenerational Epigenetics. Annual Review of Genetics, 50, 467-491.

Saze, H. (2008). Epigenetic memory transmission through mitosis and meiosis in plants. Semin Cell Dev Biol, 19(6), 527-536. doi: 10.1016/j.semcdb.2008.07.017

Schrey, A. W., Alvarez, M., Foust, C. M., Kilvitis, H. J., Lee, J. D., Liebl, A. L., . . . Robertson, M. (2013). Ecological epigenetics: beyond MS-AFLP. Integrative and comparative biology, 53(2), 340-350.

Stroud, H., Do, T., Du, J., Zhong, X., Feng, S., Johnson, L., . . . Jacobsen, S. E. (2014). Non-CG methylation patterns shape the epigenetic landscape in Arabidopsis. Nature structural & molecular biology, 21(1), 64-72.

Therneau, T. (2016). A Package for Survival Analysis in S. version 2.38; 2015. URL: http://cran. r-project. org/web/packages/survival/index. html [accessed 2016-06-17][WebCite Cache ID 6iKkaCcfO].

Verhoeven, K. J., Jansen, J. J., van Dijk, P. J., & Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. New Phytologist, 185(4), 1108-1118. doi: 10.1111/j.1469-8137.2009.03121.x

Wibowo, A., Becker, C., Marconi, G., Durr, J., Price, J., Hagmann, J., . . . Becker, J. (2016). Hyperosmotic stress memory in Arabidopsis is mediated by distinct epigenetically labile

sites in the genome and is restricted in the male germline by DNA glycosylase activity. eLife, 5, e13546.

Zas, R., Cendán, C., & Sampedro, L. (2013). Mediation of seed provisioning in the transmission of environmental maternal effects in Maritime pine (Pinus pinaster Aiton). Heredity, 111(3), 248-255.

Zhang, Y. Y., Fischer, M., Colot, V., & Bossdorf, O. (2013). Epigenetic variation creates potential for evolution of plant phenotypic plasticity. New Phytologist, 197(1), 314-322.

Zilberman, D., Gehring, M., Tran, R. K., Ballinger, T., & Henikoff, S. (2007). Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nature genetics, 39(1), 61-69.

Figures and Tables

Table 4.1. Results of linear model for growth traits in *A. thaliana* maternal plants. Height and rosette diameter were tested with a Gaussian distribution and report *F* statistics and significance. The remaining traits were tested with a negative binomial distribution and report χ^2 statistics and significance; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Source	df	Fruits		Flowers		Branches		Height		Rosette diameter	Leaves in rosette	
Genotype	2	1.903		1.098		5.705		2.293		0.640	5.828	
NaCl	1	18.835	***	30.310	***	38.823	***	14.626	***	0.983	11.220	***
SA	1	0.080		0.039		0.745		1.380		0.063	0.908	
Genotype*NaCl	2	0.692		0.403		1.386		0.026		0.107	3.035	
Genotype*SA	2	0.364		0.450		1.243		0.263		1.534	2.900	
NaCl*SA	1	0.306		1.109		6.284		0.026		0.388	0.001	
Genotype*NaCl*SA	2	1.596		9.654	**	2.955		0.253		0.131	2.945	

Table 4.2. Results of linear model for growth traits in *A. thaliana*, second generation. We tested rosette diameter with a Gaussian distribution and report F statistics and significance. We tested the number of leaves in the rosette with a negative binomial distribution and report χ^2 statistics and significance; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Source	df	Rosette diameter		Leaves in rosette	
Genotype	2	0.6509		13.9443	***
NaCl	1	24.6849	***	3.8817	*
SA	1	0.0775		0.2867	
Genotype*NaCl	2	1.1505		2.9995	
Genotype*SA	2	0.2989		0.7584	
NaCl*SA	1	0.0586		0.5185	
Genotype*NaCl*SA	2	0.5274		0.3499	

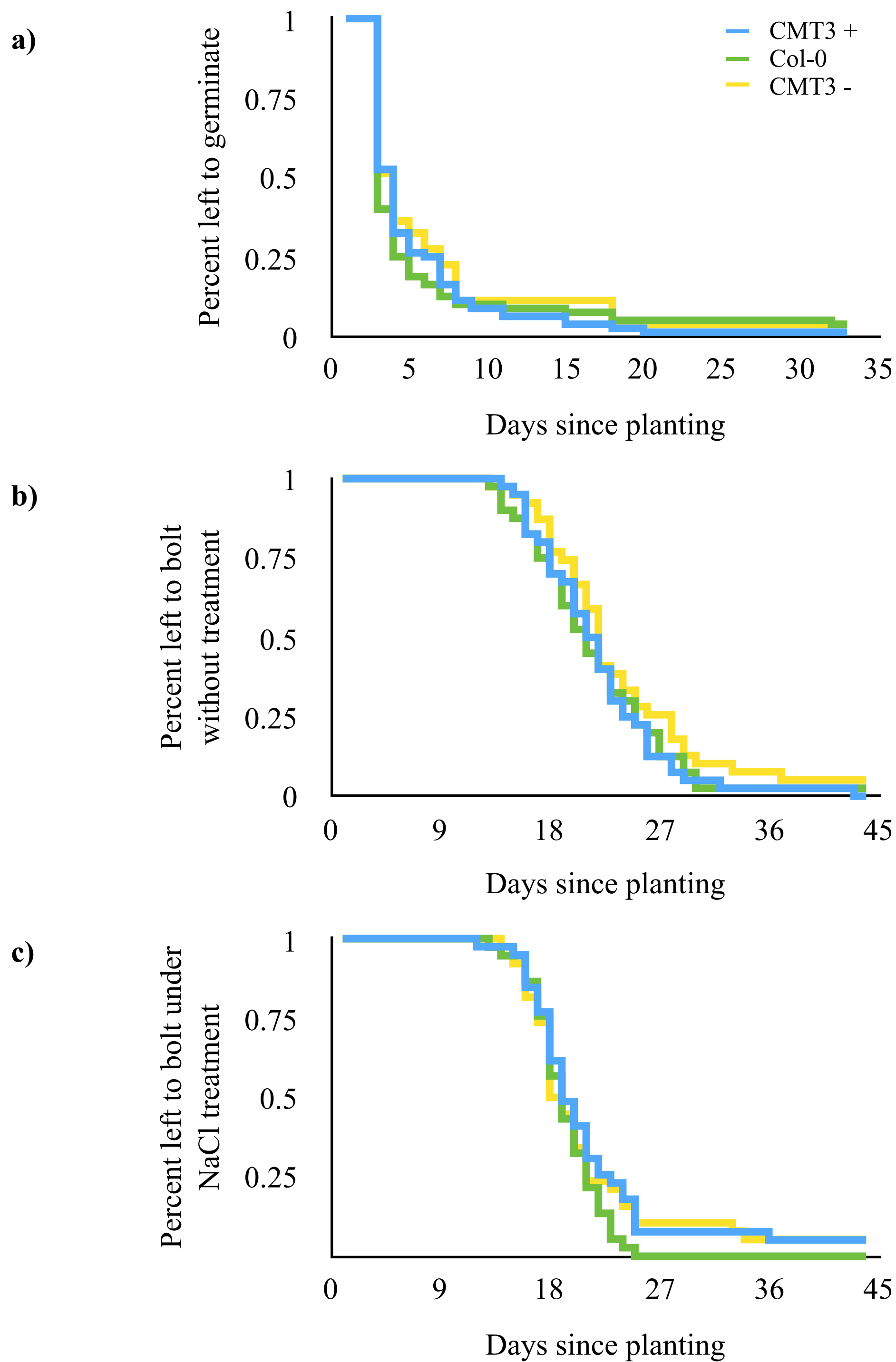
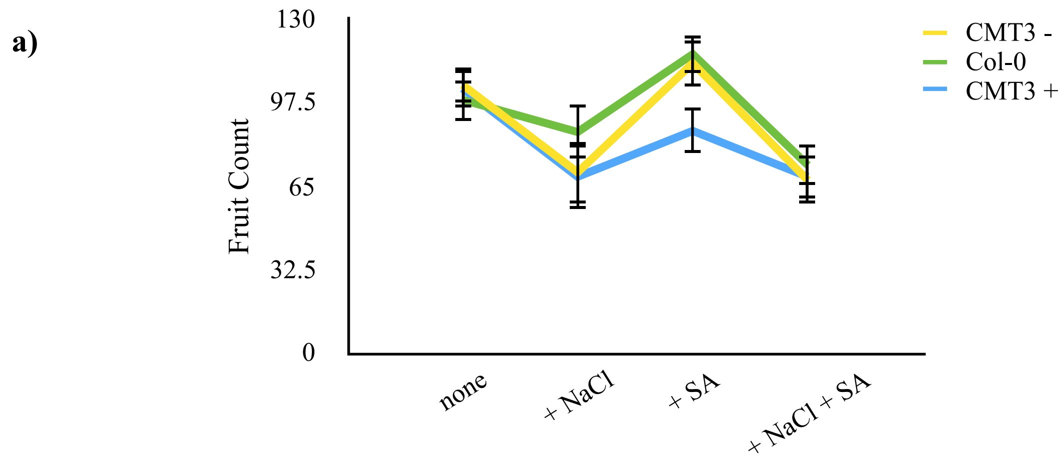
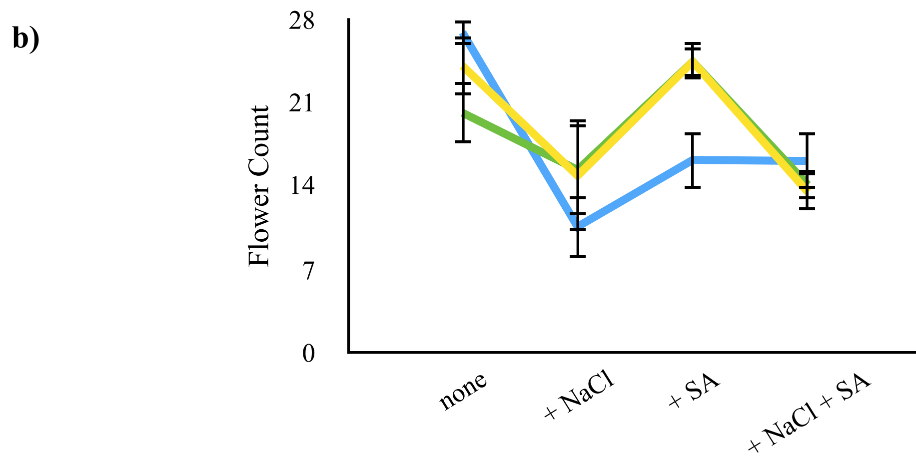


Figure 4.1. Time to **a)** germination and bolting **b)** without treatment and **c)** under salt stress. CMT3 over expression lines shown in blue, wild-type (Col-0) shown in green, and CMT3 knockout shown in yellow.

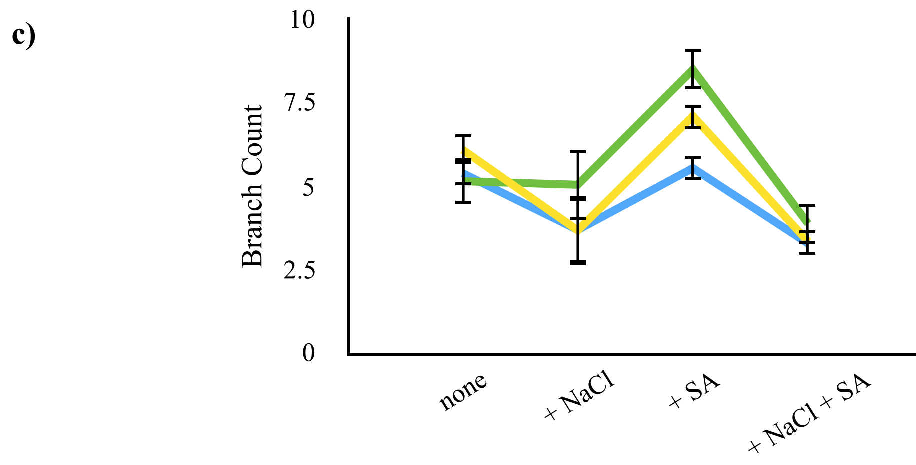
a)



b)



c)



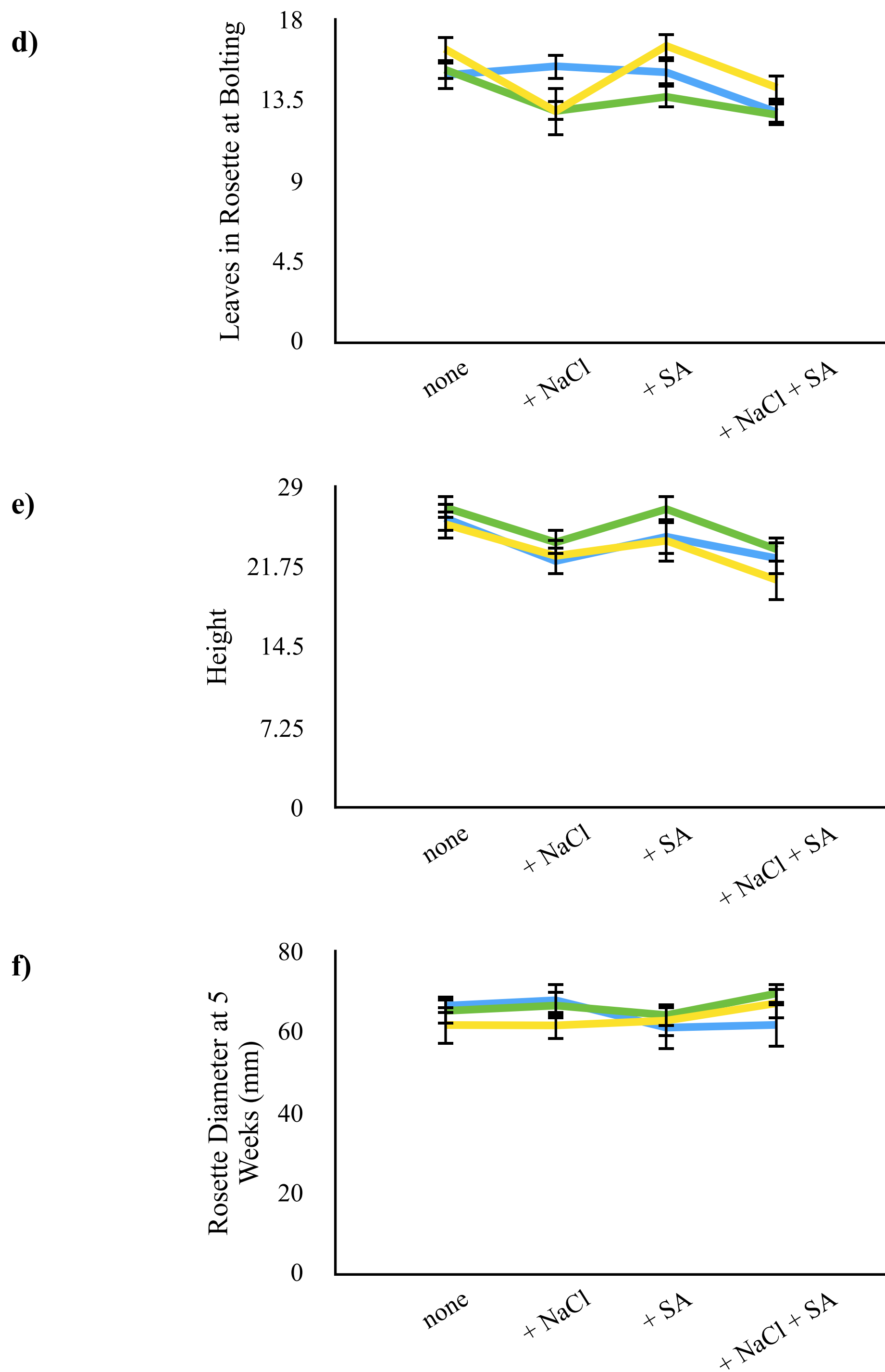


Figure 4.2. Reaction norms for **a)** number of fruits, **b)** flowers, **c)** branches and **d)** leaves in the rosette at bolting; **e)** final height; and **f)** rosette diameter at five weeks (means ± s.e.) averaged over each genotype of *A. thaliana* in each treatment in the maternal generation. CMT3 over expression lines shown in blue, wild-type (Col-0) shown in green, and CMT3 knockout shown in yellow.

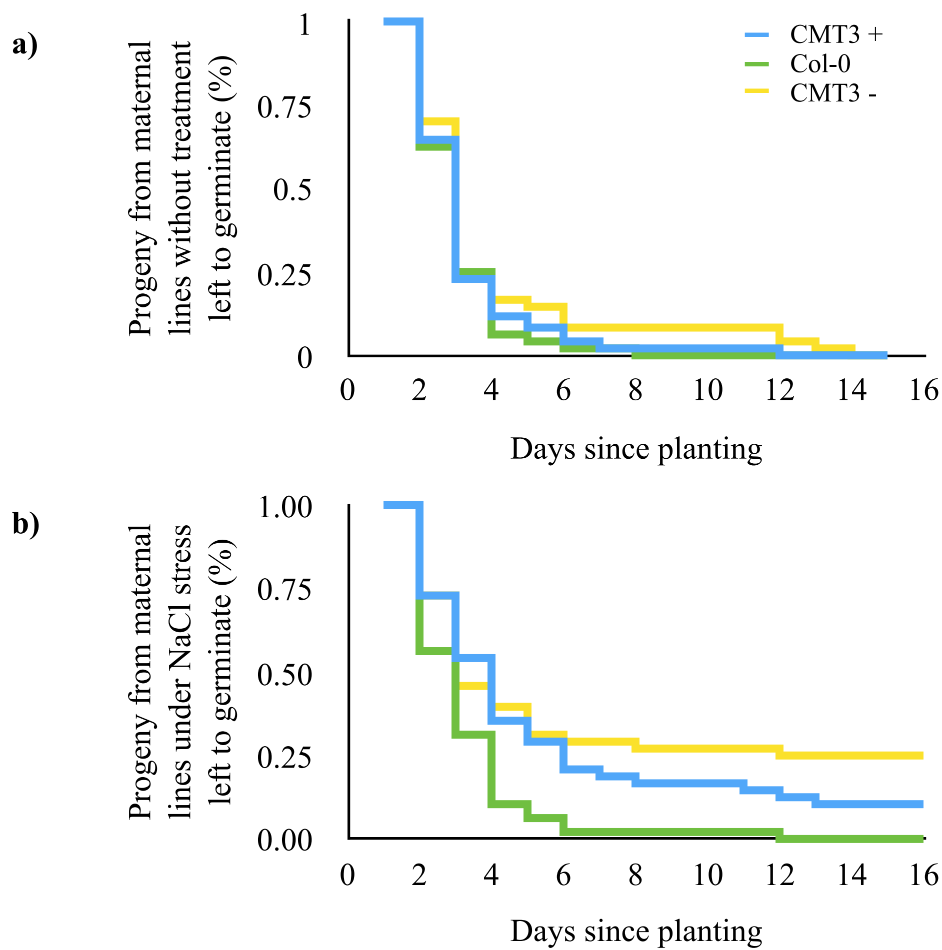


Figure 4.3. Time to germination in seeds from **a)** maternal lines without treatment and **b)** maternal lines under salt stress. CMT3 over expression lines shown in blue, wild-type (Col-0) shown in green, and CMT3 knockout shown in yellow.

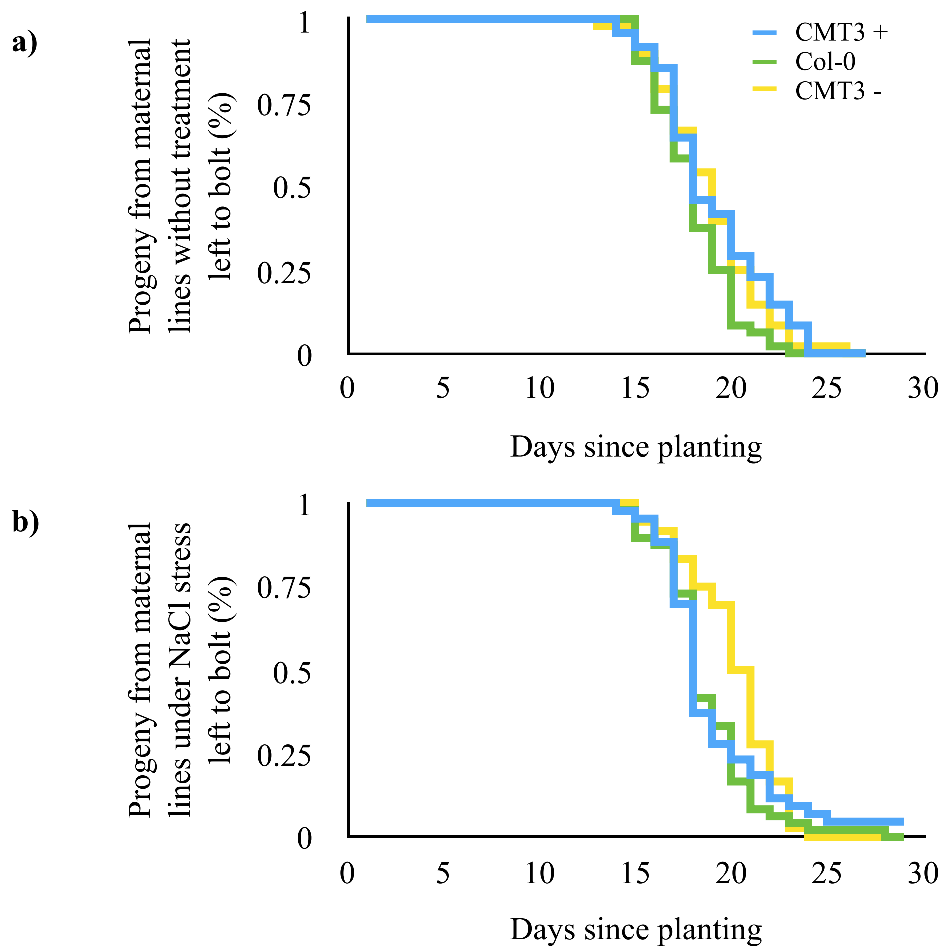


Figure 4.4. Time to bolting in seeds from **a)** maternal lines without treatment and **b)** maternal lines under salt stress. CMT3 over expression lines shown in blue, wild-type (Col-0) shown in green, and CMT3 knockout shown in yellow.

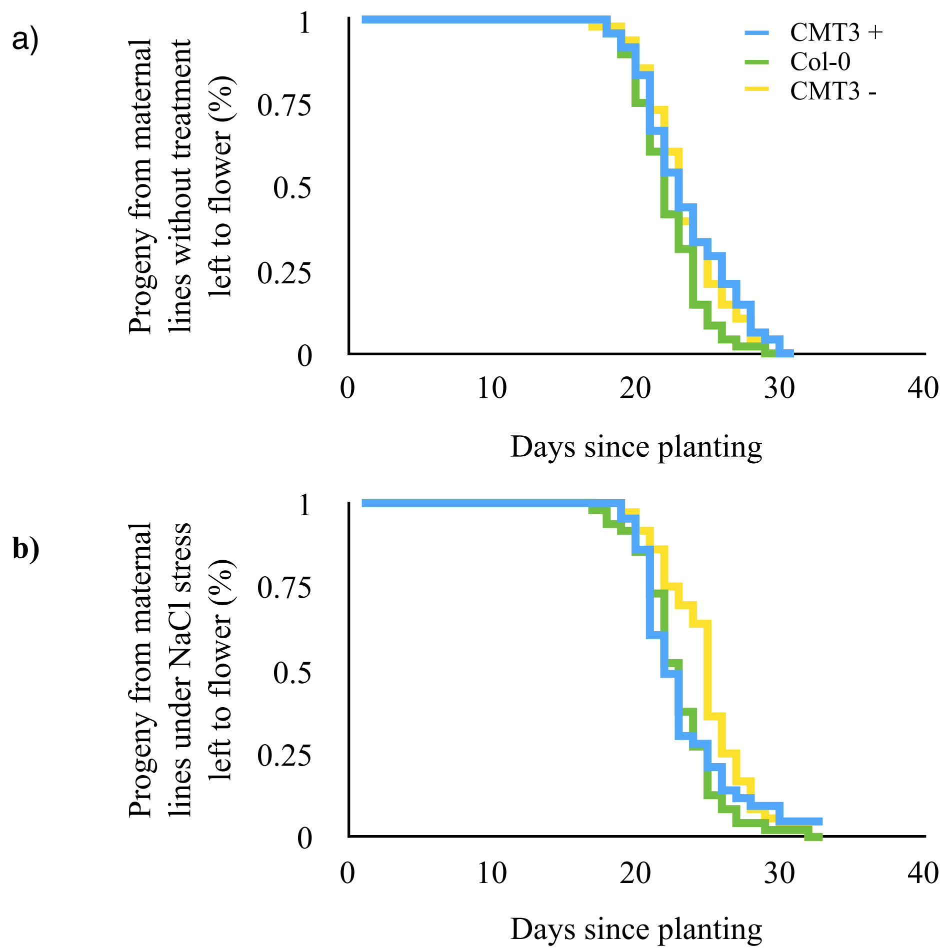


Figure 4.5. Time to flowering in seeds from **a)** maternal lines without treatment and **b)** maternal lines under salt stress. CMT3 over expression lines shown in blue, wild-type (Col-0) shown in green, and CMT3 knockout shown in yellow.

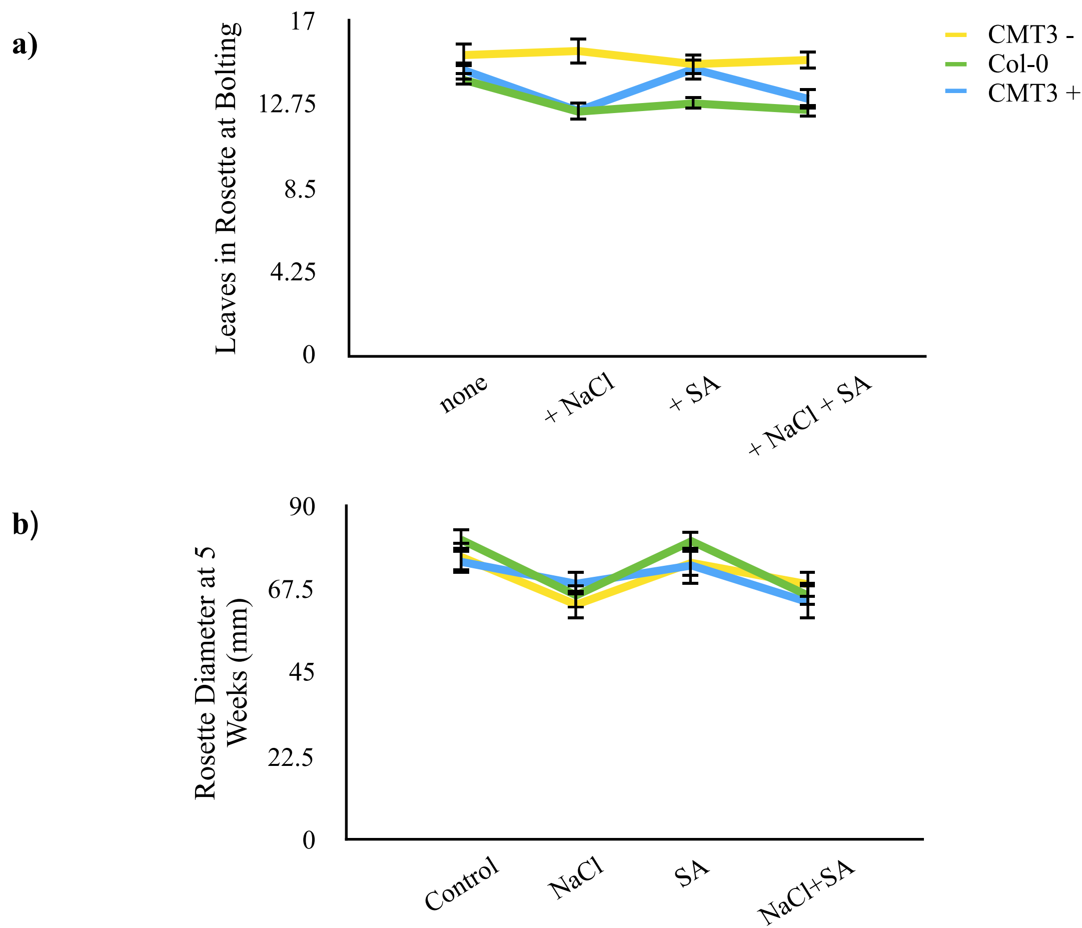


Figure 4.6. Reaction norms for **a)** number of leaves in the rosette at bolting and **b)** rosette diameter at five weeks (means ± s.e.) averaged over each genotype of *A. thaliana* in each treatment in the second generation. CMT3 over expression lines shown in blue, wild-type (Col-0) shown in green, and CMT3 knockout shown in yellow.