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UNIVERSITY OF MIAMI

THE ROLE OF PHENOTYPIC PLASTICITY IN THE ADAPTIVE EVOLUTION OF *FUNDULUS HETEROCLITUS*

By

David Isaac Dayan

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

August 2016

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THE ROLE OF PHENOTYPIC PLASTICITY IN THE ADAPTIVE EVOLUTION OF *FUNDULUS HETEROCLITUS*

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DAYAN, DAVID ISAAC (Ph.D., Marine Biology and Ecology)
The Role of Phenotypic Plasticity in the Adaptive Evolution of *Fundulus heteroclitus* (August 2016)

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Associate Professor Marjorie Oleksiak.
No. of pages in text. (214)

Phenotypic plasticity is the ability of a single genotype to produce multiple phenotypes in response to the environment. Because phenotypic plasticity mediates the relationship between genetic variation and the traits that are ultimately subjected to selection, phenotypic plasticity has the potential to influence evolutionary trajectories and contribute to the accumulation and release of cryptic genetic variation. This dissertation investigates both of these themes using a combination of trait level approaches and population genomics.

First, the dissertation focuses on variation at the trait level in chapters 2 and 3. Chapter two uses a genome wide association study to demonstrates that the genetic architecture of an ecologically relevant trait, thermal tolerance, varies across environments. For the large-effect loci detectable using the GWAS approach in this study, no loci explain variation in thermal tolerance in more than one thermal acclimation environment. These findings suggest that gene-by-environment interactions can contribute to the accumulation and release of cryptic genetic variation through conditional neutrality. Chapter three demonstrates that phenotypic plasticity contributes to adaptive divergence by characterizing gene expression using a microarray analysis of gene expression. Under a phylogenetic comparative approach, patterns of adaptive gene-

by-environment interaction are common at many genes between two distantly related populations that are locally adapted to different thermal environment. Where adaptive differences and shared plastic responses were observed for the same genes, a countergradiant pattern of expression was common, suggestive of genetic compensation. Furthermore, the majority of adaptive differences between populations are apparent under only certain environmental conditions, indicating that gene-by-environment interactions are critical in adaptive evolution.

In the next two chapters, the dissertation investigates whether extensions to current evolutionary theory, such as the effect of plasticity on evolutionary trajectories, are necessary by examining the genomic signature of selection across two disparate temporal and spatial scales. Chapter 4 examines recent thermal adaptation across spatial scales where demography has little contribution to genetic variation. This chapter identifies population genetic structure that is not parsimoniously attributable to neutral evolution and suggests that thermal adaptation in two populations exposed to coastal power plant thermal effluents proceeds from subtle shifts in allele frequency from the standing genetic variation. Finally, chapter 5 considers adaptive variation across the full extent of the study species range, where both neutral and adaptive divergence has been ongoing for tens of thousands of years. This chapter first characterizes the neutral population genetic structure of *Fundulus heteroclitus*, then it uses an environmental association analysis to identify loci allele frequency that correlates with environmental temperature across the species range in a statistical framework that parses neutral from potentially adaptive shifts in allele frequency. At this scale, allele frequency shifts are also subtle.

Together this dissertation demonstrates that the environmentally sensitivity of phenotypes, from gene expression to organismal performance, may have important evolutionary impacts that have the potential to answer many longstanding and pressing questions in biology; from the missing heritability of complex human disease to potential of rapid evolution from the standing genetic variation in response to global climate change.

TABLE OF CONTENTS

List of Figures

List of Tables

Chapter 1: Introduction

Overview

Biologists' conception of phenotypic plasticity has shifted from that of a nuisance or distraction to a central paradigm in our understanding of the origin of biological variation and evolutionary adaptation. Attempts to understand the genotype to phenotype map as a straightforward one-to-one relationship have collapsed under a wealth of investigations that can explain only a small portion of trait variation with such a model (Flint & Mackay 2009; Manolio *et al.* 2009; Rockman 2012; Zuk *et al.* 2012). At the same time, the study of phenotypic plasticity has developed rapidly and is beginning to fill the voids in this gene-centric perspective of trait variation and evolution. The field has shifted its focus away from contentious early debates regarding (i) whether plasticity evolves independently of traits in unique environments, (ii) whether there are plasticity genes *per se* (Pigliucci 2001; Schlichting & Pigliucci 1998), (iii) whether plasticity retards adaptive evolution by shielding deleterious genetic variation from selection or accelerates evolution by allowing population to persist and reach new adaptive peaks (Baldwin 1896; Price *et al.* 2003a; Wright 1931), and (iv) the respective roles of heterozygosity, epistasis and regulatory genes in producing plasticity (Scheiner 1993). Instead, the current phenotypic plasticity field takes a more nuanced view that incorporates specific patterns of environmental heterogeneity (Snell-Rood *et al.* 2010; Van Dyken & Wade 2010), genetic characteristics of populations (Crispo 2008; Ledon-Rettig *et al.* 2014), trait genetic architectures (Anderson *et al.* 2013; Des Marais *et al.* 2013; Snell-Rood *et al.* 2010), historical and contemporary patterns of selection

(Ghalambor *et al.* 2015; Ghalambor *et al.* 2007; Masel 2006), and species interactions (Agrawal 2001; Fordyce 2006).

We are on the cusp of integrating these diverse factors into a framework that predicts how environmentally induced variation interacts with genetic variation to influence traits and evolutionary trajectories. Furthermore, increasing recognition of the importance of phenotypic plasticity has contributed to our understanding of genetic variation in natural populations (Gibson & Dworkin 2004; Hermisson & Wagner 2004; Paaby & Rockman 2014). This dissertation investigates both of these consequences of phenotypic plasticity. First, a genome-wide association study (Ch. 2) and a microarray analysis of gene expression (Ch. 3) investigate how plasticity influences the genetic architecture of traits and the adaptive divergence of natural populations. Then, the dissertation considers the population genetic signature of both recent thermal adaptation (Ch. 4) and clinal divergence across a species range (Ch. 5) in the context of phenotypic plasticity's potential effects on genetic variation. Finally, by examining how candidate alleles implicated in the adaptation and genetic architecture of plastic traits (Chs. 2 and 3) segregate among locally adapted populations (Chs. 4 and 5), the dissertation synthesizes these two themes to investigate the phenotypic plasticity's role in the adaptive evolution of natural populations.

The role of phenotypic plasticity in evolution and maintenance of biological variation

The genotype to phenotype map

Understanding the origin of biological variation is one of the principal goals of biology. The modern synthesis provided an explanation of the genesis and evolution of biological variation by reconciling Mendel's genetic mode of inheritance with Darwin's

theory of natural selection. Crucial to the successes of this synthesis was a focus on the gene as the sole determinant of not only inheritance, but in the production of phenotypes themselves (Crick 1958) – the central dogma. As a consequence, the modern synthesis has led us to a point where evolution is understood almost entirely in terms of genes, yet what we seek to explain is the evolution of phenotypes (Lewontin 1974; West-Eberhard 2003). Thus the genotype to phenotype map of the modern synthesis has largely been viewed as linear and deterministic, with little role for environmental or non-additive effects in the production of phenotypes or their evolution.

This gene-centric view is proving increasingly incapable of explaining the diversity and evolution of phenotypes observed in nature. Perhaps the most striking evidence of this assertion comes from the quantitative trait nucleotide (QTN) or genome-wide association study (GWAS) program of research (Rockman 2012). The goal of this massive research effort is to identify the causal variants that underlie phenotypic variation, yet we remain unable to explain the majority of variation in even extensively studied and highly heritable traits (Visscher *et al.* 2012a). For example, 60 to 80% of schizophrenia risk is heritable, which suggests that the genetic component of the schizophrenia phenotype is very large, yet the causal variants discovered by GWAS to date can explain just 1% of this heritable variation (Visscher *et al.* 2012b). One explanation of this missing heritability problem is that we are simply querying the wrong type of genetic variation: by focusing on SNPs and QTL of large effect, we are ignoring structural variation, rare and small effect variants, microRNAs, and others (Eichler *et al.* 2010; Manolio *et al.* 2009). However this view ignores the central role of the environment in determining phenotypes (West-Eberhard 2003). If the environment not

only influences phenotypes directly, but interacts with genetic variation in a non-additive fashion, then we would expect the variants discovered by GWAS to fail to explain the extent of heritable variation in phenotypes (Flint & Mackay 2009; Zuk *et al.* 2012). Shifting focus from the production of phenotypes to their evolution, we observe a similar pattern. With the advent of next-generation sequencing we have begun to identify the genetic variation that drives adaptive evolution in natural populations, but do not understand how this adaptive genetic variation is translated into adaptive variation in phenotypes (Berg & Coop 2014; Zhang *et al.* 2013). For example, the additive genetic variance for traits that are under selection in natural populations cannot account for the rates of adaptive evolution of these traits in new environments or for the genetic basis of major transitions in the evolutionary history of lineages (Killermann *et al.* 2006; McGuigan & Sgro 2009; Moczek 2008).

Phenotypic Plasticity's Role

Phenotypic plasticity is the ability of a single genotype to produce different phenotypes in response to the environment (Whitman & Agrawal 2009). Phenotypic plasticity is a nearly ubiquitous and perhaps defining feature of living organisms (DeWitt & Scheiner 2004). Furthermore, phenotypic plasticity can be considered a trait in its own right (de Jong 2005), and genetic variation in phenotypic plasticity, usually viewed as gene-by-environment interaction (GxE), is common (Schlichting & Pigliucci 1998). The consequences of GxE are twofold. First, by providing the variation for plasticity on which selection can act, GxE permits the evolution of plasticity itself (Pigliucci 2001). Perhaps more importantly, the existence of GxE means that genetic variation is expressed differently across environmental contexts (Bradshaw 1965). Traditional perspectives hold that phenotypic plasticity functions in evolution only to shield genotypes from selection,

thus reducing the effectiveness of directional selection and slowing adaptation (Jong 1995; Orr 1999; Wright 1931). Yet, there has been a revival of theoretical and empirical research based on early evidence (Baldwin 1896; Morgan 1896; Schmalhausen 1949; Waddington 1953) that phenotypic plasticity may play a major role in shaping evolutionary trajectories because it mediates the relationship between phenotypes and selection.

Theoretical models of evolution that incorporate phenotypic plasticity fall into three broad conceptual frameworks depending on whether phenotypic plasticity: (i) reduces the cost of selection in novel environments and allows populations to persist, (ii) determines the phenotypes on which selection can act (genetic accommodation) (West-Eberhard 2003), or (iii) alters the genetic variation available to selection. These models have been hypothesized to play a role in many evolutionary phenomena with varying levels of empirical support including: adaptation to new environments (Ghalambor *et al.* 2007), the evolutionary origin of complex traits and novel innovations (Moczek 2008; Moczek *et al.* 2011; Schlichting & Wund 2014), and the divergence of specialized ecotypes, speciation and adaptive radiations (Fitzpatrick 2012b; Pfennig *et al.* 2010; Thibert-Plante & Hendry 2011). These models are briefly reviewed below.

The Models

Much of the theory on phenotypic plasticity's role in evolution is focused on whether plasticity allows populations to persist in novel environments or after an environmental shift. This perspective is the result of a focus on adaptive evolution and the emphasis on studying adaptive phenotypic plasticity over maladaptive or passive plasticity that arises because of the inescapable effects of environmental variables such as temperature and pH on biological reaction rates (Ghalambor *et al.* 2007; Grether 2005).

As a consequence, phenotypic plasticity is often viewed as a mechanism promoting adaptive evolution because it reduces the cost of selection (Haldane 1957) after an environmental shift and allows populations to avoid extinction and subsequently adapt through either new mutations or selection on the standing genetic variation (Baldwin 1896; Chevin & Lande 2011; Price *et al.* 2003a; Robinson & Dukas 1999). Although there is substantial overlap between this model and those that fall under "genetic accommodation" (reviewed below) because subsequent selection can optimize the initially plastic phenotype, this model is distinct from others because it is focused on plasticity's role in reducing the risk of extinction in new environments when the plastic phenotype increases population-wide fitness. For example, in the model of Chevin and Lande (2010), a population with a partially adaptive plastic response can maintain higher population size relative to a population without plasticity, reducing the probability of extinction and therefore increasing the rate of evolutionary adaptation. Extending these predictions to scenarios of a heterogeneous environment with gene flow produces similar results. Gene flow from populations in the ancestral habitat reduces mean fitness and imposes a selection cost in the population experiencing the new conditions, but this cost is offset by adaptive plasticity (Chevin & Lande 2011; Thibert-Plante & Hendry 2011). From this perspective, phenotypic plasticity flattens the adaptive landscape *sensu* (Simpson 1944) and provides a valley crossing mechanism, allowing populations to explore adaptive peaks other than the local optimum (Price *et al.* 2003a). This role of plasticity in promoting adaptive evolution may be especially important for populations with high rates of migration and gene flow from ancestral habitats (Crispo 2008; Sultan *et al.* 2002) or for traits with moderately beneficial plasticity (Price *et al.* 2003a).

After populations are exposed to new environmental conditions, phenotypic plasticity may also influence evolutionary trajectories through genetic accommodation (West-Eberhard 2003). This model views "phenotypes as leaders and genes as followers in evolution" (West-Eberhard 2003); novel phenotypes arise in a population, either because of new mutations or exposure to a different environmental conditions, and are subsequently refined by selection through quantitative genetic changes. Although phenotypic variants can arise because of either genetic changes or phenotypic plasticity, where the latter produces phenotypic changes, genetic accommodation may have a major influence on the rate of adaptive evolution because the emergence of the phenotype occurs within a single generation at high frequency in the population and across diverse genetic backgrounds (West-Eberhard 2003). Thus, populations do not need to wait for the emergence of a single adaptive mutation to arise, escape potential loss due to genetic drift, and spread in the population (Orr 1998; Phillips 1996). On the contrary, because the environment plays a role in both the production of and selection on the trait of interest, adaptation can occur through quantitative genetic changes from the standing genetic variation that adaptively refines the regulatory architecture of the trait's expression (Ehrenreich & Pfennig 2015; Moczek *et al.* 2011; Pfennig *et al.* 2010; Wund 2012). This refinement (accommodation) is possible because plastic phenotypes frequently have complex genetic architectures that not only provide many genetic targets for selection but are also likely to exhibit substantial genetic variation owing to their conditional expression (Aubin-Horth & Renn 2009; Hodgins-Davis & Townsend 2009; Snell-Rood *et al.* 2010; Van Dyken & Wade 2010; Windig *et al.* 2004). Furthermore, phenotypic plasticity promotes increased genetic variation in populations experiencing novel

conditions because adaptive plasticity can promote increased gene flow from other populations (Colautti & Barrett 2011; Crispo 2008).

Genetic accommodation of environmentally sensitive phenotypes can have several outcomes depending on how the regulation of the phenotype is altered. A loss of plasticity or decreased threshold of induction for the phenotype such that it is constitutively expressed in the new environment may occur if the plastic response is costly to produce or alternate environments are rare, *i.e.,* genetic assimilation (Pigliucci *et al.* 2006; Waddington 1953). Alternatively, selection may favor enhanced phenotypic plasticity in the direction of the trait optimum. In this case accommodation reduces the costs and limits of phenotypic plasticity (Murren *et al.* 2015; Suzuki & Nijhout 2006). Finally, not all phenotypic plasticity is adaptive. In fact maladaptive phenotypic plasticity that arises as a consequence of passive responses to the environment by biological molecules may be the primary form of plasticity (Schulte *et al.* 2011; Van Kleunen & Fischer 2005). Thus genetic accommodation may function to reduce the effects of maladaptive phenotypic plasticity, such that local adaptation of populations through accommodation leads to the stabilization of phenotypes across heterogeneous environments. This pattern is frequently observed in nature and is termed countergradiant variation because the direction of plastic and genetic effects on traits are in opposite directions with respect to the environment (Conover & Schultz 1995). Genetic accommodation of this form is distinguished from others under the term genetic compensation (Grether 2005). Genetic compensation may be particularly relevant to plasticity's role in promoting divergence, because it establishes reproductive barriers to gene flow between locally adapted populations (Fitzpatrick 2012b).

In the final model, phenotypic plasticity is predicted to impact evolutionary adaptation because it promotes the accumulation and release of genetic variation (Gibson & Dworkin 2004; Hermisson & Wagner 2004; Le Rouzic & Carlborg 2008; McGuigan & Sgro 2009; Paaby & Rockman 2014). Phenotypic plasticity contributes to an increase in genetic variation in two ways. First, homeostatic mechanisms exerted through phenotypic plasticity buffer the effects of new mutations, reducing genetic constraints (Moczek 2008). The most well studied instance of this phenomenon is the heat shock protein Hsp90's function as a "genetic capacitor" (Rutherford & Lindquist 1998). Thermal induction of Hsp90 canalizes the outcome of protein folding during gene expression and stabilizes protein populations in the cell, thereby simultaneously reducing the effects of environmental and genetic perturbation and promoting the accumulation of genetic variation (Queitsch *et al.* 2002). Such genetic capacitors may be common (Sangster *et al.* 2008). Second, phenotypic plasticity may lead to the conditional expression of genetic variation across space and time such that some genetic variation has an effect in only a subset of individuals or populations (Des Marais *et al.* 2013; Snell-Rood *et al.* 2011). This conditional expression can result in relaxed selection in the non-inducing environment and an increase in polymorphism at these loci because purifying selection can only remove deleterious alleles in the subset of individuals experiencing the inducing conditions (Kawecki 1994; Lahti *et al.* 2009; Snell-Rood *et al.* 2010; Van Dyken & Wade 2010).

The genetic variation that accumulates by these mechanisms is studied under the phenomenon of cryptic genetic variation (CGV) (Gibson & Dworkin 2004; Hermisson & Wagner 2004; Paaby & Rockman 2014). While such variation is simply part of the

standing genetic variation, it is considered cryptic where the inducing conditions are uncommon in the evolutionary history of the species. The release of CGV under rarely encountered or novel conditions is likely to have a major impact on the rate of evolution because it is usually accompanied by an increase of heritable variation in phenotypes (*i.e*., genetic variance) (Hoffmann & Merila 1999; Rokholm *et al.* 2011; Waddington 1953), and the alleles that form the basis of this variation can be present at intermediate frequencies before the onset of selection (Fry *et al.* 1996). In this way, the release of CGV and genetic accommodation are closely linked because the large amount of genetic variation with phenotypic effects in a population experiencing novel or rare environments provides the basis for quantitative genetic changes in the refinement of environmentally induced traits through genetic accommodation. Also, because alleles are free to recombine across multiple genetic backgrounds and remain neutral until the inducing conditions are encountered, CGV might underlie the evolution of complex traits that require multiple simultaneous genetic changes (Paaby & Rockman 2014). Under the adaptive landscape metaphor, this latter process provides a mechanism for populations to escape local optima and explore broader phenotypic spaces.

The population genomic consequences of phenotypic plasticity

The traditional understanding of the adaptive evolutionary process is that of a single new adaptive mutation that arises after the onset of selection in a new environment and is subsequently driven to high frequency (Orr 1998). This process leads to a local reduction in diversity at linked, neutral loci, because of genetic hitchhiking (Smith & Haigh 1974). Genome scans that seek to identify the genetic basis of evolutionary adaptation have been dominated by approaches that search for this population genomic signature alone (Nielsen *et al.* 2005). Yet, evidence of such selective sweeps are rare in

natural populations (Druet *et al.* 2013; Hernandez *et al.* 2011; Pritchard & Di Rienzo 2010; Pritchard *et al.* 2010), but see (Fagny *et al.* 2014; Jensen 2014). Moreover, the long lag time associated with the initial phase of adaptation by this process is at odds with the prevalence of rapid adaptation by natural selection (Colautti & Barrett 2013; Eizaguirre *et al.* 2012; Ellner *et al.* 2011; Lee 2002) and rapid changes in quantitative traits under artificial selection (Falconer & Mackay 1996; Wright *et al.* 2005).

Adaptation from the standing genetic variation can account for rapid adaption to novel conditions provided there is substantial variation present in populations (Barrett & Schluter 2008), but is likely to have a different population genomic signature that is not as clearly detected (Messer & Petrov 2013; Przeworski *et al.* 2005; Teshima *et al.* 2006). Specifically, selective sweeps from the standing genetic variation are likely to be soft; the adaptive alleles driven to high frequency by selection are likely to have multiple independent origins (Hermisson & Pennings 2005). Such soft sweeps at adaptive loci have a reduced impact on surrounding linked neutral variation (Przeworski *et al.* 2005). Additionally, adaptation from the standing genetic variation may not involve a sweep at all (Pritchard & Di Rienzo 2010). On the contrary, many subtle allele frequency changes from the standing genetic variation can drive evolution and may be sufficient to explain the adaptive evolution of phenotypes (Berg & Coop 2014; Hancock *et al.* 2010a; Hancock *et al.* 2010b).

The evolutionary consequences of phenotypic plasticity discussed above may predispose populations towards evolution from the standing genetic variation rather than hard selective sweeps from a single new mutation. Yet, there is a conspicuous lack of theoretical research that explicitly links these phenomena. The parameters determining

the probability of adaptation from the standing variation are the population size (N), the mutation rate towards an adaptive allele at the relevant locus (μ) and the relative selective advantage of the mutation after the environmental shift R_α (Hermisson & Pennings 2005). Specifically, where $2N\mu = \Theta > 1/\log(1+ R_\alpha)$, we expect adaptation from the standing genetic variation to be common (Hermisson & Pennings 2005; Messer & Petrov 2013). Phenotypic plasticity influences all three of these parameters in a direction that promotes evolution from the standing genetic variation. Phenotypic plasticity increases population size (N) in new environments by reducing the cost of selection when plastic responses are adaptive under the new conditions (Chevin & Lande 2010). Phenotypic plasticity also increases the mutational target size, thereby increasing μ, because traits with phenotypic plasticity have more complex genetic architectures than constitutively expressed traits (Moczek 2008; Sultan & Stearns 2005). The most profoundly affected of these parameters, however, is likely to be R_α. As discussed above, phenotypic plasticity may be due to conditional trait expression (Des Marais *et al.* 2013), leading to accumulation of polymorphism due to conditional neutrality and relaxed selection in the ancestral environment (Snell-Rood *et al.* 2010; Van Dyken & Wade 2010). Depending on the frequency that the population has been historically exposed to the new environmental conditions, the fitness effects of genetic variation accumulated under conditional trait expression may not be symmetrically distributed. If the environmental conditions are sufficiently rare or entirely novel in the species' evolutionary history, then this body of variation may be enriched for deleterious variants, including fixed lethal variants (Kawecki 1994), thereby reducing R_α. On the other hand, previous bouts of purifying selection on this variation during prior exposures can lead to an enrichment of adaptive

alleles (Masel 2006). Taken together, it is possible that phenotypic plasticity increases R_α because it reduces deleterious effects of polymorphism in ancestral environments and increases adaptive effects in the new environment.

Scope and aims of the dissertation

Gaps in empirical evidence

Despite this rich theoretical framework, empirical evidence pointing to plasticity's role in evolution varies among the models outlined above. There is substantial evidence that phenotypic plasticity can promote population persistence in novel environments (Amarillo-Suárez & Fox 2006; Geng *et al.* 2006; Réale *et al.* 2003; Yeh & Price 2004), that adaptive phenotypic plasticity readily evolves (DeWitt & Scheiner 2004; Pigliucci 2001; Schlichting & Pigliucci 1998) and that genetic variance increases when current environmental conditions are rare in a population's history (Hoffmann & Merila 1999; Ledón-Rettig *et al.* 2010; McGuigan *et al.* 2011; Takahashi 2015). Yet many other questions remained unanswered. These questions comprise the aims of this dissertation.

First, *how common is genetic accommodation in nature* (Schlichting & Wund 2014)? There are ample examples of parallelism between adaptive divergence and patterns of adaptive phenotypic plasticity within a lineage (Gomez-Mestre & Buchholz 2006; Losos *et al.* 2000) suggesting divergence is promoted by high levels of phenotypic plasticity (Pfennig *et al.* 2010), but these studies do not establish a causal relationship between divergence and plasticity. In order to avoid this pitfall, studies investigating genetic accommodation often rely on comparisons of phenotypic plasticity between derived and extant ancestral populations (Aubret 2015; Badyaev 2009; McCairns & Bernatchez 2010; Wund *et al.* 2008; Yeh & Price 2004), or where the ancestral state of plasticity can be inferred phylogenetically (Rajakumar *et al.* 2012). These studies suggest

that adaptation through genetic accommodation may be common. However, the distinguishing feature of genetic accommodation is the initiation of phenotypic diversity followed by refinement through selection (West-Eberhard 2003). Phenotypes are the leaders, not the followers in evolution. Therefore comparisons between populations or species after the onset of selection cannot definitively demonstrate genetic accommodation, because they do not reveal how the phenotype in question arose (Moczek *et al.* 2011; Wund 2012). Stronger evidence of genetic accommodation in nature can be inferred from experimental evolution where demonstrable genetic accommodation of introduced experimental populations mimics the evolved differences in plasticity between ancestral and derived natural populations and the molecular mechanisms underlying the plastic phenotype in both the experimental and natural populations are shared. Such an analysis was recently completed by Ghalambor *et al* (2015), but similarly appropriate natural study systems may be too rare to provide easily generalizable examples (Ehrenreich & Pfennig 2015). Nevertheless, studies that assess how phenotypic plasticity contributes to adaptive divergence reveal the molecular mechanisms, genetic characteristics of populations, and patterns of environmental heterogeneity where genetic accommodation is possible. Furthermore, while the importance of phenotypic plasticity and gene-by-environment interactions (GxE) are well recognized in the field of molecular ecology and quantitative genetics, they are not as widely appreciated in the broader biological community. Therefore more studies that demonstrate that GxE is not only common but critical for producing important traits are needed to address critical questions such as the origin of human disease in modern populations or the ability of species to rapidly adapt to climate change.

Second, *what is the genetic architecture of phenotypic plasticity?* The extent to which plastic traits are determined by constitutively *versus* conditionally expressed genetic variation has profound implications for the origin and maintenance of biological variation (Colautti *et al.* 2012; Paaby & Rockman 2014). Where phenotypic plasticity is mediated by the same genes across environments (i.e. antagonistic pleiotropy), phenotypic plasticity may slow the rate of adaptation because of pleiotropic constraints (Scarcelli *et al.* 2007) or drive divergence by establishing reproductive barriers to gene flow (Kawecki & Ebert 2004). On the other hand, conditionally expressed variation leads to relaxed selection and polymorphism accumulation at conditionally expressed loci because genetic variation will only be subject to selection in a subset of individuals (Snell-Rood *et al.* 2010; Van Dyken & Wade 2010). Theoretical research into these topics is thorough, but the extent to which it is predictive has yet to be addressed across diverse study systems. For example, there is evidence that conditional expression underlies most plastic traits in selfing plant species and agricultural crops with low gene flow, but the use of the recombinant mapping crosses of highly divergent ecotypes or crop varieties in these studies may lead to an ascertainment bias that predisposes them to discover conditional expression (Anderson *et al.* 2013; Des Marais *et al.* 2013; Kassen 2002). Little is known about the genetic architecture of plasticity in highly outbred, large natural populations exemplified by the focal species of this dissertation, *Fundulus heteroclitus*.

Third, *how common is the population genomic signature of adaptive evolution mediated by phenotypic plasticity?* If adaptive evolution is dominated by hard sweeps of a single new mutation, phenotypic plasticity might simply "be a trait subject to selection,

[but not] a developmental mechanism as important as selection in evolution" (de Jong 2005). Therefore considering the population genomic signature of adaptation within the context of phenotypic plasticity's proposed effect on this signature is crucial, not because it establishes plasticity's role *per se* in evolution, but because it suggests that the current understanding of the genotype-to-phenotype map is insufficient to explain the origin and maintenance of diversity in observable phenotypes.

Specific Aims

To address these outstanding questions, the dissertation first establishes that genetic variation in phenotypic plasticity (GxE) is common at the trait level and contributes to the genetic architecture and adaptive evolution of traits in natural populations of the estuarine fish *Fundulus heteroclitus*. Then, it confirms that the population genetic signature of evolution through phenotypic plasticity is common in natural populations undergoing thermal adaptation. Finally, the synthesis briefly examines how candidate alleles implicated in the adaptation and genetic architecture of traits with GxE segregate among these locally adapted populations. These aims are accomplished in four data chapters and one synthesis chapter.

Chapter 2: Genome-wide association study of thermal tolerance and swimming performance

In this chapter two organismal performance traits, velocity of the c-start escape response and thermal tolerance, are measured in hundreds of individuals genotyped at ~10,000 polymorphic genetic markers. By measuring these traits across multiple thermal environments in the same individuals, this chapter explores the extent of genetic variation for phenotypic plasticity in two important traits. Then, this chapter investigates the genetic architecture of phenotypic plasticity with a genome-wide association study that explicitly compares effect sizes of significantly associated polymorphisms across environments.

Therefore this chapter addresses whether phenotypic plasticity has a major contribution to trait variation and asks *what is the genetic architecture of phenotypic plasticity?*

Chapter 3: Microarray Analysis of Muscle Gene Expression

Chapter 3 examines the effect of environmental variation in thousands of traits measured in multiple locally adapted populations using a microarray analysis of muscle gene expression. Through a phylogenetic comparative approach, this chapter identifies putatively adaptive trait variation. Therefore, this chapter not only permits an estimate of how common GxE effects are across many traits, it establishes whether phenotypic plasticity can contribute to adaptive divergence of populations thereby addressing *how common is genetic accommodation in nature?*

Chapter 4: Population Genomics of Rapid Thermal Adaptation

Chapter 4 takes advantage of the thermal effluents of coastal power plants to reveal the population genetic signature of very recent adaptation in two replicate exposed populations. By comparing these exposed populations to nearby reference populations, this chapter asks whether local adaptation proceeds by soft sweeps of the standing genetic variation or hard sweeps from novel mutations. Therefore this chapter asks *how common is the population genomic signature of adaptive evolution mediated by phenotypic plasticity?*

Chapter 5: Population genomics and environmental association analysis along the Fundulus heteroclitus cline

Fundulus heteroclitus are distributed along a thermal cline where both neutral population genetic structure due to demography and adaptive divergence due to natural selection contribute to genetic differences among populations. Chapter 5 models this neutral population genetic structure to uncover potentially adaptive genetic variation that significantly associates with environmental temperature variation. An analysis of this

adaptive genetic variation allows the final data chapter to again address *how common is the population genomic signature of adaptive evolution mediated by phenotypic plasticity?* In this case, however, adaptation is ongoing for thousands of generations and takes place in a complex demographic scenario.

Chapter 6: Synthesis and Conclusions

Chapter 6 synthesizes results from the previous four chapters. Many loci are shared across the SNP datasets of chapters two, four and five because the same restriction enzyme was used to create the reduced representation libraries for sequencing and genotyping individuals. Also, alignment between microarray probes and the reduced representation libraries reveals which loci are shared between chapter three and other chapters. Analysis of the same loci across chapters allows the synthesis chapter to ask if candidate loci with environment specific roles in local adaptation or thermal tolerance variation (chapters 2 and 3) are segregating in in populations undergoing recent thermal adaptation (Chapter 4) or along a thermal cline (Chapter 5). Therefore, the synthesis can address the primary question of the dissertation: what is the role of phenotypic plasticity in adaptive evolution

Chapter 2: Phenotypic plasticity of thermal tolerance is driven by conditional neutrality

Background

Understanding the origin and maintenance of variation in complex traits such as disease risk or organismal performance is a principal goal of evolutionary biology. In addition to genetic factors, complex trait variation is partly due to phenotypic plasticity - the ability of a single genotype to produce a range of phenotypes across environments. Phenotypic plasticity studies primarily focus on identifying the adaptive potential of plastic phenotypes across environments-or elucidating mechanisms of plasticity, yet, the significance of genetic variation in plasticity, often viewed as gene-by-environment interaction (GxE), has long been recognized (Bradshaw 1965; Falconer 1952). GxE interactions greatly expand the role the phenotypic plasticity may take in determining complex traits. Not only might plasticity influence evolution of these traits by altering the distribution of phenotypes on which selection acts *sensu* (West-Eberhard 2003), but by altering which genes affect complex trait variation across environments, GxE could also account for much of the missing heritability in extensively studied traits, such as obesity or height (Manolio *et al.* 2009; Zuk *et al.* 2012) or explain the preponderance of genetic variation in natural populations (Paaby & Rockman 2014). Despite this realization that GxE is potentially critical in understanding the origin and maintenance of phenotypic variation, there are still many important questions associated with GxE. The genetic architecture of traits with gene-by-environment interactions is still largely unknown, as is how the genetic architecture of GxE might influence evolution.

GxE can arise from a number of genetic architectures (Des Marais *et al.* 2013). In the first, antagonistic pleiotropy occurs when an allele has opposite effects on trait values in different environments. From a quantitative genetics perspective, antagonistic pleiotropy is demonstrated by negative across-environment genetic correlation (Falconer 1952). Alternatively, differential sensitivity occurs when an allele effects trait values in the same direction across environments, but the magnitude of effect size varies. Finally, conditional neutrality, an extreme case of differential sensitivity, occurs when alleles only participate in the architecture of a trait in some environments, with no effect in others.

Understanding the extent to which each of these architectures contributes to GxE for ecologically relevant traits is important because they may lead to different evolutionary outcomes. Crucially, conditional neutrality may contribute to cryptic genetic variation, whereas antagonistic pleiotropy does not. Because conditionally neutral loci are subject to selection in only a subset of environments in which they are expressed, conditionally neutral loci accumulate polymorphism at a faster rate than constitutively expressed of antagonistic pleiotropic loci (Kawecki 1994; Snell-Rood *et al.* 2010; Van Dyken & Wade 2010). Cryptic genetic variation that arises as a consequence of conditional neutrality may play a major role in evolution because it serves as a source of increased genetic variance under novel conditions (Gibson & Dworkin 2004; Hermisson & Wagner 2004; Hoffmann & Merila 1999) and can maintain alleles at intermediate frequencies (Fry *et al.* 1996; Gillespie & Turelli 1989), thereby promoting rapid adaptation from the standing genetic variation. Furthermore, it is possible that these loci have an asymmetric distribution of fitness effects because they have been subject to purifying selection if the inducing environment is not entirely novel (Eshel & Matessi

1998; Masel 2006). In this case, conditional neutrality may serve as a source of pre-adapted standing genetic variation in rare environments and have a major impact on adaptive evolution.

The evolutionary consequences of antagonistic pleiotropy are more complex. Where adaptive trait values are in the same direction across a heterogeneous landscape, antagonistic pleiotropy can maintain species-wide genetic variation because alternative alleles are favored in different environments (Anderson *et al.* 2013; Levene 1953). Such genetic trade-offs can constrain evolution (Mitchell-Olds 1996; Scarcelli *et al.* 2007) or promote divergence of locally adapted ecotypes or speciation (Kawecki & Ebert 2004). Where the favored trait values are in the opposite directions across environments, however, one allele is consistently favored, and antagonistic pleiotropy may promote adaptive evolution by providing genetic variation for phenotypic plasticity (Ghalambor *et al.* 2015; Pfennig *et al.* 2010).

Despite the appreciation that the genetic architecture of GxE determines how it influences evolution, few studies directly assess the relative importance of antagonistic pleiotropy and conditional neutrality. In a recent review, Des Marais *et al.* (2013) utilized the wealth of data describing QTL effects across abiotic environments in agricultural crops to demonstrate that antagonistic pleiotropy is rarely observed while conditional neutrality is common. Another approach involves estimating QTL effects in reciprocal transplant experiments of genetic mapping populations derived from locally adapted plant populations (Fournier-Level *et al.* 2011; Latta *et al.* 2010; Verhoeven *et al.* 2004). Similarly, these studies suggest that conditional neutrality is the predominant

architecture, although there is evidence of antagonistic pleiotropy at a small number of loci (Anderson *et al.* 2013; Des Marais *et al.* 2013).

There are several reasons to believe that these findings might be influenced by ascertainment biases shared by prior analyses. First, antagonistic pleiotropy requires simultaneous discovery of allelic effects in multiple environments, whereas conditional neutrality requires discovery in only one. Thus type II error will lead to over representation of conditional neutrality in studies that identify architecture with separate analyses in each environment (Des Marais *et al.* 2013). Secondly, because these studies rely on mapping crosses of locally adapted plant populations with high rates of selfing, low gene flow and little ability to alter their environment through locomotion, we do not know if these findings are readily generalizable to outbred animal species with high gene flow and large population size. Previously investigated systems may lend themselves to the discovery of conditional neutrality if (i) polymorphism is maintained at conditionally neutral loci due to the low gene flow common among inbred plant systems (Colautti *et al.* 2012; Hall *et al.* 2010), or (ii) the probability of evolving locally adapted ecotypes is related to the genetic architecture of GxE in the standing genetic variation, such that species that evolve locally adapted ecotypes are enriched for conditionally neutral loci *versus* loci demonstrating antagonistic pleiotropy (Kassen 2002; Schlichting & Pigliucci 1998; Via & Lande 1985). Indeed, in the limited examples where results in animal systems can be used to address these questions, studies make comparisons among crosses of specialized ecotypes (Hawthorne & Via 2001; Küttner *et al.* 2014).

Our analysis extends these findings beyond experimental mapping crosses of locally adapted populations. In a genome wide association study (GWAS) we investigate

how the environment influences the genetic architecture of two organismal performance traits using wild *F. heteroclitus* sampled from a single outbred population with high diversity, high gene flow, and large effective population size (Adams *et al.* 2006; Brown & Chapman 1991). Specifically, after warm or cold acclimation, we conduct GWAS for thermal tolerance and swimming performance in a population of the estuarine fish *Fundulus heteroclitus*. By comparing changes in heritable genetic variation and the genetic architecture of these traits across acclimation environments, we examine the extent to which GxE is determined by conditional neutrality or antagonistic pleiotropy. This study finds that loci with significant trait associations are only conditionally neutral: no locus had a significant association in both environments even when liberal p-values were used.

Methods

Fish

Three hundred twenty one *F. heteroclitus* were captured from a wild population at the Rutgers University Marine Field Station in Tuckerton, NJ (39°30'31.60"N, 74°19'28.11"W) and transported live to our laboratory in aerated seawater. Fieldwork was completed within publically available lands and no permission was required for access. *F. heteroclitus* does not have endangered or protected status, and do not require collecting permits for non-commercial purposes at the sampling locations. All fish were captured in minnow traps and removed within 1 hour. IACUC approved procedures were used for non-surgical tissue sampling. Fish were maintained in the laboratory in a recirculating seawater system containing less than 1 fish per gallon and fed daily in the afternoon. Salinity, ammonia and temperature were checked regularly. All protocols were approved by the institutional animal care and use committee (IACUC protocol 13-054).

Fish were individually tagged using visible implant elastomers (4 colors, four locations) (Northwest Marine Technologies, Inc., Shaw Island, Wash., U.S.). Fish were then acclimated for 6 weeks to 20°C and 15ppt salinity using artificial seawater and a 14:10 hour light:dark schedule to reduce the effect of reversible acclimatization to local field conditions.

After the initial 6 week acclimation to 20°C, fish were randomly assigned to either 12°C or 28°C for an additional six weeks and sampled for critical thermal maximum at their respective acclimation temperature as well as maximum angular velocity of the c-start escape response at both the acclimation temperature and the alternative acclimation temperature. After sampling, fish were acclimated again for six weeks to the alternate acclimation temperature and sampled a second time.

Molecular Biology and Genotyping

Caudal fin clips approximately 5 mm^2 in size were taken from individuals during elastomer tagging and stored in 270 ul of Chaos buffer (4.5M guanadinium thiocynate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCl pH 7.5, 0.2% antifoam, 0.1M β-mercaptoethanol); these samples were stored at 4°C prior to processing. Genomic DNA was isolated from fin clips using a silica column (Ivanova *et al.* 2006). DNA quality was assessed *via* gel electrophoresis, and concentrations were quantified in triplicate using Biotium AccuBlueTM Broad Range dsDNA Quantitative Solution according to manufacturer's instructions. 100 ng of DNA from each sample was dried down in 96-well plates. Samples were then hydrated overnight with 5 ul of water before restriction enzyme digestion and further processing.

GBS was performed as described (Elshire *et al.* 2011), using the restriction enzyme AseI, adaptors (0.4 pmol/sample) and 50ng of gDNA per sample. The number of individuals exceeded the number of available barcodes, so individual were randomly assigned to one of two libraries. Each of these libraries was sequenced in duplicate on an Illumina HiSeq 2500 with a 100 bp single end read (Elim Biopharmaceuticals, Inc.).

The reference genome-based GBS analysis pipeline, TASSEL (Glaubitz *et al.* 2014) was used to call SNPs using the *Fundulus heteroclitus* genome (Reid *et al.* 2105); SNPs were identified using the "Discovery Build." A log of console input for the pipeline is available upon request. We largely used default settings throughout the pipeline with the following exceptions: a minimum of 5 counts were required for retention of individual tags during the merge multiple tag count fork, and tag alignment to the reference genome was accomplished with bowtie2 using the very-sensitive-local setting.

The initial SNP dataset produced by TASSEL-GBS was filtered to remove loci and individuals with low coverage. The resultant high coverage dataset was additionally filtered for polymorphisms that may result from sequencing error or alignment of paralogous loci: loci with low minor allele frequency (<1%) were removed in the TASSEL GUI, then loci with significant departures from Hardy-Weinberg equilibrium (greater observed than expected heterozygosity) were removed (p <0.01). Hardy-Weinberg equilibrium was calculated for individual loci using Arlequin v3.5.1.2 (Excoffier *et al.* 2005) using 1,000,000 steps in the Markov chain with 100,000 dememorization steps.

Phenotypes

We measured upper thermal tolerance with the critical thermal methodology (Lutterschmidt & Hutchison 1997). The experimental chamber consisted of a 20L

aquarium on an 8cm stand within an insulated 40L aquarium containing a circulating pump. During the CTmax trial, both chambers were filled with acclimation temperature water (12°C or 28°C), then 70°C water was introduced from a header tank to the outer aquarium at a controlled rate to maintain heating at 0.28 – 0.30°C/min in the inner tank throughout the experiment. The inner chamber was aerated to reduce thermal stratification and maintain oxygen saturation during trials. After acclimation, groups of ten fish were introduced to the inner aquarium and allowed a handling recovery time of 5 minutes before the trial began. Critical thermal maxima were determined based on continuous loss of equilibrium for 5 seconds. 99% of individuals survived the critical thermal maximum trial after 1 week.

We measured escape response C-starts using high speed cinematography after acclimation to both 12°C and 28°C. Individuals were first assayed at an acute temperature the same as their acclimation temperature, then at the second acclimation temperature (*i.e.,* first at 12°C then at 28°C for 12°C acclimated fish). To perform an assay, an individual was transferred to the 20-gallon experimental chamber and placed beneath a translucent acrylic platform lit from above using a pair of spotlights. A stand supported the experimental chamber on its edges, allowing a light-generated silhouette of an individual to be reflected by a mirror resting at a 45° incline to the bottom surface of the tank. A high-speed video camera was used to record the silhouette of the individual fish at 420 hz. Escape response was elicited with a startling stimulus: a stainless steel piston within a PVC tube was positioned over the fish and released, leading to an impact on the acrylic platform directly above the fish.

High-speed video analysis was performed frame-by-frame in FLOTE (citation). Briefly, FLOTE was used to fit an ellipse on 30-200 frame slices of the c-start. Ellipse size, and bandpass filtering parameters were adjusted per video such that the angle of the anterior body midline (from the tip of the mouth to the approximate center-of-mass) could be recorded for each frame from initial angular movement until the end of stage one (sign change in angular velocity of anterior body midline). Thus, our measure of c-start conforms to definition of Domenici and Blake (1997). Following quantification of high-speed video footage in FLOTE, the data were analyzed in the R environment for statistical computing. The maximum angular velocity (°/ms) (MaxANG) of an escape response was determined by calculating the maximum change in anterior body midline for each video. The interpolated angle data was smoothed using a cubic smoothing spline. MaxANG was determined from the maximum value of the first derivative of the smoothed data during stage 1 of the c-start.

Genome-wide Association Study

To assess the effects of possible covariates on phenotypes, we applied backward stepwise model selection and penalized likelihood methods for linear mixed effect models using the *lmer* and *lmertest* packages for R. Covariates included in our selected model were included as random effects in subsequent GWAS.

To test for significant associations between SNPs and CTmax or MaxANG, we used a unified mixed model approach (MLM) as implemented in TASSEL v5.2 (Bradbury *et al.* 2007a). The MLM approach (or Q-K approach) accounts for both cryptic relatedness among sample using a kinship matrix (K) and population genetic structure (Q) by fitting the Q and K matrices as random effects (Yu *et al.* 2006). We did not detect any population genetic structure among our 321 *F. heteroclitus* using STRUCTURE, nor

do we suspect population structure within our sample because individuals were sampled from the same location. Therefore, we did not fit the Q matrix as part of our MLM analysis. The kinship matrix was estimated using the centered identity-by-state method of Endelman and Jannink (2012). As our sample size and SNP dataset were sufficiently small, we did not employ the p3d or compression options in TASSEL. We use a B-Y modified FDR (Benjamini & Yekutieli 2001) < 0.05 as a cutoff for genome-wide significance for an association. B-Y FDR balances type I and type II error for genome-wide analyses (Narum 2006). In addition to significant associations, we also fit the MLM model without genetic markers to estimate narrow sense heritability of traits using the K matrix.

Functional Analysis

We annotated significantly associated SNPs with a hierarchical approach. SNPs with significant associations were annotated first by gene model features at the *F. heteroclitus* genomic location aligned to by bowtie. If no annotation at the locus was found, BLAST alignment against the *F. heteroclitus* genome of sequence tag containing the SNP of interest was used to annotate the SNP with any gene models within 5kb. These annotations we converted to human homolog uniprot accessions using OrthoMCL, and submitted to the PANTHER Overrepresentation Test (release 20150430) for enrichment analysis using the Gene Ontology Biological Process Experimental Only database (Release 2015-08-06) against a human background with a Bonferroni cutoff of 0.1.

Results

Sequencing Results and Filtering

Genomic DNA (gDNA) was isolated from 321 individuals. These gDNA samples were used to create a reduced representation library for genotyping by sequencing (GBS) (Elshire *et al.* 2011). Libraries were sequenced across 4 Illumina HiSeq lanes. Using the TASSEL-GBS pipeline (Glaubitz *et al.* 2014), we found 1,802,658 unique, complete sequence tags containing both the *AseI* cut site and barcode and at least 5 reads among the 574,077,991 total reads. Bowtie2 aligned 861,198 (47.8%) of these tags exactly once to the *F. heteroclitus* genome; 43.8% aligned to multiple loci and 8.4% had no significant alignment. Only tags that aligned to a single locus were retained for further analysis. After alignment, genotype calling used a binomial likelihood ratio method. We identified 363,418 SNPs with this approach.

We filtered these SNPs on the basis of coverage, minor allele frequency (MAF), and Hardy-Weinberg equilibrium. First, we removed 36 individuals with poor coverage (genotype called at < 10% of SNPs); then poorly sequenced loci were removed (SNPs with calls in < 60% of remaining individuals). This coverage based filtering resulted in an initial SNP dataset with 9,451 SNPs across 285 individuals. To remove polymorphisms that may have arisen from sequencing and amplification errors or alignment across paralogs (*versus* polymorphisms between alleles) (Hosking *et al.* 2004), we then filtered the remaining SNP dataset by minor allele frequency and whether observed heterozygosity (H_o) was significantly greater than the expected heterozygosity (H_e). Of the 9451 SNPs, 143 with minor allele frequencies less than 1% were removed. Then, 182 SNPs with $H_o > H_e$ that exceeded Hardy-Weinberg equilibrium at $p < 0.01$ were

removed. Thus, the fully filtered SNP dataset consisted of 9,126 SNPs among 285 individuals.

Mean read depth per SNP per individual was 26.32 for the fully filtered SNP dataset. The median depth of read per SNP per individual was 7 (Fig. 2.1, supplemental Fig. 2.1). When considering only SNPs with a call in an individual, mean read depth per SNP per individual was 35.56 and the median was 15. The TASSEL-GBS pipeline caps the number of reads used to make a call at 127 for each allele. Therefore, the range of read depth per individual per SNP in the dataset was 0 – 254 (2*127), and the high frequency of 127 and 254 counts per SNP per individual in the read depth frequency distribution results from highly sequenced individuals and/or loci.

Critical Thermal Maximum

Our CTmax dataset consisted of 268 total individuals acclimated to either 12°C (n= 146) or 28°C (n = 242) (Table 2.1, fig. 2.2a). CTmax was recorded in both acclimation environments for 120 of the 268 individuals (fig. 2.2b). CTmax for these 120 individuals demonstrated substantial rank changing between acclimation environments (Spearman's $\rho = 0.096$, fig. 2.2b), and there was a significant individual-by-acclimation interaction (two-way ANOVA, $p < 0.05$), suggestive of GxE in CTmax.

To assess the effects of possible covariates on CTmax, we applied backward stepwise model selection and penalized likelihood methods for linear mixed effect models that included sex, weight, length, trial time, and acclimation temperature as random effects. The best model under both approaches included only acclimation and trial time as covariates. Based on prior knowledge regarding the importance of weight to CTmax (Becker & Genoway 1979) and its limited impact on AIC ($AIC_{\text{best model+weight}} - AIC_{\text{best model}} = 2$) we also chose to include weight in the model.

This model was fit with a kinship matrix as an additional random effect in TASSEL to estimate additive genetic variance, error variance, and narrow-sense heritability (table 2.1). Genetic variance, and therefore heritability, were near zero in the full dataset which included data from both acclimation temperatures. When the model was fit for each acclimation environment separately, heritability was larger among fish acclimated to 12°C (21%) than it was in fish acclimated to 28°C (13%), despite the 6-fold increase in error variance at 12°C.

SNP-CTmax Associations

The MLM analyses for 12°C acclimated fish identified 28 SNPs that significantly associate with CTmax (B-Y FDR < 0.05) (table 2.2). At 28°C, the MLM found 14 SNPs with significant associations. No SNPs had a significant association in both environments, even when the test stringency was relaxed (B-Y FDR < 1 and $p < 10^{-3}$). Q-Q plots and genomic inflation factors (λ_{median}) suggest the MLM procedure was successful at reducing systematic type I error due to kinship (supplementary fig 2); λ_{median} at both 12°C and 28°C was less than 1. No additional SNPs with significant associations were discovered using the full model (data from 12°C and 28°C, with acclimation temperature as a random effect). R^2 values for significant associations ranged from 0.1659 to 0.3644 at 12°C and from 0.0924 to 0.1799 at 28°C (table 2.2).

To compare genetic architectures across environments, we compared additive effect sizes for the subset of significant associations with significant additive effects (B-Y FDR < 0.05) (fig. 2.3). Of the 42 total SNPs with significant associations, 10 were excluded because additive effects were not estimated (TASSEL requires sampling of both homozygous genotypes in addition to the heterozygote to estimate additive effects), and 4 were excluded because the overall association was significant but the additive effect

association was not. This filtering left 15 SNPs with additive associations at 12°C, and 13 at 28°C. SNPs with significant additive effects at 12°C had little to no effect at 28°C and *vice versa*. SNPs with significant associations at cold acclimation largely had mostly non-significant effect sizes in the opposite direction at the warm acclimation (supplemental figure 3), but this tendency was not significant (Fisher Exact Test $p = 0.0961$). SNPs with significant association in the warm acclimation environment always had effect sizes in the same direction in the alternate acclimation environment.

We annotated significantly associated SNPs with a hierarchical approach (supplementary table 2.1). Thirty five of the 42 SNPs with significant associations were annotated, and twenty six of these annotation were unique and mapped to functional annotations in the GO biological process database. With Bonferroni correction ($Q < 0.10$), one GO term remained significant: neurotrophin signaling pathway.

Escape Response

After quality control, the escape response dataset consisted of 829 maximum angular velocity (MaxANG) measurements recorded in 300 individuals (table 2.1) across two acclimation and two acute temperatures. To assess the effects of possible covariates on MaxANG, we applied backward stepwise model selection and penalized likelihood methods for linear mixed effect models that included sex, weight, length, trial time, experimental deviation from designed acute temperature as random effects; acclimation and acute temperature were fit as fixed effects. The best model, by both methods, included only acute temperature as a fixed effect and sex as a covariate. Similarly, acute temperature had a significant effect on MaxANG (two-way ANOVA, $p < 2\times10^{-16}$), while neither acclimation, nor the interaction of acclimation and acute temperature had a significant effect on MaxANG (fig. 2.4).

To investigate heritability variation in MaxANG, we again fit the best model with a kinship matrix as an additional random effect; we also subset the phenotypic data according to acclimation and acute temperature to examine how environment influenced these estimates (table 2.1). Phenotypic variance was generally increased at 28°C acute temperature, but acclimation temperature had little effect on variance. Heritability for MaxANG was low or zero across all environmental subsets and the full dataset.

Escape Response Associations

No SNP-MaxANG associations reached significance in any environmental subset of the data after controlling for multiple comparisons. Q-Q plots of association p-values suggest little deviation from the null hypothesis of no association across all MLM conducted.

Individual Plasticity

Best linear unbiased predictors (BLUPs) for CTmax (after controlling for weight and time) were used to calculate within individual plasticity effects (slope of reaction norm). These reaction norm slopes were used in an MLM association analysis. No SNPs reached genome-wide significance.

BLUPs for individual acute plasticity and acclimation plasticity of swimming performance were also calculated and used to conduct a MLM association analysis of both acute and acclimation plasticity (i.e. Two additional GWAS were conducted: (1) reaction norm slope of acute effects after controlling for acclimation and covariates and (2) reaction norm slope of acclimation effects after controlling for acute plasticity and covariates). No significant associations were found.

Discussion

Rationale

Here, we consider the genetic architecture of thermal tolerance and swimming performance in an outbred *F. heteroclitus* population acclimated to two temperatures. Using an MLM approach in a sample with no population structure resulted in empirical association p-values that are not systematically inflated because of cryptic relatedness (Yu *et al.* 2006). We found 42 significant SNP-thermal tolerance associations and no SNP-swimming performance associations. Notably, no significant SNP-thermal tolerance associations were shared at both acclimation temperatures. Furthermore, after reducing statistical stringency to reduce possible type II error that might bias our analysis towards the over representation of conditionally neutral loci, we still do not find associations that have an effect in both environments; SNPs with a significant effect in one environment had little to no effect in the other. Functional analysis bolsters our confidence in the causal relationship of the SNP-trait associations because the same biological process is enriched in a study of local thermal adaptation in a closely related population ($F_{ST} < 0.01$) exposed to power station thermal effluents (effluent GBS citation if it is available at submission).

Effect of Temperature on Traits and Heritability

Acclimation temperature had a strong positive effect on thermal tolerance as assessed by CTmax. Both thermal tolerance and the effect of acclimation on thermal tolerance was closely comparable to previous measures in populations sampled across this species' range (Fangue *et al.* 2006). The high degree of rank changing in thermal tolerance for individuals across acclimation temperatures and significant individual-by-acclimation interaction suggests that there is substantial GxE in this trait.

Acclimation temperature also had a large effect on thermal tolerance variance. Cold acclimation led to a 6.5-fold increase in total phenotypic variance, and this trait variance increase was accompanied by an even larger increase in additive genetic variance, such that narrow sense heritability was increased after cold acclimation (Table 2.1). An increase in both heritability and total variance is an often observed characteristic of traits in stressful or rarely encountered environments (McGuigan *et al.* 2011; Rokholm *et al.* 2011), dating back to Waddington's early work on canalization (Waddington 1953). Such decanalization and the accompanying increase in genetic variance is generally considered evidence of the expression of cryptic genetic variation due to GxE (Hermisson & Wagner 2004; Rokholm *et al.* 2011). Yet, the extent to which acclimation temperatures used in this study represent stressful or rarely encountered environments is questionable. Both acclimation temperatures are well within the chronic thermal scope of these highly eurythermal estuarine fish (Fangue *et al.* 2006) and can hardly be considered stressful or rare. For *F. heteroclitus* in nature, however, encountering temperatures approaching the upper extremes of thermal tolerance may be a rare occurrence in individuals acclimatized to spring or fall field conditions that are similar to our cold acclimation conditions (12°C), but a common occurrence in individuals acclimatized to temperatures more similar to summer conditions.

In either case, the assertion that changes in genetic variance for thermal tolerance across acclimation temperatures is due to changes in the effect size or conditional expression of causal variants is supported by the near-zero estimate of narrow sense heritability when both datasets are combined. Where individuals greatly differ in their responses across environments, as we observe in our data, we expect a low estimate for

heritability across environments if this response variation is truly due to GxE because GxE is mistakenly being included in the error variance (Paaby & Rockman 2014; Visscher *et al.* 2008). Zero narrow sense heritability in the combined dataset suggests that few to no SNPs considered in this study have simple additive effects on thermal tolerance. On the contrary, the genetic architecture of this trait appears to be dominated by GxE.

Evidence of Conditional Neutrality in the Genetic Architecture of Thermal Tolerance

Because the genetic architecture of GxE determines how it might ultimately influence evolutionary trajectories, we examined how effect sizes of SNP-thermal tolerance associations changed across acclimation temperatures. Specifically we examined the extent to which the genetic architecture of thermal tolerance is driven by antagonistic pleiotropy or conditional neutrality.

In the simplest possible ascertainment scheme, SNPs with a significant association at one acclimation but not another, as we observe here, could be considered evidence of complete conditional neutrality. However, GWAS suffer from high type II error due to the extent of multiple comparisons inherent to their design and the likely distribution of effect sizes for complex traits (Rockman 2012). This high type II error may lead to an overestimation of the frequency of conditional neutrality *versus* antagonistic pleiotropy in the genetic architecture of GxE, because antagonistic pleiotropy requires simultaneous discovery of significant association in multiple environments (Des Marais *et al.* 2013). We took two approaches to address this ascertainment bias. In the first, we found no shared associations using a much less stringent α cutoff to reduce type II error. In a second approach, we examined if effect sizes for SNPs across environments were in the same direction if a SNP had a significant

association at one acclimation temperature. For SNPs with a significant association in the warm acclimation, non-significant effect sizes at the alternate acclimation were always in the same direction suggesting some differential sensitivity approaching conditional neutrality for these SNPs rather than strict conditional neutrality. For the cold acclimation temperature, where we observed decanalization of thermal tolerance, more SNPs had opposite effects in the alternate environment, but not significantly more than expected by chance. Taken together, (i) the absence of a shared genetic architecture across environments even under less stringent statistical inference and (ii) comparisons considering only the sign of the effect size, indicate that the genetic architecture of thermal tolerance is dominated by conditional neutrality while antagonistic pleiotropy likely has little role to play, at least for the moderate to large effect loci that we can detect using GWAS.

This finding complements other recent analyses of genetic architecture of GxE (Anderson *et al.* 2013; Fournier-Level *et al.* 2011; Hall *et al.* 2010; Küttner *et al.* 2014; Latta *et al.* 2010) because our analysis does not rely on mapping crosses between locally adapted ecotypes with low gene flow that favor the discovery of conditional neutrality over antagonistic pleiotropy (Colautti *et al.* 2012). *F. heteroclitus* populations demonstrate large population sizes, high migration and high gene flow (Adams *et al.* 2006; Brown & Chapman 1991). Also, a GWAS approach takes advantage of standing genetic variation in a single natural population, rather than relying on variation maintained by local adaptation in contrasting environments. Therefore, we would expect to find little conditional neutrality with our approach if conditional neutrality is indeed maintained by limited gene flow; with limited gene flow, adaptive alleles should sweep to

fixation resulting in a loss of GxE (Hall *et al.* 2010). The evidence here of extensive conditional neutrality in a high gene flow, large population size species suggests that conditional neutrality is maintained by other mechanisms such as relaxed selection (Van Dyken & Wade 2010).

Conclusion

Our investigation considers how the genetic architecture of an ecologically relevant, complex trait changes across environments. We find that thermal tolerance is dominated by GxE, as indicated by our heritability estimates across environments as well as the extent of individual by environment interactions. We also find that the loci that drive variation in thermal tolerance in one environment have no detectable effect in another. If such conditionally neutral alleles frequently compose traits' genetic architecture, as suggested by most analyses to date (Anderson *et al.* 2013; Fournier-Level *et al.* 2011; Hawthorne & Via 2001; Latta *et al.* 2010; Verhoeven *et al.* 2004), then phenotypic plasticity plays a central role in the origin and maintenance of biological variation. Under conditional neutrality, differences in phenotypic plasticity among genotypes leads to the accumulation of polymorphism (Kawecki 1994; Snell-Rood *et al.* 2010; Van Dyken & Wade 2010) which can have profound effects on evolutionary trajectories. Perhaps most importantly, conditional neutrality can contribute to cryptic genetic variation if some environments are encountered less often than others, thereby creating a store of standing genetic variation that facilitates rapid adaptation after a shift in the environment (Paaby & Rockman 2014). The extent to which these findings are generalizable will require further investigations of genetic architecture that explicitly take into account environmental heterogeneity in study systems with diverse patterns of historical selection and gene flow. Additional future directions include approaches that

explicitly investigate GxE genetic architecture for fitness and can discover loci of small effect.

Tables and Figures

Trait	Acclimation Temperature (°C)	Acute Temperature (°C)	Random Effects	$n_{individuals}$	$n_{observation}$	σ^2_G	σ^2_E	h^2
Ctmax	Both	NA	Time+Weight	268	388	3.66E-05	0.83367	0.0000
Ctmax	12	NA	Weight	146	146	0.35819	1.37456	0.2067
Ctmax	28	NA	Weight	242	242	0.03512	0.23064	0.1321
C-Start	Both	Both	Sex	300	829	0.12503	1.50403	0.0767
C-Start	12	Both	Sex+Acute	153	301	0.04068	1.28299	0.0307
C-Start	28	Both	Sex+Acute	273	528	0.12661	1.66705	0.0706
C-Start	12	12	Sex	151	151	1.21E-05	1.2121	0.0000
C-Start	12	28	Sex	150	150	1.37E-05	1.3722	0.0000
C-Start	28	12	Sex	257	257	1.11E-05	1.1148	0.0000
C-Start	28	28	Sex	271	271	2.80E-05	2.802	0.0000
C-Start	Both	12	Sex+Acclimation	288	408	1.31E-01	1.07621	0.1083
C-Start	Both	28	Sex+Acclimation	295	421	2.03E-05	2.03	0.0000

Table 2.1: Random effects, total number of unique individuals, total number of unique observations, additive genetic variance (σ^2_G), error variance (σ^2_E), and narrow-sense heritability (h^2) for trait data subset by acclimation and acute temperatures.

Table 2.2: Association statistics for markers (SNPs) with significant thermal tolerance associations at 12°C and 28°C acclimation. P: Unadjusted p-value; α: additive effect size; R^2: R^2 approximation for generalized least squares model; σ_G^2 : additive genetic variance ; σ_E^2: residual variance; Q: FDR adjust p-value of the association.

Marker	P 12°C	α 12°C	R^2 12°C	σ_G^2 12°C	σ_E^2 12°C	Q 12°C	P 28°C	α 28°C	R^2 28°C	σ_G^2 28°C	σ_E^2 28°C	Q 28°C
S530_204688	7.19E-11	NA	0.3644	9.83E-06	0.9828	6.24E-06	6.64E-02	0.2581	0.03103	6.33E-02	0.1733	1
S136_41983	1.35E-09	NA	0.3362	1.23E-05	1.233	3.52E-05	4.86E-01	-0.0692	0.00892	7.21E-02	0.1822	1
S789_3169221	1.40E-09	NA	0.3417	1.16E+00	1.16E-05	3.52E-05	3.93E-01	-0.193	0.01106	2.90E-06	0.2901	1
S3057_9794	1.62E-09	3.668	0.3033	1.14E-05	1.144	3.52E-05	5.11E-01	-0.301	0.00618	2.64E-06	0.264	1
S331_146864	4.46E-09	3.641	0.3208	1.12E-05	1.119	5.54E-05	3.05E-01	-0.291	0.01259	2.79E-06	0.2793	1
S10006_665330	4.98E-09	NA	0.2634	1.34E-05	1.345	5.54E-05	4.88E-01	-0.259	0.00672	2.35E-06	0.2346	1
S10006_665337	4.98E-09	NA	0.2634	1.34E-05	1.345	5.54E-05	4.88E-01	-0.259	0.00672	2.35E-06	0.2346	1
S9899_411995	5.10E-09	NA	0.2714	1.37E-05	1.368	5.54E-05	9.06E-01	NA	0.0000654	6.53E-02	0.2095	1
S1960_11208	9.90E-09	-3.72	0.3502	1.18E-05	1.182	8.71E-05	2.47E-03	0.2912	0.0706	2.39E-06	0.2386	1
S1726_24220	1.00E-08	NA	0.256	1.40E-05	1.404	8.71E-05	8.15E-01	NA	0.00023	3.13E-02	0.2304	1
S9923_1861014	1.58E-08	NA	0.2929	1.35E-05	1.35	1.25E-04	3.97E-01	0.2573	0.00941	3.67E-02	0.2286	1
S4532_2300	2.32E-08	-3.75	0.2668	1.34E-05	1.343	1.53E-04	8.45E-01	-0.0588	0.00157	2.72E-02	0.2414	1
S9878_443768	2.43E-08	3.716	0.3155	7.92E-01	0.4559	1.53E-04	2.25E-01	-0.282	0.01645	2.34E-06	0.2342	1
S2948_3181	2.47E-08	NA	0.3124	8.68E-01	0.3753	1.53E-04	8.06E-01	NA	0.00037	1.29E-02	0.2814	1
S469_816127	5.79E-08	-3.77	0.2989	1.40E-05	1.396	3.35E-04	3.59E-01	0.3185	0.01176	2.10E-06	0.21	1
S811_18034	6.91E-08	-3.7	0.272	1.39E-05	1.394	3.75E-04	2.43E-01	0.2502	0.01484	9.99E-03	0.2342	1
S9872_235502	9.68E-08	-3.72	0.2952	1.43E-05	1.429	4.95E-04	9.69E-01	-0.0356	0.000335	3.15E-03	0.2348	1
S770_82381	1.16E-07	0.0158	0.2784	1.47E-05	1.465	5.60E-04	1.88E-01	-0.236	0.01662	7.77E-02	0.1646	1
S2948_3183	1.35E-07	3.558	0.3186	8.13E-01	0.4372	6.18E-04	9.40E-01	-0.0506	0.000771	4.86E-03	0.2917	1
S10147_174121	1.67E-07	-3.72	0.2852	1.48E-05	1.484	7.24E-04	3.15E-01	0.2721	0.01194	2.86E-06	0.286	1
S78_76780	1.86E-07	-3.66	0.2955	1.11E+00	0.2747	7.34E-04	4.73E-01	0.1483	0.00807	3.09E-02	0.2272	1
S78_76783	1.86E-07	-3.66	0.2955	1.11E+00	0.2747	7.34E-04	4.73E-01	0.1483	0.00807	3.09E-02	0.2272	1
S643_187057	7.56E-07	2.349	0.2556	1.33E-05	1.326	2.85E-03	6.37E-02	0.1586	0.02971	1.93E-02	0.2352	1
S1967_17816	2.64E-06	-0.0504	0.2784	1.60E-05	1.6	9.19E-03	6.06E-01	-0.124	0.00619	4.68E-02	0.1693	1
S1967_17812	2.64E-06	-0.0504	0.2784	1.60E-05	1.6	9.19E-03	6.82E-01	-0.121	0.00474	4.67E-02	0.1697	1
S79_997537	1.41E-05	NA	0.1659	6.24E-01	0.9652	4.52E-02	4.53E-01	-0.0729	0.00825	2.40E-06	0.2404	1
S796_121497	1.46E-05	1.5	0.2564	1.31E-05	1.312	4.52E-02	6.05E-01	-0.0511	0.00605	2.71E-02	0.2598	1

S796_121498	1.46E-05	-1.5	0.2564	1.31E-05	1.312	4.52E-02	6.05E-01	0.0511	0.00605	2.71E-02	0.2598	1
S9915_790630	3.78E-01	0.1849	1.94E-02	8.58E-01	1.158	1	1.08E-06	1.254	0.1327	3.66E-02	0.1732	0.01585
S494_236878	5.73E-01	-0.199	9.55E-03	6.36E-01	1.15	1	1.81E-06	-1.23	0.1047	5.35E-02	0.1847	0.01602
S3917_330	5.95E-01	0.1595	1.15E-02	1.52E+00	0.1616	1	3.44E-07	1.251	0.1659	2.03E-02	0.1777	0.0076
S9887_89371	5.97E-01	0.2973	8.76E-03	1.23E+00	0.5749	1	5.87E-06	1.244	0.09238	4.89E-02	0.1948	0.03852
S10024_888544	6.43E-01	-0.65	1.06E-02	1.86E+00	0.1779	1	4.64E-06	-0.0482	0.1402	2.07E-06	0.2074	0.03707
S96_459941	7.60E-01	-0.185	4.74E-03	1.85E-05	1.848	1	1.42E-06	-1.21	0.1032	6.25E-02	0.1667	0.01602
S117_385667	7.76E-01	-0.243	5.55E-03	3.55E-01	1.355	1	1.76E-06	-1.23	0.1416	4.33E-02	0.1618	0.01602
S469_816103	8.64E-01	-0.283	2.98E-03	1.31E-05	1.306	1	8.04E-08	-1.26	0.1649	7.56E-02	0.1375	0.00355
S9987_617431	8.68E-01	0.2941	2.65E-03	1.14E+00	0.6924	1	6.10E-06	1.236	0.1051	2.07E-02	0.2182	0.03852
S3329_278	8.81E-01	0.2338	2.12E-03	1.57E+00	0.1701	1	5.03E-06	1.255	0.09341	3.62E-02	0.2048	0.03707
S469_816093	8.85E-01	-0.282	2.44E-03	1.30E-05	1.295	1	1.74E-07	-1.25	0.158	7.51E-02	0.143	0.00512
S494_236882	9.14E-01	-0.206	1.55E-03	5.90E-01	1.218	1	8.41E-07	-1.22	0.1104	6.03E-02	0.1762	0.01487
S64_543564	9.49E-01	-0.211	8.54E-04	5.05E-01	1.238	1	1.60E-06	-1.24	0.09996	2.48E-02	0.2163	0.01602
S941_54974	9.76E-01	0.1461	5.01E-04	1.48E+00	0.4284	1	1.67E-08	1.245	0.1799	8.47E-02	0.0952	0.00147

Table 2.2 continued

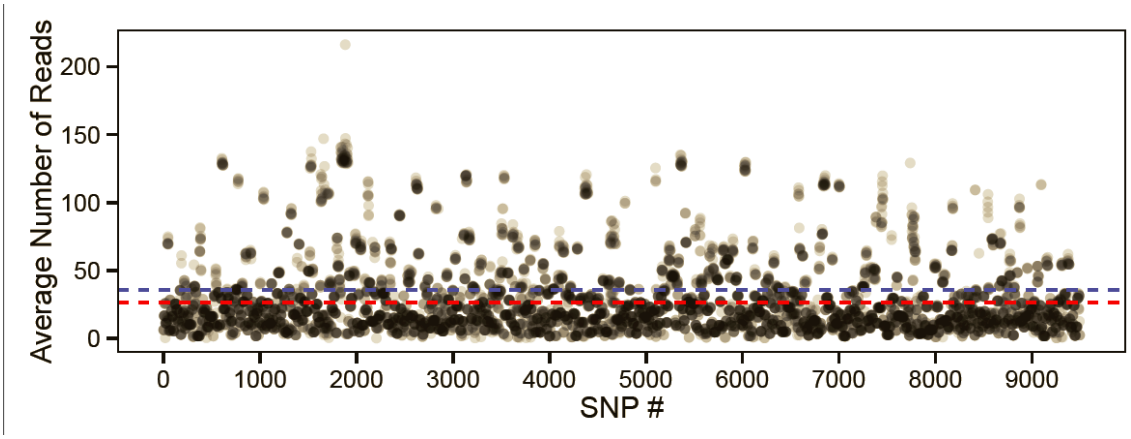


Fig. 2.1: Depth of read per individual per SNP for the 9K SNP dataset, read dashed line is the dataset-wide average, blue dashed line is the average for SNP-by-individuals with calls

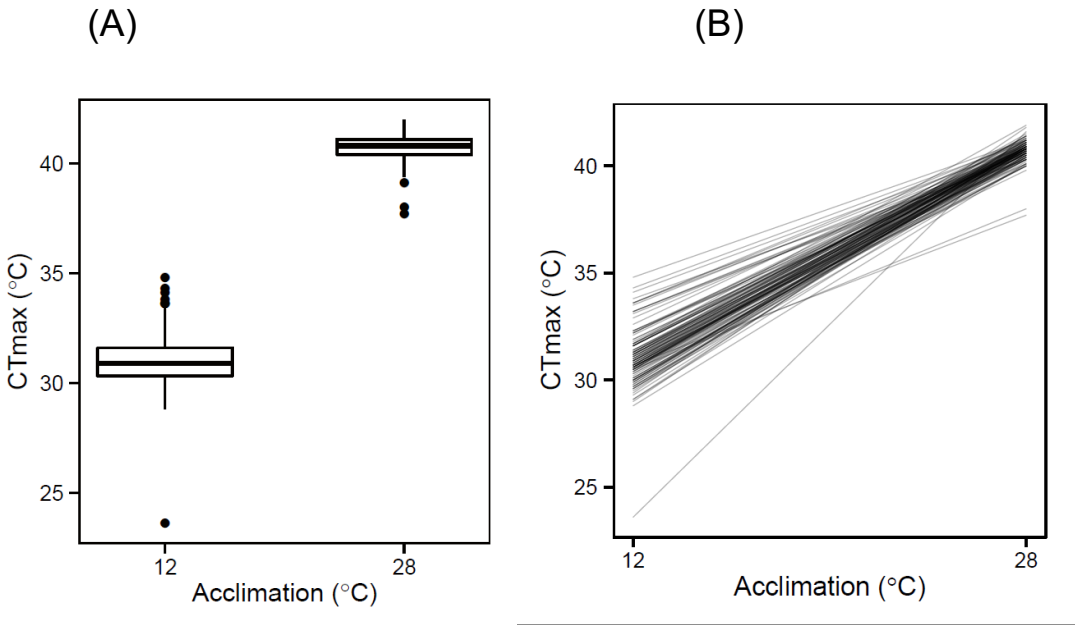


Fig. 2.2: Critical thermal maximum of 12°C and 28°C acclimated individuals. (A) Box-plot of all 268 CTmax measurements. (B) Reaction norm perspective of CTmax variation: each line demonstrates individual plasticity in CTmax with acclimation for the subset of fish (n = 120) with measurements at both acclimation temperatures

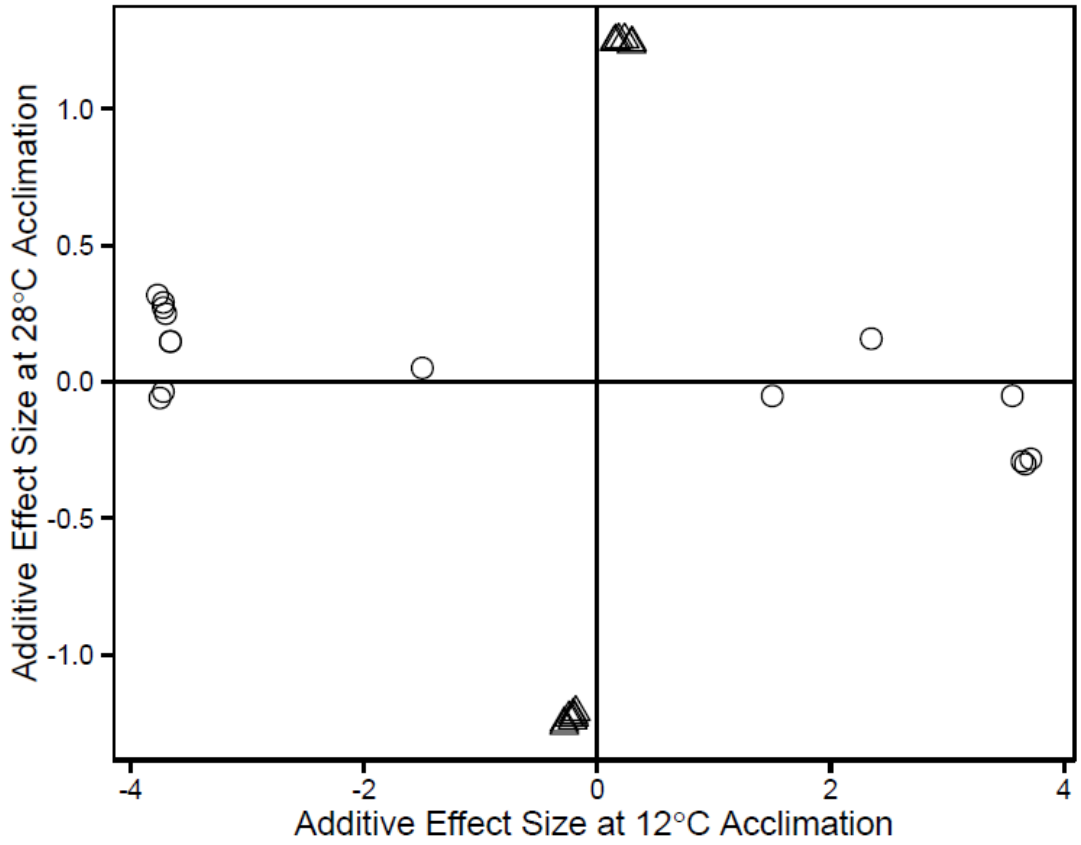


Fig. 2.3: Additive effect size of SNPs in each acclimation environment with significant associations (and significant additive effect) at 12°C (circles) or 28°C (triangles) acclimation.

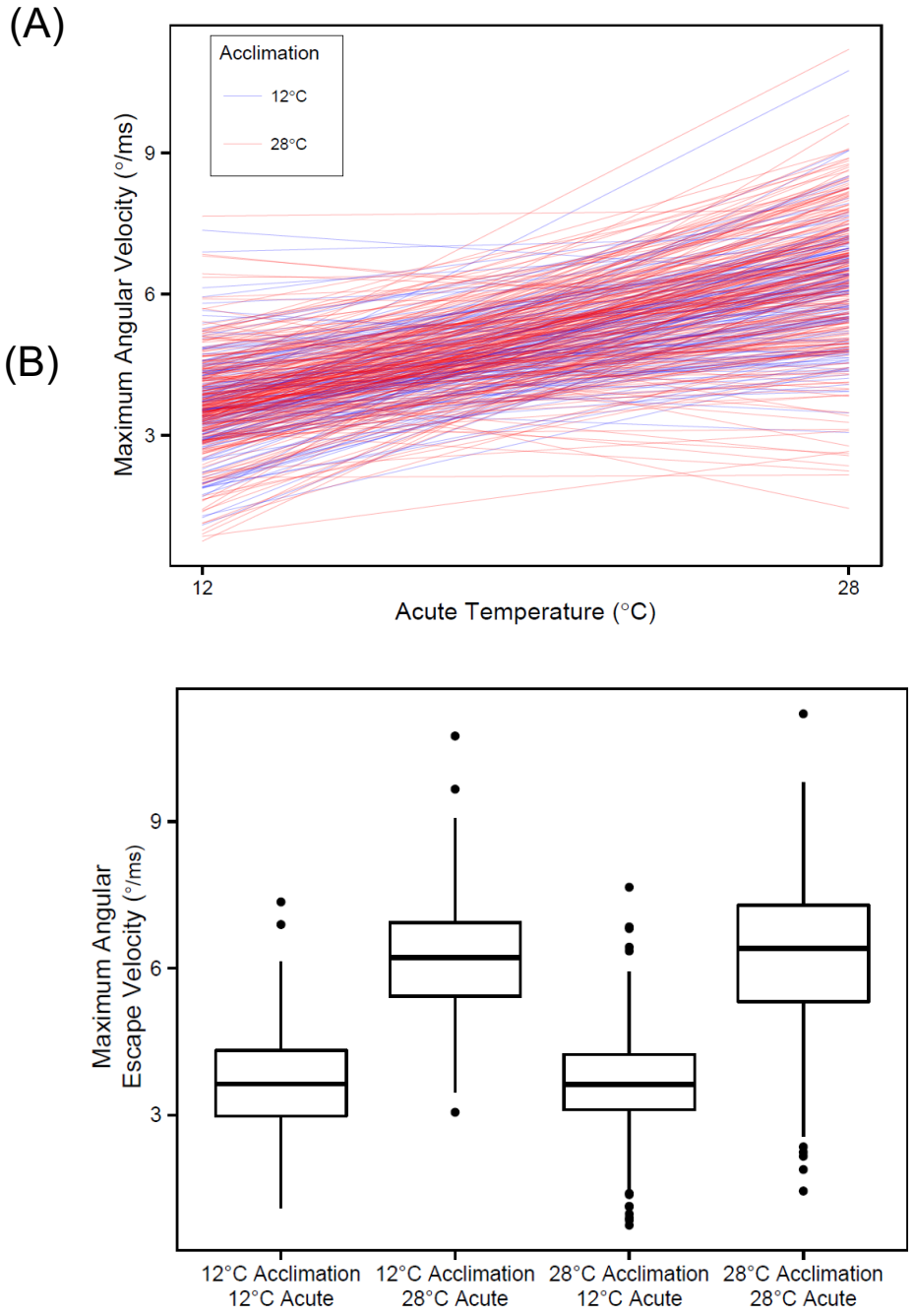


Fig. 2.4: Acclimation and acute temperature effects on escape response (MaxANG). (A) Reaction norm perspective of MaxANG. Each individual is presented as two lines: (red) MaxANG of 28°C acclimated fish assayed at 12°C and 28°C (blue) escape response of 12°C acclimated fish assayed at 12°C and 28°C. (B) Box plot of MaxANG for all combinations of acute and acclimation temperature.

Chapter 3: Phenotypic plasticity in gene expression contributes to divergence of locally adapted populations of *Fundulus heteroclitus*

Background

Phenotypic plasticity is the ability of a single genotype to produce multiple phenotypes in in response to a change in the environment. As a source of phenotypic variation, phenotypic plasticity may influence the rate and trajectory of evolutionary adaptation, but the scope of plasticity's role in evolution remains contentious. A traditional view focuses on the ability of plasticity to mask genetic variation from the force of selection, thereby impeding evolution (Wright 1931). Some authors acknowledge plasticity's role as an adaptive trait, but downplay its significance as a mechanism promoting evolutionary adaptation (de Jong 2005; Orr 1999). Recent theoretical and empirical research, however, suggest that phenotypic plasticity may facilitate important evolutionary processes such as the origin of complex traits (Moczek *et al.* 2011; Palmer 2012) speciation, diversification and macroevolution (Fitzpatrick 2012a; Pfennig *et al.* 2010; Thibert-Plante & Hendry 2011; West-Eberhard 2003) and the colonization of novel environments (Ghalambor *et al.* 2007; Lande 2009). Yet, in contrast to the great strides in elucidating the role of the gene in evolution, much less progress has been made in the incorporation of environmentally-induced variation into the modern synthesis (Pigliucci 2009).

Phenotypic plasticity alters the distribution of phenotypes in novel environments on which selection may act. Adaptive divergence that results from these shifts in phenotype distribution may be explained by at least three conceptual models. First, phenotypic plasticity that is adaptive, i.e. results in increased fitness after an environmental shift, may facilitate evolutionary adaptation by promoting population persistence in novel environments, thereby providing a mechanism to move from one

adaptive peak to another (*sensu* Wright's adaptive landscape (Wright 1932)). By reducing the cost of selection in novel environments, plasticity decreases the probability of extinction by maintaining larger population sizes and permitting the population to adapt through the standing genetic variation or novel mutants (Haldane 1957; Price *et al.* 2003b). Second, plasticity may also promote divergence through a special case of genetic accommodation in which environmentally-induced variation is converted to heritable variation. In this model, directional selection after an environmental shift favors the most plastic individuals, leading to either an extension of the plastic response or a reduced threshold of induction (genetic assimilation) (Ghalambor *et al.* 2007; Grether 2005; West-Eberhard 2003). Finally, plasticity buffers novel genetic variants from the force of purifying selection, thereby permitting the accumulation of cryptic genetic variation. The subsequent release of this cryptic genetic variation may play an important role in adaptation to novel environmental conditions (Le Rouzic & Carlborg 2008; Snell-Rood *et al.* 2010).

To explore the relationship between phenotypic plasticity and evolution, we utilize patterns of gene expression among populations of a small estuarine fish, *Fundulus heteroclitus*, acclimated to a range of ecologically relevant temperatures. Gene expression represents thousands of phenotypes, some regulated by a trans-acting factors, but most affected by gene specific cis-acting effects (Wittkopp & Kalay 2012; Wray 2007). These expression phenotypes are differentially impacted by both environmental and genetic variation, sometimes in a non-additive fashion (Gibson 2008). Many constitutive differences in gene expression have a heritable genetic basis (Gibson & Weir 2005), and evidence of selection on gene expression strongly suggests that it plays an important role

in regulating evolutionarily significant phenotypes (Crawford & Oleksiak 2007; Oleksiak *et al.* 2005; Whitehead & Crawford 2006a). Additionally, environmentally-induced shifts in gene expression are a common mechanism of phenotypic plasticity operating at higher levels of organization (Schlichting & Smith 2002). Where environmentally-induced variation occurs in a non-additive fashion with genetic variation, gene-by-environment interactions (GxE) are observed. GxE is a ubiquitous trait of gene expression and provides raw material for the evolution of adaptive phenotypic plasticity (Grishkevich & Yanai 2013; Hodgins-Davis & Townsend 2009).

Fundulus heteroclitus is a useful model to compare environmental and genetic impacts on gene expression. *F. heteroclitus* are distributed along a steep thermal cline on the Atlantic coast of North America. Populations at the northern extent of the range inhabit estuaries with mean water temperatures 12°C colder than their southern counterparts. These populations are genetically distinct (Adams *et al.* 2006) and exhibit evidence of local adaptation to temperature (Crawford *et al.* 1999; Pierce & Crawford 1997a; Whitehead & Crawford 2006a). Within these populations, individual *F. heteroclitus* also experience a seasonally variable thermal environment with mean summer temperatures greater than 10°C warmer than mean winter temperatures. In response to this seasonal thermal variation, *F. heteroclitus* demonstrate phenotypic plasticity in a number of traits, including gene expression (Burnett *et al.* 2007; Pierce & Crawford 1997b). Finally, the *Fundulus* genus has a well-established phylogeny that permits comparative analysis and identification of putatively adaptive variation among the populations (Oleksiak *et al.* 2002b).

Examining patterns of gene expression in populations of *F. heteroclitus* acclimated to different temperatures allows us to address three questions within an ecological-evolutionary framework where both adaptive phenotypic plasticity and adaptive genetic divergence are expected. (1) By examining the relative impacts of phenotypic plasticity and adaptation we evaluate whether plasticity obviates the need for genetic divergence and thereby impedes evolution. A species with near perfect plasticity experiences very little selection when exposed to new environments, and we expect very few genes with putatively adaptive, non-neutral divergence among the populations (Price *et al.* 2003b; Sultan & Spencer 2002). Alternatively, evolved differences among the populations may dominate. (2) We also evaluate whether plasticity and genetic divergence operate in parallel on the same suites of genes, or on different sets of genes entirely. If they work in parallel, the effects of genetic and environmental variation may operate in the same direction with respect to temperature, suggesting the potential of adaptive phenotypic plasticity and genetic assimilation. Alternatively, the two processes may have opposite impacts on expression of the same gene, resulting in a pattern of countergradiant variation (Conover & Schultz 1995). (3) Lastly, we evaluate the extent to which environmentally-induced variation in gene expression leads to divergence among locally-adapted, wild populations. By examining GxE interactions and comparing patterns of non-neutral variation expressed at different acclimation temperatures, we investigate how phenotypic plasticity contributes to divergence among populations and influences evolutionary trajectories.

Methods

Fundulus Populations and Acclimation.

Wild-caught individuals from three populations of *Fundulus spp.* were used: two populations of *F. heteroclitus* from Sapelo Island, Georgia and Wiscasset, Maine and one population of *F. grandis* (sister taxa to *F. heteroclitus*) from Pensacola, Florida. The southern population of *F. heteroclitus* (Georgia) and its sister taxa *F.* grandis (Florida) share similar thermal environments compared to that of the northern *F. heteroclitus* population (Maine) (fig. 3.1).

Mean summer temperatures were calculated as mean temperature during July and August from the 3 most recent years of NOAA NERRS water temperature data at Wells, Maine, Sapelo Island, Georgia and Apalachicola, Florida. The two southern taxa (*F. heteroclitus* from Georgia and *F. grandis* from Florida) form a polyphyletic grouping and together are referred to as the warm-adapted taxa, and *F. heteroclitus* from Maine are referred to as the cold-adapted taxon.

Fish were maintained in a re-circulating aquarium system with less than 2 fish/gallon and fed daily in the afternoon. Salinity, NH_4, and temperature were checked regularly. Fish were acclimated in the lab for a minimum of 4 weeks at 20°C and 15ppt salinity using artificial seawater with a 14h light 10h dark light schedule. After the initial acclimation, 90 individuals (10 per population-by-treatment group) were acclimated to 12°C, 20°C and 28°C for six additional weeks. These temperatures are ecologically relevant for all populations (NOAA NERRS, see results), beneath the threshold temperature that induces heat shock gene expression for *F. heteroclitus* (Fangue *et al.* 2006) and below the preferred temperature of Maine *F. heteroclitus* (Fangue *et al.* 2009a). Both male and female fish were used. Only post-reproductive fish in good

condition were sampled after acclimation. Condition was evaluated on the basis of physical appearance and weight; fish displaying reproductive characteristics, with visible abrasions, or weights less than 2.0 grams were excluded.

Fieldwork was completed within publically available lands, and no permission was required for access. *Fundulus heteroclitus* does not have endangered or protected status, and small marine minnows do not require colleting permits for non-commercial purposes. All fish were captured in minnow traps with little stress and removed in less than 1 hour. IACUC (Institutional Animal Care and Use Committee at University of Miami) approved procedures were used for acclimation and non-surgical tissue sampling. Fish were sacrificed using procedures approved by IACUC.

mRNA Expression.

After acclimation, total RNA was sampled from post-reproductive male and female fish in good physical condition (N=64; 12ºC: GA=9, ME=8, Fg=5; 20ºC: GA=10, ME=8, Fg=8; 28ºC: GA=6, ME=5, Fg=5; where ME are *F. heteroclitus* individuals from Maine, GA are *F. heteroclitus* individuals from Georgia and Fg are *F. grandis* individuals from Florida) by homogenizing glycolytic muscle tissue (0.01 to 0.1 g) in chaotrophic buffer (4.5M guanidinium thiocyanate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCL, pH 7.5, 0.1M β-mercaptoethanol, 0.2% antifoam A (Sigma) followed by choloroform:phenol extraction and ethanol precipitation. RNA was further purified using Qiagen RNeasy columns. Total RNA was checked spectrophotomically using a NanoDrop 1000 and examined visually by gel electrophoresis. RNA samples with 260/280 ratios > 1.8 and that were not degraded were diluted to 200μg/μl, and 1 μg was amplified using the Amino Allyl message Amp II kit from Ambion, following manufacturers protocols. Resulting aaRNA was quality checked using the Agilent

RNA6000 Nano bioanalyzer chip kit following manufacturer's protocols. aaRNA with total concentrations > 10μg were labeled. 4μg of aaRNA was labeled with 1/25th of a CyDye pack (GE Healthcare) resuspended in high quality DMSO. With the exception of the dye resuspension volume, all other manufacturers' protocols were observed. Concentration of aaRNA was determined using the NanoDrop 1000, sample pairs were combined and aaRNA concentration was adjusted to 20pmol labeled aaRNA in hybridization buffer (5x SSPE, 1% SDS, 50% formamide, 1μg/μl sheared herring sperm DNA).

Microarray Analysis

Fundulus microarrays using sequenced cDNAs isolated from cDNA libraries (Oleksiak *et al.* 2002a; Oleksiak *et al.* 2001) were used to quantify mRNA expression. Libraries used to isolate expressed sequence tags for microarray design were made from all 40 stages of *F. heteroclitus* development, immediately post-hatch whole larvae, and adult tissues. Microarrays consisted of 6,912 unique probes on the *Fundulus* array spotted onto glass slides (Corning) using an inkjet printer (Aj100, ArrayJet, Scotland). Each slide contained four spatially separated arrays of ~7,000 spots (genes) including controls. These arrays use cDNA probes that have an average length of 1.5 kb and have a technical variation of less than 5% of the mean (CV < 0.05) (Fisher & Oleksiak 2007; Oleksiak *et al.* 2002b; Oleksiak *et al.* 2005; Scott *et al.* 2009; Whitehead & Crawford 2006a). All spotted genes (probes) were sequenced and are unique. Thus, even if multiple probes were annotated identically, they were treated as unique traits in downstream analysis. Multiple probes with the same annotation do not align because: (1) they are from the same gene, but the sequences of the cDNA probe do not overlap, (2) they represent duplicate genes with different chromosomal locations, or (3) they share a high similarity

(and hence are named based on this similarity) but are not the same gene. We erred on the side of caution and treated every gene-spot as unique.

A loop design was used for the microarray hybridizations (Kerr & Churchill 2001a, b; Oleksiak *et al.* 2002a). The loop design is balanced such that every sample is labeled with both Cy3 and Cy5 (Kerr & Churchill 2001a, b; Oleksiak *et al.* 2002a). The loop consisted of 62 individuals labeled with Cy3 and Cy5 aRNAs for three populations acclimated to three temperatures (12°C, 20°C, and 28°C). The loop formed was 12GA → 20GA → 12GA → 20GA → 28GA → 12GA → 20GA → 28GA → 12GA → 20GA → 20Fg → 12GA → 20GA → 28GA → 20GA → 12GA → 12GA → 28GA → 20GAa → 12GA → 28GA → 20GA → 12GA → 20GA → 20ME → 12ME → 20ME → 12ME → 20ME → 28ME → 12ME → 20ME → 28ME → 12ME → 20ME → 28ME → 12ME → 20ME → 28ME → 12GA → 20ME → 28ME → 12ME → 20ME → 12Fg → 20Fg → 28Fg → 20Fg → 12Fg → 20Fg → 28Fg → 20Fg → 12Fg → 20Fg → 28Fg → 12Fg → 20Fg → 28Fg → 12Fg → 20Fg → 28Fg → 12ME where each arrow represents a separate hybridization (array) with the biological sample at the base of the arrow labeled with Cy3 and the biological sample at the head of the arrow labeled with Cy5. Slides were scanned using a ScanArray Express with 5μm resolution at half speed. Images were quantitated using Imagene software.

Statistical Analyses

Herring sperm spots were used to calculate background fluorescence, and this background was used to filter very lowly expressed or unexpressed mRNAs. Specifically, mRNAs with raw expression values less than the average expression +2 standard deviations for negative controls (herring sperm spots) were excluded. Of the 6,912 probes on the *Fundulus* array, 2,272 probes exceeded this low value cut off. Fluorescence values

from these 2,272 probes were $\log_2$ transformed and Loess normalized using JMP Genomics 5.1 to remove spatial variability in Cy3 and Cy5 expression data.

Analysis of mRNA expression used a mixed-model ANOVA (Jin *et al.* 2001; Kerr & Churchill 2001b; Oleksiak *et al.* 2002a; Wolfinger *et al.* 2001). All ANOVAs were implemented in JMP Genomics 5.1 (SAS, Cary, NC). Array and dye effects were removed from $\log_2$ transformed and Loess normalized data using the mixed-model of the form

$$y_{ij} = \bar{x}_{ij} + A_i + D_j + (AD)_{ij} + \varepsilon_{ij}$$

where y_{ij} is the signal from the $_i$th array with dye $_j$, $\bar{x}_{ij}$ is the sample mean, A_i and D_j are the overall variation in arrays and dyes (Cy3 and Cy5), $(AD)_{ij}$ is the array by dye interaction and ε_{ij} is the stochastic error (Jin *et al.* 2001; Wolfinger *et al.* 2001). This resulted in least squared means (LSmean) for each mRNA probe for each individual.

Three sets of ANOVAs were performed on individual LSmeans used for gene-by-gene analyses. These ANOVAs were (1) two-way ANOVA with three populations and three acclimation temperatures, 2) two-way ANOVA with cold *vs.* warm adapted taxa and three acclimation temperatures and 3) three one-way ANOVAs for cold and warm adapted taxa for each of the three acclimation temperatures.

The first analysis was a two-way ANOVA with acclimation temperature and populations as fixed effects. The model was

$$y_{ij} = \quad + \quad _i + \quad _j + \quad _{ij}$$

where y_{ij} is the $_j th$ spot intensity of treatment $_i$, is the grand mean, $_i$ is the effect of population *i*, $_j$ is the effect of acclimation temperature, and $_{ij}$ is the residual from spot

$_{ij}$. Hierarchical clustering of gene expression that varied significantly according to acclimation or population was implemented in JMP Genomics 5.1 using the Fast-Ward method (SAS, Cary, NC).

The second ANOVA was a phylogenetic comparative ANOVA comparing the cold-adapted northern population (*F. heteroclitus* from ME) to both warm-adapted southern populations (*F. heteroclitus* from GA and *F. grandis* from FL) (fig. 3.1). In contrast to the first ANOVA, which examines variation among three taxa (*F. heteroclitus* from ME, *F. heteroclitus* from GA, and *F. grandis*), this comparison examines variation between two taxa (northern *versus* the polyphyletic grouping of the southern, warm-adapted taxa (*F. heteroclitus* from GA, and *F. grandis*)). Neutral divergence among taxa is a function of genetic distance (Kreitman 1996). Thus, based on genetic distance alone, the neutral expectation is that the variation among species should be greater than the variation within species. The alternative pattern of variation, where variation is greater within species than among, is not consistent with neutral evolution. Non-neutral patterns of variation that correlate with environmental conditions experienced by genetically isolated populations may be driven by natural selection. In this study, the neutral expectation is that northern and southern populations of *F. heteroclitus* should be more similar to each other than either is to members of the sister species, *F. grandis.* However, the environmental conditions experienced by southern populations of *F. heteroclitus* and *F. grandis* are more similar than either is to conditions experienced by northern populations of *F. heteroclitus.* Thus in this study, patterns of gene expression evolved by natural selection are identified when mRNA expression is more similar between species from warm-adapted taxa (southern *F. heteroclitus* and *F. grandis*) than within the more

closely-related southern and northern *F. heteroclitus* populations. In order to find adaptive patterns of gene expression evolved by natural selection, we first compared mean gene expression of the cold-adapted northern population (ME) to the pooled mean of both warm-adapted southern populations (*F. heteroclitus* from GA and *F. grandis* from FL) using a two-way ANOVA (Oleksiak *et al.* 2002b) referred to as the phylogenetic comparative ANOVA throughout this manuscript. The model was:

$$y_{ij} = \quad + \quad_i + \quad_j + \quad_{ij}$$

where y_{ij} is the $_j$*th* spot intensity of treatment $_i$, $\quad$ is the grand mean, $\quad_i$ is the effect of local environmental conditions (cold-adapted (ME) vs. warm-adapted (GA + Fg)), $\quad_j$ is the effect of acclimation temperature, and $\quad_{ij}$ is the residual from spot $_{ij}$.

Patterns of expression most parsimoniously described as adaptive (evolved by natural selection) were determined using the ANOVA described above for northern (ME) *versus* southern (GA and *F. grandis*) taxa. The F-value on which this ANOVA depends is considered significant when there is a large difference in mean mRNA expression between the northern *F. heteroclitus versus* the pooled warm-adapted taxa (southern populations of *F. heteroclitus* and *F. grandis*) relative to the differences within northern *F. heteroclitus*, southern *F. heteroclitus* and *F. grandis, i.e.,* when between group variation is large relative to mean within group variation. For a significant F-value to occur in our analyses, intraspecies variation must exceed interspecies variation in a direction that correlates with the environmental conditions experienced by each population. The F-value will not be significant when intraspecies variation is consistent with neutral processes; in this case, within species variation is small relative to the differences among species. Notice however that there is a case that is less clear: if *F.*

heteroclitus and *F. grandis* are substantially different, but there is a small difference among northern and southern populations of *F. heteroclitus*. That is, a significant F-value could occur if the within group variance (denominator) is reduced because the average variance within ME and the interspecific variance (GA and *F. grandis*) are small relative to the numerator (variance among groups). In this case, the largest possible F-value is 2 (in the most extreme scenario, the variance within ME is 0 so that the average within group variance is (0+within southern population variance)/2. In order to avoid this problem, genes with a p-value < 0.10 between southern *F. heteroclitus* (GA) and *F. grandis* (Fg) in the first ANOVA were excluded. This relatively large p-value of 0.10 will reduce the type II error and make the phylogenetic comparative analysis more robust. Consequently, the expression of genes that vary significantly between warm and cold adapted taxa (and therefore vary within a species), but do not vary significantly between Georgian *F. heteroclitus* and Floridian *F. grandis* populations were considered adaptive.

The final ANOVA was another phylogenetic comparative ANOVA (one-way) conducted among cold and warm-adapted taxa within each acclimation temperature. The model was

$$y_{ij} = \quad + \quad _i + \quad _{ij}$$

where y_{ij} is the $_j th$ spot intensity of treatment $_i$, is the grand mean, $_i$ is the effect of taxa *i* (warm *vs*. cold-adapted), is the effect of acclimation temperature, and $_{ij}$ is the residual from spot $_{ij}$.

Variation in phenotypic plasticity among populations was conducted using a variation of the random regression method proposed by Nussey *et al* (2007) in R version 2.15.3 (http://www.r-project.org/). In brief, the package *lme4* (Bates 2013) was applied to

normalized gene expression values for acclimation temperature and population (LSmeans) to build a mixed-effect model. Variation among populations in the slope of gene expression over different acclimation temperatures was modeled as a random effect and fitted using a restricted maximum likelihood approach. Significant improvement of the model with the inclusion of population as a random effect was assessed using a log-ratio test (LRT, $p < 0.05$) and is considered evidence of variation in mean plasticity among populations.

Genes that responded to acclimation temperature and genes with putatively adaptive differences in expression were annotated and used with the Functional Annotation Clustering tool in DAVID v6.7 to test for functional enrichment (Huang *et al.* 2008)).

Results

Environmental Data

Three populations of *Fundulus* were used for this study: two populations of *F. heteroclitus* from Sapelo Island, GA and Wiscasset, ME and one population of *F. grandis* (sister taxa to *F. heteroclitus*) from Pensacola FL. The warm-adapted populations, Georgia *F. heteroclitus* and *F. grandis,* inhabit similar thermal environments with mean summer temperatures of 29.8°C and 29.6°C respectively while the cold-adapted Maine population experiences mean summer temperatures of 17.4°C (calculated from NOAA NERRS Apalachicola, FL, Wells, ME, and Sapelo Island, GA). The cold-adapted population experiences daily average seawater temperatures ranging from -1.4°C to 28°C while the warm-adapted taxa experience temperatures from 7°C to 31°C (NOAA NERRS). Thus, the 12°C, 20°C and 28°C acclimation temperatures are ecologically relevant.

Effect of Population and Acclimation

Glycolytic muscle mRNA expression was quantified within each of the three populations (northern and southern *F. heteroclitus* and the sister taxa *F. grandis*) at each acclimation temperature (12°C, 20°C, and 28°C). Of the 2,272 probes on the microarray with mean hybridization signals greater than background levels, 258 vary significantly ($p < 0.01$) in the two-way ANOVA with acclimation, population or the interaction term (fig. 3.2, supplemental table 3.1). Of these, 195 (8.6%) are significantly different among populations, 67 (2.9%) are significant for acclimation effects, 11 (0.5%) are significant for both, and 8 (0.4%) are significant for the interaction term (fig. 3.2) Of the eight genes with significant interaction terms, one gene is significant for the interaction term and the effect of population and the other seven are significant only for the interaction term. While a subset of these results may be spurious due to the multiple comparisons inherent to a gene-by-gene analysis, many more genes vary significantly than expected by chance alone (where the expected false positive would be approximately 22 genes for first order factors and 0.2 genes for second order factors). Implementing a false discovery rate (FDR) of 5%, the expression of 83 genes vary significantly among populations and 5 vary significantly among acclimation temperatures. No significant interaction were observed with a FDR less than 5%.

The statistical variance in gene expression was also investigated. Since the variance is a function of the mean, we use a standardized mean (mean equals zero) to examine the variance in expression. The average variance across populations' means (0.23) is 1. 4 fold larger than the variance across acclimation's means (0.16) (fig. 3.2D) There is a similar pattern if only the eleven genes that are significant for both population (0.24) and acclimation (0.17) are examined.

Hierarchical clustering of the 67 genes that vary significantly with acclimation temperature clusters the expression at 20ºC and 28ºC together, suggesting that gene expression is more similar at these temperatures than either is to gene expression at 12ºC. The variance for the standardized mean supports this conclusion: the average variance among the 67 acclimation significant genes for 12ºC and 20ºC (0.13), or 12ºC and 28ºC (0.26) is 1.4 or 2.8 (respectively) times greater than the variance for 20ºC and 28ºC (0.09).

Non-Neutral Variation

Of the 2,272 probes on the microarray with mean hybridization signals greater than background levels, 249 vary significantly ($p < 0.01$) in the phylogenetic comparative two-way ANOVA contrasting the cold-adapted northern *F.heteroclitus* (ME) *versus* warm-adapted southern populations (*F. heteroclitus* GA and *F. grandis* GA), acclimation temperature, or the interaction term (fig. 3.2, supplemental table 3.2). Sixty five genes vary significantly for acclimation temperature. One hundred eighty four (7.5%) genes vary significantly for the phylogenetic contrast between cold-adapted, northern *F. heteroclitus* (ME) *vs*. warm-adapted southern *F. heteroclitus* and *F. grandis* (GA + Fg).

Of these 184 genes with a significant phylogenetic contrast, the expression of 44 is significantly different between the two warm-adapted taxa (*F. heteroclitus* (GA) and *F. grandis*) using two-way ANOVA with a liberal p-value of $p < 0.1$ (10%). Even though the cold-adapted population is significantly different ($p < 0.01$) from both of the two warm-adapted populations (which includes two species), the observation that *F. heteroclitus* (GA) and *F. grandis* are also different reduces the strength of the argument that the northern level of gene expression is derived. Thus to be conservative we exclude

these 44 genes, leaving 140 genes with evolutionary significantly altered expression where the northern population has a derived pattern of gene expression.

Thus, conservatively, the expression of 140 (6.2%) genes is inconsistent with neutral evolution. In addition to these potentially adaptive genes, sixty-five vary significantly according to acclimation temperature and 14 demonstrate significant interaction terms. The magnitude of the statistical variance is 1.2-fold greater for the phylogenetic effects than acclimation temperature (fig. 3.2D). Of the 11 genes affected by both factors (phylogeny and acclimation), 3 are different between the two warm southern taxa. This leaves 8 genes with both phenotypic plasticity and non-neutral patterns of variation. Ten of the 11 genes that significantly differ for acclimation effects and in the phylogenetic contrast share a common pattern of expression. For these genes, the phylogenic contrast and acclimation temperature have opposite effects on transcription levels: the eight genes that are upregulated at cold acclimation temperatures through phenotypic plasticity are also downregulated in the cold-adapted northern population, and the opposite patterns holds for the 2 genes downregulated at 12°C (fig. 3.3)

Applying FDR < 0.05 to these comparisons results in a nearly identical pattern, but with fewer genes. The expression of 69 genes are significantly different (FDR < 0.05) for the phylogentic contrast. Of these 69 genes, 47 genes are only significantly different between cold northern population and both southern population ($p < 0.01$ with FDR < 0.05) and not different ($p < 0.1$) between southern taxa (supplemental table 3.2). Therefore, these 47 demonstrate patterns of expression inconsistent with neutral evolution with an FDR of 5%. Six genes vary significantly according to acclimation temperature. No genes demonstrate significant effect of second order factors; zero genes

have significant interaction terms, or an effect of both acclimation temperature and the phylogenetic contrast using a FDR of less than 5%.

Gene-by-Environment Interactions

Eight genes demonstrate significant interactions in the first two-way ANOVA for the effect of population and acclimation temperature, and 14 genes demonstrate significant interactions in the phylogenetic comparative ANOVA, suggestive of a gene-by-environment (G x E) interaction in gene expression. The significant interactions make it difficult to evaluate the statistical meaning of differences due to both factors. To further explore G x E interactions, we conducted phylogenetic comparative analyses at each acclimation temperature separately. Specifically, we are statistically applying the phylogenetic contrast at each temperature, and not doing multiple constrasts among acclimation temperatures (eg 12 *vs*. 20, 12 *vs*. 28,…). There are 163 genes with significant phylogentic constrasts at any of the three acclimation temperatures (one-way ANOVA, $p < 0.01$ for each acclimation temperature). Only one gene is different at all three acclimation temperatures (Cytochrome c oxidase subunit VIIa). Six genes have adaptively significant mRNA expression at two acclimation temperatures (4 at 12°C and 28°C, one each at 20°C and 12°C or 20°C and 28°C). Thus, 96% of significant genes (157 out of 163) only demonstrate adaptive divergence at one acclimation temperature. Of these, acclimation to 28°C has the most genes with adaptive divergence (97 genes, 71%). At 12°C acclimation, there are 59 (20%) significant genes. The fewest adaptively significant genes occurr at 20°C (14 genes, 9%). The number of individuals at each acclimation temperature, however, is not the same (38, 46, and 29 at 12°C, 20°C and 28°C respectively). Although there are more individuals at 20°C (where there are the fewest number of significant genes), we created a condensed data set by randomly

sampling 4 individuals per taxa (4 North and 8 South). Using this smaller data set (fewer overall individuals) the results are similar: 15 significant genes at 12°C (35%), 4 at 20°C (8%) and 53 at 28°C (57%).

Statistical differences among groups can become significant if the variation with groups is reduced *versus* increased differences among groups. Our specific concern is that the lack of significant phylogenetic difference at 20°C is due to increase in the variation among individuals. To explore whether acclimation temperatures affected the statistical variance in gene expression among individuals, we used the condensed data set with 4 individuals at each condition. For the 15 genes significantly different for the phylogenetic contrast acclimated to 12ºC, only one demonstrates significantly less variation in expression at 12ºC relative to 20ºC (F-test $p<0.05$). Similarly for the 53 genes significantly different for the phylogenetic contrast at 28ºC, 4 vary less at this temperature than at 20ºC. These observations suggest that for the majority of genes, differences in the magnitude of the adaptive transcriptional response among acclimation temperatures are not due to canalization of expression at intermediate temperatures.

Next we explicitly tested for non-neutral variation in plasticity among the 62 genes with both significant phylogenetic contrast and similar variance at each acclimation temperatures. Using a variation of the method proposed by Nussey *et al* (2007), we used random regression to test for significant variation in the slope of the gene expression reaction norm among warm adapted and cold adapted *Fundulus spp.*. Of the 62 genes considered, only 2 have significantly detectable differences in plasticity, (log-likelihood ratio test (LRT), $p < 0.05$). This method was also used on genes that had a significant interaction term in either of the two-way ANOVAs. Of these 18 genes, 8 demonstrate

significant among population variation in adaptive plasticity in a pattern consistent with evolution by natural selection (LRT, $p < 0.05$).

Gene Enrichment Analysis

We annotated genes that responded to acclimation temperature and genes with putatively adaptive differences in expression and used the Functional Annotation Clustering tool in DAVID v6.7 to test for functional enrichment (Huang *et al.* 2008)). Of the 67 genes that responded to acclimation temperature 54 have unique annotations and 51 map to functional annotations in the DAVID database. No functional annotation clusters were found in this dataset with an enrichment score above 1.3 (in these analyses, an enrichment score of 1.3 is equivalent to $p = 0.05$). Two clusters were found with less conservative enrichment scores (0.97-1.13) and included a cluster dominated by immune system related annotations, as well as a cluster related to the response to hormone stimuli. Of the 140 genes with putatively adaptive differences in expression among the populations, 106 have unique annotations and 97 map to functional annotations in the DAVID database. Four functional annotation clusters with enrichment scores above 1.3 were discovered in the dataset as well as 2 with less conservative enrichment scores (0.95 and 1.23). The conservatively enriched clusters contain annotations related to pyruvate metabolism, glycolysis, electron transport and inner mitochondrial membrane proteins, while the additional two clusters include annotations related to the ubiquitin conjugation pathway and NADH binding.

Discussion

Rationale

Examining patterns of gene expression in populations of *F. heteroclitus* acclimated to different temperatures allows us to address three questions within an ecological-evolutionary framework where both adaptive phenotypic plasticity and adaptive genetic divergence are expected. (1) Whether plasticity obviates the need for genetic divergence and thereby impedes evolution, (2) Whether plasticity and genetic divergence operate in parallel on the same suites of genes, or on different sets of genes entirely, and importantly whether cogradient or countergradient patterns of expression are common where they operate together, and (3) whether environmentally-induced variation in gene expression leads to divergence among locally-adapted, wild populations.

Putatively Adaptive Variation

Fundulus spp. demonstrate shared patterns of gene expression in response to thermal acclimation, constitutive differences in gene expression among populations and putatively adaptive, non-neutral variation between cold- and warm-adapted populations. We are assuming that the observed non-neutral patterns are heritable because they persist after acclimation to a common temperature (see below). Yet, we cannot rule out irreversible developmental plasticity or maternal effects. Where irreversible plastic effects occur in a pattern similar to the non-neutral scenario, we will be unable to differentiate these patterns from putatively adaptive variation. In fact, analyses of fish muscle gene expression demonstrate that embryonic development temperature can influence the transcriptional response to cold acclimation (Schnurr *et al.* 2014; Scott & Johnston 2012).

However, based on three assumptions, we assert that many of the significant differences in the phylogenetic comparative analysis likely have a heritable genetic basis

and are consistent with evolution by natural selection. First, muscle acclimation in fish reaches a steady state after 3-4 weeks (Heap *et al.* 1985; Sidell *et al.* 1973), and beneficial impacts of developmental plasticity on swimming performance is largely eliminated with acclimation (Scott & Johnston 2012) suggesting that maintaining fish for 4 weeks to a common temperature in advance of experimental acclimation removes the effect of reversible acclimatization to varying field conditions in the experimental populations. Second, gene expression is heritable within species (Brem & Kruglyak 2005; Gibson 2008; Gibson & Weir 2005) and demonstrates a strong phylogenetic signal among species (Brawand *et al.* 2011; Romero *et al.* 2012), reinforcing the expectations that patterns of gene expression are more similar among more closely related taxa and that many of the differences among the experimental populations are due to genetic variation. Finally, only a minority of inter-species differences in gene expression can be accounted for by epigenetic regulatory mechanisms such as histone modification (Cain *et al.* 2011; Pai *et al.* 2011). Within a species, developmental plasticity only has a significant effect on gene expression for a small portion of genes (Scott & Johnston 2012), again supporting the genetic basis of the majority of the differences among the populations.

To be concise we refer to those genes with significant phylogenetic contrasts and thus non-neutral patterns as putatively adaptive divergences even though a minority of differences may be due to irreversible acclimation and thus may not be strictly heritable effects (Crawford & Oleksiak 2007; Crawford *et al.* 1999; Loftus & Crawford 2013).

Comparing Plasticity to Adaptation

Fundulus heteroclitus are distributed along a steep thermal cline that is thought to underlie many of the adaptive differences among populations. Populations along this

cline also experience seasonal shifts in temperature similar to the temperatures difference between the northern and southern extremes of the species range. By comparing thermal acclimation to thermal adaptation, we gain insight into the relative effects of phenotypic plasticity and evolutionary adaptation in response to the same environmental variable. The expression patterns of many more genes demonstrate a significant phylogenetic contrast than due to significant acclimation effects. Furthermore, difference in the means is larger for gene expression with significant population effects than for acclimation effects. For putatively adaptive variation, the same pattern is observed: the magnitude of the differences in expression among gene with a significant phylogenetic constrast is larger than for the expression of genes with significant acclimation effect. These data suggest that plasticity does not obviate the need for an adaptive genetic response across the many thousands of phenotypes examined in our microarray analysis. Instead, differences among populations, including putatively adaptive differences, dominate the differences due to acclimation temperature. It is important to note, however, that the acclimation regime utilized in this study represents only a moderate shift in a single environmental parameter while differences among populations are determined by both neutral divergence and selection due to all the varied biotic and abiotic environmental conditions that co-vary with temperature along the range of *F. heteroclitus*.

Countergradient Variation

In addition to comparing the magnitude of phenotypic plasticity and evolutionary adaptation, we are also interested in whether these processes work in parallel on the same set of genes, or orthogonally on different genes. Of the 205 genes that demonstrate either a significant effect of acclimation temperature or putatively adaptive variation, only 8 demonstrate a significant effect of both in the phylogenetic comparative ANOVA ($p <$

0.01), and no genes demonstrate a significant effect of both using the conservative FDR of less than 5%. Therefore, for the majority of significant differences in expression, phenotypic plasticity and adaptation operate on different suites of genes. Where these processes operate on the same genes, however, there is a common pattern of negative covariance between the effect of acclimation temperature and temperature of the populations' natural habitat (fig. 3.3) Of the 2,272 genes analyzed, we expect only ~0.2 genes to show both significant phenotypic plasticity and non-neutral variation due to chance alone using the un-adjusted p-value ($p < 0.01^2$). The eight genes that share both significant plasticity and adaptive patterns of expression are 40-fold more numerous than the random expectation and therefore warrant some consideration.

The shared pattern of expression observed among genes with a significant effect of both acclimation and adaptation, where adaptive differences among populations oppose environmentally-induced variation is termed countergradient variation (Conover & Schultz 1995). Countergradiant variation may evolve through a special case of genetic accommodation referred to as genetic compensation. Genetic compensation occurs when an environmentally-induced change in phenotype results in reduced fitness in a new environment, but the plastic response is genetically constrained, as is often the case for passive plasticity (Grether 2005). Directional selection then acts to restore the optimum phenotype in the new environment by altering the trait mean and not necessarily plasticity. Thus, genetic compensation results in a stabilization of phenotypes across the species range, minimizing phenotypic variation among populations experiencing different environments. This pattern is most readily seen by comparing the larger differences among southern fish acclimated to 12°C *versus* northern fish acclimated to 28°C in

contrast to the more similar pattern of expression for southern fish acclimated to 28°C and northern fish acclimated to 12°C (fig. 3.3). Northern fish acclimated to their summer temperature of approximately 12°C have RNA expression levels similar to southern fish acclimated to their summer temperature of approximately 28°C (compare two center columns C 12°C *versus* W 28°C of fig. 3.3). These data suggest that when fish experience their "*normal"* temperatures, phenotypic plasticity and natural selection act together to produce similar levels of mRNA expression and thus reduce clinal variation among populations. A second implication of this countergradiant variation is the promotion of selective barriers to gene flow among populations owing to the relative fitness of locals over migrants. In this way, genetic compensation can lead to further divergence among populations experiencing different patterns of ecological selection (Fitzpatrick 2012a).

Gene-by-Environment Interactions

Many genes demonstrate significant population-by-environment interactions in both two-way ANOVAs. Because many of the differences in mean expression among populations are likely to have a heritable genetic basis, these interactions are suggestive of significant gene-by-environment (GxE) interactions. GxE in the phylogenetic comparative analysis indicate non-neutral differences among populations in the response to environmental change. Consequently, these significant interactions may represent examples of adaptive phenotypic plasticity in gene expression. Significant population-by-environment interactions, however, may arise as a consequence differing levels of residual variance across environments in an ANOVA (Windig *et al.* 2004). Therefore, we examined whether variance in expression is better fit by a linear mixed effect model that includes a different environmental response among the cold and warm-adapted

populations than a model with no differences in plasticity (Nussey *et al.* 2007). Using the reaction norm heuristic (Woltereck 1909), this test explicitly examines whether there is a significant difference in the slope of the gene expression reaction norm among the cold and warm-adapted taxa. Of the 18 genes with significant interaction terms in either of the two, two-way ANOVAs, 8 exhibit a significant difference in plasticity.

In order to further explore GxE interactions, we considered non-neutral differences among populations at each acclimation temperature separately. The two most salient findings of this analysis are that the majority of putatively adaptive differences in expression are unique to a single acclimation temperature and that many more of these differences are apparent at extreme relative to the moderate acclimation temperature. Thus, both the identity and magnitude of adaptive differences expressed among populations depends on the environmental conditions experienced by individuals within the populations. Such a pattern may be explained by either differences in the extent of inter-individual variation in different environments (canalization), or adaptive variation in phenotypic plasticity (slope of reaction norm) among populations (Windig *et al.* 2004). For example, environmental canalization may reduce inter-individual variation in gene expression at extreme temperatures. This canalization introduces a bias in statistical power among the tests at each acclimation temperature such that lower variation among individuals leads to higher probabilities of finding a significant adaptive variation at a given acclimation temperature. The alternative, adaptive variation in plasticity, is suggested by non-neutral differences in the slope of the reaction norm among the populations. We are unable, however, to rigorously attribute the majority of these differences in the adaptive response across environments to either canalization or

adaptive differences in phenotypic plasticity. Among the genes that demonstrate non-neutral differences at the extreme acclimation temperatures, 7% demonstrate reduced variation at the moderate acclimation temperature. This suggests that, for the majority of genes, differences in the magnitude of the transcriptional response among acclimation temperatures are not due to canalization. For the remaining genes, only 4% demonstrate adaptive variation in the slope of the reaction norm. Yet, this is in conflict with the observation that adaptive expression is a function of acclimation temperatures and is not due to statistical bias among the tests for the majority of genes.

Whether these patterns are due to canalization or adaptive phenotypic plasticity, the prevalence of GxE interactions and the varying penetrance of adaptive traits under different acclimation temperatures observed in this study highlight the increasingly important role of the environment in our understanding of the genotype-to-phenotype map and thus, phenotypic evolution. While examples of GxE interaction of gene expression are common in the literature (Grishkevich & Yanai 2013; Hodgins-Davis & Townsend 2009; Smith & Kruglyak 2008), the significance of our findings stems from the observation that GxE interactions contribute to the divergence among locally-adapted, natural populations alongside constitutive differences. The phylogenetic comparative analysis emphasizes the significance of this divergence because adaptive variation is likely to have a biological impact (Whitehead & Crawford 2006b). These environment-specific differences among natural populations may influence evolutionary trajectories because both the loci responsible for and the extent of genetic variation exposed to selection are likely to differ among the populations. Thus, environment-specific expression can result in relaxed selection and an accumulation of cryptic genetic variation

(CGV) among loci underlying plastic traits. (Snell-Rood *et al.* 2010; Van Dyken & Wade 2010). Subsequent release of this CGV in an altered genetic or environmental background results in further phenotypic diversification (Gibson & Dworkin 2004) (Le Rouzic & Carlborg 2008) and may enhance reproductive isolation among populations (Fitzpatrick 2012a; Pfennig *et al.* 2010).

Functional Analysis

Patterns of gene expression suggest an acclimation response shared across all three populations (supplemental table 3.1). Enrichment analysis reveals two over-represented clusters with annotations related to immune response and hormonal signaling. Genes with functions related to innate immunity are upregulated in cold-acclimated fish. Indeed, this is a common feature of cold acclimation in teleost fish (Bowden *et al.* 2007). While few genes that respond to acclimation form functional clusters, many have been identified as functionally important for acclimation in other studies. For example, three genes with proteolysis biological function GO terms that are associated with the ubiquitylation system are up-regulated with cold acclimation. Upregulation of ubiquitin-proteasome associated proteins is a common feature of cold acclimation in fish skeletal muscle (Cossins *et al.* 2006; Gracey *et al.* 2004). Additionally, cold-inducible nucleoside diphosphate kinase b (NDK-B) is down-regulated and cold inducible RNA binding protein (CIRBP) is upregulated after cold acclimation. Both loci have been identified as candidates important to thermal acclimation in fish (Castilho *et al.* 2009; Gracey *et al.* 2004). NDK-B has a diversity of biological functions (Crawford *et al.* 2005; Hippe *et al.* 2011; Wagner & Vu 1995). Intriguingly, it interacts with creatine kinase and glyceraldehyde-3-phosphate dehydrogenase in cardiac muscle suggesting a role in energy metabolism in this tissue

(Otero 1997). Shifts in NDK-B expression are also associated with recent adaptation to new environmental conditions (Roberge *et al.* 2005; St-Cyr *et al.* 2008). Taken together, the enhanced expression of innate immunity and proteolysis associated genes as well as the candidate loci NDK-B and CIRBP suggest that there may be increased reliance on alternative energy sources and/or increased protein turnover at low temperatures.

Patterns of gene expression also suggest functional differences among the warm adapted southern populations and the cold adapted northern population (supplemental table 3.2). Much of the inferred functional impact from gene expression variation that is putatively adaptive corroborates previous findings on adaptive divergence in this species and thermal adaptation in general (Hochachka & Somero 2001). Functional clusters containing annotations related to glycolysis (Pierce & Crawford 1997a), pyruvate metabolism (Crawford & Powers 1989), oxidative phosphorylation and the inner mitochondrial membrane (Fangue *et al.* 2009b) are significantly enriched in our phylogenetic comparative analysis. Intriguingly however, many genes that regulate thermal acclimation in other fish species diverge in their expression among our experimental populations in a non-neutral pattern. These genes include an enriched cluster associated with the ubiquitylation system, groups of genes associated with mRNA processing and translation and the high mobility group box 1a gene (HMGB1a). HMGB1a is of particular interest as it has been identified as a potential global regulator of thermal acclimation (Podrabsky & Somero 2004). The observation that mechanisms of acclimation common to many other teleost fish are instead involved in thermal adaptation in *F. heteroclitus* suggests that adaptive divergence in this species may be mediated in part by changes in phenotypic plasticity or the conversion of plastic gene expression to

constitutive differences among locally adapted populations. Alternatively, ongoing selection in response to the thermal cline experienced by these populations may simply target different loci with similar functions to those that underlie thermal acclimation. As we are unable to determine if the genes we identified as putatively adaptive are directly orthologous to acclimation related genes in other species, we are unable to differentiate between these two alternative explanations; this is an exciting approach for future research.

Conclusion

We combine a microarray analysis of gene expression across acclimation temperatures with a phylogenetic comparative analysis that can identify patterns of variation which may be adaptive. Adaptation and phenotypic plasticity (acclimation) operate for the most part on different genes. For the few genes with both significant adaptive and plastic responses, the direction of change is in opposite directions: the effect of acclimation to colder water is in the opposite direction from the effect of adaptation to colder environments. Where these two factors operate alone, putatively evolved differences in gene expression dominate phenotypic plasticity. The expression of many more genes is affected by adaptation than phenotypic plasticity and the mean effect of adaptation is larger than mean plastic effects. However, the putatively adaptive differences are dependent on the acclimation temperature. There are many genes with a significant interactions between adaptation and acclimation temperature. Additionally, more and different genes have putatively different patterns of expression at 12° and 28°C than at 20°C, although we cannot explain if this pattern is due to canalization of expression or changes in the slope of the reaction norm. Taken together, the extent of GxE interactions and the observation of countergradient variation in gene expression

suggest that environmentally-induced differences are an important component of adaptive divergence among populations. Future studies on these phenomena should conservatively demonstrate that genes with GxE interaction in gene expression are indeed adaptive. For example, a similar approach using RNAseq can simultaneously identify putatively adaptive patterns of GxE in gene expression while taking advantage of data at the sequence level to search for the genomic signature of selection near these genes.

Tables and Figures

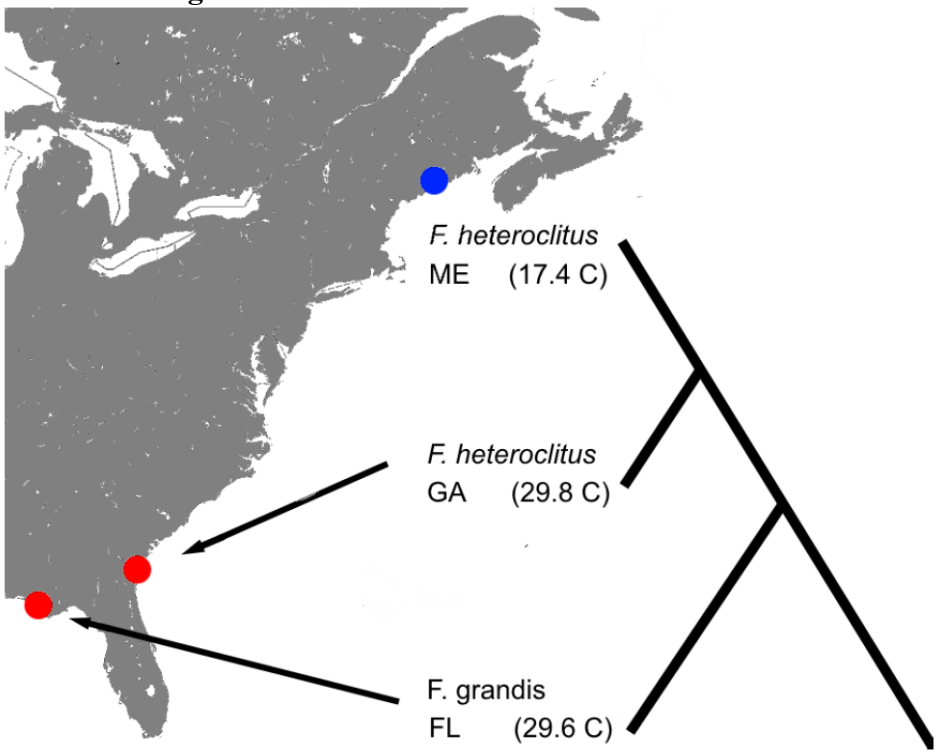
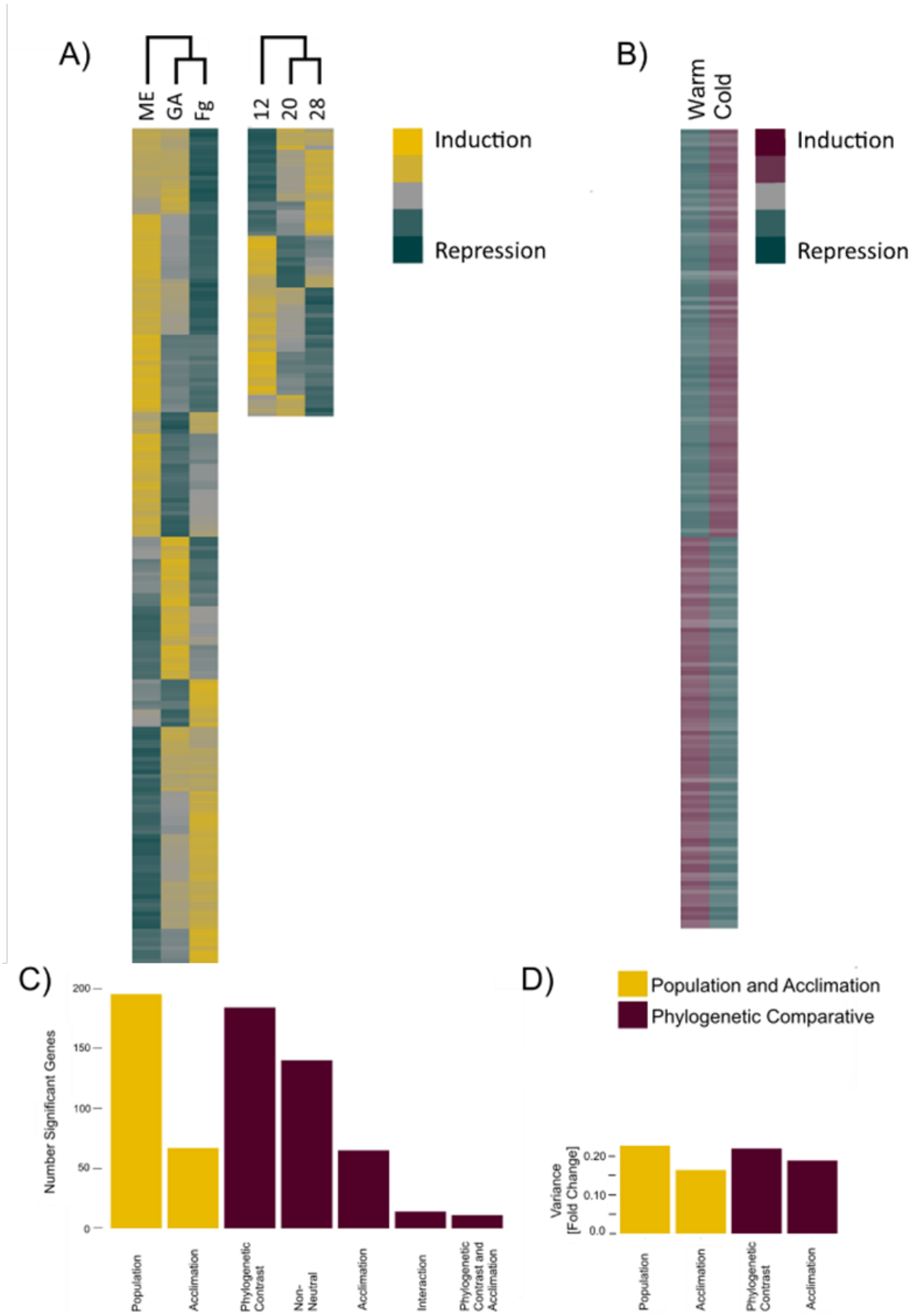


Fig. 3.1: Phylogenetic relationships and mean summer temperatures among experimental populations. The genetic distance between the two *Fundulus heteroclitus* populations (Maine and Georgia) is less than the genetic distance between either and *Fundulus grandis*. Thus, the neutral expectation is that Maine and Georgia populations of *F. heteroclitus* are more similar to each other than either is to *F. grandis*. Significant differences in gene expression between Maine and both Georgia and *F. grandis* do not fit the neutral expectation and are most parsimoniously described as adaptive. Furthermore, because Georgia *F. heteroclitus* and *F. grandis* inhabit similar thermal environments, this suggests that these gene expression changes could be due to thermal adaptation.

Fig. 3.2: (next page) (A) Hierarchical-clustered heatmap of gene expression for genes with significant differences among populations (GA – Georgia;ME – Maine; and Fg – Fundulus grandis) or acclimation temperatures (12, 20 and 28 °C) according to two-way gene-by-gene ANOVA ($P < 0.01$) (gold and blue coloration). Gene expression is standardized with mean equal to zero and variance equal to 1 for each gene. (B) Hierarchical-clustered heatmap of gene expression for genes with significant differences among cold- and warm-adapted taxa (cold-adapted: Maine; warm-adapted: Georgia and F. grandis) in the phylogenetic comparative, two-way ANOVA (purple and blue coloration – throughout manuscript) (C) Number of genes with expression that varies significantly due to factors in the population vs. acclimation two-way ANOVA (gold bars) and the phylogenetic comparative ANOVA (purple bars). (D) Mean variance among factor means for all genes with a significant effect of population, acclimation or the phylogenetic contrast in the population vs. acclimation two-way ANOVA (gold bars) and the phylogenetic comparative ANOVA (purple bars).


A)
ME
GA
Fg
12
20
28
Induction
Repression
B)
Warm
Cold
Induction
Repression
C)
Number Significant Genes
200
150
100
50
0
Population
Acclimation
Phylogenetic Contrast
Non-Neutral
Acclimation
Interaction
Phylogenetic Contrast and Acclimation
D)
Population and Acclimation
Phylogenetic Comparative
Variance [Fold Change]
0.20
0.10
0.0
Population
Acclimation
Phylogenetic Contrast
Acclimation

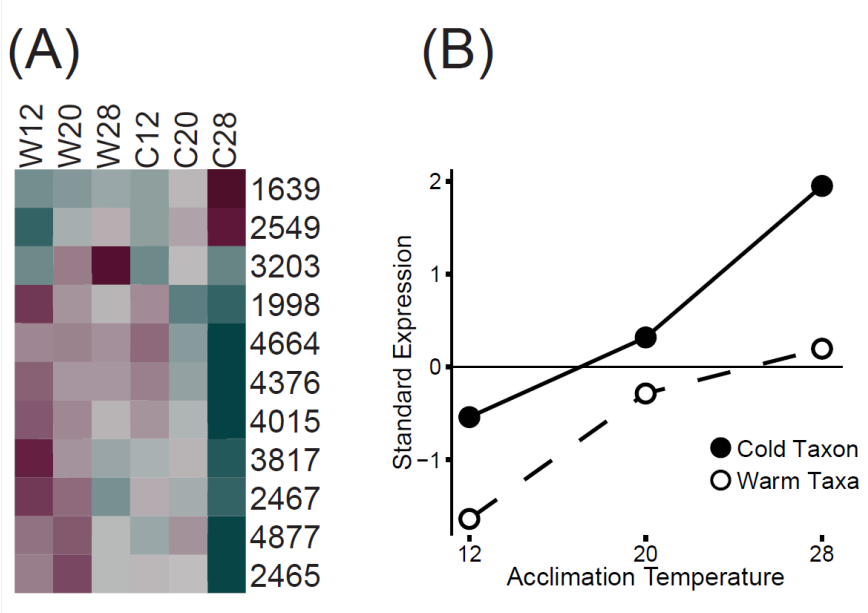


Fig. 3.3: Countergradient variation. (A) Hierarchical-clustered heatmap of gene expression for the 11 genes with a significant effect of both acclimation and taxa in the phylogenetic comparative ANOVA (W: warm-adapted taxon; C: cold-adapted taxon; 12, 20 and 28 °C: acclimation temperature). Annotation print numbers are provided for identification (see Supplement). Gene expression is standardized with mean equal to zero and variance equal to 1 for each gene. With the exception of gene 3203, expression is most similar between warm-adapted fish at the warm acclimation temperature (W28) and cold-adapted fish at the cold acclimation temperature (C12). (B) Representative countergradient variation in the reaction norm of expression for gene 2549 (open circles, dashed line: warm-adapted taxon; closed circles, solid line: cold-adapted taxon). The phylogenetic contrast opposes the effect of acclimation; warm-adapted fish demonstrate repression of gene 2549, while warm acclimation results in induction in both taxa.

Chapter 4: Population genomics of rapid evolution: the importance of soft, polygenic adaptation

Background

Environmental temperature is one of the most important factors affecting the distribution and physiology of organisms. Much of our understanding of thermal adaptation is drawn from comparisons among species that diverged millions of generations ago, or among populations that diverged thousands of years ago (Angilletta 2009). Yet, many of the most salient evolutionary questions today, and perhaps historically, occur on much more rapid time scales, *e.g.* adaptation to novel environments during species introductions (Bock *et al.* 2015) and in spatially restricted populations coping with global climate change (Schloss *et al.* 2012). While an exception to this generalization comes from rapid evolution of resistance to pesticides or environmental pollutants, these circumstances are fundamentally different from thermal adaptation. Selection to a single chemical or related chemicals is often mediated through a single pathway or even a single genetic locus (Menozzi *et al.* 2004; Oleksiak *et al.* 2011), and this specificity lends itself to a narrow genomic basis of adaptation (Jensen 2014). In contrast, temperature broadly impacts all physiological systems through its effects on biochemical reaction rates and biomolecular structures (Hochachka & Somero 2001). Accordingly, rapid thermal adaptation is likely to have a different genomic basis than other well-understood examples of rapid evolution because the selection target is so broad. If selection operates primarily on highly integrated, complex performance traits such as thermal tolerance (Irschick *et al.* 2008; Rockman 2012), understanding the genomic basis of recent thermal adaptation provides critical insight into the adaptation of natural populations.

There are several competing population genetic models of adaptation, and each produces a unique genomic signature. In the hard sweep model, a new mutation borne on a single haplotype is driven to high frequency by selection, leading to a reduction in genetic variation at linked neutral loci (Smith & Haigh 1974). Genome scans for hard sweep signatures have dominated our understanding of adaptation, yet there is increasing theoretical evidence that alternative models such as soft sweeps and polygenic adaptation may also be common, especially in the case of recent selection in large natural populations (Barrett & Schluter 2008; Hermisson & Pennings 2005). In the soft sweep model, the adaptive alleles swept to high frequency are borne on multiple haplotypes, either because they arise independently or they are present in the standing genetic variation long enough to become unlinked from nearby variation, resulting in a weaker signal at nearby neutral loci (Messer & Petrov 2013). Finally, because most ecologically important traits are likely to have a polygenic basis (Rockman 2012), quantitative genetics predicts that recent adaptation should occur through subtle shifts in allele frequencies at many loci (Falconer & Mackay 1996). Importantly, polygenic adaptation and soft sweeps share many characteristics, because in both cases, adaptive alleles can be located on multiple haplotypes resulting in reduced hitchhiking of linked variation during selection. While these models have been the subject of considerable theoretical discussion, there is a dearth of empirical examination. In this investigation, we evaluate the extent to which each adaptation model contributes to recent thermal adaptation in large, outbred populations.

We sampled populations of the estuarine fish *F. heteroclitus* near the effluents of two power stations: Oyster Creek nuclear generating station in New Jersey and Brayton Point generating station in Massachusetts. Thermal effluents produced by coastal power stations provide a recent source of environmental variation that is both localized and well quantified. Effluents from both power plants have produced significant thermal impacts since the beginning of their operation in the late 1960s. The Oyster Creek thermal effluent is discharged along a modified river for approximately 3km into the intracoastal Barnegat Bay. Temperatures range from 10-13°C above ambient at the discharge site and 4-5°C above ambient where Oyster Creek joins Barnegat Bay. Beyond this point, the effluent's thermal influence is limited to a ~2km radius from the mouth of Oyster Creek (Kennish & Olsson 1975). Documented ecological impacts of thermal input at Oyster Creek include maintenance of non-native, warm-adapted species not found elsewhere in the region (Hoagland & Turner 1980) as well as decreased growth rates and failed spawning events in benthic molluscs (Kennish & Olsson 1975). At Brayton Point, effluent is released into the surrounding estuary, Mount Hope Bay, at 7-16°C above ambient temperatures. This effluent leads to ~1°C temperature anomaly throughout Mount Hope Bay (Mustard *et al.* 1999), but has varied thermal impacts on smaller spatial scales due to incomplete mixing and advection of the thermal plume (Swanson *et al.* 2006). There are few predicted ecological impacts of the thermal effluent at Brayton Point (DeAlteris *et al.* 2006; O'Neill *et al.* 2006), although increased temperature may interact with other anthropogenic stressors in this region (Calabretta & Oviatt 2008).

These thermal effects lead to thermal adaptation among exposed populations. Indeed, comparisons of natural populations living in or near thermal effluents have

traditionally been used to parse adaptive genetic variation from neutral genetic variation. Allelic selection among electrophoretic variants of candidate loci is frequently reported in populations exposed to thermal effluents (Nevo *et al.* 1977; Nyman 1975; Yardley *et al.* 1974). In *F. heteroclitus*, a northern thermal effluent population has allele frequencies more similar to distant, warm-adapted southern populations than more closely-related northern populations at several allozyme loci (Mitton & Koehn 1975). Selection due to thermal effluents can also elicit rapid phenotypic evolution. Largemouth bass (*Micropterus salmoides*) living in effluent ponds demonstrate increased frequency of more thermally stable isozymes after fifteen generations and allele frequencies at these loci return to ancestral levels after just ten (Smith *et al.* 1983).

Our investigation extends these allozyme data by directly examining variation at thousands of genotyping-by-sequencing derived genetic markers. We provide population genomic and functional evidence of adaptation in natural *Fundulus heteroclitus* populations exposed to thermal effluents. We then consider the genetic variation patterns near adaptive loci to establish the roles of hard *vs.* soft sweeps as well as polygenic adaptation in recent thermal adaptation.

Methods

Populations

F. heteroclitus were collected from six sites. The six locations form two replicate triads, where a triad contains a single population subjected to thermal effluents (TE) as well as northern and southern reference populations (fig. 4.1). For the Oyster Creek triad, the TE population was sampled along Oyster Creek, in Forked River, NJ (39°48'31.40"N, 74°11'3.72"W), the northern reference population was sampled at Mantoloking, NJ (40°3'0.02"N, 74°4'4.92"W), and the southern reference population was sampled at the

Rutgers University marine field station in Tuckerton, NJ (39°30'31.60"N, 74°19'28.11"W). For the Brayton Point triad, the TE population was sampled at a marsh ~1km from the effluent canal (41°42'44.99"N, 71°11'9.74"W), the northern reference population was sampled at Horseneck Beach, MA (41°30'16.16"N, 71° 1'32.03"W), and the southern reference was sampled at Matunuck, RI (41°22'56.45"N, 71°31'32.04"W). Fish were captured using wire mesh minnow traps. Fin clips were taken for GBS library preparation; other fish from Oyster Creek and Mantoloking, NJ were transported live to the laboratory in aerated seawater for later critical thermal maximum analyses. Fieldwork was completed within publically available lands and no permission was required for access. *F. heteroclitus* does not have endangered or protected status, and do not require collecting permits for non-commercial purposes in the sampling locations. All fish were captured in minnow traps and removed within 1 hour. IACUC approved procedures were used for non-surgical tissue sampling.

GBS Library Preparation and Population Genetic Analysis

Caudal fin clips approximately 5 mm^2 in size were taken from individuals in the field and stored in 270 ul of Chaos buffer (4.5M guanadinium thiocynate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCl pH 7.5, 0.2% antifoam, 0.1M β-mercaptoethanol); these samples were stored at 4°C prior to processing. Genomic DNA was isolated from fin clips using a silica column (Ivanova *et al.* 2006). DNA quality was assessed *via* gel electrophoresis and concentrations were quantified in triplicate using Biotium AccuBlueTM Broad Range dsDNA Quantitative Solution according to manufacturer's instructions. 100 ng of DNA from each sample was dried down in 96-well plates. Samples were then hydrated overnight with 5 ul of water before restriction enzyme digestion and further processing.

GBS was performed as described (Elshire *et al.* 2011), using the restriction enzyme AseI, adaptors (0.4 pmol/sample) and 50ng of gDNA per sample. The library was created in duplicate with barcode assignment of individuals randomized across both replicate libraries. The GBS libraries were sequenced on two lanes of an Illumina HiSeq 2500 with a 100 bp single end read (Elim Biopharmaceuticals, Inc.).

The reference genome-based GBS analysis pipeline, TASSEL (Bradbury *et al.* 2007b) was used to call SNPs using the *Fundulus heteroclitus* genome (Reid *et al.* 2105); SNPs were identified using the "Discovery Build." A log of console input for the pipeline is available upon request. We largely used default settings throughout the pipeline with the following exceptions: a minimum of 5 counts were required for retention of individual tags during the merge multiple tag count fork, and tag alignment to the reference genome was accomplished with bowtie2 using the very-sensitive-local setting.

The initial SNP dataset produced by TASSEL-GBS was filtered to remove loci and individuals with low coverage. The resultant high coverage dataset was additionally filtered for polymorphisms that may result from sequencing error or alignment of paralogous loci: loci with low minor allele frequency (<1%) were removed in the TASSEL GUI, then loci with significant departures from Hardy-Weinberg equilibrium (greater observed than expected heterozygosity) were removed (p <0.01). Hardy-Weinberg equilibrium was calculated for individual loci using Arlequin v3.5.1.2 (Excoffier *et al.* 2005) using 1,000,000 steps in the Markov chain with 100,000 dememorization steps.

Outlier scans were conducted with FDIST2 (Beaumont & Nichols 1996) as implemented in LOSITAN (Antao *et al.* 2008). For all pairwise population comparisons we used the

same settings. We culled loci that are potential outliers to more narrowly estimate initial mean F_{ST} values (neutral mean F_{ST} option) and used the bisection approximation algorithm to estimate mean F_{ST} values (force mean F_{ST} option). After these steps, we conducted 50k simulations. To control for multiple comparisons, we adjusted empirical p-values provided by LOSITAN with a modified FDR of 5% (Benjamini & Yekutieli 2001; Narum 2006).

Population genetic parameters were calculated in a variety of statistical packages. Isolation by distance was tested using a Mantel test (9,999 simulations) in the ade4 R package (Chessel *et al.* 2004). Arlequin v3.5.1.2 was used to calculate the proportion of inter-population pairwise differences, intra-population estimates of nucleotide diversity (π), and AMOVA (99,999 permutations).

Population genetic structure inference was made using STRUCTURE (Falush *et al.* 2003) and DAPC (Jombart *et al.* 2010). STRUCTURE analyses were performed on a SNP dataset consisting of all pairwise outliers within a triad (effluent population + two reference populations). For all analyses, we used a burn in of 10k steps and Monte Carlo Markov chain (MKMC) of 20k steps with at least 7 replicates for each *k* value and varied *k* from 1 to 6. Replicate individual *k* runs were merged and the ΔK method for identifying the optimal number of clusters (Evanno *et al.* 2005) was calculated using CLUMPAK (Kopelman *et al.* 2015). For DAPC, we used the full SNP dataset. The number of principal components retained in the analyses is detailed in the results.

Critical Thermal Maximum

Using separate *F. heteroclitus* collections from the Oyster Creek TE population (n = 51) and its northern reference population (Mantoloking, NJ) (n = 47), we measured upper thermal tolerance with the critical thermal methodology (Lutterschmidt &

Hutchison 1997). Fish were maintained in the laboratory in a recirculating seawater system containing less than 1 fish per gallon and fed daily in the afternoon. Salinity, ammonia and temperature were checked regularly. All protocols were approved by the institutional animal care and use committee (IACUC protocol 13-054). Fish were acclimated for 8 weeks to 28°C and 15ppt salinity using artificial seawater and a 14:10 hour light:dark schedule to reduce the effect of reversible acclimatization to local field conditions.

The experimental chamber consisted of a 20L aquarium within an insulated 40L aquarium. Both chambers were filled with acclimation temperature water (28°C), then 70°C water was introduced from a header tank to the outer aquarium at a controlled rate to maintain heating at 0.28 – 0.30°C/min in the inner tank throughout the experiment. The inner chamber was aerated to reduce thermal stratification during trials and maintain oxygen saturation. After acclimation, groups of six fish were introduced to the inner aquarium. Critical thermal maxima were determined based on continuous loss of equilibrium for 5 seconds. 99% of individuals survived the critical thermal maximum trial after 1 week.

Results

Samples and Experimental Design

F. heteroclitus populations were sampled in two replicate "triads" (Williams & Oleksiak 2008), each consisting of a single thermal effluent (TE) site bordered on either side along the coast by a reference site (fig. 4.1). Final sample sizes per location are listed in Table 4.1. The two TE populations are Oyster Creek and Brayton Point.

Sequencing Results and Filtering

Genomic DNA (gDNA) was isolated from 296 individuals. These gDNA samples were individually-barcoded and used to create a reduced representation library for genotyping by sequencing (GBS) (Elshire *et al.* 2011). The libraries were sequenced on 2 Illumina Hi-Seq lanes. We found 1,451,801 unique sequence tags that contained both the barcode and *AseI* cut site. Bowtie aligned 1,142,340 (78.7%) of these tags to unique loci in the *F. heteroclitus* genome; 159,591 (11.0%) sequence tags aligned to multiple loci, and 149,870 (10.3%) had no alignment. The latter two tag sets were excluded from further analysis. Heterozygotes were called using a binomial likelihood ratio based approach of quantitative genotype calling, as implemented in the TASSEL-GBS discovery pipeline (Glaubitz *et al.* 2014). Among the 1.1 million tags that singly aligned to the *F. heteroclitus* genome we identified 314,746 SNPs.

We filtered the 314,746 SNPs identified by the TASSEL discovery pipeline among all 296 individuals in the library. We removed fifty-seven individuals missing more than 12.5% of SNPs. Next we retained SNPs that were called in at least 85% of the remaining 239 individuals, resulting in 5,907 retained SNPs. To remove polymorphisms that may have arisen from sequencing and amplification errors or alignment across paralogs (*versus* polymorphisms between alleles) (Hosking *et al.* 2004), we then filtered the remaining SNP dataset by minor allele frequency and whether observed heterozygosity (H_o) was significantly greater than the expected heterozygosity (H_e). Of the 5,907 SNPs in 239 individuals, 110 with minor allele frequencies less than 1% were removed. Then, 348 SNPs with $H_o > H_e$ that exceeded Hardy-Weinberg equilibrium at $p < 0.01$ were removed. Thus, the fully filtered SNP dataset consisted of 5,449 SNPs among 239 individuals. Mean read depth per SNP per individual was 26.29 ± 0.43 for the 5.4k SNP

dataset (Fig. 4.2, supplemental Fig. 4.1). Most (64.3%) SNPs in the final 5.4k SNP dataset have at least 10 reads in all individuals. The TASSEL-GBS pipeline caps the number of reads used to make a call at 127 for each allele. Therefore, the range of read depth per individual per SNP in the dataset was 0 – 254 (2*127), and the high frequency of 127 and 254 counts per SNP per individual in the read depth frequency distribution results from highly sequenced individuals and/or loci.

Genome-Wide Diversity Estimates

We identified substantial genetic diversity both within and among populations (Tables 4.1 and 4.2). Mean estimated pairwise F_{ST} values across all 5.4k SNPs ranged from 0.008 - 0.126. Mean genetic diversity (π) estimates ranged from 0.103 – 0.148 among populations and 0.097 – 0.152 within each population (Table 4.1). Note, however, that these π estimates are inflated relative to true genome-wide averages because they are calculated using only SNPs from polymorphic sequence tags used to generate our SNP dataset.

AMOVA partitioned 9.6% of the total genotypic variation to differences among triads, 0.7% among populations within a triad and the remainder (89.7%) is found within each population. The variance component among triads in the AMOVA was not significant while the other variance components were significant, suggesting that although the portion of genetic variation among triads is large, much of this variation is attributable to among populations structure; *i.e.,* the larger grouping (triads) is an artificial product of the hierarchical population sampling we employed.

Isolation by distance (IBD) among populations can lead to an increase in spurious results in outlier analyses and be mistaken for population genetic structure due to adaptive

processes (Meirmans 2012). Therefore we examined populations within each triad for IBD (Fig. 4.3). While there is significant IBD across all populations among triads ($p < 0.01$, Mantel test, 9999 simulations), neither triad showed significant IBD among its populations (Mantel test, $p > 0.5$, 9999 simulations). Therefore, outlier analyses will be more reliable when pairwise comparisons are made only within a triad.

Outlier Analyses

To distinguish loci that have neutral divergence patterns from those loci evolving by natural selection among populations within a triad, we conducted outlier analyses using the FDIST2 algorithm implemented in Lositan (Antao *et al.* 2008). An outlier analysis uses empirical data to simulate a neutral distribution of F_{ST} values for a given level of expected heterozygosity. Loci with F_{ST} values that significantly exceed the simulated distribution with a modified FDR of 5% (Benjamini & Yekutieli 2001; Narum 2006) are considered outliers. The numbers of significant outliers in any single pairwise comparison among populations ranged from 3.2% to 5.7% of the total 5.4k SNPs (Fig. 4.4) and were distributed across the observed heterozygosity range (supplemental Fig. 4.2). Within the Oyster Creek triad, 624 SNPs were identified as outlier loci in any of the three pairwise comparisons; 619 were identified in the Brayton Point triad (Fig. 4.4). We identify putatively adaptive SNPs among these outliers using the triad experimental design. Specifically, we refer to SNPs that were identified as outliers in both pairwise comparisons of TE *vs.* reference populations, but not in the reference *vs.* reference comparison as adaptive outlier loci (see Fig. 4.4) (Williams & Oleksiak 2008). This approach reveals 94 adaptive outlier loci in the Oyster Creek TE population and 36

adaptive outlier loci in the Brayton Point TE population where population differentiation may be due to directional selection at the effluent site.

Population Genetic Structure Inference

Genetic structure among populations within a triad was inferred using two methods: a model-based Bayesian approach (STRUCTURE) and a non-model-based multivariate approach (discriminant analysis of principal components (DAPC)) (fig. 4.5, supplementary fig. 4.3). Given a number of ancestral populations or genetic clusters (*k*), STRUCTURE estimates the probability that an individual derives its ancestry from a particular genetic cluster. DAPC maximizes among population differences while minimizing within group variation by combining principal components of genetic variation.

STRUCTURE identified no genetic substructure within a triad when all 5.4k SNPs were used, *i.e.* best *k* was 1. We also ran STRUCUTRE using only SNPs that demonstrated substantial differentiation among populations. This STRUCTURE analysis used the union of outlier SNPs from all three pairwise comparisons within a triad (624 SNPs for the Oyster Creek triad and 619 for the Brayton Point triad) at a range of putative population clusters between 1 and 6. STRUCTURE analysis produced different results between the two triads. For the Oyster Creek triad, $K = 2$ captured most of the structure among the populations (Fig. 4.5a). Likelihood scores (Pr(X|K)) beyond $K = 2$ increase at a decreasing rate (supplementary Fig. 4.3a), and the ΔK method identifies $K = 2$ as the optimal number of population clusters (supplementary Fig. 4.3c). Using two population clusters, the two reference populations group with one another, separate from the TE population. While there are admixed individuals in all populations, the two

reference populations were dominated by a single cluster while the TE population was dominated by a second cluster. Group membership in the first cluster was 75% and 68% for the two reference populations and 5% for Oyster Creek (TE). Population genetic structure at increasing *K* values are qualitatively similar; the identity of a population as reference or effluent-affected predicts the major inferred ancestry cluster. For the Brayton Point triad, $K = 3$ explains most of the structure among populations (Fig. 4.5b). Although the ΔK method identifies $K = 4$ as the optimal number of population clusters, likelihood scores (Pr(X|K)) plateau at $K = 3$ (supplementary Fig. 4.3b and d), and results at *K* higher than 3 are qualitatively similar. The first genetic split ($K = 2$) separated the southern reference population (Matunuck, RI) from both the TE and the northern reference populations (Horseneck Beach, MA). At $K = 3$ and $K = 4$ each population is dominated by its own cluster, such that each population is unique.

DAPC performed on the full 5.4k SNP dataset identified similar patterns of population structure for both triads. For the Brayton Point triad, we identified 2 as the optimal number of principal components (PCs) using the *a*-score method (supplementary Fig. 4.3h) to maximize discriminatory power while avoiding overfitting, although using up to 40 PCs produced qualitatively similar results. DAPC perfectly matched inferred genetic clusters with the three populations using these 2 PCs. Furthermore, the Bayesian information criterion (BIC) of K-means clustering from $K = 1\text{-}12$ demonstrated an inflection point at $K = 3$ (supplementary Fig. 4.5f). These results remained consistent when up to 40 PCs were used. The DAPC presented here for the Brayton Point triad used 2 PCs. The first discriminant function in DAPC represents the major axis of genetic structure among populations. The three populations were distributed along this axis of

genetic variation in a pattern consistent with their geographic distributions; the TE population is intermediate to the two references (Fig. 4.5d). The second major axis of genetic variation used to discriminate between the populations explained less variation (eigenvalues: 297.8 *vs.* 169.6) and revealed a population genetic structure pattern that is not consistent with their geographic distribution. Along the second major axis of genetic variation the two reference populations demonstrated substantial overlap while the TE population is distinct.

DAPC revealed similar population structure patterns in Oyster Creek and Brayton Point triads. However, the Oyster Creek triad *a*-score was low from 1-90 retained PCs; *i.e.,* individuals from single populations did not reliably fall into single genetic clusters (supplementary Fig. 4.5g). Similarly, the BIC for successive *K*-means clustering suggested 1 as the appropriate number of genetic clusters to describe the data (supplementary Fig. 4.5e), despite the extent of population differences indicated by pairwise genome-wide F_{ST} value estimates (Table 4.1) and significant among population differences in the AMOVA (Table 4.2). As our goal was to use DAPC to describe the genetic structure among these populations along orthogonal axes of genetic variation rather than to establish the extent of population differences, we performed DAPC using population as the grouping factor rather than inferred genetic clusters, as is common. This analysis maximizes the differences among *a priori* assigned populations rather than among the putative genetic clusters contained in our dataset. Including more or less PCs in this DAPC did not change the relationship among populations from 5-90 PCs, but populations were more distinct with more PCs and therefore more genetic variation was incorporated into the DAPC. Using the first 32 PCs, DAPC revealed the same population

genetic structure as in the Brayton Point triad. The TE population is intermediate to the two reference populations (Fig. 4.5c) along the first major axis of genetic variation. The second major axis (eigenvalues: 84.4 *vs.* 51.2) separates the two reference populations from the effluent-affected Oyster Creek population. Thus, for both triads the second major axis of genetic differences among populations for all 5.4k loci in the SNP dataset does not fit the neutral expectation: the two reference populations are more similar to each other than either is to the TE population.

Adaptive Allele Frequency Changes are Small

We examined the distribution of all SNP's allele frequency differences between reference and TE populations (Fig. 4.6a and b). Major allele frequencies are similar for both TE and references population (Fig 4.6). There were no fixed differences between populations. In fact, all alleles at fixation in any one population were the major allele overall, both within and among the two triads. For each TE population and its two references, change in allele frequencies for all SNPs have a maximum of 33% change for Oyster Creek and a maximum of 45% for Brayton Point. At the majority of loci (90%), allele frequency differences are less than 10% between the Oyster Creek TE population and the mean of both Oyster Creek reference populations. Similarly, 92% of loci have less than 10% allele frequency differences for the same comparison in the Brayton Point triad.

For the adaptive outlier loci, the allele frequencies changes are also small. Among adaptive outlier loci, the maximum allele frequency change is 8% between the TE population and both reference populations for both the Oyster Creek and the Brayton

Point triads. Furthermore, all of these differences have allele frequency = 1.0 (fixed) in either the reference or TE population (Fig. 4.6c and d). Thus, allele frequency changes identified as adaptive in our analysis are small shifts from near fixation in both reference populations to fixation in the TE population (67% of adaptive outlier loci) or fixed in both reference populations to less than 1.0 in the TE population (33% of adaptive outlier loci). In the latter case, where the adaptive shift includes a minor allele observed only observed within the TE population within a triad, the adaptive allele is also observed as a minor allele in the alternative triad for all but two loci. In other words, adaptive alleles are rarely private alleles globally because most are the major allele overall and become fixed in the TE population, or the adaptive minor allele in the TE population is the minor allele in the other triad.

No extended blocks of Elevated F_{ST} values around outliers

We surveyed individual pairwise F_{ST} values within a triad for SNPs physically close to adaptive outlier loci (fig. 4.7). Where an adaptive outlier SNP and another SNP occurs on the same scaffold in the *F. heteroclitus* genome assembly, we calculated the physical distance between the pair. The maximum distance observed between any such pair in our dataset is ~5 Mb, yet most pairs are much closer together. Among the pairs, 57% are separated by 64 bp or less for Brayton Point adaptive outliers (58% for Oyster Creek adaptive outliers). Many of these pairs are likely to be occur within the same 64bp sequence tags used to generate our dataset. Surprisingly, the genomic regions with elevated FST values surrounding adaptive outlier loci are exceptionally small: < 25bp. That is, the FST values of SNPs near adaptive outliers become indistinguishable from genome-wide mean FST values or a random permutation of the FST values after 12bp and 25bp, respectively, for the Oyster Creek triad (fig 4.7a). For Brayton Point, elevated

FST value regions near adaptive outliers are smaller; they are indistinguishable from genome-wide mean FST values and the random permutation of the pairwise distance data after ~6bp (fig 4.7b).

Functional Enrichment

We conducted a functional enrichment analysis to assess whether loci identified as adaptive outliers have roles in biological pathways or processes that are canonically involved in thermal adaptation. Adaptive outlier loci were annotated using the *F. heteroclitus* genome (Reid *et al.* 2105) where gene annotations are based on expression and homology evidence. Using the Functional Annotation Clustering tool in DAVID v6.7 (Huang *et al.* 2008), we find significantly enriched functional clusters among the adaptive outliers from both triads. The statistical significance of functional annotation clusters is evaluated on the basis of an EASE score, the mean value of the log-transformed, FDR corrected p-values of enrichment for all genes included in that cluster. Approximately half of sequence tag with adaptive SNPs mapped with genes in the DAVID database: 42 of 94 adaptive outliers for Oyster Creek map to the database and 21 of 36 for Brayton Point. At the highest stringency for annotation clusters, 1 cluster in both triads is enriched with an EASE score > 1.3. However, many functional gene clusters are enriched at lower EASE scores (0.5-1.2) and are salient because of their association with thermal adaptation (supplemental tables 4.1 and 4.2). Functional clusters for synaptic transmission and neuronal morphogenesis are enriched in both triads. The Oyster Creek adaptive outliers are also enriched for macromolecular complex assembly, immunoglobulin genes, exocytosis and kinase activity. Brayton Point is additionally enriched for GTPase activity, apoptosis and plasma membrane processes.

Critical Thermal Maximum

To assess whether exposure to thermal effluents has led to an increase in thermal tolerance, we measured critical thermal maxima (CT_{max}) in *F. heteroclitus* collected from Oyster Creek and its northern reference population. Oyster Creek individuals demonstrated significantly increased thermal tolerance relative to the northern reference population (ANOVA, $p = 0.0483$, $N = 98$). The critical thermal maximum for Oyster Creek was 39.7 ± 0.07 (mean ± s.e.) while the critical thermal maximum for the northern reference was 39.5 ± 0.07.

Discussion

Rationale

Much of our theoretical understanding of adaptive evolution assumes a new mutation that rises to fixation quickly after the onset of selection (Smith & Haigh 1974). Yet, much of adaptive evolution may involve neither new alleles, nor fixation of a single allele (Pritchard & Di Rienzo 2010). This latter scenario is particularly likely in natural populations with large effective population sizes that have been recently subjected to environmental changes with broad physiological impacts (Pennings & Hermisson 2006; Wilson *et al.* 2014). To provide empirical data for this debate, we provide analyze changes in allele frequencies associated with recent thermal adaptation in two populations.

Evidence of Recent Adaptation in Response to Thermal Effluents

Population genetic structure

In our sampling design, each effluent-effected population is flanked by two reference populations. This triad sampling design allows us to infer adaptive patterns in genetic variation. The neutral or demographic expectation is that the two reference populations will be more distantly related to each other than either is to the intermediately located TE

population. Population genetic structure where the two more distantly located reference populations are more similar to each other than either is to the TE population is indicative of selection unique to the TE population (Williams & Oleksiak 2008). While we attribute this non-neutral divergence between TE and both reference populations to the temperature changes near the thermal effluents, other environmental or ecological factors could also be important.

We evaluate the neutral assumption of population genetic structure using two approaches: first, a model-based Bayesian approach (STRUCTURE) (Falush *et al.* 2003) and second, a discrimination analysis (DAPC) (Jombart *et al.* 2010) that maximizes the weighting of allele frequencies among principal components to discover differences among populations. These approaches use different SNP datasets. We utilize STRUCTURE to examine population genetic structure using the loci identified as outliers in any one of the pairwise comparisons within a triad while we use the full 5.4K SNP dataset with DAPC to characterize the differences among populations described by orthogonal principal components.

For the Oyster Creek triad there are two genetic clusters inferred by STRUCTURE. Individuals from the two reference populations primarily derive ancestry from one cluster, while the TE population is dominated by a second cluster. This pattern is consistent across the range of ancestral genetic clusters that we model and leads us to reject the neutral hypothesis for genetic variation among the most differentiated loci. The DAPC analyses support this conclusion. The second principal component in DAPC using all 5.4K SNPs indicates a non-neutral pattern where the TE population is distinct from both reference populations with little distinction between the two reference populations.

This divergence in the TE population in the second principal component is different from the primary axis of genetic variation that follows a neutral pattern, where the position along the genetic axis of a population correlates with its geographic distribution. These patterns among all 5.4K SNPS are expected; we expect a mosaic of historic evolutionary forces to drive the differences observed among 5.4K SNPs randomly sampled along the genome, with older, neutral forces shaping the majority of variation.

In the Brayton Point triad, populations are more strongly differentiated (F_{ST} values ~ 0.03), and STRUCTURE analysis using the most differentiated loci suggests that each population is unique. When we examine all loci for the Brayton Point triad using DAPC, however, we find a similar pattern as in the Oyster Creek triad. The major axis of genetic variation separates each of the populations in a pattern consistent with its geographic position, but the two reference populations are more similar to each other along the second major axis of genetic variation than either is to the TE population. In both the Oyster Creek triad and Brayton Point triad, the second largest component of genetic variation among the populations occurs in a pattern that is not consistent with neutral evolution. Therefore we interpret the DAPC results as evidence of selection in the TE populations.

Critical thermal maxima

Although the three populations within a triad demonstrate little genetic divergence, critical thermal maximum (CT_{max}) of individuals from the effluent impacted habitat was significantly higher than that of individuals from the northern reference population. These data suggest that selection due to thermal effluents has led to an adaptive increase in thermal tolerance. Compensatory variation in CT_{max} is observed in *F. heteroclitus* populations separated by 1,000s of kilometers (New Hampshire and Georgia populations,

~0.6°C change in thermal tolerance) when individuals are acclimated to similar temperatures as those used in our analysis (Fangue *et al.* 2006). Thus, our results are best interpreted with a little caution because our nearby southern *F. heteroclitus* population is expected to be marginally more thermally tolerant because is it a few kilometers south of the reference population. Additionally, our analysis cannot rule out irreversible thermal acclimation or developmental plasticity. However, it seems unlikely that clinal adaptation would lead to the observed variation between TE and the northern reference population, which is only separated by approximately 30 Km, when the magnitude of this difference is approximately one third of the difference among populations separated by thousands of kilometers . Instead, we suggest that the Oyster Creek population CT_{max} difference is more likely due to thermal effluent.

Signature of Recent Selection in Response to Thermal Effluents

Adaptive outlier loci identification

Separating true signals of directional selection from the extreme tails of neutral variation is a persistent challenge associated with genomic outlier scans (Teshima *et al.* 2006). To more conservatively identify adaptive divergence in TE populations, we again utilize the triad sampling design (Fig. 4.1). In our analysis we define adaptive outlier loci as those that are significant outliers in both TE population *versus* reference population comparisons but are not outliers between the reference populations. Our definition of adaptive outlier loci then combines the typical F_{ST}-based outlier approach with additional requirements. For the Oyster Creek triad, there were 626 outlier loci, with 94 identified as adaptive (Fig. 4.4). For the Brayton Point triad, there were 622 outlier loci, with 36 identified as adaptive (Fig. 4.4).

Outlier scans suffer from both Type I and II error to varying extents depending on the demographic history of the populations in question. In particular, departures from the island model of migration such as spatial autocorrelation of allele frequencies due to isolation by distance (IBD) or expansion from refugia can lead to high false positive rates (Lotterhos & Whitlock 2014; Meirmans 2012). We do not observe significant IBD within a triad although there is significant IBD across all six populations (Fig. 4.3). This observation suggests that SNPs are providing measures of genetic distance due to the well-established genetic cline in *F. heteroclitus* (47), and that this clinal variation in allele frequencies is not significantly large within a triad. The mean F_{ST} value among the three populations within a triad using all 5.4K SNPs is small but significant according to AMOVA ($F_{ST} \sim 0.01$ for the Oyster Creek triad, and 0.03 for the Brayton Point triad). Thus, these data demonstrate that populations within a triad are not well isolated and have sufficient migration that would strongly inhibit genetic divergence due to drift.

To assess whether adaptive outlier loci have annotations that provide insights into the genes responsible for thermal adaptation, we conducted an enrichment analysis. Two observations bolster the conclusion that variation at the adaptive outlier loci may lead to increased thermal tolerance. First, adaptive outlier loci are enriched for several functional clusters of genes that are canonically associated with thermal adaptation. For example, immunoglobulin, apoptosis, and plasma membrane structure genes are consistently observed as thermal adaptation targets in fish (Hochachka & Somero 2001; Podrabsky & Somero 2004) and are enriched among the adaptive outlier loci. Second, there is some evidence of adaptive convergence in gene functions enriched in both TE populations. Adaptive outlier loci from both triads demonstrate non-significant enrichment for genes

associated with synaptic transmission and neuronal morphogenesis. Interestingly, these functions have typically not been implicated in fish thermal adaptation and highlight the advantage of taking a functionally agnostic approach to investigating thermal adaptation.

The lack of significant IBD within a triad, small divergence among populations (as estimated with genome-wide F_{ST} values), enrichment of gene functions canonically associated with thermal adaptation and the additional requirements imposed in the triad design reduces the probability of spurious outliers in our identification of loci undergoing adaptive divergence. Furthermore, these data are based on sufficient sequencing depth to accurately call heterozygotes at most SNPs for most individuals. This sequencing depth, combined with the requirement for 1% minor allele frequency across all 239 individuals and filtering of loci with excess observed heterozygosity, indicates that SNPs are unlikely to be sequencing, genotyping or alignment errors (Hosking *et al.* 2004).

Next, we consider variation patterns at and around these adaptive outlier loci to examine the genomic signature of recent selection due to thermal effluents and assess the roles of hard and soft sweeps as well as polygenic adaptation. In particular we address three questions (i) are adaptive shifts in allele frequency between reference and effluent-effected populations large or small, (ii) are adaptive alleles common or rare in the standing genetic variation, and (iii) are adaptive outlier loci embedded in extended genomic regions of elevated F_{ST} values consistent with the model of a hard selective sweep of a single adaptive haplotype?

Allele Frequencies at Adaptive Loci Vary Subtly From the Standing Genetic Variation

In the classical paradigm, adaptation proceeds through selective sweeps that drive an advantageous allele from low to very high frequency. However, evidence from

quantitative genetics (Falconer & Mackay 1996), population genetics (Coop *et al.* 2009; Hancock *et al.* 2010b) and the GWAS program of research (Manolio *et al.* 2009) suggests that subtle allele frequency shifts across many variants (polygenic adaptation) also play an important role in evolution. Our findings support this view in the case of recent selection in large natural populations.

Owing to the low degree of differentiation among populations within a triad, allele frequencies of the reference and TE populations are highly correlated at most loci. Indeed, among the 5.4K SNPs in this dataset, allele frequencies at ~90% of loci differ between these populations by less than 10%. While F_{ST} based outlier analyses perform well with respect to type I error when the underlying population genetic structure is properly accounted for, they are not as successful with respect to type II error (Lotterhos & Whitlock 2014; Narum & Hess 2011) and are generally biased towards finding large changes in allele frequencies (Hancock *et al.* 2010a). Therefore, we expect that loci identified as adaptive outliers in our F_{ST} based approach should be among the small portion of loci where allele frequency differences between reference and TE population are large. However, for the data presented here, adaptive outlier loci demonstrate only small allele frequency shifts between reference and TE populations (Fig. 6).

This finding is contrary to the expected results of a hard selective sweep, yet there is considerable evidence of adaptation to thermal effluent environments in both the population genetic structure and critical thermal maxima. These observations can be reconciled by one of three scenarios. First, natural selection has acted on alleles segregating at appreciable frequency in the standing genetic variation at the onset of selection. Second, adaptive outlier loci are in linkage disequilibrium with, but far from,

those loci actually driving selection, leading to patterns of polymorphism consistent with soft sweeps (Schrider *et al.* 2015). Third, gene flow from populations under selection has driven alleles to high frequency in nearby populations where they are in migration-drift equilibrium. We address the first scenario in detail below (subsection *Soft polygenic adaptation*). The second scenario is not supported by the data: we find little evidence of linkage disequilibrium (as assessed by correlation of F_{ST} values at nearby SNPs) at distances greater than the distance covered by the sequence tags used to generate our SNPs (Fig. 4.7). The third scenario suggests that the allele in the TE population was fixed due to natural selection, migrating into the reference populations and altering the neutral frequencies. This scenario cannot account for alleles that are fixed in the reference populations but present at lower frequencies in the TE populations (*i.e.,* directional selection in favor of the minor allele). Furthermore, in the minority of cases where the minor allele is putatively advantageous in the TE environment (minor allele has higher frequency in the TE population among 33% of adaptive outlier loci), it is rarely a private allele among all six populations in both triads, and is therefore likely available in the standing genetic variation given the only moderate genetic distance between populations in different triads. We conclude that at 98% of adaptive outlier loci, the adaptive allele is likely present in the standing genetic variation, either because it is the major allele overall or it is present at an appreciable frequency in relatively nearby populations.

No Extended Blocks of Elevated F_{ST} around Adaptive Outliers

In addition to investigating the allele frequency shifts at loci identified as adaptive outliers, we also considered the genomic regions near these loci. In our GBS methodology, all SNPs in our dataset occur within 64 base sequence tags, and we have high levels of coverage within about twice this distance at many of our adaptive outlier

loci. We expected that if variation at adaptive outlier loci is due to a hard selective sweep, adaptive outliers would lie in large regions with elevated F_{ST} values due to the fixation of the single haplotype bearing the adaptive allele (Kaplan *et al.* 1989). Our data does not fit this pattern: the average region surrounding an adaptive outlier where the F_{ST} value exceeds the genome-wide average extends only 6-12 base pairs. We do not have an example of an elevated F_{ST} value region due to a hard sweep in *F. heteroclitus* to compare this to, but observations in other species suggest that the LD distance we find is orders of magnitude shorter than any predicted unit of adaptive hitchhiking due a recent hard selective sweep. For the frequently invoked example of a hard sweep involving the Duffy locus, the advantageous SNP lies in an elevated F_{ST} value region that extends at least 10 kb in adapted populations (Hamblin *et al.* 2002). An empirical estimate of the average unit of adaptive hitchhiking in humans is 20 kb, where F_{ST} values are highly significantly correlated (Akey *et al.* 2002). Furthermore, genome scans that rely on a moving average of F_{ST} values commonly utilize windows of 100 kb or more (*e.g.,* (Hohenlohe *et al.* 2010)). We find no extended blocks of high linkage disequilibrium surrounding adaptive alleles due to adaptive hitchhiking that would lead to large regions with elevated signals of population differentiation. Rather, adaptive alleles occur in regions with polymorphism similar to genome-wide averages, suggesting that selection has acted on adaptive alleles within genomic regions that coalesce well before the onset of selection.

Soft, polygenic selection

In a soft sweep, multiple haplotypes bearing the adaptive alleles rise to high frequency at a locus under selection. Under polygenic adaptation, selection drives many subtle shifts in allele frequency until a new phenotypic optimum is reached (Pritchard *et al.* 2010).

Our data strongly implicate a model of adaptive evolution where selection has acted on previously segregating mutations to produce small changes in allele frequency at many loci: adaptive alleles are present at appreciable frequency in nearby populations, adaptive variation in allele frequency is small, and there are only very short regions of elevated F_{ST} values surrounding these alleles. Therefore, recent thermal adaptation in these populations is both soft and polygenic in that adaptive alleles are borne on multiple haplotypes that coalesce before the onset of selection, but the adaptive alleles themselves are not swept to high frequency.

While the parameter space where soft sweeps become feasible may be narrow (Jensen 2014), there are a number of reasons to expect that adaptation to the Oyster Creek and Brayton Point thermal effluents should produce the soft, polygenic adaptation from standing genetic variation that we observe in the 5.4K SNP dataset. First, selection due to thermal effluents has occurred for approximately 50 generations, and adaptive polymorphisms are more likely to be derived from the standing genetic variation when selection is quite recent because there has been insufficient time for new mutations to arise (Barrett & Schluter 2008; Hermisson & Pennings 2005). Soft evolution is also more likely to occur where the effective population size is large (Pennings & Hermisson 2006). Estimates of *Fundulus heteroclitus* effective population sizes are large, ranging from $2X10^4$ (Powers & Place 1978) to $3X10^5$ (Adams *et al.* 2006). Effective population size in TE populations should be similar to these estimates because we do not find reduced genetic diversity relative to the overall diversity. Finally, soft evolution is only likely where the adaptive allele is nearly neutral and present at an appreciable frequency before the onset of selection (Przeworski *et al.* 2005). Most of our SNPs identified as adaptive in

this analysis are the major allele overall in all six populations, and those that are not occur at moderate frequency in moderately distant populations. Finally, unlike other cases of rapid evolution that are predicted to produce local hard sweeps because of the limited mutational target size (Jensen 2014; Menozzi *et al.* 2004), the mutational target size of thermal adaptation is quite large. Consequently, the effective value of $\theta = 2N_e\mu_a$ (where is adaptive mutation rate) and therefore the probability of adaptation from the standing genetic variation, is increased in the case of thermal adaptation (Wilson *et al.* 2014).

It is important to also emphasize that our data do not preclude the possibility of hard sweeps as the final outcome of selection in these populations due to thermal effluents. First, absence of evidence is not necessarily evidence of absence. We surveyed the genetic variation at a small subset of the total polymorphisms present and cannot refute other patterns of variation at loci that our dataset does not query. Second, it is possible we do not observe a sweep, hard or soft, because selection in these populations is ongoing and there has been insufficient time for the fixation of adaptive alleles (Fitzpatrick *et al.* 2012). However, for the 5.4K SNPs in our dataset, we find both functional and population genetic evidence of adaptation and a genomic selection signature that indicates soft, polygenic adaptation.

Conclusion

We combined a population sampling regime that allows us to identify potentially adaptive variation with an analysis of population genetic structure and FST-based outlier scans. We used this approach to assess the hypothesis that populations exposed to thermal effluents near coastal power stations have undergone thermal adaptation and then examined the genomic signature of recent selection to investigate the evolutionary history

of adaptive alleles. We conclude that fish living near thermal effluents have rapidly evolved from the standing genetic variation through small allele frequency changes at many loci in a pattern consistent with a soft, polygenic model of evolution. Overall, we suggest that evolution through these mechanisms may be a common feature early in the adaptive process in large, outbred, natural populations exposed to environmental changes with broad physiological impacts. However, the reliance on genome scans for the signature of hard sweeps may limit our ability to discover the genetic variation that underlies adaptive evolution. Therefore, future studies should rely on additional, complementary approaches such as environmental association analysis as well as trait-centered studies to discern the extent to which adaptation in natural populations is driven by rare, novel variants or the standing genetic variation.

Tables and Figures

	N	*RB*	***OC***	*Mg*	*SR*	***BP***	*HB*
RB	***40***	**0.1518**	0.0095	0.0081	0.1089	0.1256	0.1107
OC	***36***	0.1198	**0.0973**	0.0104	0.107	0.1261	0.1095
Mg	***37***	0.1483	0.1184	**0.1479**	0.0891	0.1063	0.0908
SR	***41***	0.1402	0.1111	0.1353	**0.1055**	0.0252	0.0318
BP	***47***	0.1418	0.1125	0.1367	0.1048	**0.1026**	0.0272
HB	***38***	0.138	0.1093	0.1331	0.1048	0.1028	**0.1015**

Table 4.1: Pairwise estimates of nucleotide diversity and population differentiation among experimental populations. Values above the diagonal are mean pairwise F_{ST} values across all 5.4k SNPs. Values along the diagonal (bold) are mean nucleotide diversity (π) within populations. Values below the diagonal are mean proportion of pairwise differences (π). Population abbreviations: Oyster Creek Triad (Southern Reference – Rutgers Basin (RB), TE Population – Oyster Creek Generating Station (OC), Northern Reference – Mantoloking, New Jersey (Mg)) Brayton Point Triad (Southern Reference – Succotash Marsh, Matunuck, Rhode Island (SR), TE Population – Brayton Point Generating Station (BP), Northern Reference – Horseneck Beach, Massachusetts (HB)).

Variance Component	df	% variation	Φ Statistic	P
Among triads	1	9.59	Φ_{CT} = 0.09593	p = 0.099
Among populations within triads	4	0.69	Φ_{SC} =0.00762*	p < 0.00001
Among individuals within populations	472	89.72	Φ_{ST} =0.10282*	p < 0.00001
Total	477			

Table 4.2: AMOVA Results

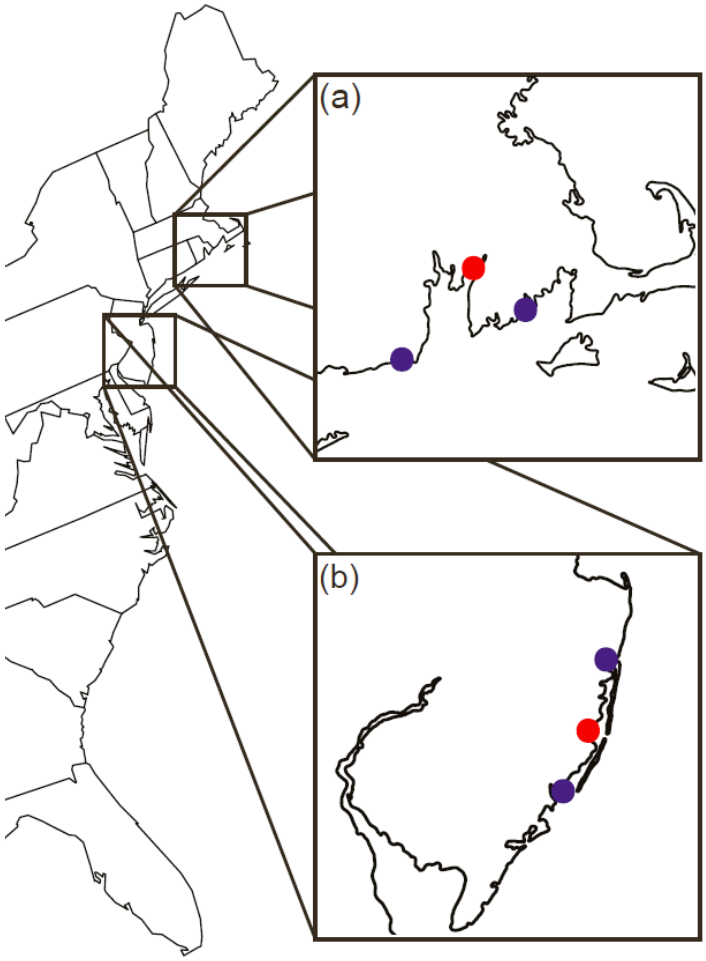


Fig. 4.1: Sampling locations and triad design.
Each thermal effluent population (red markers) is surrounded by two reference populations (blue markers). The northern triad (a) is the Brayton Point generating station population and its references. The southern triad (b) consists of the Oyster Creek nuclear generating station population and its references.

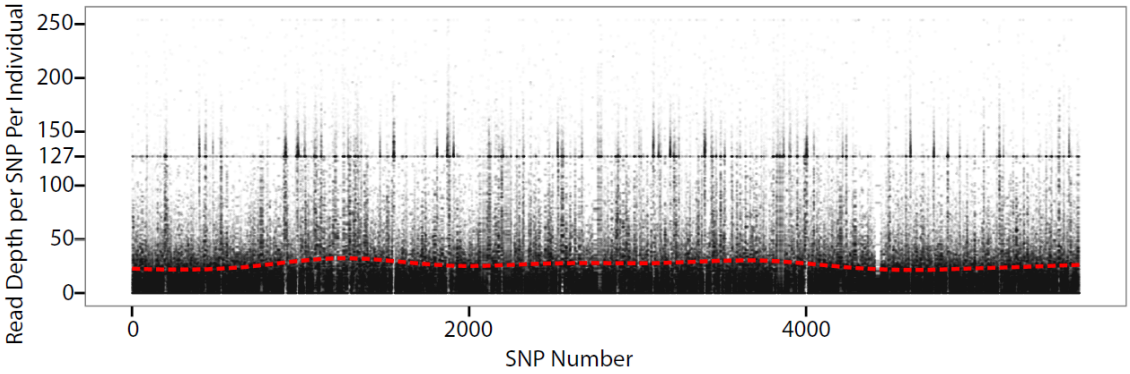


Fig 4.2: Depth of read per individual at each SNP in the 5.4k SNP dataset.
Mean depth across individuals represented by red line.

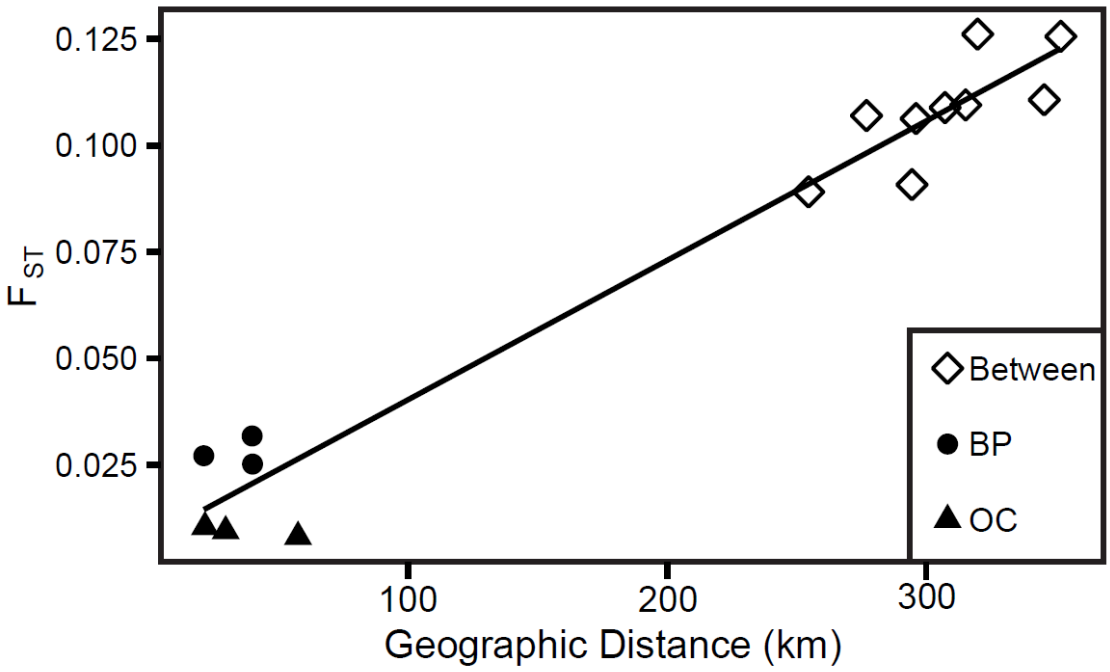


Fig 4.3: Isolation by distance.
Geographic distance along the coast *vs*. genetic distance as estimated by mean genome-wide F_{ST} value for comparisons within the Brayton Point (BP, filled circles) and Oyster Creek (OC, filled triangles) triads and comparisons between triads (open diamonds). There is significant isolation by distance for all comparisons ($p < 0.01$, Mantel test, 9999 simulations), but not within triads ($p > 0.5$, Mantel test, 9999 simulations).

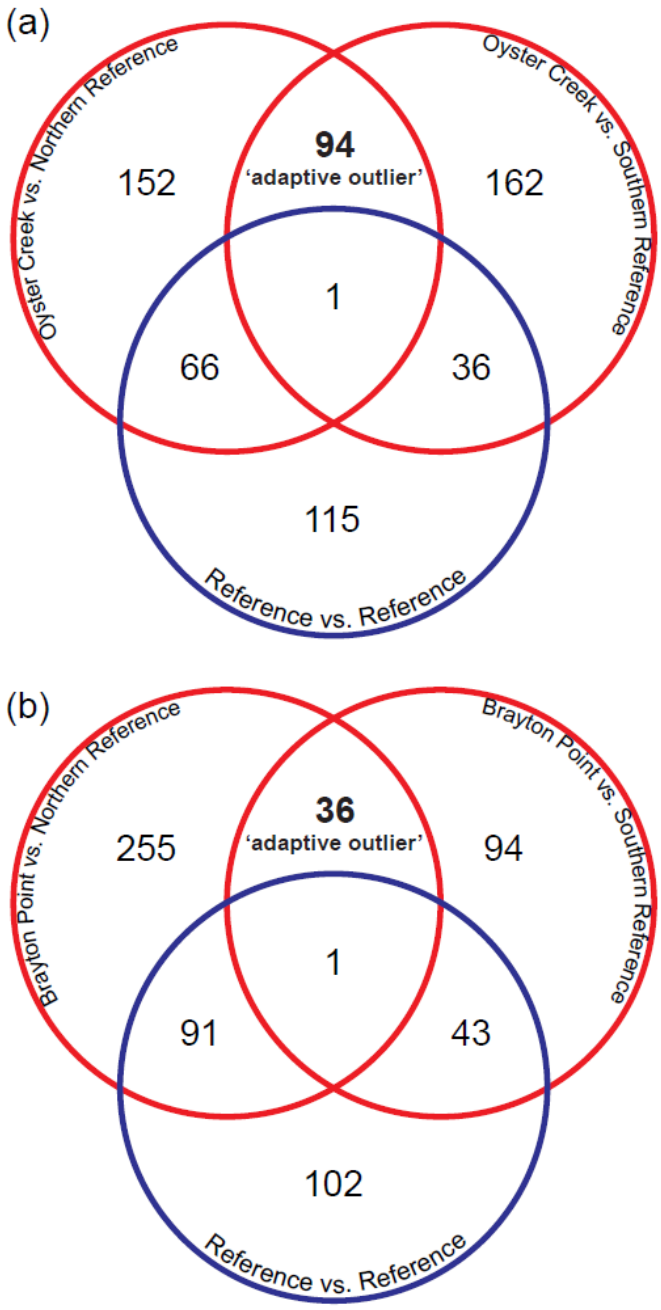


Fig. 4.4: Adaptive Outliers
Number of significant outliers in pairwise comparisons of thermal effluent (TE) populations *vs.* reference populations (red circles) and pairwise comparisons of reference *vs.* reference populations (blue circles) for the Brayton Point (BP) and the Oyster Creek (OC) triads. Adaptive outlier loci for each triad ('adaptive outlier') are those that are significant outliers in both TE *vs.* reference comparisons (red circles), but not in the reference *vs*. reference comparison (blue).

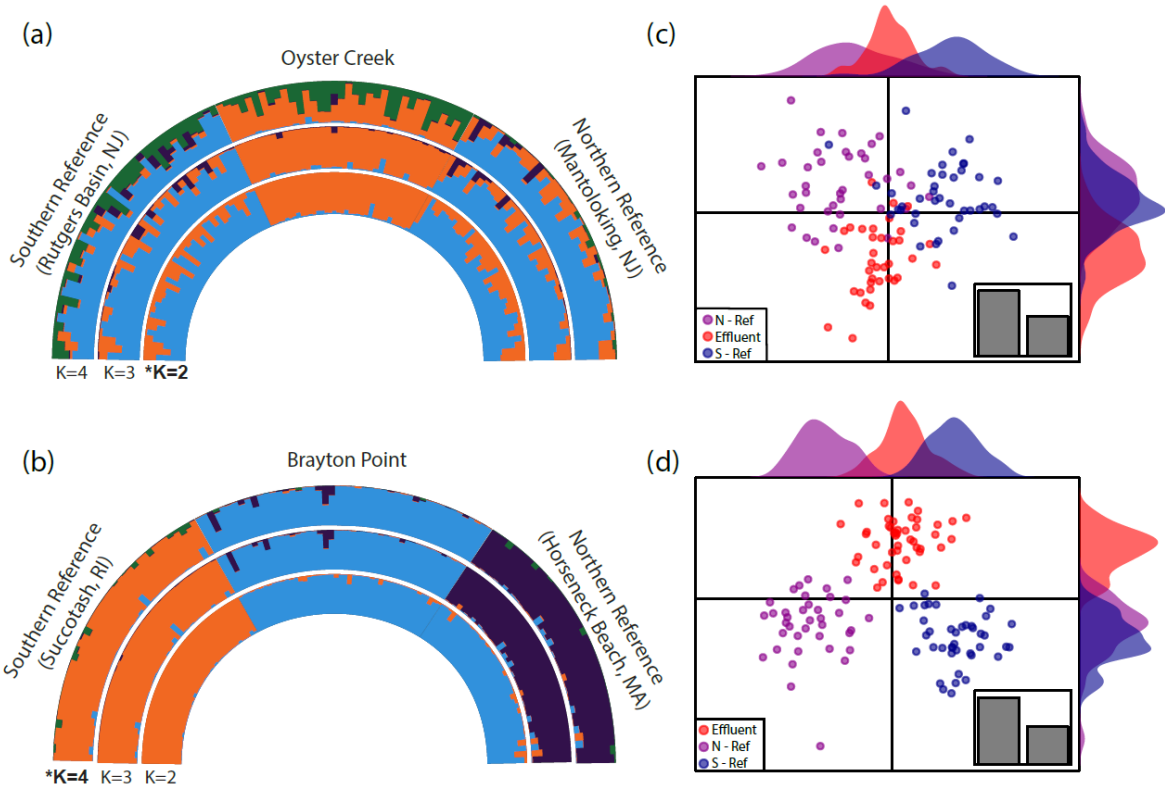


Fig. 4.5: Population genetic structure within triads.

STRUCTURE plots for Oyster Creek (a) and Brayton Point (b) triads. Each individual is represented with a radial line that is partitioned into colors according to modeled admixture proportions for *k* ancestral populations. Results for $k = 2 – 4$ are presented with best *k* denoted by an asterisk. DAPC plots for Oyster Creek (c) and Brayton Point (d) triads. Each individual's position along the first two principal components (discriminant functions) is shown with a point, with populations identified by color. The relative eigenvalues of the first (horizontal) and second (vertical) principal components are shown in the bar plot at the bottom right of each figure.

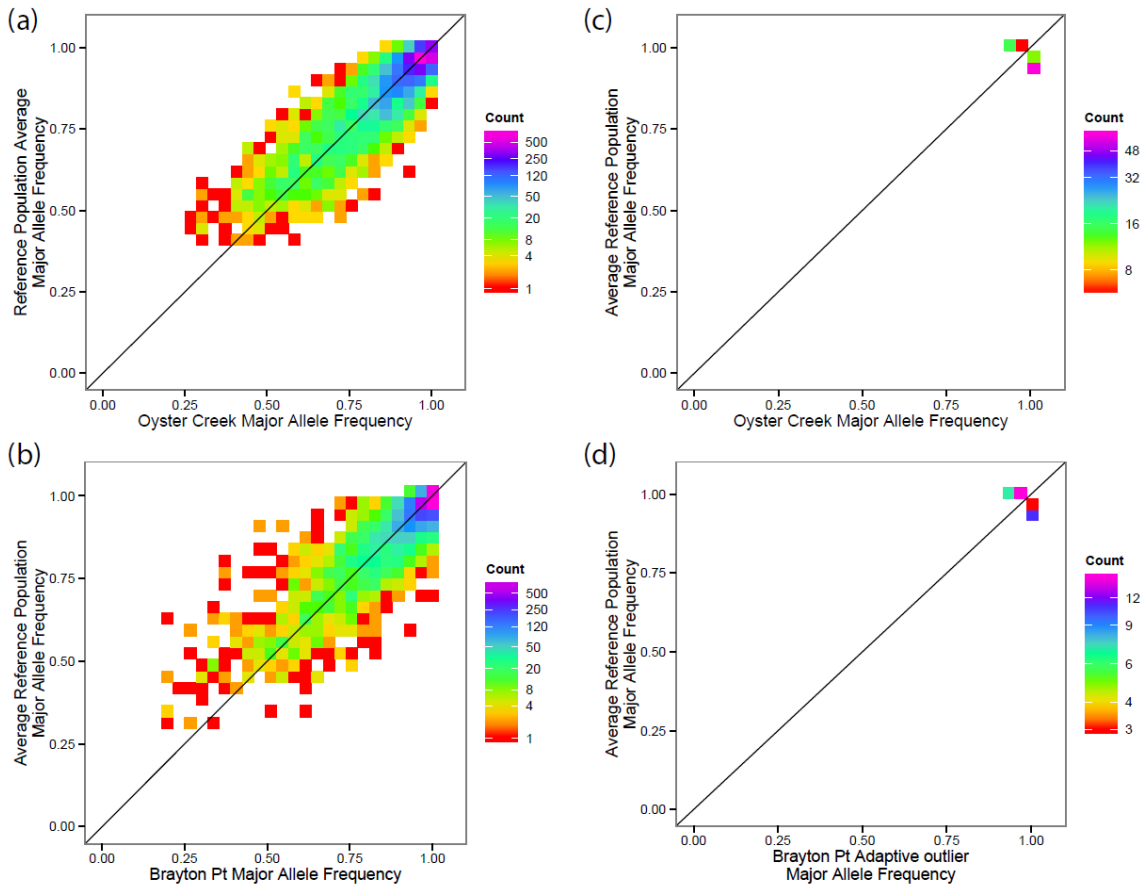


Fig. 4.6: Allele Frequency Change Histograms
Allele frequency of the global major allele (of all 6 populations) in TE populations *vs.* the mean of both reference populations for all 5.4k SNPs for the Oyster Creek triad (a) and the Brayton Point triad (b). The same for only the 94 adaptive outlier loci in Oyster Creek (c) and the 36 adaptive outlier loci in Brayton Point (d).

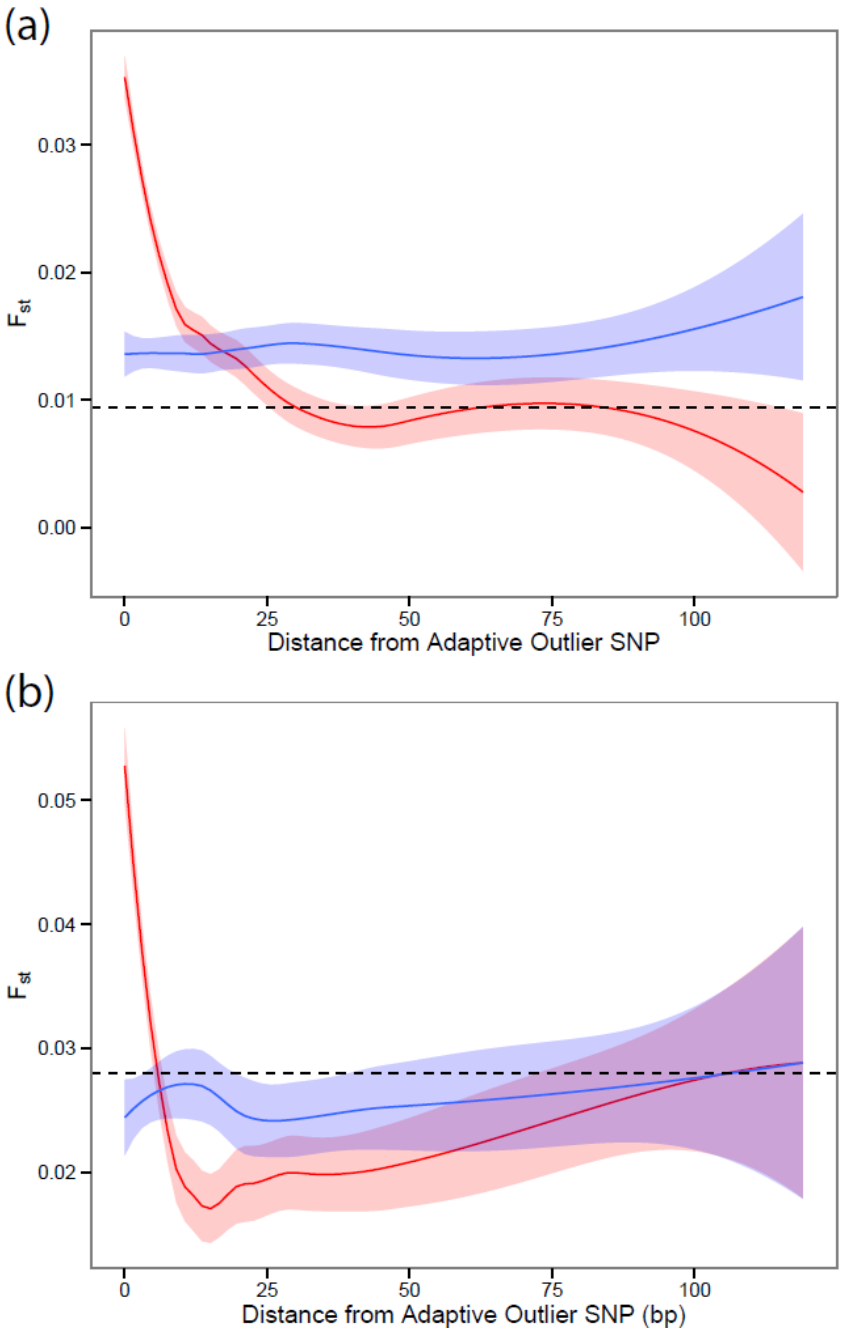


Fig. 4.7: F_{ST} values near adaptive SNPs. Mean F_{ST} values at SNPs physically near adaptive outlier loci for the Oyster Creek triad (a) and Brayton Point triad (b). Distance is presented in base pairs from adaptive outlier locus *vs.* Loess-smoothed mean F_{ST} value with 95% confidence intervals for all pairwise comparisons within a triad (red), randomly permutated data (blue) and the mean genome-wide F_{ST} value estimate (dashed black line) within a triad.

Chapter 5: Polygenic adaptation along the *F. heteroclitus* cline

Background

Identifying the genetic variation that underlies local adaptation is a key component of understanding evolution by natural selection. This goal is often accomplished by identifying genomic regions that significantly deviate from genome-wide patterns in population genetic parameters. For example, F_{ST}-based outlier approaches identify genomic regions where populations demonstrate unexpected levels of genetic differentiation that may be due to selection. Yet, such genome scans methods exhibit significant limitations. F_{ST} based approaches may demonstrate high false positive rates because of the confounding influence of neutral genetic variation that accumulates through demography (Charlesworth *et al.* 2003; Lotterhos & Whitlock 2014). Alternative genome scans methods are less sensitive to population structure (Pavlidis *et al.* 2010) but specifically search for genomic regions that may have been subject to a hard selective sweep (Nielsen *et al.* 2005), where a single new mutation that arises after the onset of selection is driven to high frequency leading to reduction in linked neutral variation (Przeworski *et al.* 2005; Smith & Haigh 1974). By focusing on genomic regions bearing the signature of hard selective sweeps or on demographic scenarios where we can effectively utilize F_{ST}-based approaches, we may be ignoring a significant portion of adaptive genetic variation in natural populations. Specifically, genome scans will have reduced ability to discover adaptive variation due to soft selective sweeps, where the origin of adaptive alleles predates the onset of selection (Hermisson & Pennings 2005; Messer & Petrov 2013), adaptive variation that arises through subtle shifts in allele frequency at many loci of small effect, *i.e.,* polygenic adaptation (Hancock *et al.* 2010a; Le Corre & Kremer 2012; Pritchard & Di Rienzo 2010), and adaptive variation within

species with confounding demographies (Lotterhos & Whitlock 2014). These weaknesses are especially relevant given increasing evidence that hard sweeps may be rare in nature (Cao *et al.* 2011; Hernandez *et al.* 2011; Pritchard *et al.* 2010).

Environmental association analyses (EAAs) provide an alternative to genome scans. EAAs associate environmental parameters with shifts in allele frequencies, but provide the opportunity to conduct the association in a statistical framework that accounts for demography as a random effect (Rellstab *et al.* 2015). The extent to which this is successful depends on the spatial orientation of the environmental gradient with respect to the orientation of genetic variation, demography, and sampling schemes (de Villemereuil *et al.* 2014; Frichot *et al.* 2015; Lotterhos & Whitlock 2015; Mita *et al.* 2013). EAAs demonstrate increased power to identify adaptive genetic variation due to polygenic selection or soft selective sweeps relative to genome scan methods (Berg & Coop 2014; Coop *et al.* 2010; Günther & Coop 2013). Therefore EAAs complement genome scan approaches by expanding the body of adaptive genetic variation discernable to investigators.

To identify adaptive genetic variation with this approach, we used SNP data collected across the species range of the small estuarine fish *Fundulus heteroclitus*. *F. heteroclitus* inhabit coastal salt marshes from Florida to Newfoundland. The *F. heteroclitus* range coincides with a steep thermal cline where mean water temperatures decreases about 1 °C per degree latitude, such that the southernmost populations experience mean annual temperatures ~13°C warmer than their northern counterparts. Adult habitat temperature reflects lifetime exposure because development occurs from demersal eggs without a pelagic larval stage, and there is limited dispersal among adults

(Brown & Chapman 1991; Sweeney *et al.* 1998; Teo & Able 2003). This distribution and life history has made *F. heteroclitus* species range a useful system for investigating genetic clines, especially clinal adaptation to temperature (Burnett *et al.* 2007). Analysis of both neutral and adaptive patterns of genetic variation across the species range suggest that secondary recontact of northern and southern refugia populations since the last glacial maximum has led to substantial phylogeographical structuring of extant populations (Adams *et al.* 2006; Duvernell *et al.* 2008; Gonzalez-Vilasenor & Powers 1990). Therefore *F. heteroclitus* populations are separated into two distinct clades: a northern cold-adapted clade and a southern warm-adapted clade.

There are several barriers to parsing neutral from adaptive genetic variation along the *F. heteroclitus* range. First, because the species is distributed along a thermal cline, much of the environmental heterogeneity which may drive directional selection is coincident with neutral variation due to both contemporary and historic restriction of gene flow among populations. Thus, there is a high degree of expected covariance between neutral and adaptive genetic structure. Second, while migration-drift non-equilibrium conditions observed even on small spatial scales in this species (Duvernell *et al.* 2008) may permit local adaptation (Slatkin 1994), the particular demographic history of *F. heteroclitus* is likely to produce spurious results among allele frequency (F_{ST}) outlier based genome scans used to identify adaptive genetic variation (see refugia models of (Lotterhos & Whitlock 2014; Lotterhos & Whitlock 2015)). Indeed, a simulation study of the allele frequencies due only to the secondary recontact between northern and southern *F. heteroclitus* clades produced clinal patterns of genetic variation highly similar to empirically derived allele frequencies at 42% of simulated loci (Strand

et al. 2012a). Perhaps most problematically, these simulations predict fixation of alternative alleles with little to no intergradation for some loci. F_{ST} outlier approaches will suffer from a high false discovery rate in this case, where all genetic variation at a locus is distributed among, rather than within, populations and this variation is due to demography rather than selection.

In this investigation we reconsider the neutral population genetic structure of the *Fundulus heteroclitus* species range using ~9 thousand genotyping-by-sequencing derived genetic markers; then we conduct an EAA that accounts for this population genetic structure to identify loci that may underlie *F. heteroclitus* thermal adaptation.

Methods

Environmental data and populations

F. heteroclitus populations were sampled at 16 locations along the species range spanning 2,123 km along the North American Atlantic coast (table 5.1, fig. 5.1). Fieldwork was completed within publically available lands and no permission was required for access. *F. heteroclitus* does not have endangered or protected status, and collecting permits are not required for non-commercial purposes at the sampling locations. All fish were captured in minnow traps and removed within 1 hour.

We extracted 10 years of daily sea surface temperatures on a 0.25° grid from NOAA High Resolution SST data provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, USA (Reynolds *et al.* 2007) using custom R scripts (available at lukemiller.org). Three derived temperature variables were calculated for each of the sampling locations from these data: mean temperature, mean annual minimum, and mean annual maximum. These data were centered, scaled and then summarized with principal

component analysis using the *PRCOMP* function in R and analyzed using biplots (Legendre & Legendre 2012).

Genotyping

Caudal fin clips approximately 5 mm^2 in size were taken from individuals in the field and stored in 270 ul of Chaos buffer (4.5M guanadinium thiocynate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCl pH 7.5, 0.2% antifoam, 0.1M β-mercaptoethanol). IACUC approved procedures were used for non-surgical tissue sampling. These samples were stored at 4°C prior to processing. Genomic DNA was isolated from fin clips using a silica column (Ivanova et al. 2006). DNA quality was assessed via gel electrophoresis and concentrations were quantified in triplicate using Biotium AccuBlueTM Broad Range dsDNA Quantitative Solution according to manufacturer's instructions. 100 ng of DNA from each sample was dried down in 96-well plates. Samples were then hydrated overnight with 5 ul of water before restriction enzyme digestion and further processing.

GBS was performed as described (Elshire et al. 2011), using the restriction enzyme AseI, adaptors (0.4 pmol/sample) and 50ng of gDNA per sample. GBS libraries were sequenced on two lanes of an Illumina HiSeq 2500 with a 100 bp single end read (Elim Biopharmaceuticals, Inc.).

The reference genome-based GBS analysis pipeline, TASSEL 4.0 (Bradbury et al. 2007) was used to call SNPs using the *Fundulus heteroclitus* genome (genome Citation); SNPs were identified using the "Discovery Build." We largely used default settings throughout the pipeline with the following exceptions: a minimum of 5 counts were required for retention of individual tags during the merge multiple tag count fork, and tag

alignment to the reference genome was accomplished with bowtie2 using the very-sensitive-local setting.

The initial SNP dataset produced by TASSEL-GBS was filtered to remove loci and individuals with low coverage. The resultant high coverage dataset was additionally filtered for polymorphisms that may result from sequencing error or alignment of paralogous loci: loci with low minor allele frequency (<1%) were removed in the TASSEL GUI; then loci with significant departures from Hardy-Weinberg equilibrium (greater observed than expected heterozygosity) were removed ($p < 0.01$). Hardy-Weinberg equilibrium was calculated for individual loci using Arlequin v3.5.1.2 (Excoffier et al. 2005) using 1,000,000 steps in the Markov chain with 100,000 dememorization steps.

Population Genomics

Pairwise genetic distance between populations (F_{ST}) was estimated as Weir and Cochran's θ in the R package diveRsity (Keenan *et al.* 2013). These genetic distances were used with pairwise geographic distances to investigate isolation by distance (IBD). To estimate geographic distance among populations, we used the R package marmap (Pante & Simon-Bouhet 2013). As *F. heteroclitus* are estuarine fish with limited spatial dispersal (Brown & Chapman 1991), we calculated the shortest pairwise distances between sampling locations constrained by the 20m isocline. This depth was chosen based on evidence that the mouth of the Chesapeake Bay (greatest depth ~20m) does not significantly reduce gene flow between neighboring populations, while the mouth of the Delaware Bay does (greatest depth ~40m) (Duvernell *et al.* 2008). IBD was assessed using Mantel tests (10,000 simulations) for all pairwise comparisons and within both the northern and southern clades. To test if patterns of IBD varied across these comparisons,

we used stepwise model comparison of linear models that included varying slopes and intercepts for the comparisons within and among clades.

Arlequin v3.5.1.2 (Excoffier *et al.* 2005) was used to calculate within population heterozygosity and conduct analysis of molecular variance (AMOVA). AMOVA utilized 10,000 permutations and genetic distances estimated from the SNP dataset according to (Tajima & Nei 1984). Mean population-wide θ ($=4N_e\mu$), as well as per locus estimates of nucleotide diversity (π and θ_w) and Tajima's D were calculated in Tassel 4.0. Average nucleotide diversity for all SNPs within 10kbp was calculated from these data for candidate SNPs identified in the EAA and for 500 randomly sampled SNPs. Comparisons were also made against a control SNP set that matched the candidate loci in the ratio of genic to non-genic SNPs. This control SNP set was created by identifying all SNPs as genic or non-genic according to current *F. heteroclitus* genome identification and then randomly sampling genic and non-genic SNPs at the same ratio identified in the candidate SNP list.

Population genetic structure inference was made using STRUCTURE (Falush *et al.* 2003). For all *k* values, both F_{ST} and α converged between approximately 400 and 1,000 iterations. Given the size of the dataset and the rapid convergence of these parameters, we prioritized replicate runs at each *k* value over a large burnin or number of MCMC steps. Therefore we used a burn in of 1,500k steps and Monte Carlo Markov chain (MKMC) of 6,000 steps with at 12 replicates for each *k* value and varied *k* from 1 to 10. Replicate individual *k* runs were merged and the ΔK method for identifying the optimal number of clusters (Evanno *et al.* 2005) was calculated using CLUMPAK (Kopelman *et al.* 2015). The optimal number of clusters was also assessed using the

SNMF function of the R package LEA. We calculated average cross entropy for 10 replicate runs of SNMF at each value of *k* from 1 to 16.

Environmental Association Analysis

EAA was completed with a latent factor mixed model (Frichot *et al.* 2013) as implemented in the R package LEA (Frichot & François 2015). To find candidate loci driven by thermal adaptation we identified SNPs in the 9k SNP dataset that were associated with the first principal component of temperature summary variables calculated for the 16 sampling locations along the *F. heteroclitus* species range. We fit the latent factor mixed model at a range of putative ancestry clusters (*k*) with 10 replicate runs of 5,000 burnin steps and 10,000 dememorization steps. Association z-scores from replicate runs at each *k* were combined using the Stouffer method (Whitlock 2005), then adjusted using the genomic inflation factor (Devlin & Roeder 1999) and used to calculate raw p-values. These p-values were adjusted for multiple comparisons using B-H FDR (Benjamini & Hochberg 1995).

Results

Environmental Data

We extracted 10 years of daily optimum interpolated sea surface temperatures (OISSTs) from NOAA/OAR/ESRL PSD (Reynolds *et al.* 2007) for each of the sampling locations and calculated three derived environmental variables: mean temperature, mean annual minimum and mean annual maximum (table 5.1, fig. 5.1). The first principal component in a principal component analysis captured 90.1% of the variance among these variables, and all three variables were highly correlated as indicated by the biplot (supplementary fig. 5.1). While mean temperature and mean annual maximum demonstrate a linear relationship with latitude (R^2 = 0.87 and 0.97; slope = -0.97 and -

1.1°C per ° latitude, respectively), minimum temperature is constrained by the freezing temperature of seawater and plateaued beyond 39 degrees of latitude. The value of the first temperature principal component and position along the shore for the sampling locations are also strongly correlated ($R^2 = 0.94$, $p < 10^{-9}$ ANOVA).

Sequencing and Filtering

We collected sequencing data generated across several GBS libraries prepared for *F. heteroclitus* using the same restriction enzyme and combined them in a single SNP discovery pipeline. Briefly, genomic DNA (gDNA) was isolated from 1,446 individuals. These gDNA samples were digested and individually-barcoded in 10 unique reduced representation libraries for GBS (Elshire *et al.* 2011). The libraries were sequenced on 17 Illumina Hi-Seq lanes. We found 7,075,338 unique sequence tags that contained both the barcode and *AseI* cut site. Bowtie2 aligned 3,502,524 (49.50%) of these tags to unique loci in the *F. heteroclitus* genome. 2,977,712 (42.09%) tags aligned to multiple loci, and 595,102 (8.41%) had no alignment. The latter two tag sets were excluded from further analysis. We identified 624,902 SNPs among these tags. Genotypes were called using a binomial likelihood ratio based approach, as implemented in the TASSEL-GBS discovery pipeline (Glaubitz *et al.* 2014).

The 625k SNP dataset was subsequently filtered by minor allele frequency, Hardy-Weinberg equilibrium and coverage. We removed 143 poorly sequenced individuals with calls at less than 5% of the 625k SNPs. Next, we retained SNPs that were called in at least 65% of the remaining individuals with minor allele frequency greater than 1%, resulting in 9,307 retained SNPs in 1,304 individuals. To remove SNPs that may due to paralogous alignment or sequencing errors, we further filtered this dataset by Hardy-Weinberg equilibrium. Four hundred fifty eight SNPs with greater observed

than expected heterozygosity ($p < 0.01$) were excluded. Two populations had many individuals (table 5.2) so we randomly sampled 200 individuals from each. Finally, we removed 2 populations that may have undergone recent thermal adaptation, as evidenced by separate analyses (chapter 4). The final SNP dataset contained 8,819 loci across 956 individuals.

Genome-wide estimates of genetic diversity and divergence

We estimated expected heterozygosity (H_e) and mean θ ($= 4N_e\mu$) per base pair for each population (table 2, fig. 5.2). H_e ranged from 0.1046 to 0.1531. H_e was significantly increased among southern populations (t-test, $p < 0.05$) and was highest among the three populations near the zone of proposed secondary intergradation near 40°N latitude (fig. 5.2). θ was also increased in southern populations (figure 5.2, $p = 0.0016$, t-test).

We examined population differentiation using Weir and Cochran's θ (henceforth referred to as F_{ST}) for all pairwise comparisons among populations (table 5.2, fig. 5.3). F_{ST} ranged from 0.0031 to 0.2672. Using alongshore distances constrained by the 20m isocline (fig. 5.1), there was significant isolation by distance (IBD) across the species range using these genetic distance estimates (Mantel test, 100,000 repetitions, $p < 10^{-6}$) (fig. 5.3). There was also significant IBD within both the northern (Mantel test, 100,000 repetitions, $p = 0.00015$) and southern (Mantel test, 100,000 repetitions, $p = 3 \times 10^{-6}$) clades individually. The rate at which genetic distance increased with alongshore distance varied significantly among comparisons within the southern clade, within the northern clade and between clades (stepwise model selection $p < 10^{-13}$) (fig. 5.3).

An analysis of molecular variance (AMOVA) was conducted to examine the distribution of genetic variation within and among populations, as well between southern and northern clades (table 5.3). Most of the variation (90.1%) was distributed within

populations, but substantial portions of the genetic variation were distributed among northern and southern regions (6.4%) and among populations within regions (3.5%). All components of variation were significant at the 10^{-5} level (AMOVA, 10,000 permutations).

Population genetic structure

The optimal number of genetic clusters (*k*) according to STRUCTURE data was 2 (supplementary fig. 5.2). At this level, STRUCTURE clearly discriminates populations from north or south of the Hudson River (fig. 5.4). Admixture between these two genetic clusters is limited in most populations. The average membership in the minor ancestral cluster for all populations is 6%, but is higher for the three populations near the zone of putative secondary recontact along the New Jersey coast. For these three populations (SH, RB, MG), average membership in the minor ancestry cluster is increased: 14.6%, 12.3%, 22.5%, respectively. Populations in Long Island Sound also demonstrate elevated admixture (CT and SR). Membership in the minor ancestry cluster is 7.2% and 10.2% respectively. Excluding these 5 admixed population membership in the minor ancestry cluster across the remaining populations is 2.5%. At $k = 3$, the two northernmost populations are largely distinguished from other populations, but increasing *k* values suggest isolation by distance rather than clear distinctions between populations.

Genome wide population genetic structure as estimated by the SNMF function of LEA suggested a different optimum number of genetic clusters. The average cross-entropy criterion and genomic inflation factors across replicate runs at $k = 1 – 16$ suggest that using $k = 7$ will best describe the population genetic structure along the species range. The difference in best *k* across these analyses probably reflects different priorities

in the tradeoff between accounting for as much structure as possible and voiding overfitting of the data such that all populations are completely unique.

Environmental Association Analysis

We conducted an environmental association analysis (EAA) using a latent factor mixed model (LFMM) (Frichot *et al.* 2013) in the LEA package for R (Frichot & François 2015). To identify the best number of latent factors (*K*) we considered the best *K* estimated from the cross entropy criterion of SNMF (supplemental fig. 5.3), best K from STRUCTURE results (supplemental fig. 5.2) as well as the number of latent factors that minimized the genomic inflation factor λ. We used 7 latent factors for our EAA which produced λ of 2.18. After adjusting these p-values by λ and correcting for multiple comparisons, we identified 238 SNPs with Q values < 0.10 (B-H FDR). The median $-\log_{10}(Q)$ value of the association between these candidate SNPs and environmental temperature was 3.53.

We examined the GBS sequence tags from which we identified the 238 candidate SNPs. There were 67 unique sets of tag alignments among the 238 candidate SNPs. Thus the 238 SNPs are located across at most 67 unique genomic loci. Linkage disequilibrium (LD) among candidate SNPs was limited in our dataset, even among SNPs found within the same 64bp tags. Average R^2 for candidate loci within a tag was 0.19, whereas average R^2 across tags was 0.03 (supplementary fig. 5.3). Similarly, there was significant LD for 34% of pairwise SNPs comparison within tags ($p < 0.01$), whereas 1% were significant between SNPs on separate tags. These estimates are similar to others based on GBS-derived genetic markers in this species (publication in preparation).

Thirty eight (57%) of these tags aligned within annotated gene models (65% of SNPs were within annotated genic regions) in the *F. heteroclitus* genome (genome

citation). Candidate SNPs were significantly enriched for genic alignments (Fisher's exact test, $p = 0.0027$), compared to a randomly drawn sample of 500 SNPs. We assigned a functional annotation to 45 of the 67 candidate loci that aligned within 1kb of a genic region in the *F. heteroclitus* genome. We then conducted functional enrichment analysis of OrthoMCL informed human homologs of these 45 loci. Using the functional clustering tool of DAVID gene enrichment, we found 3 clusters of enriched gene functions (EASE score > 1): (i) protein catabolism, (ii) endocytosis and vesicle mediated transport, and (iii) cell morphogenesis.

Nucleotide diversity near candidate loci

To examine if candidate SNPs are located within genomic regions with reduced genetic diversity we calculated average nucleotide diversity within 10kbp of the 238 candidate SNPs and 500 control SNPs randomly drawn without replacement the total dataset. Mean nucleotide diversity (π) was significantly reduced in genomic regions surrounding candidate SNPs *vs.* control SNPs (one tailed Wilcoxon rank sum test, $p = 4.822 \times 10^{-11}$). However, candidate SNPs are significantly enriched for genic loci relative to the 500 random SNPs. Therefore, we randomly sampled SNPs non-genic and genic SNPs to create a random SNP dataset that matched the candidate list in the proportion of genic to non-genic SNPs and compared nucleotide diversity again. The candidate SNPs also demonstrated significantly less nucleotide diversity than this control dataset ($p = 1.07 \times 10^{-9}$) (fig. 5.5). We also compared Tajima D values for the 10kbp windows across candidate SNPs and the genic to non-genic ratio matched control SNPs (fig. 5.5). Candidate SNPs demonstrate significantly lower Tajima D values (one tailed Wilcoxon ranked sum test, $p = 9.156 \times 10^{-10}$).

Adaptive Allele Frequency Shifts

We compared allele frequencies for candidate SNPs across populations. There were no fixed differences between clades at candidate SNPs. On the contrary, allele frequency differences between the clades at candidate SNPs were generally small (median = 1.1%, mean = 4.6%) (fig. 5.6). When examined on a by-population basis, the maximum allele frequency differences between any pair of populations for candidate SNPs were also dominated by subtle effects. The median for maximum allele frequency shifts was 14%, and the mean was 24%. 9.6% of candidate SNPs demonstrated fixed differences in at least one pair of populations. Allele frequency *vs.* the environmental gradient (first principal component of thermal variation) is presented in fig. 5.7. Some representative examples of allele frequency shifts across the environmental gradient are provided in supplementary figure 5.4.

Discussion

Rationale

By focusing on genomic regions bearing the signature of hard selective sweeps or on demographic scenarios where we can effectively utilize F_{ST}-based approaches, current approaches to identifying the adaptive genetic variation underlying adaptation may be limited. The *Fundulus heteroclitus* species range is situated along a strong cline owing to historic vicariance among northern and southern clades, but this clinal genetic variation is superimposed on a nearly linear change with geographic distance in a major selective environmental variable, temperature. These factors limit the success of genome scan approaches to identify the genomic regions responsible for the adaptive divergence along the species range (Strand *et al.* 2012b). By identifying allele frequency changes that strongly correlate with an environmental variable underlying selection, temperature, in a

statistical framework that allows us to parse neutral structure from other patterns, the EAA approach taken here extends our ability to find such adaptive changes.

Population genomic structure corroborates previous studies

While the population genetic structure along the *F. heteroclitus* cline has been thoroughly investigated with microsatellites and mitochondrial haplotypes (Adams *et al.* 2006; Bernardi *et al.* 1993; Duvernell *et al.* 2008; Gonzalez-Vilasenor & Powers 1990), few studies have investigated whether structure at these loci reflects genome-wide trends, but see (Williams *et al.* 2010). Our analysis based on ~9,000 SNPs generally supports previous descriptions of population genetic structure in this species: there is a sharp population genetic boundary at the mouth of the Hudson River, isolation by distance is significant both within and between northern and southern clades, and southern populations harbor increased genetic diversity.

The most striking demographic feature of the *F. heteroclitus* species range is the genetic divergence between the southern and northern clades. There is a strong pattern of isolation by distance (IBD) along the cline, with nearly twice the proportion of overall genetic variation described by AMOVA found between populations north and south of the Hudson River than between populations within these groups. Similarly, mean F_{ST} between populations on either side of Hudson Bay is nearly three times greater than mean F_{ST} within a clade. These observations are in close agreement with estimates by previous analyses (Duvernell *et al.* 2008). Observed heterozygosity is greatest among populations where these two clades meet, suggesting some intergradation near the zone of proposed secondary recontact. These findings are corroborated by STRUCTURE. Individuals from most populations demonstrate little admixture, with the most informative number of ancestry clusters splitting populations into distinct northern and southern groups on either

side of the Hudson River. The exceptions to this pattern are the populations on the New Jersey coast. These three populations demonstrate some admixture from the northern ancestry cluster, with the extent of northern ancestry increasing from south to north, suggesting a zone of intergradation in this region.

In addition to IBD driven by the putative recontact of southern and northern refugia populations, our analysis identifies significant IBD within each clade and increased genetic diversity in southern relative to northern populations (Adams *et al.* 2006; Duvernell *et al.* 2008; Williams *et al.* 2010). In contrast to previous studies, however, the slope of the relationship between genetic and geographic distance was different between the two clades. Northern populations demonstrate increased genetic divergence over the same geographic distances when compared to southern populations. Given the evidence that within-clade IBD patterns are likely to have been largely established prior to glacial retreat (Adams *et al.* 2006; Duvernell *et al.* 2008), the difference in the slope of IBD between the clades suggests that variation in genetic diversity and population size is likely to be longstanding in the evolution history of these populations. Historical variation in population size has important implications for studies dealing with adaptive divergence among the two clades (Charlesworth 2009).

Adaptive Genetic Variation

Identifying adaptive genetic variation across the *F. heteroclitus* range is complicated by the strong correlation between environmental variation and genetic distance among populations. Substantial population genetic structure due to historical vicariance may lead to narrow clines in allele frequency at many loci near the secondary recontact zone of southern and northern clades. For example, in simulations conducted by Strand *et al.* (Strand *et al.* 2012a), secondary recontact of southern and northern clades

leads to a clinal pattern in allele frequency at 42% of all loci. Furthermore, these findings are robust to a wide parameter space for gene flow (N_em = 0.4 – 40). F_{ST} based outlier analyses exhibit very high false positive rates under this demographic scenario (Beaumont & Nichols 1996; Lotterhos & Whitlock 2014). Therefore, their usefulness in identifying adaptive genetic variation is limited because the most parsimonious explanation of outliers from these analyses is the neutral population genetic structure.

While F_{ST} based approaches may be confounded by false positives in this species, genome scans that search for the signature of hard sweeps may instead suffer from high rates of false negatives given the population genetics of *F. heteroclitus*. Specifically, large effective population size observed in *F. heteroclitus* increases the probability of evolution from the standing genetic variation that reduces the strength of the effects on linked neutral variation used by genome scans (Hermisson & Pennings 2005; Messer & Petrov 2013). Empirical estimates of neighborhood size from mark-recapture studies and estimates derived from microsatellite data point to effective population sizes of 10^4 or greater in *F. heteroclitus* (Adams *et al.* 2006; Sweeney *et al.* 1998). We do not estimate population size from our data, because SNPs are called only for polymorphic sequence tags; therefore genome-wide estimates of nucleotide diversity used to estimate population size are comparable only between SNPs within this dataset and are not more broadly interpretable. However, our estimates of expected heterozygosity are similar to previous studies, suggesting that genome-wide effective population size should be comparable to previous estimates from microsatellite data and that evolution from standing genetic variation including both soft sweeps and polygenic adaptation potentially play an important part in the evolution of *F. heteroclitus*.

To identify genetic variation that underlies adaptation along the *F. heteroclitus* range without relying on the approaches above, we utilize an environmental association analysis that simultaneously accounts for neutral population genetic structure and discovers SNPs with significant associations with the environment. 238 SNPs at 67 unique loci demonstrate strong associations with environmental temperature after controlling for population genetic structure with 7 latent factors. Several lines of evidence suggest these candidate SNPs contain or are linked to the true targets of selection due to temperature or a correlated environmental parameter along the *F. heteroclitus* range. First, these candidate SNPs are significantly enriched for genic regions relative to random SNPs drawn from the dataset. Second, the candidate SNPs are enriched for shared biological functions including functions canonically involved in thermal adaptation such as ubiquitin mediated protein modification, and membrane organization (Gracey *et al.* 2004; Podrabsky & Somero 2004). Finally, candidate SNPs lie in genomic regions with reduced nucleotide diversity and lower Tajima's D values than randomly drawn SNPs, even after accounting for systematic differences in these parameters due to the enrichment of genic SNPs.

The majority of candidate SNPs demonstrate subtle shifts in allele frequency associated with environmental temperature changes across the species range. In the polygenic selection model of evolution, traits are determined by many of loci of small effect that are present in the standing genetic variation, and adaptation proceeds through subtle, correlated shifts in allele frequency across many loci until the optimum trait value is reached (Hancock *et al.* 2010a; Pavlidis *et al.* 2012; Pritchard & Di Rienzo 2010; Turchin *et al.* 2012). This is in contrast with the prediction of a hard or soft sweep, where

the adaptive allele(s) become fixed. Therefore evidence of subtle, adaptive allele frequency changes, as observed here, suggests that polygenic selection plays a role in the adaptive evolution of this species. However, the signature of these partial sweeps may be the result of fixation of linked adaptive alleles, *i.e.,* genetic draft (Gillespie 2001). In this scenario, small allele frequency shifts at the candidate loci are driven by incomplete linkage with adaptive alleles that have undergone a selective sweep (Kim & Stephan 2003; Przeworski 2002). The spatial pattern of genetic variation at a locus that underwent a hard sweep will resemble the neutral population genetic structure in this species (Strand *et al.* 2012a) and subsequently will not be detected in our EAA analysis. Our candidate loci may therefore simply lie at the border of genomic regions where genetic diversity and allele frequencies are driven by selective sweeps. However the finding that linkage disequilibrium is limited even among SNPs within the same 64bp tags and the high probability that the selective pressures driving this differentiation are tens of thousands of generations old (Haney *et al.* 2009) suggests otherwise. The lack of correlation in allele frequency between SNPs 10s of base pairs apart suggests that many of the candidate SNPs identified in our EAA lie in regions with exceptionally low recombination rates or are very old. In both cases it is unlikely that we detect adaptive patterns at these loci because of linkage to nearby alleles driven to fixation (Kim & Stephan 2003; Przeworski 2002). Rather, the small LD around candidate SNPs suggests that many are the causative loci underlying selection.

Another set of observations complicates the interpretation that variation at candidate SNPs is due to strictly to polygenic selection. The reduced genetic diversity (π) and shift in the allele frequency spectrum away from intermediate-frequency alleles

(Tajima's D) near candidate SNPs are considered hallmarks of hard sweeps (Braverman *et al.* 1995; Nielsen *et al.* 2005). Yet, recent theoretical work that relaxes assumptions away from strictly hard sweep models predict complex relationships between selection and these parameters under polygenic and soft sweep models of evolution (Cutter & Payseur 2013). Specifically, high rates of partial sweeps (polygenic selection) can produce skews in the allele frequency spectrum and genetic diversity depending on the allele frequency trajectories at the onset of selection (Chevin & Hospital 2008; Coop & Ralph 2012; Pavlidis *et al.* 2012). Therefore these parameters may be better used as evidence ~~of~~ that selection is acting generally at these loci than as evidence of hard sweeps over polygenic selection or soft sweeps.

Conclusions

We assess the population genetic structure of the *F. heteroclitus* species range using a genome-wide 9k SNP dataset. Previous findings as ascertained through analyses of microsatellite and mtDNA markers are largely corroborated: there is a discrete break between northern and southern populations coincident with a narrow intergradation zone suggesting recent recontact between populations that have experienced historical vicariance; within each of these groups there is a pattern of isolation by distance, and genetic diversity is decreased in northern relative to southern populations. We then take advantage of these population genetic inferences to inform our search for loci that associate with the environmental gradient across the species range in an environmental association analysis.

Taken together, the evidence of association with environmental temperature after controlling for population structure, enrichment of both genic region and functional clusters, small LD, and skews in genetic diversity and the allele frequency spectrum

suggest that genetic variation at candidate SNPs has been shaped by selection. Furthermore, the subtle allele frequency shifts with environmental temperature that characterize candidate SNPs implicate a polygenic mode of adaptation. Candidate SNPs represent nearly 3% of all SNPs queried, but given the low power of EAAs two identify adaptive loci under a demographic scenarios like that observed in *F. heteroclitus* (Lotterhos & Whitlock 2015), this number probably represents a lower bound to the portion of the genome that is evolving under polygenic adaptation along the *F. heteroclitus* range. It is important to recall that because adaptive loci evolving through hard or soft sweeps would likely be driven to near fixation at opposite ends of the *F. heteroclitus* range, our analysis has an ascertainment bias towards discovering loci that evolve under polygenic selection rather than sweeps because neutral variation due to demography in this species can produce strong clinal patterns in allele frequency and are unlikely to be identified in our analysis. For example, there is strong evidence from candidate genes such as lactate dehydrogenase B that directional selection drove alternative alleles to fixation at alternate ends of the *F. heteroclitus* range (Crawford & Powers 1989), yet EAA analyses will not find these loci with clinal allele frequency patterns. In contrast, our findings suggest that polygenic adaptation, in addition to selective sweeps, plays an important role in *F. heteroclitus* adaptive evolution. Future studies should investigate the functional role of candidate loci implicated in both genome scan and EAA approaches to better understand that this genetic variation influences fitness. For example, combining the effects of many subtle allele frequency changes using multivariate approaches such as polygenic risk scores for fitness traits in independent populations may provide strong evidence that the candidate loci which

significantly correlate with the selective environment actually underlie fitness differences between populations.

Tables and Figures

Population	Longitude	Latitude	Mean Temperature	Mean Annual Maximum	Mean Annual Minimum	1st Principal Component
MDIBL	68°17.7' W	44°25.7' N	8.30	14.87	2.72	-2.47
ME	69°39.9' W	43°59.8' N	9.19	18.41	2.07	-1.90
NBH	70°54.5' W	41°37.5' N	11.83	22.27	1.78	-0.98
M	70°48.9' W	41°39.5' N	11.83	22.27	1.78	-0.98
SLC	70°58.3' W	41°31.4' N	11.83	22.27	1.78	-0.98
HB	71°1.5' W	41°30.3' N	11.98	22.19	2.95	-0.82
SR	71°31.5' W	41°23.0' N	11.94	22.08	2.96	-0.84
CT	72°31.5' W	41°16.7' N	12.21	23.59	0.90	-0.85
MG	74°4.2' W	40°3.0' N	13.50	24.66	3.72	-0.13
RB	74°19.5' W	39°30.5' N	13.72	25.13	2.66	-0.16
SH	74°45.9' W	39°3.4' N	13.66	25.37	1.77	-0.25
KC	76°25.5' W	37°17.9' N	15.56	27.04	3.26	0.47
NC	75°40.0' W	35°54.0' N	18.67	27.65	9.87	1.88
WNC	77°55.2' W	34°3.0' N	21.36	28.94	12.86	2.86
SC	79°55.0' W	32°45.5' N	22.33	29.44	14.97	3.35
GA	81°21.3' W	31°27.0' N	21.35	29.65	10.92	2.71

Table 5.1: Locations and environmental data for populations.

	n	MDIBL	ME	NBH	M	SLC	HB	SR	CT	MG	RB	SH	KC	NC	WNC	SC	GA
MDIBL	**23**	**0.1095**															
ME	**26**	0.0675	**0.1046**														
NBH	**149**	0.0904	0.0895	**0.1338**													
M	**31**	0.098	0.0961	0.0031	**0.1346**												
SLC	**30**	0.0977	0.0949	0.0062	0.0074	**0.1341**											
HB	**48**	0.1059	0.1019	0.0193	0.0202	0.013	**0.1283**										
SR	**48**	0.096	0.092	0.0355	0.0366	0.0343	0.0223	**0.1346**									
CT	**21**	0.0922	0.0776	0.0448	0.0445	0.0419	0.0445	0.0344	**0.1167**								
MG	**200**	0.1146	0.1224	0.0664	0.0622	0.0626	0.0634	0.063	0.075	**0.15061**							
RB	**200**	0.1327	0.1371	0.0799	0.0752	0.0753	0.0762	0.0773	0.0839	0.0065	**0.1487**						
SH	**61**	0.1387	0.1446	0.0807	0.0752	0.0767	0.078	0.0786	0.0938	0.004	0.004	**0.1531**					
KC	**23**	0.1989	0.1939	0.1206	0.1191	0.1166	0.1205	0.12	0.1271	0.0382	0.0279	0.0338	**0.1336**				
NC	**15**	0.2387	0.235	0.146	0.1446	0.1439	0.1573	0.1524	0.1556	0.0551	0.0364	0.051	0.0285	**0.1278**			
WNC	**27**	0.2105	0.2088	0.1337	0.1312	0.1309	0.1357	0.1379	0.1414	0.0529	0.0432	0.0483	0.045	0.0312	**0.1373**		
SC	**15**	0.2657	0.2672	0.1656	0.1649	0.1637	0.1713	0.1733	0.1832	0.0753	0.0564	0.0693	0.047	0.0339	0.0175	**0.1225**	
GA	**39**	0.222	0.2241	0.1569	0.1542	0.154	0.1588	0.1582	0.1744	0.0639	0.0597	0.0556	0.0612	0.0653	0.0326	0.0367	**0.1426**

Table 5.2: Sample size (***n***), expected heterozygosity (along the diagonal), and F_{ST} (Weir and Cochran's θ) for all pairwise comparisons. Populations are ordered north to south

Component of Variance	Φ-Statistic	Variance	% of total	*p*
Among regions/clades	$\Phi_{CT} = 0.064$	46.1	6.41	$< 10^{-5}$
Among populations	$\Phi_{SC} = 0.037$	24.8	3.45	$< 10^{-5}$
Within population	$\Phi_{ST} = 0.099$	647.5	90.13	$< 10^{-5}$

Table 5.3: AMOVA results

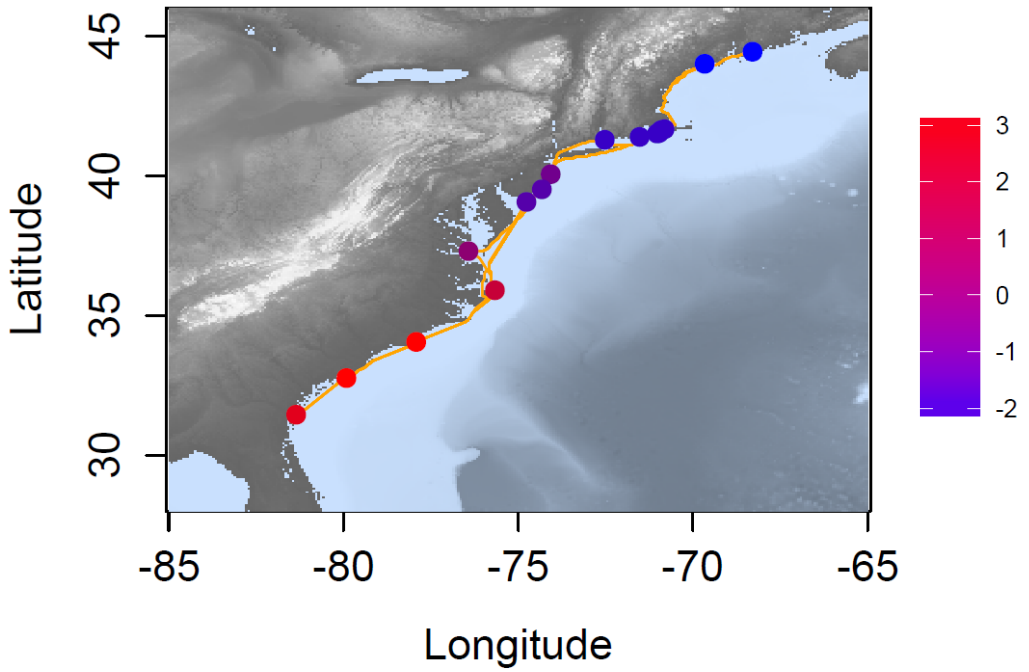


Fig. 5.1: Sampling locations and environmental temperature. Colors points represent sampling locations with colors corresponding to the value of the 1st principal component of temperature variation. Gold lines connecting points are the minimum alongshore distances between populations constrained by 20m depth.

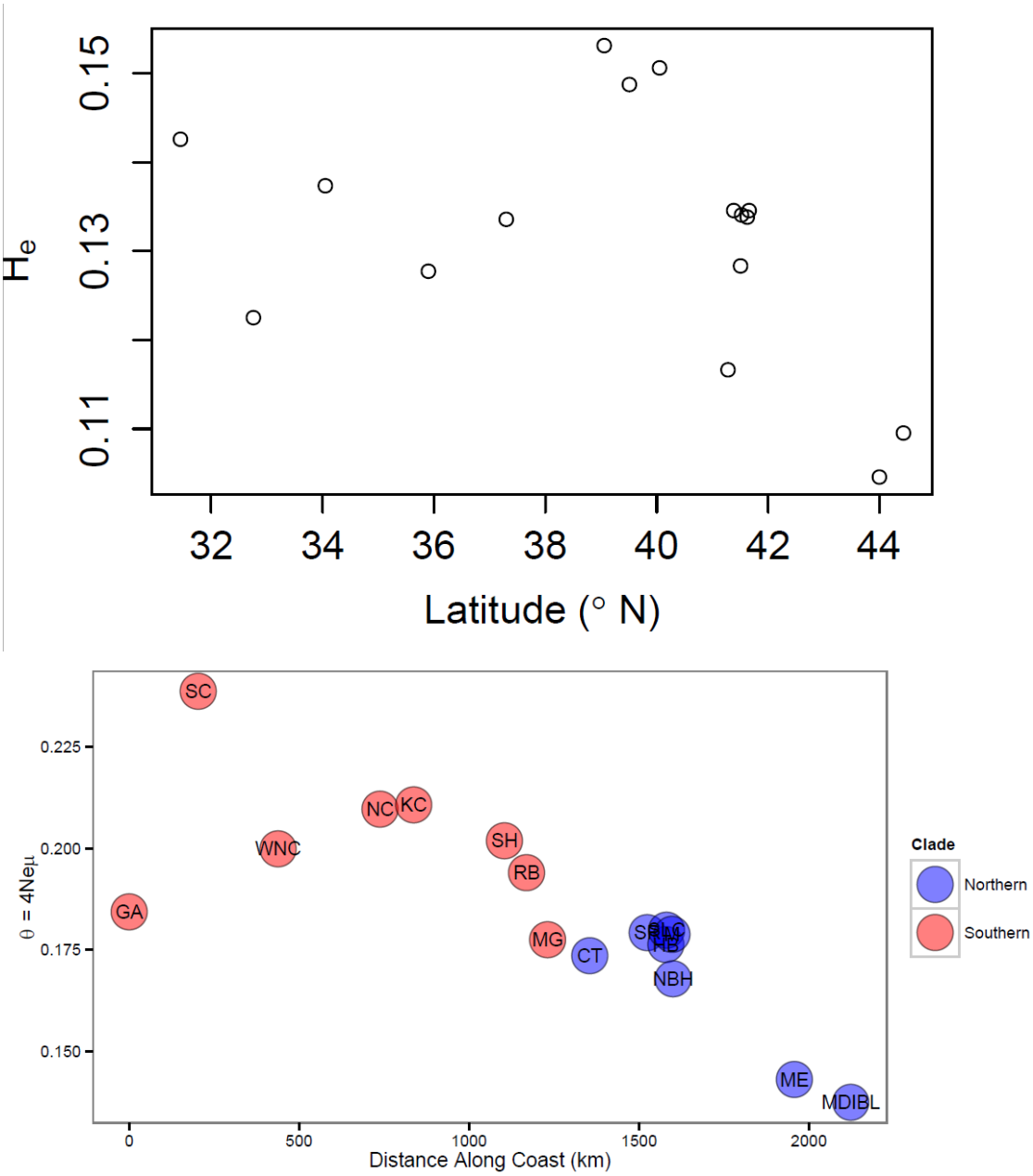


Fig. 5.2: Genetic diversity across the species range.
(Top Panel) Expected heterozygosity *vs.* latitude. (Bottom Panel) Mean per locus estimate of θ_w; colors correspond to whether populations are from north or south of the Hudson River. Population labels are as table 5.1.

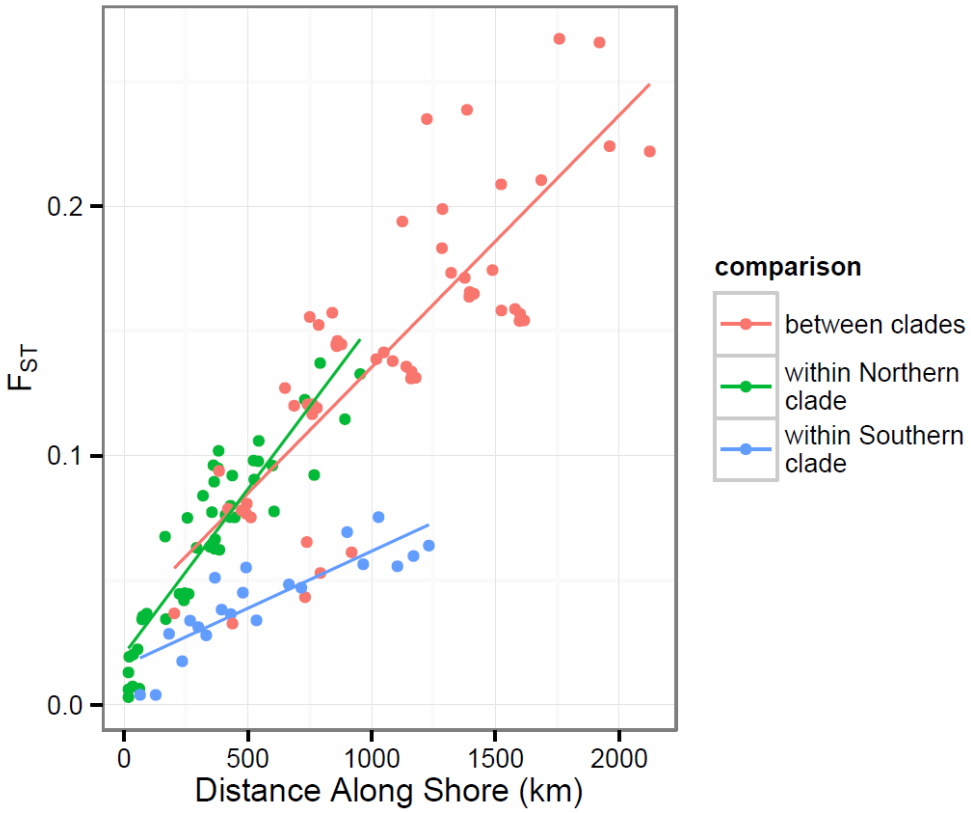
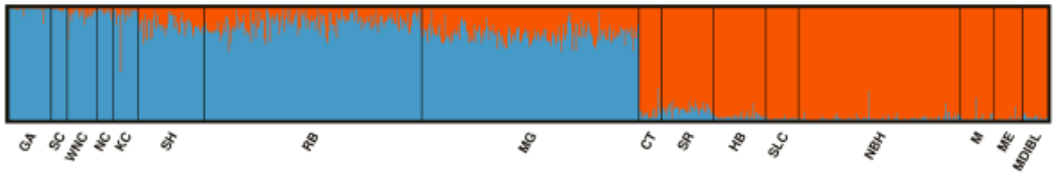


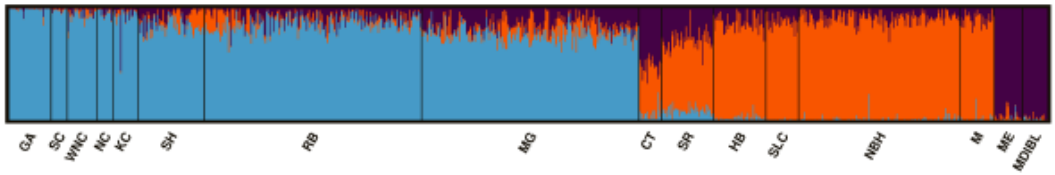
Fig. 5.3: Isolation by distance.
Pairwise genetic distance as estimated by Weir and Cochran's θ (F_{ST}) *vs.* geographic between all population pairs. Linear regressions and colors are for all pairwise comparisons within southern or northern populations (blue and green, respectively) or between northern and southern populations (red)

Fig. 5.4: Structure plot. (next page) Each individual is represented with a vertical line that is partitioned into colors according to modeled admixture proportions for *k* ancestral populations. Results for *k* = 2-7 are presented; best *k* was 2. Population labels are as in table 1.

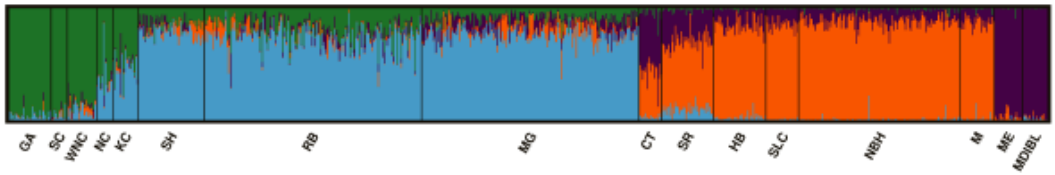
K=2



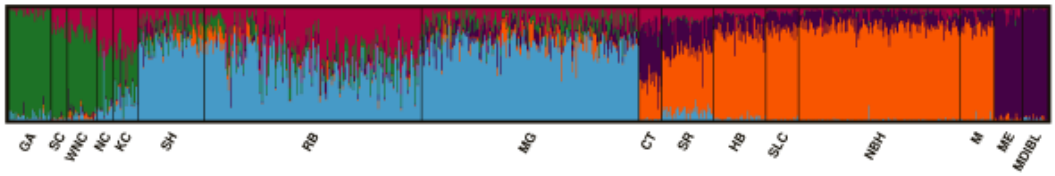
K=3



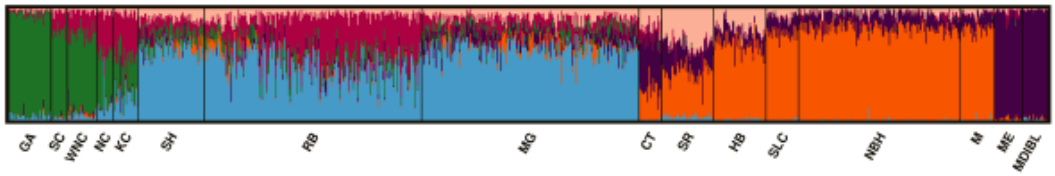
K=4



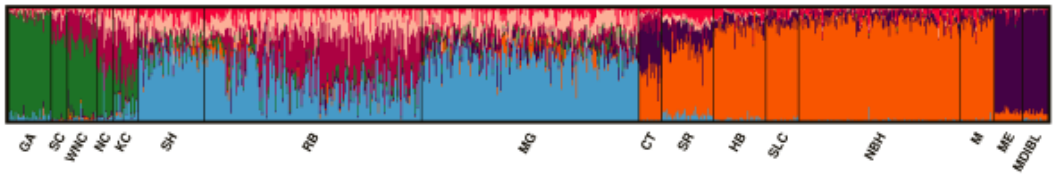
K=5



K=6



K=7



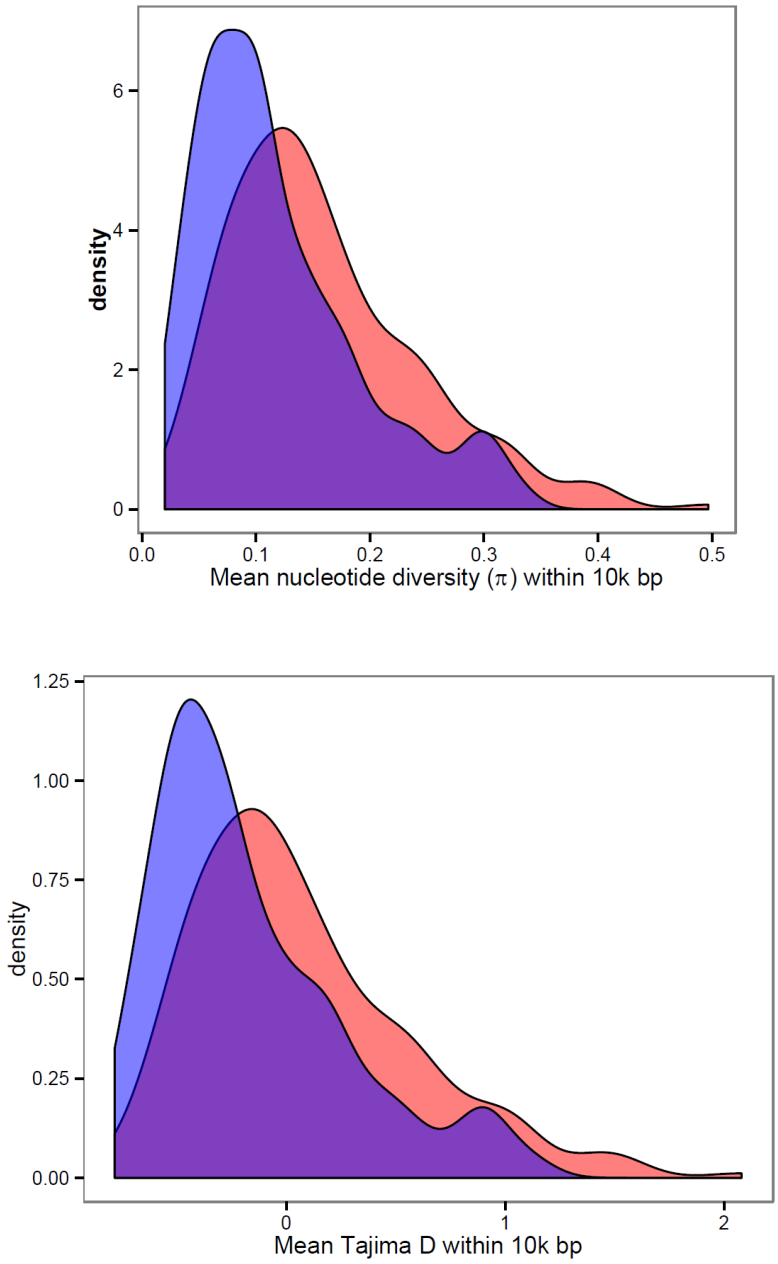


Fig 5.5: Nucleotide diversity and allele frequency spectrum near candidate SNPs. (Top) Density plot of nucleotide diversity (π) for all SNPs within 10kb of candidate SNPs (blue) *vs*. control SNPs matched for genic to non-genic ratio (red). (Bottom) Density plot of Tajima D for all SNPs within 10kb of candidate SNPs (blue) *vs*. control SNPs matched for genic to non-genic ratio (red). Candidate SNPs demonstrate a significant difference in nucleotide diversity ($p = 1.07 \times 10^{-9}$) and allele frequency spectrum ($p = 9.156 \times 10^{-10}$) relative to control SNPs.

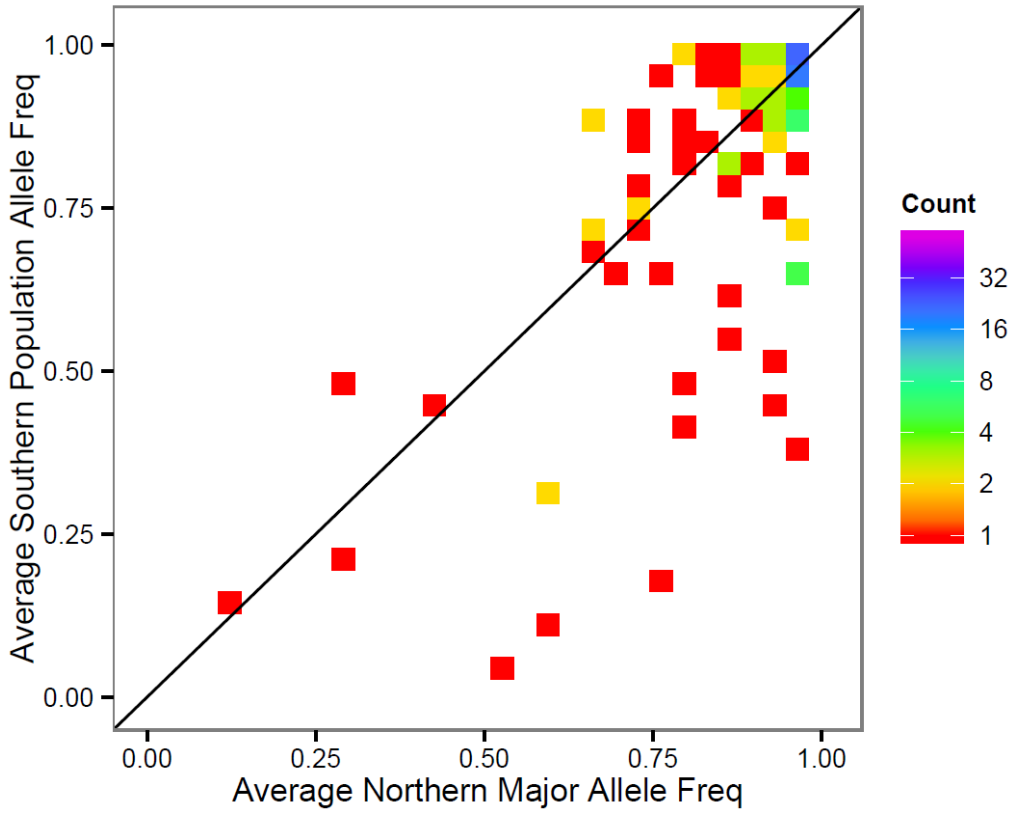


Fig. 5.6: Allele Frequency Shift Histogram Frequency histogram of candidate SNP average major allele frequency for southern populations *vs.* southern populations. Major allele for all populations defined as the major allele in the northernmost population. Color scale is logarithmic.

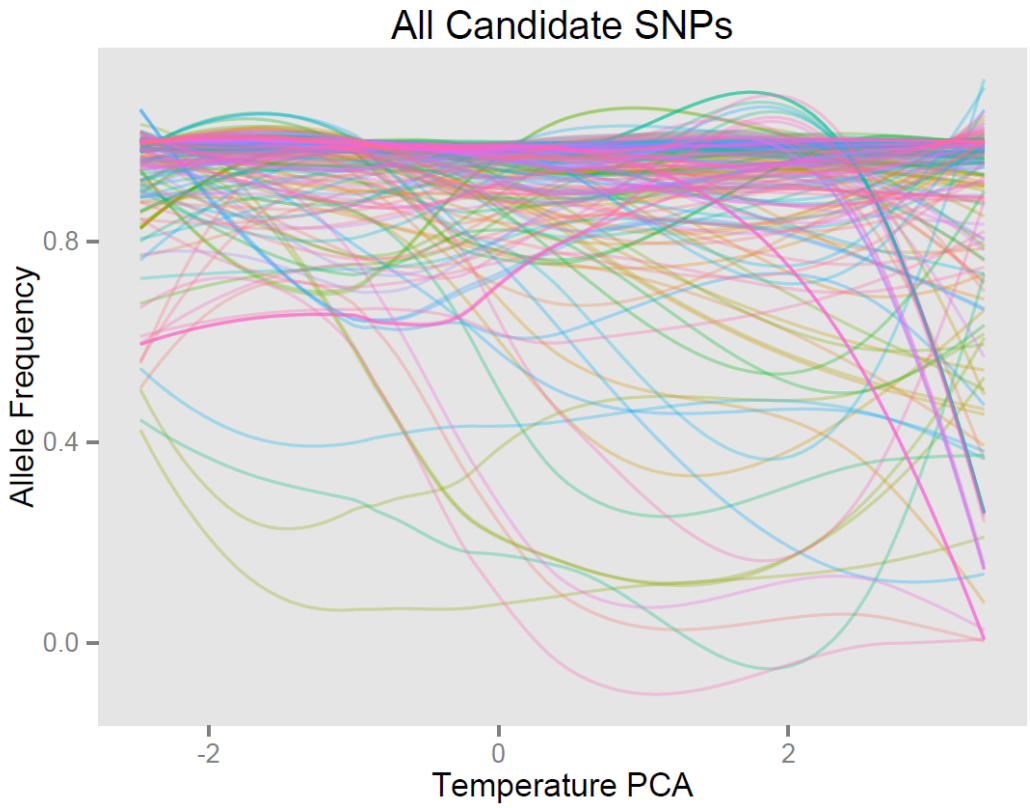


Fig. 5.7: Allele Frequencies Associations. Loess smoothed allele frequency *vs*. 1st principal component of temperature variation for candidate SNPs. The plotted allele is the major allele in the northernmost

Chapter 6: Synthesis

Revealing the mechanisms and signature of adaptive evolution relies on querying variation in the environment and the genotypes and phenotypes of organisms. This dissertation combines these factors in four different approaches to study how phenotypic plasticity influences adaptive evolution (fig. 6.1). Chapter two conducts a genome-wide association study to reveal how genetic variation underlies phenotypes and how this relationship changes across environments. Chapter three investigates how environmentally sensitive gene expression affects adaptive divergence by characterizing thousands of phenotypes with a microarray analysis. Chapter four examines genetic variation directly to identify population genomic signature of recent selection near thermal effluents. Finally, chapter four identifies genetic variation that associates with changes in the environment across the *F. heteroclitus* species range. These four chapters are united by three questions: (i) *what is the genetic architecture of phenotypic plasticity, (ii) how common is genetic accommodation in nature, and (iii) how common is the population genomic signature of adaptation mediated by phenotypic plasticity?*

What is the genetic architecture of phenotypic plasticity?

Phenotypic plasticity can result from a variety of genetic architectures (Des Marais *et al.* 2013). Briefly (see chapter 2 for more in-depth background), phenotypic plasticity can result from differential sensitivity where allelic effects are in the same direction, but have different magnitudes across environments. An extreme case of differential sensitivity, conditional neutrality, occurs when an allele that has an effect in one environment has no effect in another. Alternatively, plasticity can be driven by antagonistic pleiotropy, where the same allele has opposite effects across environments.

The extent of conditional neutrality or antagonistic pleiotropy in plastic traits determines how plasticity influences (i) the accumulation of genetic variation within a population and (ii) gene flow between locally adapted populations from disparate environments. Conditional neutrality leads to relaxed selection and thereby promotes the accumulation of genetic variation within a population (Lahti *et al.* 2009; Snell-Rood *et al.* 2010; Van Dyken & Wade 2010). Where traits with conditionally neutral architecture are locally adaptive, conditional neutrality also promotes gene flow among populations from disparate environments, because locally adaptive, but conditionally neutral alleles will have little effect outside the environments where they evolved (Anderson *et al.* 2013; Crispo 2008; Fournier-Level *et al.* 2011; Hall *et al.* 2010). If gene flow is high enough, it can ultimately lead to the fixation of conditionally neutral alleles and the production of generalist genotypes. On the other hand, antagonistic pleiotropy results in fitness tradeoffs between locally adapted populations at individual loci (Kawecki & Ebert 2004). Because alternate alleles are under selection across environments, antagonistic pleiotropy discourages gene flow between locally adapted populations and reduces within population genetic variation, thereby promoting genetic divergence and genetic diversity across the species range (Nosil *et al.* 2009; Weinig *et al.* 2003).

The dissertation investigates the genetic architecture of phenotypic plasticity directly using a genome-wide association study (GWAS), but data from the microarray analysis of gene expression also yields insights into this question. Furthermore, examining how the alleles that underlie variation in plastic traits segregate along the species range and between locally adapted populations suggests how plasticity influences gene flow and genetic variation.

The GWAS for thermal tolerance (Ch. 2) suggests that phenotypic plasticity is primarily driven by conditional neutrality rather than antagonistic pleiotropy. These results corroborate most studies to date that investigate this question (Anderson *et al.* 2013; Fournier-Level *et al.* 2011; Hall *et al.* 2010; Hawthorne & Via 2001; Latta *et al.* 2010; Verhoeven *et al.* 2004; Weinig *et al.* 2003). The results presented in Ch. 2 are especially relevant, because the population characteristics of our study system should encourage antagonistic pleiotropy over conditional neutrality; the large outbreeding populations of *F. heteroclitus* should both permit the fixation of conditionally neutral alleles and encourage adaptive divergence among populations through genetic tradeoffs. Therefore the discovery of conditional neutrality in *F. heteroclitus* suggests that the results of previous studies are not due to an ascertainment bias owing to the enrichment of conditionally neutral alleles in species with low gene flow or population size (Colautti & Barrett 2013; Hall *et al.* 2010).

Identifying conditional neutrality using the phylogenetic comparative analysis of the microarray analysis (Ch. 3) complements the results of the GWAS (Ch. 2), because while GWAS queries genetic architecture directly, the phylogenetic comparative analysis identifies conditionally neutral differences in adaptive traits. Conditional neutrality in the microarray data stems from two observations. First, for genes with significant phylogenetic contrast-by-acclimation interaction terms (*i.e.,* where there is significant inferred adaptive GxE in gene expression) we observe no evidence of antagonistic pleiotropy. Instead, expression differs between warm adapted and cold adapted populations at only one of the three acclimation temperatures for these genes; there is no rank changing in the reaction norm (fig. 6.2). Second, when we examine gene expression

within each acclimation temperature individually, there is very little overlap in the sets of genes that demonstrate adaptive divergence between southern warm-adapted populations and the northern cold adapted population. Putatively adaptive genetic differences between northern and southern *F. heteroclitus* only have phenotypic effects under a subset of environmental conditions.

Together, the data presented in Ch. 2 and 3 suggest that the genetic architecture of phenotypic plasticity is dominated by conditional neutrality, both at the level of individual loci and for adaptive traits.

Do we observe the predicted impacts of conditional neutrality?

Examining the loci identified in these chapters also allows us to investigate the predicted impacts of conditional neutrality on gene flow and genetic variation. First, we can ask if cryptic genetic variation is released under rarely encountered environmental conditions. In chapter 2, there is greater genetic variance for thermal tolerance at 12°C acclimation than at 28°C. Therefore there is not only increased trait variation, but this increase has a heritable genetic basis. Acute environmental temperatures approaching the upper thermal tolerance may be a rare event for fish acclimatized to environmental conditions similar to our 12°C acclimation. Although we do not have the environmental data to directly address the novelty of this environmental challenge, we assume that fish in nature experience temperatures near their upper limits more frequently during the warm season than during the cold. Therefore the increased genetic variance at 12°C might be considered a release of cryptic genetic variation. Similarly, in chapter 3 we observe greater magnitude of adaptive differences at extreme acclimation temperatures relative to moderate, although we are not able to rigorously attribute these differences in expression among populations to either decanalization at extreme temperatures within

individuals or adaptive variation in phenotypic plasticity among populations (Dayan *et al.* 2015).

Second, is there increased genetic variation at conditionally neutral alleles? In the GWAS, we did not find any associations that have consistent effects across acclimation temperatures. Thermal tolerance was dominated by GxE, both at the trait level and for allelic effects of significantly associated causal variants. Therefore, we cannot directly ask whether conditionally neutral alleles accumulate genetic variation at a faster rate than constitutively expressed genetic variation. For example, nucleotide diversity (π) is significantly reduced at SNPs with significant thermal tolerance associations relative to genome-wide distributions, but this is expected for any SNPs that are functionally constrained and subject to purifying selection, even if this functional constraint is reduced by conditional neutrality. Similarly, genetic diversity at genes with adaptive variation in plasticity cannot be rigorously investigated because few gene from the microarray analysis (Ch. 3) are near SNPs in the GBS-derived genetic datasets (Chs. 2, 4, and 5) (see appendix 1). However, one gene with a change in strength of plasticity between northern and southern populations is near SNPs from a GBS-derived genetic dataset. Interstingly, this gene demonstrates conditional neutrality in expression and lies in a genomic region with significantly elevated nucleotide diversity (θ) compared to the genome wide average.

Finally, how do conditionally neutral alleles segregate among populations? None of the SNPs with significant associations with thermal tolerance are shared with Ch. 4 (appendix 1). However for 105 SNPs with associations under a less stringent FDR (FDR $Q < 1$), 5 are queried in Ch. 4. These 5 SNPs demonstrate very little differentiation

between locally adapted populations exposed to thermal effluents and closely related reference populations (fig. A.3), suggesting they do not play a role in rapid evolution in response to elevated temperatures. Similarly, the 12 SNPs that have significant associations with thermal tolerance and are shared with the clinal SNP dataset (Ch. 6) demonstrate neither clinal nor adaptive shifts in allele frequency along the species range (fig. A.4). While it is possible that conditional neutrality has permitted high gene flow for these loci (Crispo 2008; Hall *et al.* 2010), it is unlikely that allele frequency would be homogenized across the entire species range, given the extent of differentiation for completely neutral loci (Strand *et al.* 2012a). A more parsimonious explanation is that SNPs that effect critical thermal maximum under laboratory conditions have limited fitness effects in natural populations because critical thermal maximum is a poor proxy for thermal tolerance in the wild (Terblanche *et al.* 2007), or that conditionally neutral genetic variation in general has limited impact on fitness at this large spatial scale.

How common is genetic accommodation in nature?

Researchers have made substantial inroads into the study of genetic accommodation. There is substantial evidence of that phenotypic plasticity promotes population persistence (Badyaev 2009; Geng *et al.* 2006; Novy *et al.* 2013; Réale *et al.* 2003; Yeh & Price 2004), leads to the accumulation and release of genetic variation (Hoffmann & Merila 1999; McGuigan *et al.* 2011), and that reaction norms readily evolve (Pigliucci 2001; Van Kleunen & Fischer 2005). However, understanding the extent to which ecologically relevant phenotypes have evolved by genetic accommodation in nature is challenging because in most study systems, putatively accommodated phenotypes have already undergone selection. In other words, once a trait has evolved, it becomes impossible to directly observe its evolution. One solution to this

problem is experimental evolution using introductions (Ghalambor *et al.* 2015; Scoville & Pfrender 2010) or resurrection studies (Sultan *et al.* 2013), where germplasm from ancestral lineages is propagated under novel environment conditions. This approach allows the observation of genetic accommodation *in situ,* but species introductions and resurrection studies may be poor analogs for natural cases of accommodation and are limited in the number of study systems where they are feasible (Ehrenreich & Pfennig 2015; Merilä & Hendry 2014; Schlichting & Wund 2014).

Instead, researchers utilizing natural study systems are only able to infer genetic accommodation. Levis and Pfennig (2016) outline four criteria for evidence of genetic accommodation that when observed together, strongly implicates genetic accommodation despite the absence of its observation *in situ.* These criteria are based on comparisons drawn between adaptive derived phenotypes and their inferred ancestral state. The phylogenetic comparative analysis we use in Ch. 3 takes just such an approach. By identifying gene expression that is similar among a southern, warm-adapted *F. heteroclitus* population and a warm-adapted, closely related outgroup (*F. grandis*), but is different between this polyphyletic group (*F. grandis* + southern *F. heteroclitus*) and a northern, cold-adapted *F. heteroclitus* population, we can infer which patterns of expression are evolutionarily derived in the northern populations relative to the ancestral state. Furthermore, rather than conducting this analysis on a single focal trait, making these phylogenetically informed contrasts for patterns of expression at thousands of genes allows us to explore the mosaic of mechanisms that underlie genetic accommodation and address the first major question of this dissertation: *how common is genetic accommodation*?

The four criteria outlined by Levis and Pfennig (2016) are (i) ancestral plasticity in the trait, (ii) release of cryptic genetic variation by the ancestral population in the novel environment, (iii) a shift in the reaction norm between derived and ancestral lineages, and (iv) adaptive refinement of the trait in the derived population. Re-examining the microarray data (Ch. 3) under this framework suggests that expression of many (12%) ~~of~~ genes with significant thermal plasticity has evolved through genetic accommodation. First we consider only genes that meet criterion (iii), a shift in the reaction norm between derived and ancestral lineages. The set of genes with both significant phylogenetic contrasts and effects of acclimation (Ch. 3 fig. 3a) and the set of genes with significant interaction between these terms demonstrate a change in the intercept and slope, respectively, of the reaction norm between the derived (northern *F. heteroclitus*) and inferred ancestral state (warm adapted *F. heteroclitus* and *F. grandis*). Some of these genes also meet criterion (i). Specifically, genes that demonstrate significant shared acclimation effects among the warm adapted populations are suggestive of ancestral plasticity. We inferred ancestral plasticity for 6 of the 11 genes (ANOVA, $P < 0.05$), with both significant phylogenetic contrasts and a significant effect of acclimation, but for only 2 of the 14 genes with significant interaction terms (ANOVA, $P < 0.05$). To examine criterion (ii) we consider the cold environment (12°C acclimation) as the novel environment with respect to the warm-adapted ancestral-proxy populations and compare the variance of gene expression between the inferred ancestral and derived populations. For the 8 genes that meet criteria (i) and (iii), there is on average, greater expression variance among ancestral-proxy populations than the derived population under novel conditions (ratio ancestral variance: derived variance = 1.34). This pattern is not

significant (Wilcoxon rank sum test $p = 0.22$), nor is there significantly greater variance among comparisons within any one gene (F-tests, $p > 0.05$), although there is limited power to conduct these tests. Finally, criterion (iv) is arguably met for these genes because differences in gene expression between the ancestral-proxy populations and the derived population correlate with differences in environmental temperature that drive local adaptation in this species (Ch. 6)(Burnett *et al.* 2007).

Taken together, our microarray analysis suggests that temperature dependent expression of many genes (~0.4% of all genes analyzed, 12% of genes with phenotypic plasticity in expression) has evolved through genetic accommodation because there is putative ancestral plasticity, an increase in variance in the novel environment, and a potentially adaptive change in the reaction norm between derived and inferred ancestral populations. It is also worth noting that the acclimation temperatures used in this study are modest relative to the seasonal and clinal variation in temperature experienced by these populations (Healy & Schulte 2015). Using a wider temperature range most likely would result in a stronger signal of phenotypic plasticity in ancestral-proxy populations because much of phenotypic plasticity is threshold dependent (Schlichting & Pigliucci 1998) and the extent of variance in ancestral-proxy populations would likely increase because colder acclimatization temperatures are less common the evolutionary history of warm-adapted populations (Ghalambor *et al.* 2007; Healy & Schulte 2015; Wund 2012). Therefore the proportion of genes from the microarray analysis that meet the 4 criteria probably represents a lower bound of the total number of genes that have potentially evolved through genetic accommodation.

The microarray analysis provides a second insight into the process of genetic accommodation. Ultimately, genetic accommodation results in a change in the reaction norm of a trait, but this change can take several forms (Grether 2005). If the initially environmentally induced trait is stabilized such that it has a reduced threshold of expression, or it is constitutively expressed, genetic accommodation results in genetic assimilation. In this case, the initial plastic response is in the direction of the new adaptive optimum, and accommodation refines this plasticity such that it is more frequently expressed in the derived population. Accommodation can also enhance initial plasticity so that there is a steeper reaction norm. There has been a historic focus on these two forms of plasticity in the study of phenotypic plasticity's impact on adaptive divergence because early theoretical work considered the effects of only adaptive phenotypic plasticity on populations (Baldwin 1896) and many mechanistic studies examine the origins of plasticity as an adaptive trait (Ghalambor *et al.* 2007). However, most phenotypic plasticity is likely to be maladaptive (Grether 2005; Van Kleunen & Fischer 2005). Furthermore, maladaptive plasticity is likely to be under stronger directional selection than adaptive plasticity (Grether 2005; Orr 1998; Wright 1931). Genetic accommodation acting on traits that arise through maladaptive plasticity can result in a pattern of countergradient variation (or genetic compensation), where adaptive genetic differences along an environmental gradient opposes environmentally induced trait variation such that trait values are stabilized across the species range. The microarray data (Ch. 3) suggests genetic accommodation is driven primarily by genetic compensation. Of the 8 genes that meet the criteria for genetic accommodation outlined above, 5 demonstrate genetic compensation, one demonstrates enhanced plasticity and

none are consistent with genetic assimilation. Furthermore, of the genes that demonstrate derived changes in the extent of plasticity (slope of the reaction norm, 14 genes with significant phylogenetic contrast-by-acclimation interaction terms) we infer ancestral plasticity at only two genes (Healy & Schulte 2015). Rather, adaptive phenotypic plasticity evolves from a less plastic ancestor. These conclusions corroborate those made by (Handelsman *et al.* 2013) and (Ghalambor *et al.* 2015) which suggests genetic accommodation functions largely to reduce the impacts of maladaptive, environmentally induced traits.

How common is the population genomic signature of adaptive evolution mediated by phenotypic plasticity?

Phenotypic plasticity promotes adaptation by the soft sweep and polygenic models of evolution rather than hard sweeps of single novel variants. Plasticity can increase effective population size and genetic variance in new environments, and the adaptive mutation rate at relevant loci (see introduction). These effects encourage adaptation from alleles at moderate frequency in the standing genetic variation rather than rare or novel variants, which suggests that adaptation through genetic accommodation of initially plastic traits is polygenic and soft. The third question addressed by this dissertation investigates whether the genomic signature of polygenic adaptation and soft sweeps is common in nature.

Chapters four and five reveal the population genomic signature of adaptation at two different spatial and temporal scales. Chapter four considers very recent adaptation across a spatial scale where the effects of genetic drift are likely to be minimal. Chapter five represents the other extreme; selection has been ongoing for tens of thousands of generations and the effects of genetic drift and demography over the entirety of the *F.*

heteroclitus species range strongly obscure the genomic signal of adaptation (Strand *et al.* 2012a). In both cases, the temporal and spatial scales used probably prevent the discovery of hard sweeps. In chapter four, selection has acted for approximately 50 generations. This time frame is shorter than the expected lag time required for a genetic variant at low frequency or a novel mutant to arise and sweep to high frequency in the population, even under very strong selection (Burke *et al.* 2010; Stephan *et al.* 1992). In chapter five, there is ample times for rare variants to sweep to fixation, but censoring genetic variation that is similar to neutral structure in our search for alleles that associate with environmental temperature obviates the possibility of finding fixed differences between northern, cold-adapted and southern, warm-adapted populations.

Yet, our intention was not to characterize the relative importance of hard sweeps *vs.* other models of adaptive evolution. Hard sweeps may underlie many of the fixed differences that characterize the clinal, thermal adaptation of *F. heteroclitus* (Crawford & Powers 1989). On the contrary, our goal was to demonstrate that the hard sweep model of evolution is insufficient to explain the total of adaptive genetic variation in natural populations by demonstrating that other models have a role to play. Chapter four demonstrates that the standing genetic variation can respond rapidly to selection and produce adaptive shifts in organismal performance. Chapter five shows that subtle shifts in allele frequency associate with changes in environmental temperature and that these subtle shifts are most parsimoniously described as adaptive. In this way, chapters four and five demonstrate that polygenic adaptation from the standing genetic variation plays a significant role in evolution and warrants further investigation.

Importantly, while phenotypic plasticity may promote evolution by polygenic adaptation and soft sweeps, examples of polygenic adaptation and soft sweeps are not evidence that phenotypic plasticity is an important facet of evolution. These phenomena can occur regardless of the environmental sensitivity of phenotypes. Rather, the results of chapters four and five suggest that current understanding of the genotype-to-phenotype map is insufficient to explain the origin and maintenance of diversity in observable phenotypes. The roles of relatively unexplored concepts in evolutionary biology, including phenotypic plasticity, but also epigenetic inheritance, niche construction theory and developmental bias should be investigated to create an extended evolutionary synthesis that more completely explains phenotypes and their evolution (Laland *et al.* 2014) in the face of mounting evidence such as that presented in chapters four and five.

Conclusions

Phenotypic plasticity can interact with genetic variation in the production of phenotypes and therefore influence evolutionary trajectories (West-Eberhard 2003). Phenotypic plasticity can also affect how genetic variation itself is produced and maintained (Paaby & Rockman 2014). This dissertation investigates both of these themes of phenotypic plasticity's potential role in adaptive evolution. First, chapters two and three establish that genetic variation in phenotypic plasticity (gene-by-environment interaction) is common for traits at the levels of gene expression and organismal performance, neutral under some environmental conditions, and contributes to the trait genetic architecture and adaptive divergence of natural populations. Then, chapters four and five demonstrate that evolutionary adaptation in natural populations across a wide range of spatial and temporal scales can be caused by subtle shifts in allele frequency from the standing genetic variation, consistent with

phenotypic plasticity's proposed impacts on the production and maintenance of genetic variation.

The gene-centric focus of the modern synthesis in evolutionary biology is proving increasingly incapable of explaining evolution and diversity of phenotypes. In its place, an extended evolutionary synthesis needs to be constructed that does not discount the role of the gene in evolution, but shifts our focus back to phenotypes. Together, the data presented in this dissertation suggests that phenotypic plasticity will play a major role in this extended evolutionary synthesis and warrants continued empirical investigation.

Tables and Figures

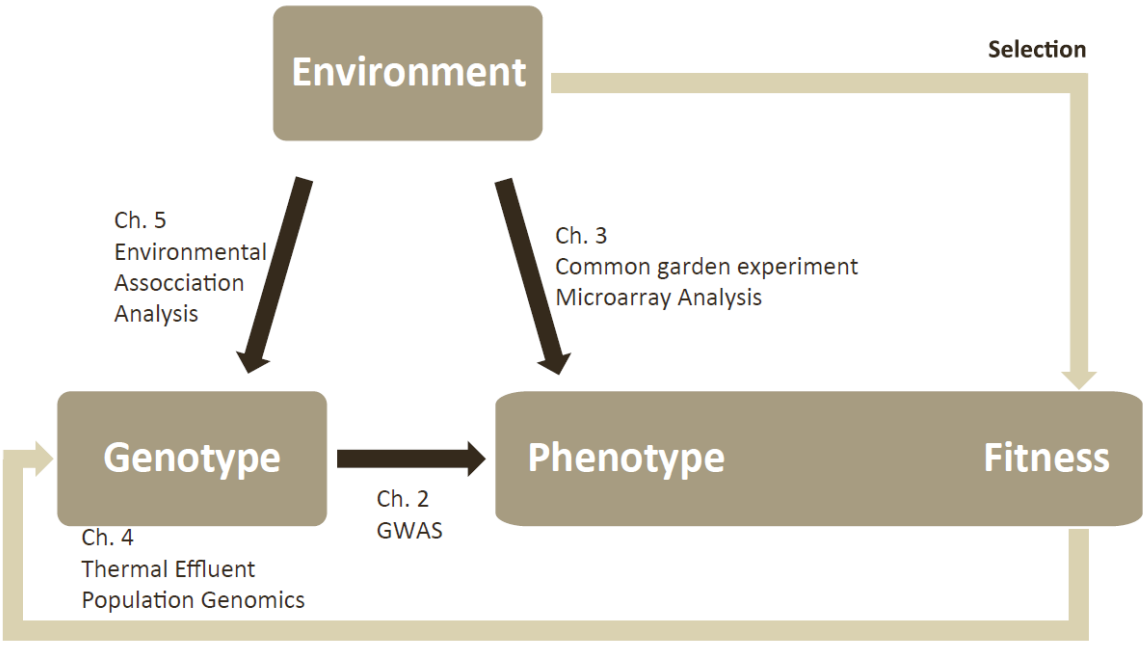


Fig. 6.1: Chapters of the dissertation how they query biological variation. Modified from (Rellstab *et al.* 2015)

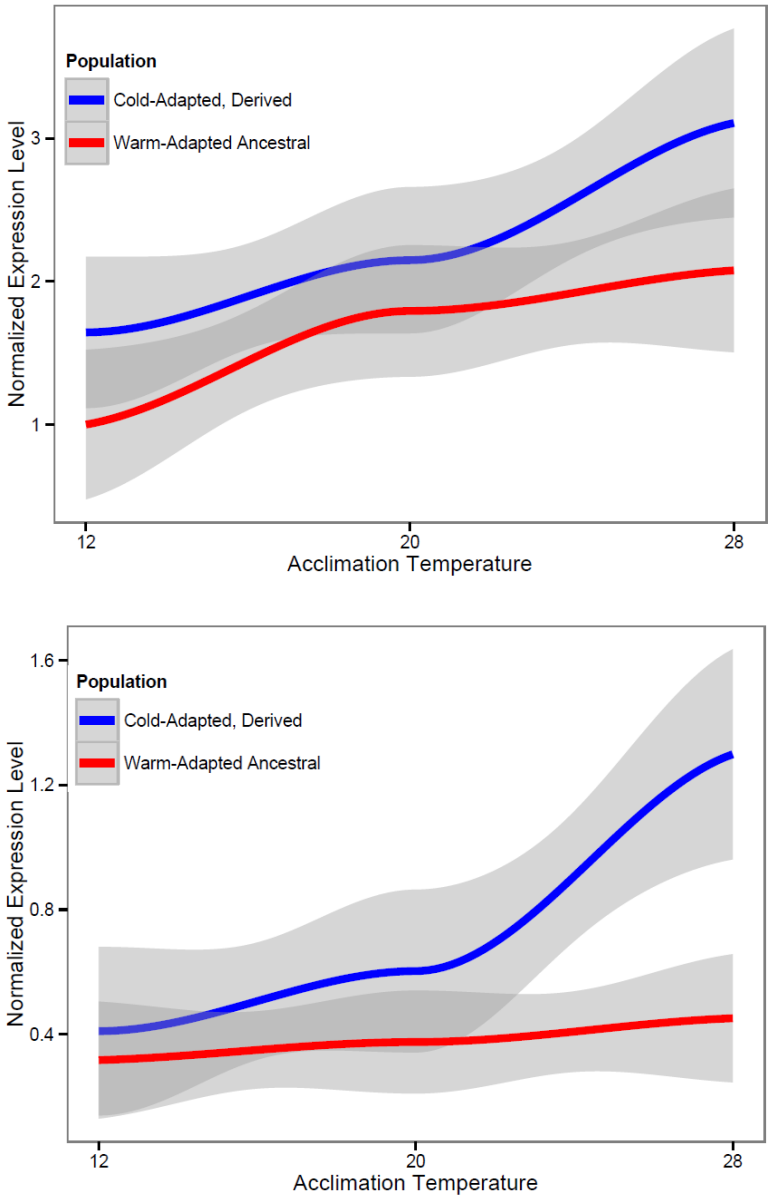


Fig. 6.2: Representative reaction norm plots. Loess smoothed, normalized gene expression *vs.* acclimation temperature for two representative genes. Gene 2,549 (top panel) is significantly impacted by acclimation and the phylogenetic contrast and demonstrates countergradient variation. Gene 1,639 (bottom panel) has a significant interaction between these two factors and demonstrates conditional neutrality of adaptive divergence in gene expression.

Appendix 1: Directly comparing data among chapters

The dissertation consists of four chapters. Three of these four chapters query genetic variation at the same loci because the same restriction enzyme was used to produce the reduced representation libraries for genotyping-by-sequencing. Also, alignments between microarray probe sequences and the shared set of GBS derived tag sequences reveal some overlap between these datasets. By synthesizing these results we are able to ask how any given locus contributes to trait variation, plays a role in rapid thermal adaptation, varies in allele frequency across the species range, and demonstrates adaptive patterns of phenotypic plasticity. Thus we may be able integrate population genomic signals of adaptation and divergence with allelic effect sizes and estimates of phenotypic plasticity to ask very specific questions such as is there evidence that relaxed selection leads to the accumulation of polymorphism in non-inducing environments? Or, does selection operate on the same targets over tens of generations and tens of thousands of generations? This appendix contains the results of this analysis and are discussed, where relevant, in the synthesis (Ch. 6)

Shared Loci

There should be substantial overlap of loci across GBS derived datasets because the libraries of the same restriction enzyme digest fragments are used across experiments. However, read depth among these restriction digest fragments will vary among the experiments due to (i) stochastic effects such as variation in amplification and sequencing among tags and (ii) systematic differences such as non-independence of cut site variation and population sampling across experiments. Regardless of the underlying cause, fewer SNPs than might be expected are shared across the analyses because only a small portion of SNPs are retained after filtering, and filtering is based primarily on read depth, (fig.

A1.1). For example, 11% of the 5,450 SNPs used in Ch. 4 (thermal effluents) are also used in both Chs. 2 and 5, and 28% of the SNPs from Ch. 2 are shared with Ch. 5 (fig. A1.1).

We also consider whether genes with non-neutral gene expression profiles identified in Ch. 3 are near any SNPs in the datasets of Chs. 2, 4 and 5 (table A1.1). Briefly, microarray probe sequence for the 139 SNPs with significantly non-neutral patterns in the phylogenetic comparative analysis of Ch. 3 were aligned to the *F. heteroclitus* genome using BLAST with an e-value cutoff of 10^{-20}. Of the 139 probe sequences, 123 aligned once to the genome, 9 had no alignments above the cutoff, and 7 multiply aligned. We then compared genomic positions of the 123 microarray probes with single alignments with GBS derived SNPs from other chapters. Few microarray probe alignments are within 20kb of a SNP (11), and only one probe is within 1kb of a SNP. Five of the probes are within 20kb of a SNP from multiple datasets and 1 probe is within 1kb of SNP from multiple datasets.

Few genes with significant phenotypic plasticity or population-by-environment interactions in gene expression aligned near GBS derived SNPs. Of the 76 genes with significant acclimation or population-by-acclimation interactions terms from chapter 3, 60 align to one location on the *F. heteroclitus* genome, 6 multiply align and 10 have no significant alignment. None of these 60 singly aligning genes are within 20kb of any SNP in the datasets of Chs. 2 or 5, and 2 align within 20kb of SNPs in the dataset of Ch. 4. Similarly 1 gene with a significant phylogenetic contrast-by-environment interaction in gene expression aligned near GBS derived SNPs from Ch. 4.

Segregation of conditionally neutral alleles among effluent populations

To investigate how loci with environment specific allelic effects on thermal tolerance segregate among populations undergoing rapid thermal adaptation, we compare results from Chs. 2 and 4. These two studies utilize 9,126 and 5,449 GBS derived SNPs, respectively, and 1,085 SNPs are shared across the analyses. Among these 1,085 SNPs, there are data for both allelic effect sizes and F_{ST} estimates among reference and thermal effluent populations for 749 SNPs. Only 8 of the 130 adaptive outlier SNPs from Ch. 4 and none of the 42 SNPs with significant thermal tolerance associations from Ch. 2 are contained in the overlapping datasets. Therefore no SNPs in the overlapping dataset are both adaptive outliers in the effluent populations and have significant associations with thermal tolerance. Nor are any adaptive outliers and association SNPs within distance that we observe LD in this species (<1 kb). However, given that F_{ST} based outlier scans and genome-wide association studies suffer from high rates of type II error, we also considered if there is a relationship between conditional expression of allelic effects and differentiation between reference and thermal effluent populations at non-significant loci (fig. A1.2).

Rather than relying on the conservative methods in Chs. 2 and 4 to identify important SNPs, we compared the distribution of population differentiation (F_{ST} values) between reference and effluent populations with the distribution of the difference in allelic effect sizes across environments. These F_{ST} distributions used here are less conservative than the process for identifying adaptive outliers in Ch. 4 because they consider the average level of differentiation between effluent and reference populations; therefore SNPs with high levels of differentiation between the two reference populations are not excluded. Similarly, the distribution examined for effect sizes is less conservative

than the mixed model framework used in Ch. 2. Rather, the distribution plotted along the x-axis in fig. A1.2 is the difference in normalized effect sizes across acclimation temperatures; therefore SNPs that fall in the tail of the distribution demonstrate large differences in effect sizes across environments due to either conditional neutrality or antagonistic pleiotropy, but do not necessarily have low p-values for the association after controlling for kinship in the sampled population.

Since conditionally neutral SNPs should fall in the tail of the effect size distribution in fig. A1.2 if there is a relationship between the extent of conditional neutrality and the level of differentiation between effluent and reference populations, then the tails of these distributions should substantially overlap. This hypothesis was tested by examining if the tail (greater than 95^{th} percentile) of the F_{ST} distribution is significantly enriched for SNPs in the tail (greater than 95^{th} percentile) of the effect size difference distribution. No SNPs for the Brayton Point triad overlapped in the tails, and six SNPs overlapped for the Oyster Creek triad (purple loci, fig. A1.2, top panel). Six SNPs is more than expected by chance alone (odds-ratio: 3.2), but not significantly so (fisher exact test, $p = 0.28$).

Similarly, when we relax the stringent p-value cutoff and examine where SNPs with thermal tolerance association FDR Q value < 1.0 (red loci, fig. A1.2) lie in the F_{ST} distribution, we find that SNPs with non-significant thermal associations demonstrate (i) large differences in effect sizes between environments relative to the rest of the distribution, similar to significant associations and (ii) very low differentiation between effluent and reference populations

Segregation of conditionally neutral alleles along the species cline

We also investigate how loci with environment specific allelic effects on thermal tolerance (Ch. 2) segregate along the *F. heteroclitus* species range (Ch. 5). These two chapters have a larger number of shared SNPs. Of the 9,129 SNPs from Ch. 2 and 8,748 SNPs from Ch. 5, 3191 are shared, including 12 of the 42 SNPS with significant thermal tolerance associations. None of these 12 SNPs were also candidate SNPs in the adaptive clinal divergence among *F. heteroclitus* populations. However, 3 of the 42 thermal tolerance association SNPs are within 500 bp of candidate clinal adaptation SNPs (S9987_617431, S10006_665330 and S10006_665337). The spatial distribution of allele frequency for the 12 thermal tolerance association SNPs is presented in figure A1.3. None of the SNPs demonstrate clinal allele frequency distributions across the species range, where alternative alleles are at or near fixation at alternate ends of the range. Also, the distributions of nucleotide diversity (both π and θ) and Tajima's D for these 12 SNPs are not significantly different than the genome wide distribution of these parameters in the cline SNP dataset (Ch. 6) (Wilcoxon rank sum test, $p > 0.10$).

Gene expression vs. population genetics

Too few genes with significant phenotypic plasticity identified by microarray analysis of gene expression (Ch. 3) align near SNPs in the other datasets to test predictions about the role of phenotypic plasticity in adaptive evolution. We can draw no insights from the clinal genetic variation nor assess how allelic effects on thermal tolerance and gene expression are related because no loci are shared among Chs. 2, 3, and 5.

Two genes with significant plasticity in gene expression (annotation print numbers: 710 and 2061) are near SNPs in the thermal effluents dataset (Ch. 4).

Interestingly one of these genes is within 1kb of an adaptive outlier at the Brayton Point triad. One gene with a significant phylogenetic contrast-by-acclimation interaction term (annotation print number: 274) is near SNPs in the thermal effluents dataset (Ch. 4). The median nucleotide diversity within a 10kb window within these SNPs is less than the genome wide average. For π this trend was non-significant, but for θ it was (Wilcoxon rank sum test, $p = 0.0041$).

Tables and Figures

Chapter	Short chapter Name	within 20kb	within 1kb
2	GWAS	5	1
4	Thermal Effluents	8	0
5	Clinal Adaptation	4	1

Table A.1: Number of the 140 genes with significantly non-neutral patterns from Ch. 3 that align near SNPs from GBS derived datasets

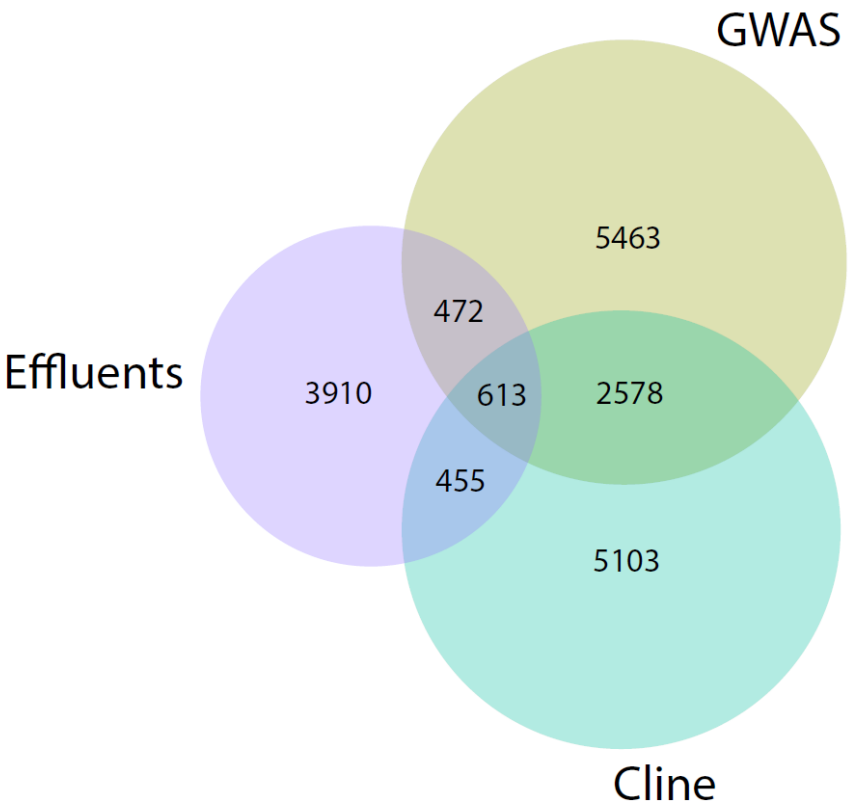
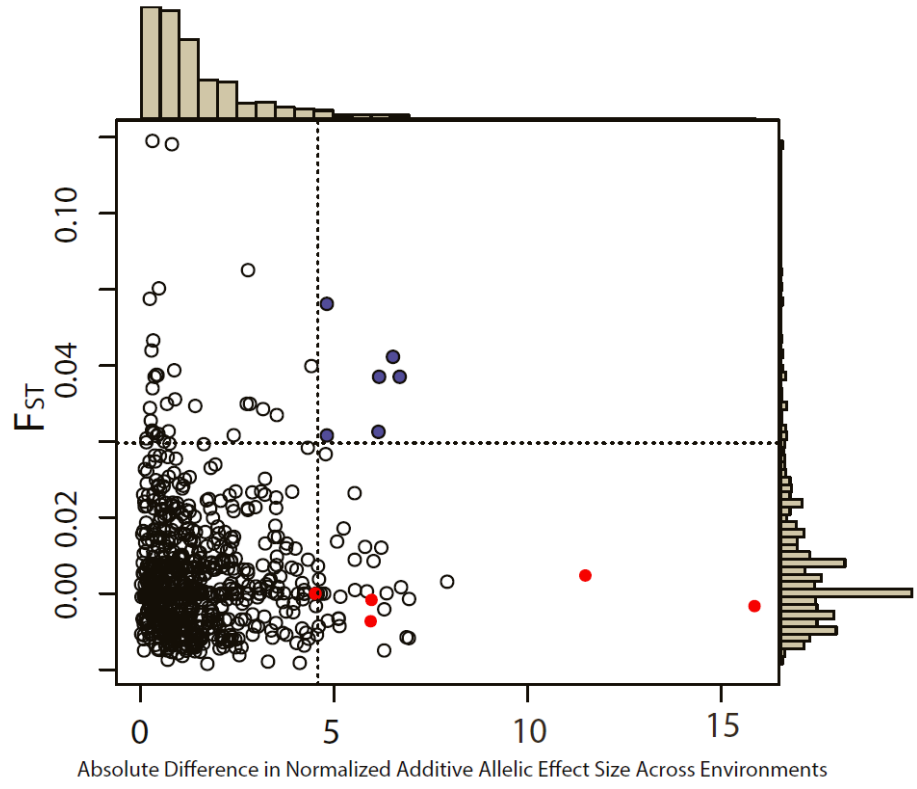
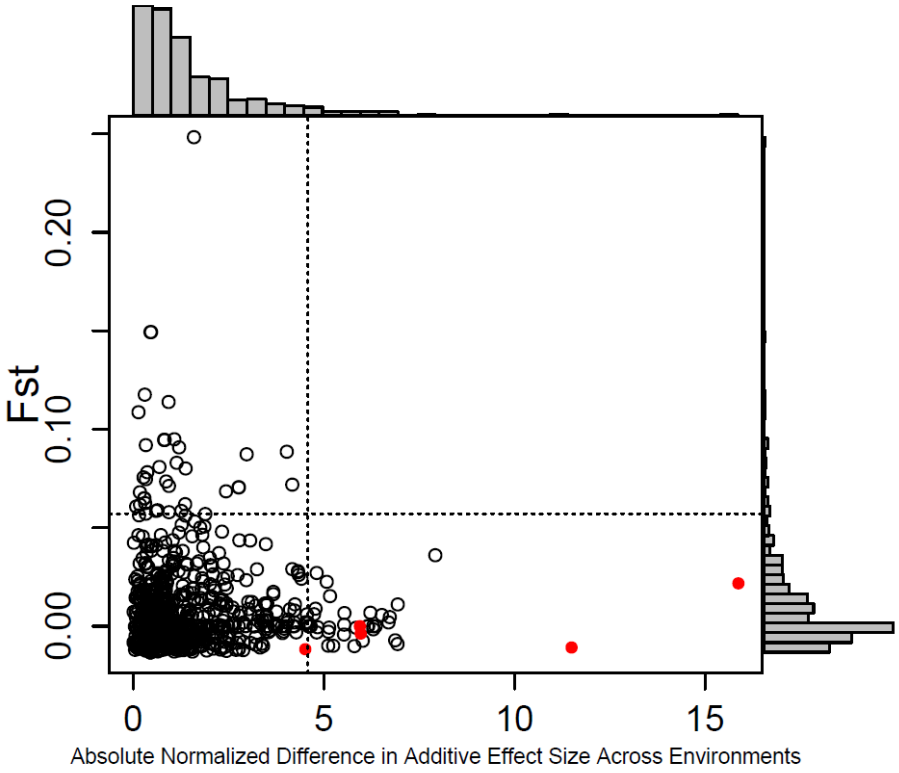


Figure A.1: Euler diagram of GBS data. Exact overlap of SNPs between the three chapters (GWAS – Ch. 2; Effluents – Ch. 4; Cline – Ch. 5) that use GBS derived SNP data. Circles and their intersections are scaled to number of SNPs therein.

Figure A.2: (next page) **Segregation of conditionally neutral alleles across effluent triads.** In the main body of the figure, mean F_{ST} per locus of effluent exposed vs. reference populations (Ch. 4) is plotted against the absolute difference between allelic effect sizes at 12 and 28°C acclimation temperatures for the 749 SNPs with both effect size and F_{ST} estimates. The distributions of these two parameters are reflected across their respective axes, with the 95th percentile represented by a dashed line. Red colored loci have a strong, but non-significant association with thermal tolerance (top 105 loci, FDR < 1), purple colored loci fall in the upper 5% tails of both distributions. The top panel is for the Oyster Creek triad, lower is for Brayton Point.


F_{ST}
0.00
0.02
0.04
0.10
0
5
10
15
Absolute Difference in Normalized Additive Allelic Effect Size Across Environments


Fst
0.00
0.10
0.20
0
5
10
15
Absolute Normalized Difference in Additive Effect Size Across Environments

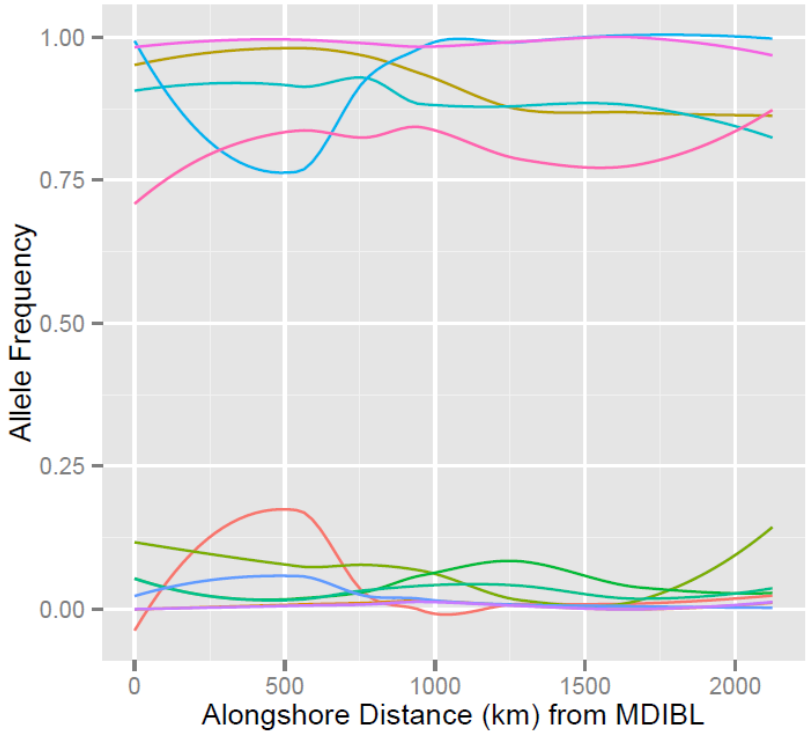
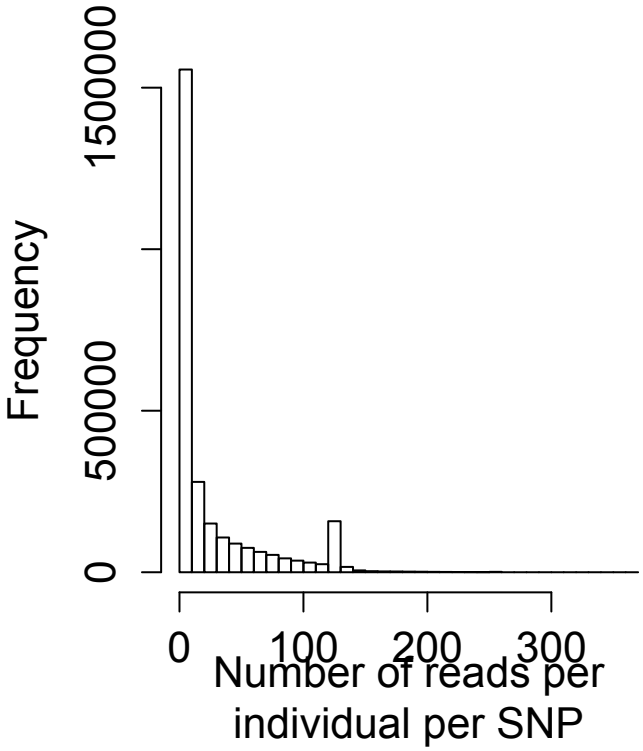
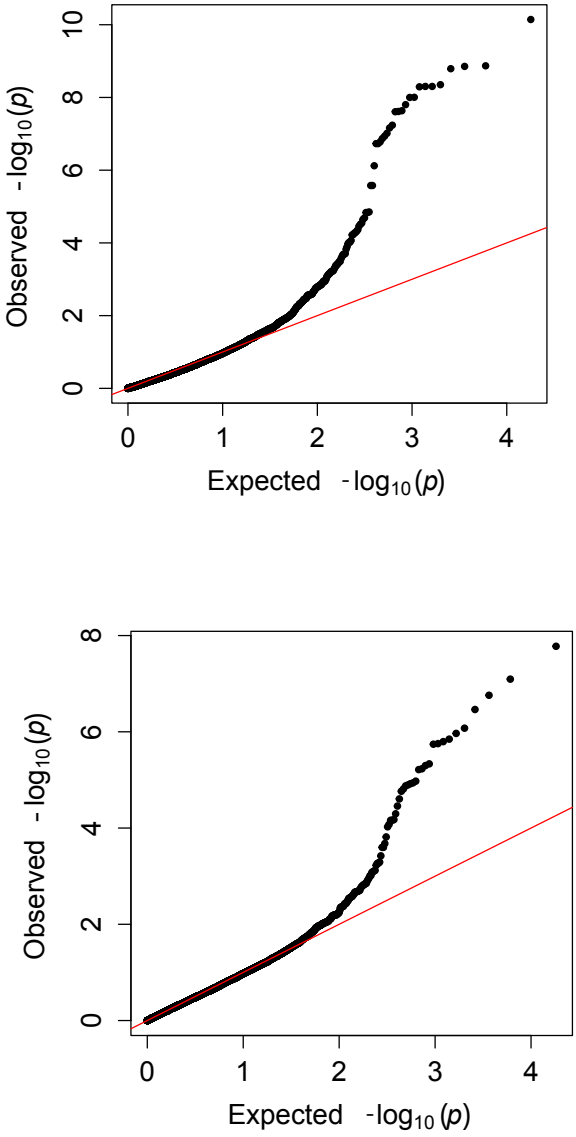


Fig. A.3: Segregation of conditionally neutral alleles along the species range. Loess smoothed allele frequency is plotted against the position along the cline (km alongshore from the northernmost population - MDIBL) for the 12 SNPs that demonstrate both significant thermal tolerance associations (Ch. 2) and are queried in the clinal genetic dataset (Ch. 5)

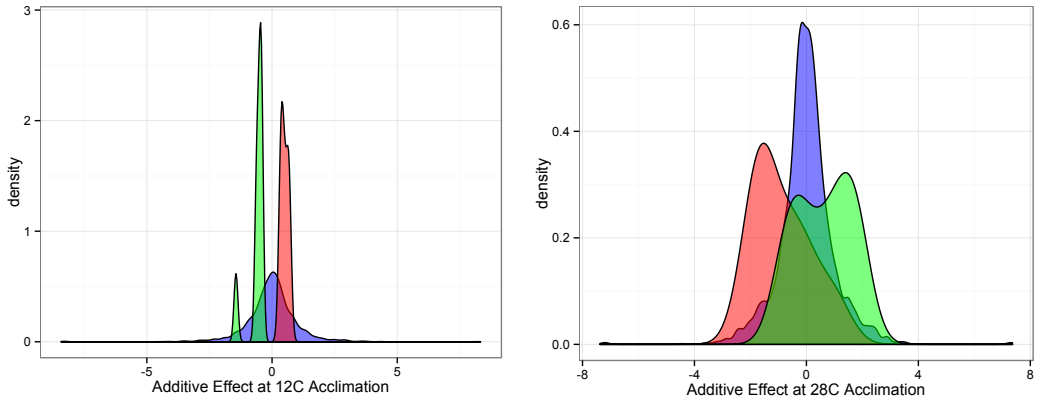
Appendix 2: Data Supplement



Supplemental Fig. 2.1: Read depth distribution for the filtered SNP dataset



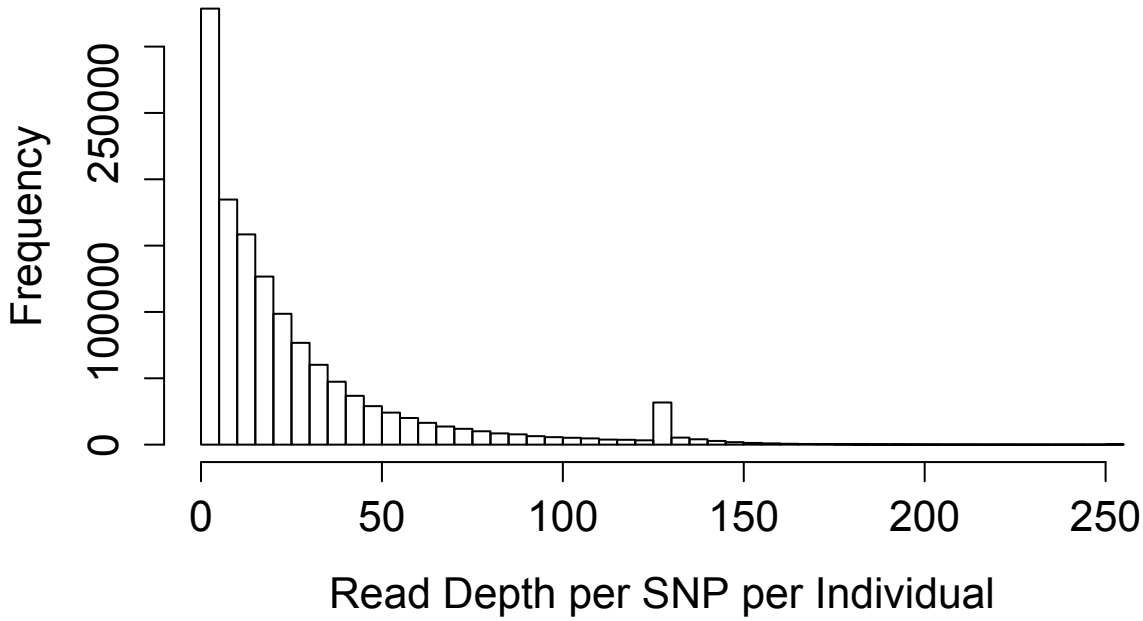
Supplemental Fig. 2.2: Q-Q plots for the MLM procedure at 12°C (left) and 28°C (right)



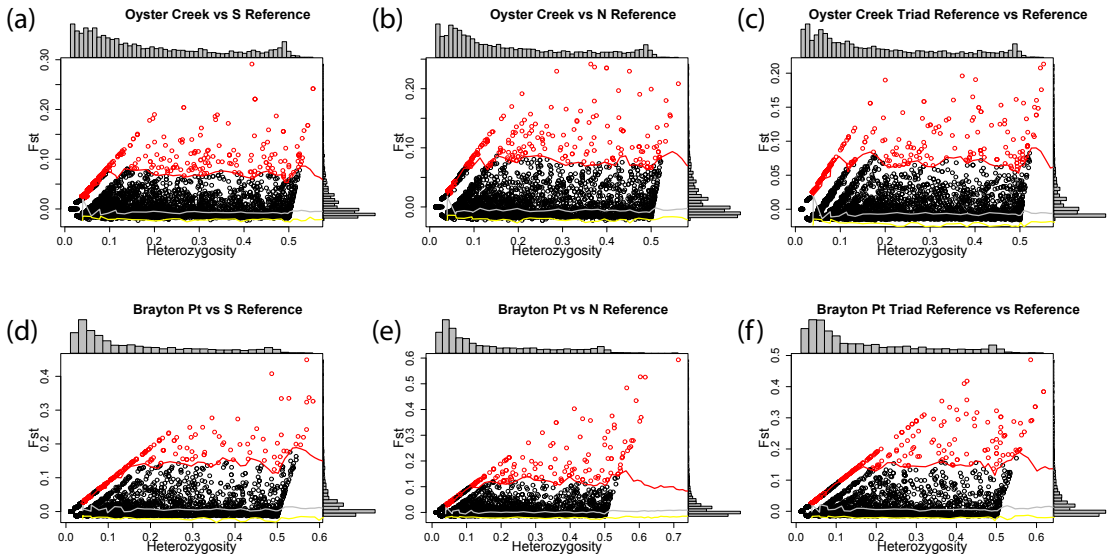
Supplemental Fig. 2.3: Density plots of all effect sizes (normalized with mean 0 and s.d. 1) at an acclimation temperature (blue), with the effect size for SNPs with signifcant associations at the opposite temperature, where SNPs that are positive in the alternate acclimation are red and those with negative are green. Therefore antagonistic pleiotropy exists but is limited by censoring if green values fall on the right extreme of the total distribution while red values fall on the left extreme. Left panel is thedensity plot in this format for significant association at warm acclimation (but their effect sizes at cold acclimation) and right panel is for cold acclimation

Marker	Acclimation	UniProt Accesion	Gene Symbol	Gene Name
S10006_665330	28	Q7SXE9	NDUFA11	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11
S10006_665337	28	Q7SXE9	NDUFA11	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11
S10024_888544	12	E7FGS9	B3GALT1	Beta-1,3-galactosyltransferase 1
S10147_174121	28	F1QW70	CHD6	Chromodomain-helicase-DNA-binding protein 6
S117_385667	12	E7FFB2	LRRTM4	Leucine-rich repeat transmembrane neuronal protein 4
S136_41983	28	F1R3V0	TACR3	Neuromedin-K receptor
S1726_24220	28			
S1960_11208	28	E7F442	MKL2	MKL/myocardin-like protein 2
S1967_17812	28	Q1MTC7	HLA-DQA2	HLA class II histocompatibility antigen, DQ alpha 2 chain
S1967_17816	28	Q1MTC7	HLA-DQA2	HLA class II histocompatibility antigen, DQ alpha 2 chain
S2948_3181	28			
S2948_3183	28			
S3057_9794	28			
S331_146864	28	Q5RFV2	GALNT14	Polypeptide N-acetylgalactosaminyltransferase 14
S3329_278	12	E7FFL9	STEAP2	Metalloreductase STEAP2
S3917_330	12			
S4532_2300	28			
S469_816093	12		FAM64A	Protein FAM64A
S469_816103	12		FAM64A	Protein FAM64A
S469_816127	28		FAM64A	Protein FAM64A
S494_236878	12	F1QVL5	NLGN3	Neuroligin-3
S494_236882	12	F1QVL5	NLGN3	Neuroligin-3
S530_204688	28		EFHD1	EF-hand domain-containing protein D1
S64_543564	12	Q1LVD7	RIMS4	Regulating synaptic membrane exocytosis protein 4
S643_187057	28			
S770_82381	28	F1R9Y1	COL14A1	Collagen alpha-1(XIV) chain (Undulin)
S78_76780	28	F1Q6F2	KMT2C	Histone-lysine N-methyltransferase 2C
S78_76783	28	F1Q6F2	KMT2C	Histone-lysine N-methyltransferase 2C
S789_3169221	28	A5WUR1	WASF1	Wiskott-Aldrich syndrome protein family member 1
S79_997537	28			
S796_121497	28	F1Q6V2	TRIM25	E3 ubiquitin/ISG15 ligase TRIM25
S796_121498	28	F1Q6V2	TRIM25	E3 ubiquitin/ISG15 ligase TRIM25
S811_18034	28	H9GYN2	CLCC1	Chloride channel CLIC-like protein 1
S941_54974	12	F1R9M4	LOC100148190	Uncharacterized protein
S96_459941	12	F1QKJ0	NTRK3	Neurotrophic tyrosine kinase receptor type 3
S9872_235502	28	Q6AZB8	HARBI1	Harbinger transposase-derived nuclease
S9878_443768	28	A1L144	MPRIP	Myosin phosphatase Rho-interacting protein
S9887_89371	12	Q08BC0	PIM2	Serine/threonine-protein kinase pim-2
S9899_411995	28	Q6AZB8	HARBI1	Harbinger transposase-derived nuclease
S9915_790630	12	E7F4B2	RAPGEF1	Rap guanine nucleotide exchange factor 1
S9923_1861014	28	E7F110	CHST8	Carbohydrate sulfotransferase 8
S9987_617431	12	F1R1R1	HOMER3	Homer protein homolog 3

Supplemental Table 2.1: (Previous page) Functional annotation used for enrichment analysis for significantly associated loci.

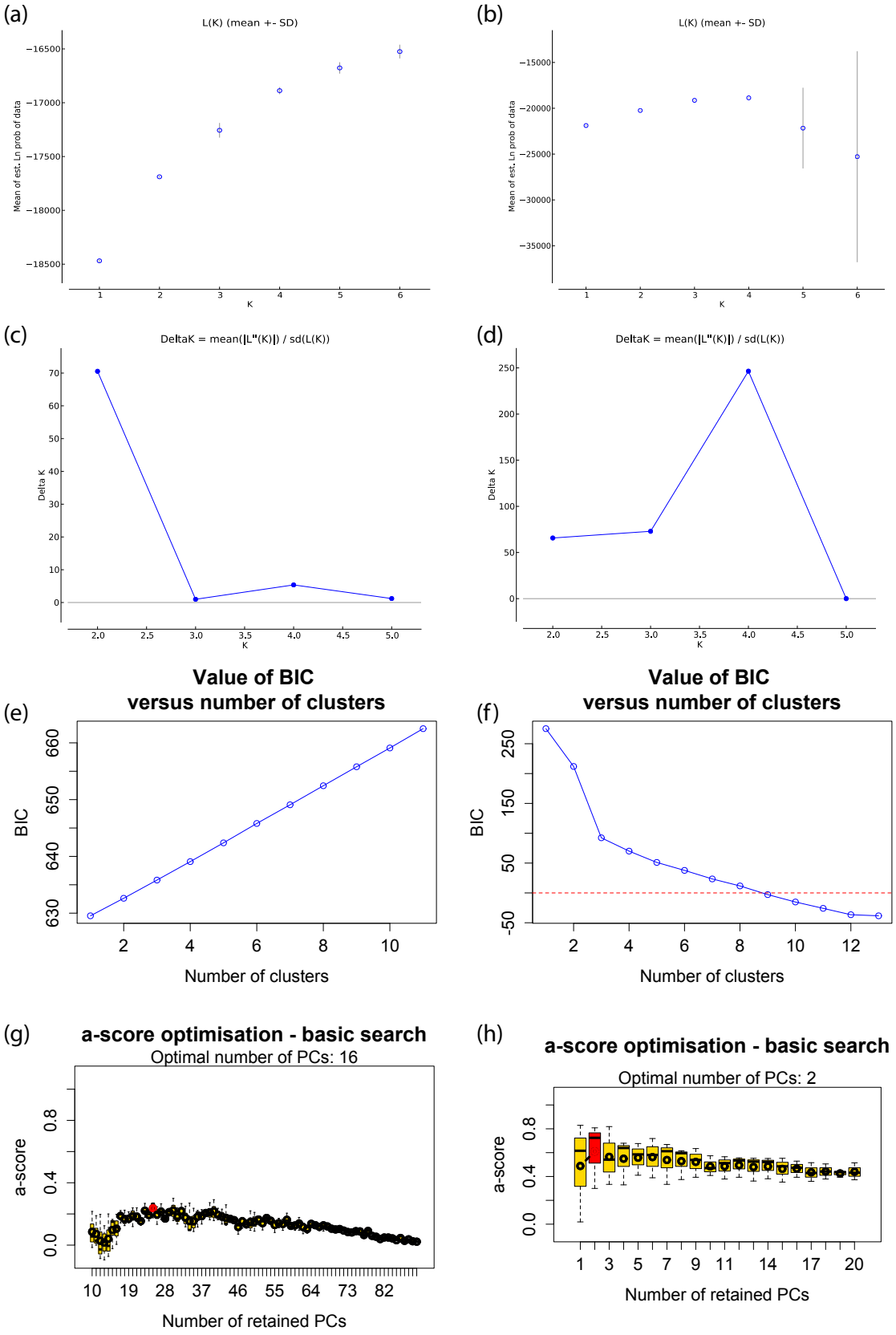


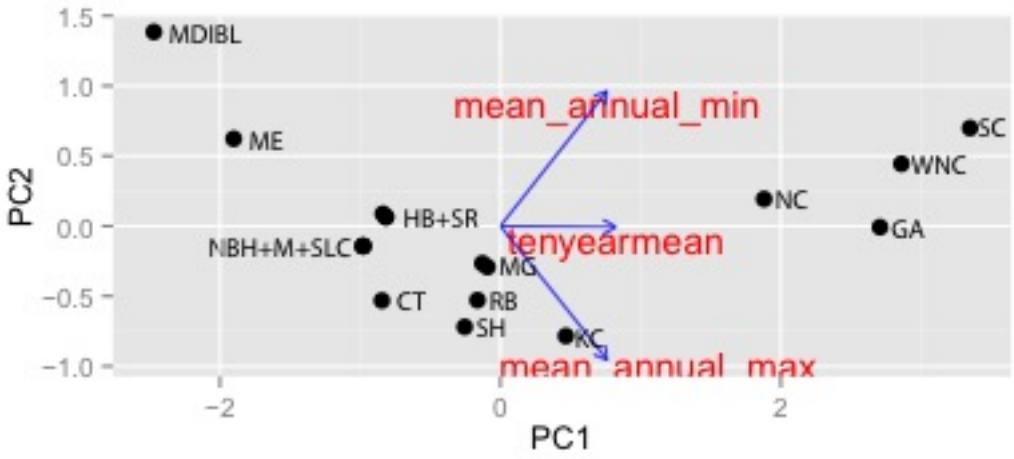
Supplemental Fig. 4.1: Read depth for the fully filtered SNP dataset



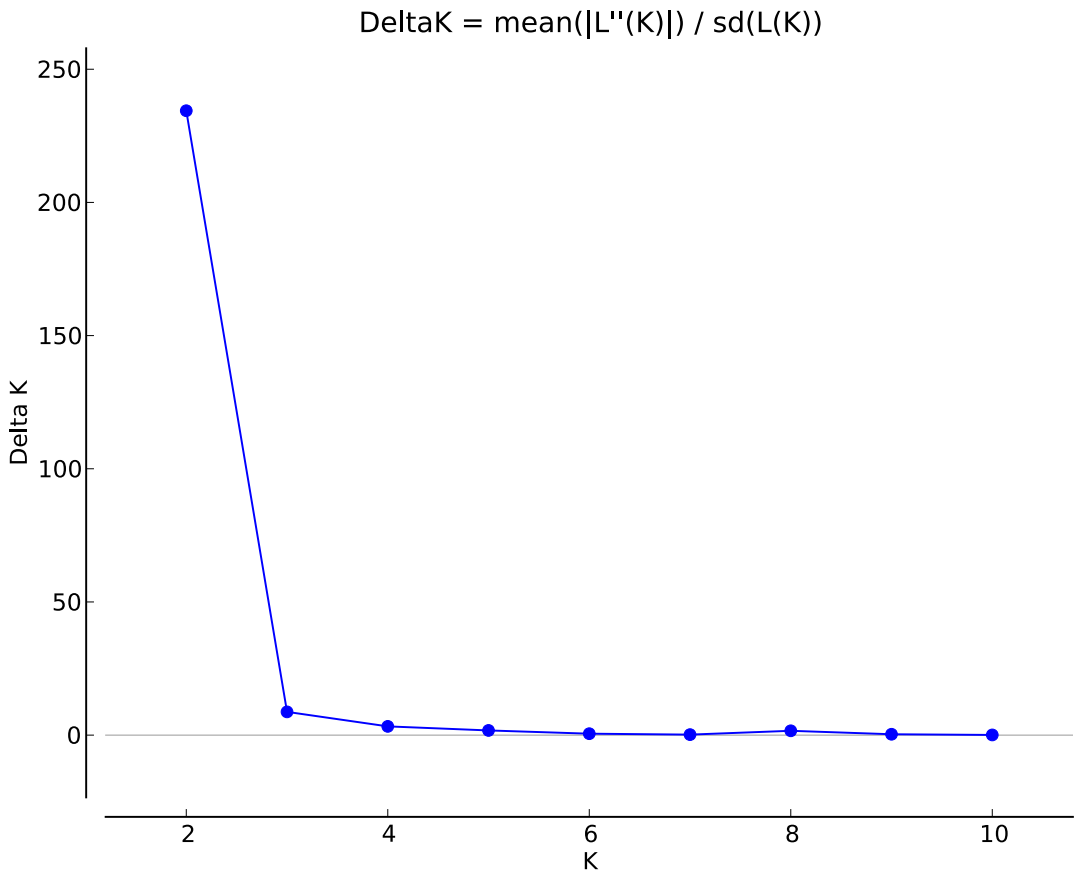
Supplemental Fig. 4.2: Pairwise outlier analysis (lositan) for all comparison within Oyster Creek (a-c) and Brayton Point (d-f) triads. Red loci are outliers before FDR correction, black loci are neutral, frequency distribution for F_{ST} and heterozygosity are reflected across respective axes.

Supplemental Fig. 4.3: (next page) determining ancestral genetic clusters. (a,c,e,g) Oyster Creek; (b,d,f,h) Brayton Point. (a and b) ln likelihood scores from STRUCTURE run across different K. (c and d) Evanno-plot for optimum number of ancestral clusters. (e and f) Bayesian information criterion across genetic clusters from DAPC analysis. (g and h) a-score from DAPC versus numbers of principal components retained in discriminate analysis.

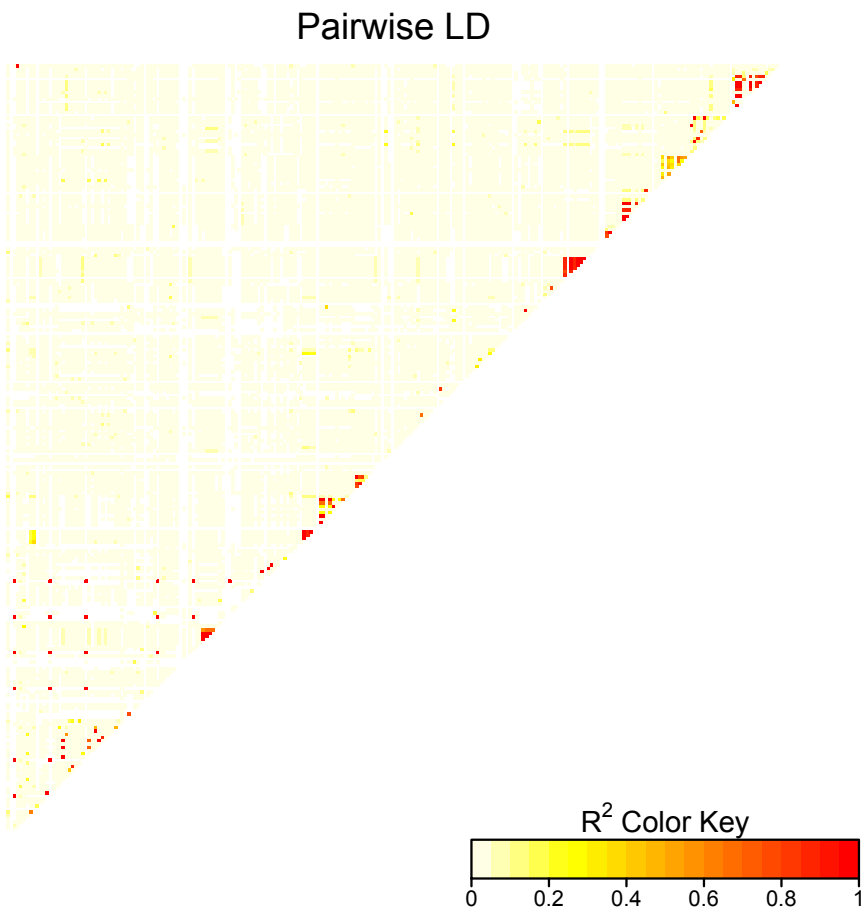

(a)
L(K) (mean +- SD)
Mean of est. Ln prob of data
K
(b)
L(K) (mean +- SD)
Mean of est. Ln prob of data
K
(c)
DeltaK = mean(|L"(K)|) / sd(L(K))
Delta K
K
(d)
DeltaK = mean(|L"(K)|) / sd(L(K))
Delta K
K
(e)
Value of BIC
versus number of clusters
BIC
Number of clusters
(f)
Value of BIC
versus number of clusters
BIC
Number of clusters
(g)
a-score optimisation - basic search
Optimal number of PCs: 16
a-score
Number of retained PCs
(h)
a-score optimisation - basic search
Optimal number of PCs: 2
a-score
Number of retained PCs



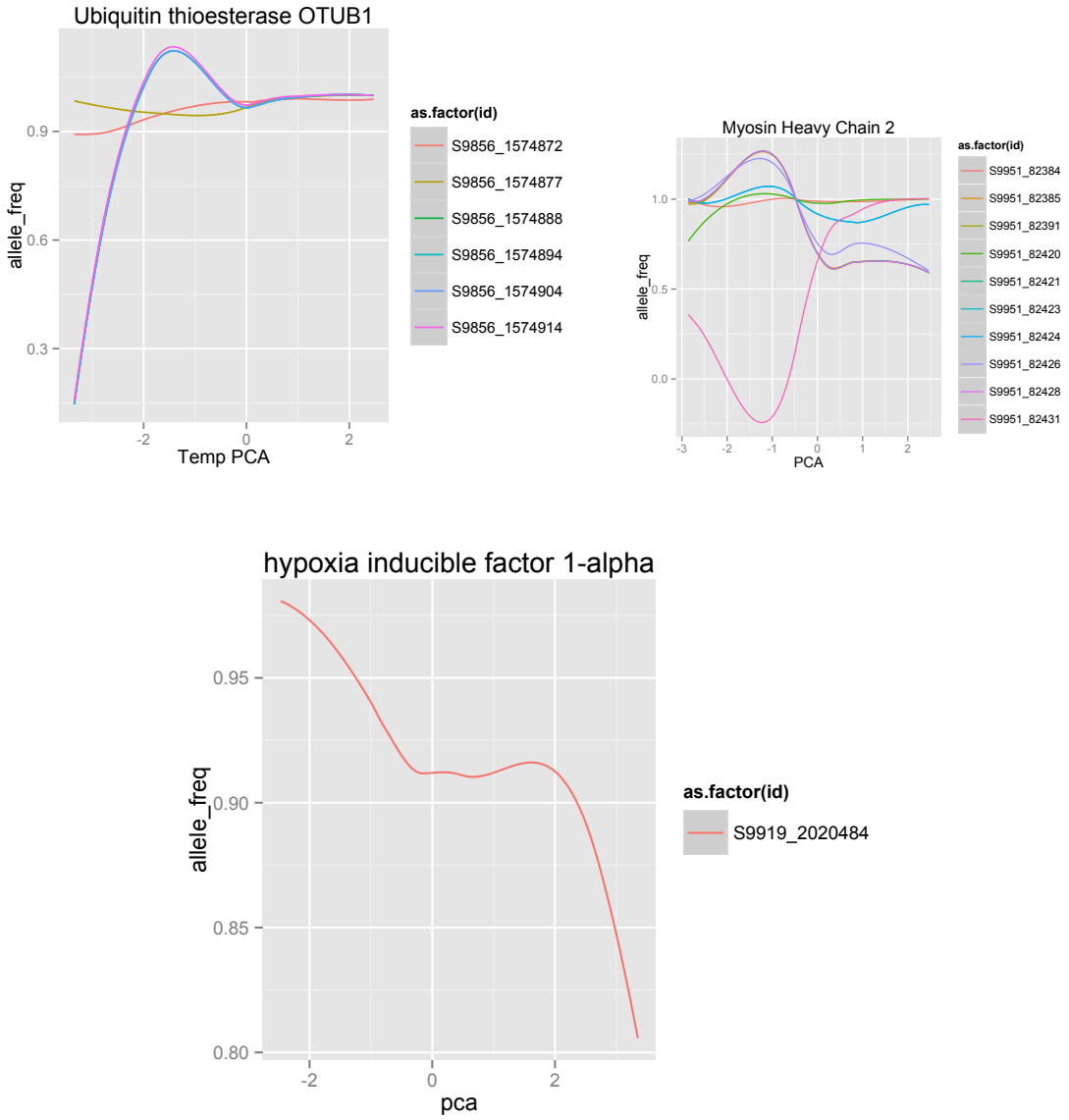
Supplemental Fig. 5.1: Biplot of environmental summary variables, population abbreviations are as in table 5.1.



Supplemental Fig 5.2: Evanno-style plot of best number of ancestral clusters for the species range.



Supplemental Fig. 5.3: Linkage disequilibrium between candidate loci



Supplemental Figure 5.4: Three representative plots of allele frequency shifts across the environmental gradient. Major allele frequency is adjusted so the Northern major allele is plotted as the major allele overall.

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