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Evolution of Two Genomes: Impact of Sequence Divergence on Mitochondrial Function

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EVOLUTION OF TWO GENOMES: IMPACT OF SEQUENCE DIVERGENCE ON MITOCHONDRIAL FUNCTION

By
Tara Zeynep Baris

A DISSERTATION

Submitted to the Faculty
of the University of Miami
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the degree of Doctor of Philosophy

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The oxidative phosphorylation pathway (OxPhos) is the only pathway where proteins are encoded by both the mitochondrial and nuclear genomes. It is also responsible for creating the majority of ATP used by animal cells. The critical role this pathway plays in animal cells, and it’s reliance on two genomes makes it a perfect system to study the co-evolution of the mitochondrial and nuclear genomes. Using Fundulus heteroclitus, an ectotherm inhabiting a steep thermal cline along the eastern coast of the United States, I explore the effect of temperature on the evolution of this important pathway, as well as the associations between nuclear genes and specific mitochondrial haplotypes present in different populations.
I dedicate this first and foremost to Margie Oleksiak and Doug Crawford. I am so fortunate to have had such encouraging, intelligent, supportive, patient, and passionate advisors.

To my family, who have been my rock and biggest source of support.

To all of my friends, near and far, who have provided me with endless amounts of morale boosting events, conversations, and general happiness.

For Celine, you are, and always will be, my source of inspiration.
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CHAPTER 1. INTRODUCTION

1.1 BACKGROUND

Environmental pressures affecting individuals’ abilities to survive and thrive drive adaptive changes in genomes. Although it is simpler to think about how one gene affects adaptation, many biological processes depend on interactions among proteins to work properly. This is especially evident in the OxPhos pathway, which involves 89 different proteins that make up the 5 enzyme complexes needed to make ATP. This pathway is unique because the proteins are encoded by two different genomes, the mitochondrial and nuclear genomes. The mitochondrial genome encodes 13 genes that are part of the OxPhos pathway, and the nuclear genome encodes the other 76 (Table 1.1). Proteins encoded by these two genomes must work together and therefore both the mitochondrial and nuclear genomes must evolve together when faced with new environmental challenges.

Evolved changes in one protein can alter the adaptive landscape, which can then favor a compensatory genetic variation in an interacting protein (4, 35). This may occur more often in OxPhos because the enzymes in this pathway depend on two genomes with different rates of evolution [1,2]. The mitochondrial genome has an especially high mutation rate and greater intra-specific divergence [1-3] than the nuclear genome. This rapid mtDNA evolution is due to high mutation rates, relaxed selective constraints, and limited mtDNA repair mechanisms [3]. The proteins encoded by the mitochondrial genome are part of one of the most essential biological processes in a eukaryotic cell, cellular energy production, and thus changes in the proteins it encodes may alter the evolution of the nuclear genome. These mitochondrial-nuclear interactions serve as a
unique model to study how pathways evolve, and it is this idea that catalyzed the research in this thesis.

What makes these interactions more interesting is that not all OxPhos enzyme complexes have the same reliance on both genomes. Some complexes have primarily mitochondrial-encoded catalytic domains, and other complexes have only nuclear-encoded catalytic domains. Additionally, the mito-nuclear interactions are unique in that there are many differences between the mitochondrial and nuclear genomes. The nuclear genome has two alleles per locus and could have evolved greater functional variation because there is less linkage among nuclear OxPhos genes. Mitochondria have a relatively high mutation rate, strict maternal inheritance, and no recombination, and thus evolution is constrained, forcing selection to act on all 13 protein-encoding genes.

My thesis focuses on the OxPhos pathway, which is integral for proper cell and organismal function. My goal is to better understand how interactions between nuclear and mitochondrial genomes evolve and natural selection's role in shaping these genomes. My research should enhance our understanding of how evolution shapes complex phenotypic traits like metabolism.

1.2 The Mitochondria

The mitochondrion’s unique characteristics influence it’s biology and how evolution by natural selection acts on it. The mitochondrial genome differs from the nuclear genome in that it is circular and only encodes 13 genes that are part of a single metabolic pathway (OxPhos pathway); it also codes for 24 genes which are part of the translational machinery of the mtDNA (22 tRNAs, and 2 rRNAs) (Fig. 1.1) [4]. The
remaining genes required for mitochondrial function (apoptosis, nucleotide biosynthesis, fatty acids metabolism, etc) are encoded by the nuclear genome [5]. The rest of the mitochondrial genome is non-coding, including the D-loop, which contains the heavy and light strand promoters, as well as the origin of replication of the heavy strand. Another shorter non-coding region encompasses the light strand origin of replication [6].

Additionally, the mitochondrial genome does not undergo recombination [7]. This means that all sites on the genome are physically linked, and if selection favors one part of the genome, it affects all other DNA variations in the genome. Therefore, deleterious or selectively important mutations in one protein will reduce the amount of functional variation in the mitochondrial genome through either background selection or selective sweeps, respectively [8,9]. Due to these characteristics, mitochondria have long been thought to accumulate neutral mutations and have been used as neutral markers for determining population structure and evolutionary history. More recently, there have been studies that challenge this idea of mitochondria as a strictly neutral marker and have suggested that mitochondria may be playing a more active role in evolution, with changes in mitochondrial sequences affecting fitness [4,10-16].

Finally, mitochondrial genomes are haploid and only maternally inherited. This means that for every mtDNA passed on to the next generation, four nuclear genomes are passed along. Therefore the effective population size (Ne) for the mitochondrial genome is about a quarter of that of the nuclear genome.

These mitochondrial specific genomic traits (higher mutation rate, few genes that are involved in a single pathway, circular genome without recombination, smaller effective population size) will alter the rate of evolution relative to the nuclear genome.
With a difference between mitochondrial and nuclear genome evolution, there is an expectation that evolution by natural selection will favor compensatory changes in one or both genomes. My thesis examines this concept by quantifying OxPhos physiology within and between populations.

1.3 Oxidative Phosphorylation

Mitochondria provide cells with the majority of energy through the oxidative phosphorylation (OxPhos) pathway. OxPhos is the only pathway where the proteins that form the pathway’s enzymes are encoded by both mitochondrial and nuclear genomes. The OxPhos pathway has 13 mitochondrial-encoded proteins and 76 nuclear-encoded proteins that interact to make up the five enzyme complexes in this pathway (Table 1.1) [17]. Reduced electron carriers (NADH₂, FADH₂) from the TCA cycle provide electrons which enter the pathway, either though Complex I or Complex II. As these electrons are transferred along the chain of complexes, Complexes I, III, and IV pump protons across the inner mitochondrial membrane, building up an electrochemical gradient. Complex V, or ATP synthase, uses this electrochemical force energy to eventually phosphorylate ADP into ATP.

Complex I, NADH dehydrogenase (E.C.: 1.6.5.3), is the first enzyme in the OxPhos pathway. Complex I in vertebrates has 45 protein subunits: 7 mitochondrial and 38 nuclear encoded subunits [18,19]. There are 14 essential catalytic domains; seven are encoded by the mitochondrial genome and 7 are encoded by the nuclear genome [20,21]. Due to the large number of subunits, both mitochondrial and nuclear encoded Complex I subunits could potentially accumulate many polymorphisms. Cooperation of
mitochondrial and nuclear encoded catalytic subunits may affect the evolution of the complex and of the individual subunits [22].

Complex II, Succinate dehydrogenase (EC:1.3.5.1), is the second enzyme in the OxPhos pathway and provides an alternative entry for reduction equivalents. It is the smallest enzyme complex, consisting of only 4 nuclear encoded subunits. This complex is solely dependent on nuclear encoded proteins, and we therefore predict that its evolution will be less affected by mitochondrial-nuclear interactions than the other complexes, which require cooperation between the two genomes.

Complex III, Ubiquinol cytochrome c reductase (EC 1.10.2.2), is the third enzyme in the OxPhos pathway and has three essential catalytic subunits [21,23]. Only one of the three essential catalytic subunits (cytochrome b) is encoded by the mitochondrial genome [21,23]. Since catalytic domains are more conserved than other subunits, there may be greater differences in gene frequencies in the other subunits due to genetic drift.

Complex IV, Cytochrome c oxidase (EC:1.9.3.1), is the terminal enzyme of the electron-transport chain. In Complex IV, only the mitochondrial-encoded subunits perform the electron transfer and proton pumping activities. The functions of the nuclear-encoded subunits are accessory and may play a role in the regulation and assembly of the complex [24]. These nuclear-encoded subunits are less constrained, have less conservation among other organisms, and may accumulate a larger number of polymorphisms. These polymorphisms may be adaptively important.

Complex V, ATP synthase (EC 3.6.3.14), is the last enzyme in the OxPhos pathway and is responsible for ATP synthesis using the energy from the proton gradient created by the other complexes. This complex is made up of 2 mitochondrial proteins and
14 nuclear proteins. Complex V is highly conserved and constrained due to its complex structure and function.

Studying the OxPhos pathway, which is dependent on these two genomes, gives us a better understanding of how interactions between nuclear and mitochondrial genomes evolve and the role of natural selection in shaping these genomes.

1.4 Fundulus heteroclitus

To investigate the evolutionary processes affecting OxPhos, I utilize the adaptive divergence among populations of the teleost fish *Fundulus heteroclitus*. *F. heteroclitus* is distributed along the east coast of the United States where there is a steep thermal cline (approx. 1°C/degree latitude) and northern populations experience temperatures more than 12°C colder than southern populations (Fig. 1.2, Fig. 1.3) [25]. Temperature is one of the most important factors in determining the distribution of ectothermic organisms and directly affects physiological and biochemical processes including catalytic capacity of whole mitochondria and key enzymes in respiration [26-28].

The temperature difference along the thermal cline is thought to drive the adaptive divergence in biochemical and physiological processes of the *F. heteroclitus* populations along the coast. Northern and southern *F. heteroclitus* populations harbor two distinct mitochondrial haplotypes (mt-haplotypes), a “northern” mt-haplotype and a “southern” mt-haplotype differing by five amino acid replacements, (one in ATPase8, two in ND1, one in ND2, and one in ND5) [29]. This genetic divergence between mt-haplotypes is influenced by a historical break at the Hudson River due to the last glaciation [25], enhancing the nucleotide differences between northern and southern populations [30]. In addition to mt-haplotype divergence among *F. heteroclitus* populations, there are
differences in the nuclear genome that alter enzymes and enzyme expression and subsequently affect metabolism [25,31-38]. *F. heteroclitus* has large populations with low migration rates and are adapted to local environmental conditions [39,40]. These demographic and environmental features are likely to affect the nuclear and mitochondrial divergences among *Fundulus* populations [25].

I take advantage of the distinct *Fundulus* population structures to understand the physiological differences between divergent mitochondrial and nuclear genomes and the underlying genetic cause.

To separate demographic processes that will affect the evolution of many genes, from specific interactions between the mitochondrial and nuclear genomes, I focus on a single population with two mt-haplotypes. In a single population where an individual's ancestry does not affect the genetic frequencies of nuclear-mitochondrial genes, there should be a random association between nuclear alleles and mt-haplotype. My research utilizes this within population comparison to investigate how the nuclear alleles influence OxPhos physiology.

**1.5 Temperature**

In ectothermic organisms, environmental temperature plays an important role in shaping enzymatic processes. There are always tradeoffs that exist with adaptation to either warm or cold environments. At cold temperatures, slower enzymatic rates, and slower oxygen diffusion rates are counteracted by an increase in basal metabolic rate [41-44]. In warm environments, basal metabolic rates are slower [45-47]. *F. heteroclitus* populations living along a steep thermal cline provide us with an excellent opportunity to understand adaptive differences within the OxPhos pathway. In *Fundulus* many studies
have provided insight into how acute and acclimation temperature change can alter OxPhos physiology [47-49]. Northern and Southern *F. heteroclitus* populations respond differently to acclimation and acute temperature changes [50]. For example, at low acclimation temperatures, there are minimal differences in mitochondrial activity between the two populations at low assay temperatures, whereas at high assay temperatures, southern individuals have much higher activity. This difference in mitochondrial activity between assay temperatures disappears at high acclimation temperatures [50]. This is a clear example of how the interaction between the environment and the genotype (G x E) (either mitochondrial or nuclear) influences the evolution of OxPhos proteins [51-59]. However, it is also important to consider that the interaction of two genomes with each other and the environment (G x G x E) adds even more complexity to the evolution of this pathway [60-63]. In this dissertation, I try to understand the nuances of G x G x E by exploring how acclimation and acute temperature affect OxPhos physiology both within and between populations. This allows me to tease apart how the interaction of the nuclear and mitochondrial genomes with each other and the environment are contributing to thermal tolerance.

**1.6 mtDNA Effect on Fitness**

Mito-nuclear interactions and their effects on fitness has been an important area of research [1]. This research typically involves comparison among species or among disparate populations. In these comparisons, mtDNA polymorphisms affect life-history traits and fitness. A study among *Drosophila* populations by Ballard *et al.* 2007 [64] showed that genetic variation in the mitochondria was linked to COX activity and affected fitness traits such as egg size and fecundity. Other studies on *D. simulans* have shown that
differing mtDNA types contribute to different levels of pro-oxidant and antioxidant activity with age [65] and differences in proton leak, ATPase activity and mitochondrial cytochrome content [66]. Trans-specific replacement of a mitochondrial haplotype from *D. melanogaster* with that of *D. melanogaster* or *D. simulans* significantly affected life span through epistatic effects. The severity of the effect increased with increased divergence [67]. In studies with the intertidal copepod, *Tigriopsis californicus*, OxPhos disruption and consequent loss of fitness occurs in F3 hybrids from population crosses. OxPhos function is completely restored by backcrossing to the maternal populations and restoring the original mitochondrial background [68]. In wasps, hybridization of *Nasonia giraulti* and *Nasonia vitripennis* resulted in mito-nuclear incompatibilities leading to increased mortality.

As mentioned above, in ectotherms, enzymatic functions are temperature sensitive, and many studies have shown that thermal environments impose selection on mito-nuclear co-adaptation. In *D. simulans*, one mt-haplotype allows for faster recovery from cold coma whereas another is more resistant to starvation [64]. Willett and Burton have shown that in *T. californicus* exposure to different temperature regimes influences selection of the cytochrome c genotype [69]. They suggest that coadaptation and selection influences the outcome of natural hybridizations between populations. For seed beetles, *Callosobruchus maculatus*, the temperature at which beetles are reared influences the time it takes for eggs to develop into adults for certain mitochondrial haplotypes.

These studies show how cooperation between nuclear and mitochondrial genomes affect evolutionary fitness. These studies focus on how species specific or divergent population mitochondria are disrupted when placed in a different nuclear background.
Very few studies have examined whether mito-nuclear interactions within a population alter OxPhos. This avenue of research is important because it provides insights into the evolutionary significance of mito-nuclear interactions without the confounding effect of demography and neutral drift. Additionally, it is more likely to reflect the human condition where many different mt-haplotypes occur in a single population [70].

1.7 Objectives

This dissertation aims to elucidate how mitochondrial polymorphisms with different nuclear backgrounds affect one of the most important pathways, OxPhos. 1) I measure physiological differences in OxPhos between populations to understand how environmental factors have shaped the evolution of the pathway. 2) I measure physiological differences in OxPhos in a single population with two mt-haplotypes, to understand the influence of mt-haplotype. 3) I explore how mitochondrial nuclear interactions can alter allele frequencies in the nuclear genome and how this affects OxPhos function.

Chapter 2

In chapter 2, my thesis explores differences in mitochondrial activity between two distant populations that are subjected to very different thermal environments. Northern and Southern *F. heteroclitus* populations from ME and GA, respectively, were used to explore differences within species, and the sister taxa, *F. grandis* was used to explore differences between species. Northern populations are exposed to temperatures 12°C colder than southern populations. *F. grandis* populations experience similar temperatures to southern *F. heteroclitus* populations. *Fundulus* populations living along a thermal cline
are predicted to have biologically adaptive differences that enable optimal OxPhos function at different temperatures.

My research seeks to understand how genetic differences, acclimation temperature, and acute temperature changes interact in the OxPhos pathway in these populations and how the OxPhos pathway as a whole has adapted to different thermal environments. In short, is there adaptive divergence between mitochondria? My expectation was that the *F. grandis* population and the southern *F. heteroclitus* populations would have similar adaptations to warm temperatures and would therefore have similar patterns of OxPhos activity. What I found instead, was that the two *F. heteroclitus* populations had more similar OxPhos activities than the *F. grandis* population. Environmental factors, such as acclimation and acute temperature, were more important in influencing OxPhos levels than population. This finding is in contrast with my research within a population (Chapters 3 and 4). Thus, in hindsight, the lack of difference between the colder northern Maine population and the warmer southern Georgia population, which have two different mt-haplotypes, most likely involves compensatory changes in the nuclear genome to reach optimum activity in their respective environments.

**Chapter 3**

Chapter 2 examined OxPhos between two divergent populations, and showed that population differences minimally affected OxPhos physiology. In this chapter, a single interbreeding population with two mitochondrial haplotypes was studied. Examining both mt-haplotypes in a single population removed demographic effects and randomized the nuclear-mitochondrial linkage in the two latitudinal extreme populations. That is, the Maine and Georgia populations have two different mitochondrial haplotypes that exist
with many different nuclear changes, which have evolved by neutral processes, and potentially many environmental adaptive changes, and thus there are many causes affecting both genomes in addition to mito-nuclear interactions. A single well mixed population is a better system to examine nuclear-mitochondrial interactions because nuclear allelic variation should be randomly associated with mt-haplotype. In the single New Jersey population, the mt-haplotype has a significant effect on OxPhos metabolism. This difference in OxPhos activity between individuals with different mt-haplotypes was most evident when individuals were acclimated to 28°C and measured at three different acute temperatures, indicating a strong G x E interaction. Overall, there was a pattern of higher OxPhos activity for individuals with the southern mt-haplotype, with this difference being more evident at a higher acclimation temperature and higher acute measurement temperature. I therefore concluded that differences between mt-haplotypes are most apparent within a population, where there may not be any compensatory changes within the nuclear genome.

**Chapter 4**

OxPhos between individuals with different mitochondrial haplotypes in an interbreeding population is not solely reliant on the mitochondria but also includes the effect of nuclear genes on this pathway. To explore if associations existed between the mt-haplotype and the nuclear genome, I examined the genetic variation at 11,705 loci using genotyping by sequencing (GBS) [71]. Among these eleven thousand single nucleotide polymorphisms (SNPs) were 349 SNPs that had a significant association with mt-haplotype. This association was not due to recent admixture (demography) and is most parsimoniously explained by evolution by natural selection. Importantly, these 349 SNPs
explained a significant fraction of OxPhos function: OxPhos activity of individuals with these two mt-haplotypes depends on the alleles at these 349 loci \((G \times G \times E)\). These significantly associated genes are acting upstream of OxPhos, and I postulate that these associations are due to epistatic selection.

1.8 Overall

The research in my thesis demonstrates population differences in OxPhos physiology that are a function of acclimation temperature and acute assay temperatures. These data suggest that the evolution of physiological processes creates complex changes that are dependent on both the recent thermal environment (acclimation) and the acute changes in the environment. Importantly, this gene by environmental interaction is modulated by interactions between the genome. Specifically, individuals with northern mt-haplotypes have lower OxPhos activity at high temperatures, but this is modulated by the allelic variation at 349 nuclear loci. Surprisingly, these 349 allelic variants are not part of the 89 OxPhos proteins but instead are upstream of OxPhos pathway. These data point out the complexity of evolution and evolutionary adaptation and suggest that adaptation involves many genes with substantial standing genetic variation.
Figure 1.1 Mitochondrial DNA
Circular mitochondrial DNA containing 13 genes that are part of the OxPhos pathway, two rRNAs, and D-loop containing the origin of replication [12].
Figure 1.2 Map of *Fundulus heteroclitus* populations

Four *Fundulus* populations with mean annual temperatures. *F. heteroclitus* populations are from Maine (ME) and Georgia (GA), and New Jersey (NJ). *F. grandis* is from the Florida panhandle in the Gulf of Mexico.

![Map of Fundulus heteroclitus populations](image)

Figure 1.3 Thermal Cline along the east coast of United States

There is a linear relationship where for every degree latitude change there is a 1°C temperature change.
CHAPTER 2. ACCLIMATION AND ACUTE TEMPERATURE EFFECTS ON POPULATION DIFFERENCES IN OXIDATIVE PHOSPHORYLATION

2.1 SUMMARY

Temperature changes affect metabolism on acute, acclimatory and evolutionary time scales. To better understand temperature’s affect on metabolism at these different time scales, we quantified cardiac oxidative phosphorylation (OxPhos) in three Fundulus taxa acclimated to 12°C and 28°C and measured at three acute temperatures (12°C, 20°C, and 28°C). The Fundulus taxa (northern Maine and southern Georgia F. heteroclitus, and a sister taxa, F. grandis) were used to identify evolved changes in OxPhos. Cardiac OxPhos metabolism was quantified by measuring six traits: State 3 (ADP and substrate dependent mitochondrial respiration), E State (uncoupled mitochondrial activity), Complex I, II, and IV activities, and LEAK ratio. Acute temperature affected all OxPhos traits. Acclimation only significantly affected State 3 and LEAK ratio. Populations were significantly different for State 3. In addition to direct effects, there were significant interactions between acclimation and population for Complex I and between population and acute temperature for State 3. Further analyses suggest that acclimation alters the acute temperature response for State 3, E State, and Complexes I and II: at the low acclimation temperature, the acute response was dampened at low assay temperatures, and at the high acclimation temperature, the acute response was dampened at high assay temperatures. Closer examination of the data also suggests that differences in State 3 respiration and Complex I activity between populations were greatest between fish acclimated to low temperatures when assayed at high temperatures, suggesting that differences between the populations become more apparent at the edges of their thermal range.
2.2 INTRODUCTORY REMARKS

Mitochondria produce the majority of cellular ATP through the oxidative phosphorylation (OxPhos) pathway. The OxPhos pathway comprises five enzyme complexes; these complexes contain 89 proteins encoded by 76 nuclear and all 13 mitochondrial protein genes [72]. A diversity of ectotherms have adaptive nucleotide substitutions in the mitochondrial OxPhos proteins, which are affected by thermal environments [51,53,54,56,57,73-76]. OxPhos function is also affected by adaptive differences in mRNA expression in *F. heteroclitus* [34,77,78]. The mRNA expression changes are related to changes in protein expression [79] and explain up to 82% of the variation in cardiac metabolism [35]. These data showing adaptive changes in animals from flies to humans highlight OxPhos' biological importance. Yet, OxPhos' adaptive importance is a function of environmental conditions, especially temperature [44,80], and these environmental conditions could modulate the importance of evolved differences among populations [81].

Lower temperatures decrease chemical reactions and diffusion rates. Thus, at cold temperatures, ectotherms need to counteract slower enzymatic reactions and oxygen diffusion rates to maintain normal physiological function [31,41,42]. In contrast, warm temperatures increase basal metabolic rates, which results in a higher oxygen demand and leads to reduced aerobic scope [44]. Consequently, tradeoffs exist for responses to cold and warm environmental temperatures. In cold environments, organisms tend to increase their basal metabolic rate, while warm environments require a decreased basal metabolic rate [45-47]. These acclimation differences (increases at cold and decreases at warm acclimation temperatures) can affect acute responses; specifically, acclimation alters the
temperature response curve [82-84]: warm acclimation shifts the preferred temperatures to the right [85], increases the critical thermal maximum (CTmax) [86] and reduces the effect (decrease in the slope) of acute temperature for mitochondrial respiration [47,87] or critical swimming speeds [50]. These thermal performance differences reflect metabolic changes due to genetic differences among populations as well as both acclimation and acute physiological responses [82,84].

Metabolic rate depends on mitochondrial function, which is affected by both physiological acclimation and evolved changes. *F. heteroclitus* is distributed along a steep thermal cline (approx. 1°C/degree latitude; Fig. 2.1) where two major mitochondrial haplotypes with five non-synonymous substitutions co-occur. A “northern” haplotype, common in populations north of the Hudson River, and a “southern” haplotype, common in populations south of the Hudson River [88]. In previous studies, cold temperature acclimation enhanced northern *F. heteroclitus* mitochondrial respiration levels more than that of its warmer southern counterpart, and acclimation altered the acute temperature effect [87]. These data support earlier findings [36,89], that physiological acclimation has little phylogenetic constraint, and further suggest that physiological adjustment alters the acute response. Based on these observations, one should expect significant interaction between acute temperature change, acclimation, and evolved differences among populations as related to OxPhos metabolism.

Differences between organisms in cold and warm environments should be reflected in OxPhos because of its importance in ATP production. Thus, we expect *F. heteroclitus* individuals living along a thermal cline to have biologically adaptive differences that enable optimal OxPhos function at different temperatures. To better understand OxPhos
function in different thermal environments, we investigated acclimation and acute
temperature effects in different *Fundulus* populations. Specifically, we investigated the
effect of acclimation to 12°C and 28°C in three *Fundulus* taxa (populations or species
groups): northern and southern *F. heteroclitus* populations and a *F. grandis* population.
Northern and southern *F. heteroclitus* populations were used to explore differences within
species, and *F. grandis* was included to explore differences between species (Fig. 2.1).
We investigated how acclimation temperature modulates acute temperature effects by
quantifying OxPhos function in heart ventricles at three assay temperatures (12°C, 20°C
and 28°C). These three temperatures represent the mean spring and summer temperature
range for natural *Fundulus* populations. Because *F. grandis* populations naturally
experience temperatures similar to the *F. heteroclitus* GA populations, we expected
similar acclimation and acute temperature effects despite the greater phylogenetic
distance. Heart mitochondrial function is an important indicator of an organism’s ability to
adapt to different temperatures and constrains thermal range expansion [90,91]. The data
presented here add to the understanding of temperature's effect on mitochondrial
respiration by providing data on six OxPhos respiration parameters (State 3, E State,
Complexes I, II and IV, and LEAK ratio). Furthermore, similar to previous published data
on mitochondrial respiration [47,87], these data demonstrate that acclimation temperature
affects acute temperature response. There are two surprising results from our studies not
captured in previous reviews [82,84]: 1) while evolved differences between populations
alter thermal response, these differences are only evident at specific acclimation
temperatures (for State 3, E State and Complex I) and most importantly, 2) the acute
temperature response is muted for temperatures similar to the acclimation temperature (for
State 3, E State, and Complexes I and II). These results help explain how acclimation tends to mitigate acute responses [84].

2.3 MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Adult *F. heteroclitus* were collected during the summer months from northern and southern locations. The northern individuals were collected from two locations, Wiscasset, ME (43° 57’ 15.10”N, 69° 43’ 13.64”W) and Mount Desert Island, ME (44° 25’ 29.04”N, 68° 19’38.83”W), but these two collections were treated as one northern population. The southern population was collected from Sapelo Island, GA (31° 27’ 13.39”N 81° 21’47.65”W). The sister taxa, *F. grandis* was collected from Crawfordville, FL (30 04’48.72” N, 84 10’43.47”W). The southern 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 northern Maine population experiences mean summer temperatures of 17.4°C (calculated from NOAA NERRS Sapelo Island, GA, Apalachicola, FL and Wells, ME). The warm-adapted southern populations experience daily average seawater temperatures ranging from 7°C to 31°C while the cold-adapted northern population experiences temperatures ranging from -1.4°C to 28°C (NOAA NERRS).

All fish were kept at 20°C for 4 weeks, then acclimated to either 12°C or 28°C for 4 weeks. They were exposed to a 14 hour light cycle, kept at 15ppt salinity and fed twice a day, 7 days a week. Housing and protocols were in compliance with the University of Miami Institutional Animal Care and Use Committee (IACUC) guidelines.
Fieldwork was completed within publically available lands and no permission was required for access. *F. heteroclitus* does not have endangered or protected status, and small marine minnows do not require collecting permits for non-commercial purposes. All fish were captured in minnow traps with little stress and removed in less than 1 hour. IACUC approved procedures were used for acclimation and non-surgical tissue sampling. Fish were sacrificed by pithing and decapitation, using procedures approved by IACUC.

**HIGH-RESOLUTION RESPIROMETRY**

**Tissue Permeabilization**

Heart ventricles were dissected, cut into quarters, and three quarters were placed into a muscle relaxation solution (10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl₂, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) [92]. The last quarter was saved for future RNA work. The tissues were too small to accurately weigh. Hearts were cut anteriorly/posteriorly and dorsally/ventrally, and pieces were randomized for each assay. The amount of tissue in the respirometer was estimated by determining the DNA concentration for each quarter (see below). Prior to measuring respiration, each tissue was permeabilized using 0.25% saponin solution for 15 minutes, followed by 4 washes in relaxation solution for 5 minutes each [93]. Once permeabilized, tissues were immediately transferred to the respirometry chambers containing a respiration medium (5mM EGTA, 3mM MgCl₂.6H₂O, 60mM K-lactobionate 20mM Taurine, 10mM KH₂PO₄, 20mM HEPES, 110mM Sucrose, 0.1% BSA).
**OxPhos Determinations**

The acute temperature effect on mitochondrial activity was measured at three temperatures, 12°C, 20°C, and 28°C in Miro5 media (EGTA, 0.5 mM; MgCl₂, 3 mM; K-lactobionate, 60 mM; taurine, 20 mM; KH₂PO₄, 10 mM; Hepes, 20 mM, pH 7.1; sucrose, 110 mM; BSA, essentially fatty acid free, 0.1%). The pH of Hepes at 20°C was 7.1, and the pH changed by approximately 0.11 pH unit at 12°C (+0.11) and 28°C (-0.11). Activity was measured and analyzed using the Oxygraph 2-k and DatLab software (Oroboros Instruments, Innsbruck, Austria). Oxygen sensors were calibrated by marking air saturation and zero oxygen concentration before all assays. Air saturation for calibration was achieved by adding Miro5 medium then allowing oxygen concentration to stabilize. Zero oxygen concentration was achieved by adding Na-dithionite. One quarter of the heart from each fish was used to measure activity at each of the three assay temperatures. Therefore, each fish was measured at all assay temperatures. For measurements, the order of the population, acclimation temperature, and acute temperature were all randomized.

After tissue addition to the respiration chamber, pyruvate (5 mM), glutamate (10mM), and succinate (10mM) were added to reach State 2 levels. This was followed by the addition of ADP (5mM, State 3), cytochrome c (10µM, to check mitochondrial membrane integrity), oligomycin (2µg/ml, to block Complex V), FCCP (a mitochondrial uncoupler used to reach E State; 0.5µM was added sequentially until activity no longer increased), rotenone (0.5µM to block Complex I), malonate (5mM to block Complex II), Antimycin A (2.5µM to block Complex III), and finally TMPD and ascorbate (artificial substrate for Complex IV, 0.5mM TMPD and 0.2mM ascorbate; these were kept in separate tubes) (Table 2.1). Background oxygen consumption levels that could arise from
TMDP and ascorbate were not measured and thus, if significant, could mask significant effects. For our study, we are measuring “LEAK ratio”, which we define as State 2/State 3. Alternatively it can also be calculated as State 4/State 3 since State 2 and State 4 are equivalent (no ADP, depletion of ADP, respectively) [94].

Cytochrome c addition tests whether mitochondrial membranes were damaged during tissue isolation and cell permeabilization and was performed for each assay. Assays in which cytochrome c levels differed from State 3 by more than 10% were discarded. Although chambers were not made hyperoxic, oxygen levels remained above 100 nMole/ml, and these lower oxygen levels had no effect on State 3 respiration (n = 7, paired t-Test p-val > 0.25, where the pairs comparison is between the same individual at normoxia versus 20% oxygen).

The tissue from each chamber was recovered after respiration assays, homogenized, and total DNA was quantified from the homogenate using AccuBlue high sensitivity dsDNA quantitation solution (BIOTIUM, Hayward, CA). All activity was normalized by ng of DNA. OxPhos metabolic rates are reported as pmol O₂ s⁻¹ ml⁻¹ per ng DNA. Although DNA concentration, protein concentration and cell count have a linear relationship, DNA has been found to be the most consistent way of normalizing metabolomics data across a range of cell numbers [95]. Since we had small amounts of tissue that we permeabilized before assays, we recovered the tissue from the respiration chambers as the most accurate way to quantify tissue amount. Since our tissues were too small to accurately weigh, and because the respiration medium contained BSA (which interferes with protein assays), we used DNA amount to normalize.
E State activity, induced by the uncoupler FCCP, unexpectedly did not reach State 3 levels if oligomycin had been used to block Complex V (ATP synthase) activity. Four different types of oligomycin and three different types of FCCP were tested for optimum E State activity. Oligomycin A from two different companies (Enzo lifesciences and TOCRIS bioscience), oligomycin B (ENZO lifesciences), and a mixture of oligomycin A, B and C (Sigma Aldrich) were tested in combination with FCCP from three different companies (Enzo lifesciences, TOCRIS biosciences and Sigma Aldrich). The oligomycin mixture and the FCCP from Sigma Aldrich were the most successful combination that brought FCCP levels close to, or higher, than State 3. The other combinations had lower E States relative to State 3. Therefore, for our study we used the mixture of oligomycin and the FCCP from Sigma Aldrich. The reason E State did not exceed State 3 is likely due to the interaction with oligomycin; when FCCP is added without oligomycin, respiration levels always equaled or exceeded State 3 levels (data not shown). FCCP starting concentrations were not too high, as FCCP was sequentially added with observed increases in E State after each addition. All data used in analyses had E State values that were at least 80% of ADP levels. Contributions to OxPhos by enzyme complexes (I, II, and IV) were measured by sequential addition of complex specific poisons. However, the starting rate is that of the E State (Complex I = E State – rotenone) (Table 2.1); thus these measures may reflect the additional effect of oligomycin (less than 20% of State 3).

The respiratory control ratio (RCR) is used to quality check tissues used for respiration analysis. Appropriate RCR ratios change depending on organism and tissue type [96]. We included all data with an RCR of 2.5 or higher (Fig. 2.2). These RCR values are consistent with other publications using heart tissues in ectotherms [97,98].
**Statistical Analyses**

A 3-way mixed model analysis was performed with all factors: population, acclimation temperature, and assay temperature (JMP, SAS, Cary NC). Body mass (Table 2.2, 2.3) often affects metabolic physiology [99,100], and to correct for variation in body masses, we included body mass as a covariate in our mixed model.

The analysis was performed on six important traits: State 3 (substrates and ADP), E State (uncoupled mitochondrial activity with FCCP), Complex I activity, Complex II activity, Complex IV activity and LEAK ratio. LEAK reflects the number of protons that “leak” across the membrane where the creation of a proton gradient consumes O₂ but does not produce ATP [94]. In our study LEAK ratio is measured as State 2/State3 (Table 2.1).

Although the 3-way mixed model analysis gives us a comprehensive overview (Table 2.4, Fig. 2.3), to explore the more intricate patterns that exist between treatments, we performed separate analyses to examine patterns between two treatments with the third factor as a covariate or random factor. Notice that we are using 2-way ANOVAs with one treatment as a covariate to better understand the data, yet with these analyses, there are three ANOVAs for acute temperature effects, three ANOVAs for population, and two ANOVAs for acclimation. If we were to provide multiple correction for these separate ANOVAs, a Bonferroni’s corrected critical p-value of 0.05, would equal 0.017 for acute and population effects or 0.025 for acclimation. There is no appreciable difference in our results using this more stringent p-value.

In the figures, the data for these analyses are presented with untransformed numbers for clarity, but statistical analyses were performed with body mass and other factors as covariates. A linear regression was used to analyze each population's response to acute
temperature change. Clearly, acute responses typically have an exponential rise [82,84], but for our limited range of acute temperatures a linear model was found to be the best fit. 

Post-hoc analyses were performed using Tukey’s test, which corrects for multiple comparisons, to identify which means are different.

2.4 RESULTS

The six OxPhos functions are quantified by measuring oxygen consumption rates with substrate addition, or poison inhibition (Table 2.1): State 3 (ADP stimulated respiration), E State (uncoupled mitochondrial activity with the dissolution of the $H^+$ gradient), Complexes I, II and IV, and LEAK ratio (respiration limited by the leakage of $H^+$ back into the mitochondrial matrix). DNA concentration was used to correct for differences in the amount of tissue used for each assay.

Cytochrome c addition did not affect respiration [the average CytC/State 3 ratio was 1.03, (sem. 0.009)], indicating the functional integrity of the outer mitochondrial membranes. A separate measure of functional coupling for OxPhos is RCR (State3/State2). RCR determinations, which measure respiration dependency on ADP, are shown in figure 2.2. The mean RCR was 4.14 (sem. 0.26) across all groups, indicating intact OxPhos respiratory chain.

Table 2.2 provides the sample sizes (for individuals used in analysis) and body masses for the three populations: ME and GA $F$. heteroclitus and $F$. grandis from the Gulf of Mexico (Fg). Body masses ranged from 3.2 to 25.9 grams. Body mass often affects metabolic physiology [99,100], and to correct for variation in body masses, we included body mass as a covariate in our mixed model. The effect of body mass was only significant for Complex I (p-val < 0.05), and the p-values for body mass effects for State 3
and E State are between 0.05 and 0.1 (Table 2.3). We use body mass as a covariate in order to avoid potential population bias due to body mass, and to keep all analyses consistent.

**OXPHOS FUNCTIONS**

We performed a mixed model analysis using acclimation temperature, population and acute temperature change and their interactions (Table 2.3). Acute temperature significantly affected all six OxPhos determinations. Acclimation and population had significant effects for State 3, and additionally, acclimation had a significant effect on LEAK ratio. The interaction of acclimation and population was significant for Complex I and the interaction of population and assay temperature was significant for State 3. All data is present in figure 2.3. Tukey’s post-hoc test reveals significant differences among all three acute temperatures for all six OxPhos functions except LEAK. For LEAK, 12°C is different from 28°C.

**Acute and Acclimation Temperature Interactions**

To better understand the effect of acclimation temperature on the acute response, we minimized the effect of populations by using mixed model residuals with population as a covariate and compared the effect of acute temperature for each of the two acclimation temperatures. The p-values for these analyses are shown in Figure 2.4, but the data graphed are raw values (i.e., graphs do not use residual values, and graphs with residuals are not substantially different). For State 3, E State and Complexes I and II, these analyses show a consistent pattern: assays at 12°C and 20°C are similar at the low acclimation temperature, and assays at 20°C and 28°C are similar at the high acclimation temperature (see “aab”/”dcc” significance patterns, Fig. 2.4). Complex IV and LEAK show a change
with acute temperature, but with little difference between acclimation temperatures. These patterns for State 3, E State and Complexes I and II suggest that with cold acclimation, cooler assay temperatures have less effect and with warm acclimation, warmer assay temperatures have less effect. If we include population in the analysis (Fig. 2.3), we can see that this pattern is still evident for most populations at each measured trait. Thus, even though acclimation only significantly affects State 3 and LEAK in our mixed model (Table 2.3), acclimation may play a role in OxPhos sensitivity to acute temperature changes.

**Acute Temperature and Population Interactions**

To better understand the difference among populations, we statistically minimized the acclimation effect by using the mixed model residuals with acclimation as a covariate. While the graphs (Fig. 2.5) display raw values (i.e., not using the residuals), the p-values reflect differences in slopes between each population using these acclimation residuals. In general, acute temperature change affected all six OxPhos traits (Table 2.3). However, when broken down by population, acute response differences were observed among populations. This was determined by comparing linear regression slopes between assay temperature and population. The acute response for *F. grandis* was significantly stronger for State 3 and Complex I, but the trend is seen in all other traits, excluding LEAK. For the ME *F. heteroclitus* population, the acute temperature change slope was similar to the slope for the GA *F. heteroclitus* population. Complex II was the only trait where, unlike for GA *F. heteroclitus* and *F. grandis*, the slope for ME *F. heteroclitus* was marginally insignificant. With the exception of LEAK, *F. grandis* always had the steeper slope with
the largest deviation from the two *F. heteroclitus* populations occurring at the 28°C measurement.

**Acclimation and Population Interactions**

Acclimation effects were significant for State 3 and LEAK (Table 2.3). Because acclimation and population showed a significant interaction for Complex I, we examined their relationship separately by using residuals from assay temperature. This approach is similar to correcting for body mass, and appears to be appropriate because these residuals are unaffected by assay temperatures. The significance of the differences among populations or between acclimation temperatures is shown in Table 2.4. Significant OxPhos differences among populations occur only at the 12°C acclimation temperature; there are no significant differences among populations at 28°C acclimation (Table 2.4A, Fig. 2.6). At 12°C acclimation, three OxPhos functions are significantly different among populations: State 3, E State and Complex I. In all cases ME is less than Fg, and GA is intermediate (Fig. 2.6). Thus, differences among populations are a function of acclimation temperature.

When looking at how acclimation affects the different populations, there was no significant acclimation effect on OxPhos respiration in the two *F. heteroclitus* populations (Table 2.4B). However, acclimation did significantly affect State 3 and Complex I in the *F. grandis* population; therefore *F. grandis* is the driving force for the significant differences between acclimation temperatures.
2.5 DISCUSSION

To investigate the effect of temperature on adaptation of cardiac metabolism, we examined six different OxPhos functions (Table 2.1 and 2.3) among three Fundulus populations (northern and southern F. heteroclitus and F. grandis) acclimated to two temperatures and assayed at three acute temperatures. In general, acute temperature affected OxPhos function (Fig. 2.3, Table 2.3), and acclimation temperature had a significant effect on State 3 and LEAK ratio, while population only had a significant effect on State 3. There were also significant interactions between acclimation and population for Complex I and between population and acute temperature for State 3. Yet, the influence of acclimation on both acute temperature sensitivity and differences among populations was seen in subsequent ANOVA analyses, where only acclimation and acute temperature were considered (Fig. 2.4 and 2.6). These analyses revealed that when population is removed as a parameter (through the use of residuals), the acute temperature response curve shape was dependent on acclimation temperature (Fig. 2.4). In addition, significant population differences are restricted to low acclimation temperatures (Fig. 2.6), are larger at higher acute temperatures (Fig. 2.5), and mostly reflect genetic distance: ME F. heteroclitus < GA F. heteroclitus < F. grandis. These patterns are similar to those for State 3 measured in livers: there are few differences among fish acclimated to 5°, 15° and 25°C, and differences between ME and GA populations were more apparent when acclimated to low temperatures and assayed at 25°C and above [87]. Chung et al. [47], showed that in a northern population from Nova Scotia, acclimated to 33°C, State 3 respiration is reduced overall, and acclimation to this high temperature reduced the acute temperature effects.
State 3 showed an interaction between population and acute temperature in our mixed model analysis (Table 2.3). When acclimation was removed as a parameter (using residuals), the slope of the acute response was different between *F. heteroclitus* (ME and GA) and *F. grandis* (Fig. 2.4, Fig. 2.5, Table 2.4B) although this is only significant for State 3 when all parameters were included (Table 2.3, Fig. 2.3). Acclimation only had a significant effect on OxPhos function for State 3 and Leak ratio (Fig. 2.3, Tables 2.3 and 2.4). These data suggest that both acclimation and differences among populations account for changes in acute responses such that in the ME *F. heteroclitus* population, a rapid or acute temperature change results in a less dramatic increase in metabolic demand at seasonal low temperatures than it would for *F. grandis*. Our supposition is that these conditions (low, long-term or seasonal temperatures with high acute temperatures) expose potential evolutionary differences that define a species' thermal niche space.

**ACUTE TEMPERATURE RESPONSES**

One of the more important observations presented here is how acclimation alters the acute response curve shape. Thermal performance curves provide insights into the environmental-genetic interactions that shape an organism’s physiology [82-84]. Schulte makes an important distinction between acute and acclimation thermal response: acute responses provide insight into the biochemistry and biophysics of temperature effects, and acclimation tends to mitigate these responses [82,84]. That is, acclimation is a physiological process that compensates for the chemical and physical temperature effects. Our data support this supposition. For the data presented here, we only have three acute temperatures, all within the normal range of *Fundulus’* thermal environment. While these three acute temperatures do not provide the breadth of temperatures needed to fully
describe a thermal performance curve [83,84], they do provide insight on how acclimation alters an acute response.

Acclimation, while rarely a significant factor for the six OxPhos functions, mitigated acute temperature effects when measured near the acclimation temperatures (Fig. 2.4). Thus, there are no significant acute temperature effects at low temperatures using cardiac tissue from fish acclimated to low temperature, nor between high acute temperatures when fish were acclimated to high temperature. Specifically, acute temperature significantly affected all six OxPhos functions, yet for State 3, E State and Complexes I and II, there is little difference between 12°C and 20°C OxPhos function when fish were acclimated to 12°C, nor did acute temperature have much affect on 20°C and 28°C OxPhos function when fish were acclimated to 28°C. Although the statistics were performed using residuals from population, a similar pattern is seen when considering all parameters (Fig. 2.3). These data reflect a similar pattern seen in _F. heteroclitus_ whole metabolic rates, where there is no acute effect between 5° and 10°C when fish were acclimated to 5°C, and the steepest acute effects occur above 15°C [87]. Yet, our data on cardiac State 3 respiration are different from liver State 3 respiration, where the response was dampened only at high acute temperatures at all acclimation temperatures [47,87]. The reduced acute response is not due to technical problems since this pattern occurs in all three populations, and the dampened acute response happens at both low and high assay temperatures depending on the acclimation conditions. Additionally, these patterns are observed in many individuals in three separate populations, making them unlikely to be spurious. Yet, these patterns are different from those observed in similar _F. heteroclitus_ studies that measure OxPhos State 3 metabolism.
These other studies used pooled livers from several individuals and examined a wider range of acclimation (5°C, 15°C and either 25°C or 33°C) and acute temperatures, providing a more thorough description of temperature performance curves. Thus, the acute response dampening we observed is unlikely to be due to the difference in the range of temperature *per se*, but may reflect differences among tissues. We used cardiac tissue because, along with neural tissue, it is most susceptible to deleterious mutations in OxPhos that affect human health [101]. Cardiac tissue has also been shown to affect teleosts' thermal ranges as they have narrow thermal limits [90,91]. This sensitivity to alteration in OxPhos proteins likely reflects dependency of high aerobic metabolism. If the different acute responses are due to tissue specific responses, it suggests that acclimation more strongly buffers acute response in cardiac tissues than in liver tissues. How this is achieved could provide insights into mitigating deleterious effects on OxPhos metabolism.

*OxPhos Metabolism Reliance On Enzyme Complexes*

State 3 measures OxPhos metabolism – mitochondrial respiration with substrates and ADP. State 3 respiration depends on Complex V (ATP synthase) depleting the inter-membrane proton gradient, thus allowing hydrogen pumping and electrons to pass from Complex I or Complex II through Complex III and then to Complex IV, which reduces O₂ to H₂O. It should be noted that State 3 also has a “leak” component, which is incorporated in all our measurements; protons may still leak through the membrane and enhance O₂ consumption (this should not be confused with our measured “LEAK ratio”). With little limitation by Complex V activity, changes in State 3 will be mirrored in E State (respiration unconstrained by Complex V, ATP synthase). In our data, State 3 and E State are tightly correlated (correlation coefficient 0.977). Thus, for our data we can investigate
how State 3 or E State depends on Complexes I-IV. Significant differences among populations for State 3 and E State are associated with changes only in Complex I (Table 2.4B): Complex II and IV were not significantly different among populations. The lack of significant differences in Complexes II and IV when there is a significant difference in State 3 and E State suggests that changes in these enzyme complexes are less important. Although acute temperature changes and population differences affect Complex II similarly to Complex I, the lack of significance in Complex II activity reflects a lower mean without a similar decrease in individual variation. Complex IV is unlike State 3, E State and Complex I: how acclimation and population affect the acute temperature response shows a different and non-significant pattern for Complex IV. Importantly, similar to Complex II, individual variation is also larger (standard deviation > 2-fold) for Complex IV. These data suggest that many differences in OxPhos function among populations and treatments are more dependent on Complex I than the other enzyme complexes, and the lack of significant difference for Complexes II and IV is related to individual or technical variation.

A linear regression is a statistically strong approach to define the relationship between State 3 and the enzyme complexes and LEAK. A linear regression between State 3 and these other OxPhos factors shows a significant relationship independent of acute temperatures. We removed the effect of acute temperatures because similar OxPhos responses to acute temperatures could create spurious relationships. Complexes I, II, IV and LEAK have a significant relationship with State 3 (p < 0.05 linear regression). The relative importance of each of these OxPhos functions reflects their statistical significances (the $R^2$ for Complex I (69%) > Complex II (62%) > Complex IV (23%) >
LEAK (3%). Thus, most variation in State 3 is explained by the variation in Complexes I and II. This relative importance is reflected in a stepwise regression: Complexes I and II are the only significant factors in the stepwise regression, and together they explain 90% of the State 3 variance (p-value to be included in the equation < 0.0001). Thus, similar to the ANOVA, Complex I is the most important OxPhos function affecting OxPhos respiration (State 3), followed by Complex II.

The dependence of OxPhos metabolism, measured as State 3 respiration, on Complex I is similar to the importance of this enzyme complex when *Fundulus* is acclimated to 33°C – at this high acclimation temperature, State 3 suppression depends on Complex I [47]. Complex I's importance is seen across acclimation temperatures and among populations, and thus represents environmental and evolutionary impacts on OxPhos. The strong association between Complex I and State 3 is different from investigations of metabolic control, which suggest that control coefficients are spread among the respiratory complex (review: [102]). These studies focus on a single vertebrate and tissue specific differences. Integrating these observations with our data suggests that the variation in Complex I is more likely to effect an evolutionary change. This makes sense if much adaptive variation is from standing genetic variation because Complex I has 46 protein subunits (versus < 20 for the other Complexes) and thus would have more targets for functional polymorphisms to occur. That is, with more proteins there are more nucleotides that could contain important polymorphisms. Complex I

**Evolved Population Differences**

Population differences were a function of both assay and acclimation temperatures (Fig. 2.5 and 2.6). State 3, E State and Complex I were greater in *F. grandis* than Maine *F.*
heteroclitus, and the Georgia F. heteroclitus population was intermediate between these two populations. Yet, these differences only occur at the 12°C acclimation temperature (Table 2.4A, Fig. 2.6) and were largest at the high acute temperature (Fig. 2.5). Although acclimation temperature influences the significant difference between populations, it had little effect on the F. heteroclitus populations (Table 2.4B). These results are similar to differences among Drosophila simulans haplotypes, which are apparent only at low acute temperatures [76]. Additionally, Fangue et al [87] found differences between northern and southern F. heteroclitus populations at all acclimation temperatures, but only at certain assay temperatures. Our assay temperatures did not include temperatures below 12°C, and we therefore did not observe differences between populations at the lower assay temperatures (2.5-10°C) that were observed by Fangue, Richards et al [87]. Also consistent with their data, the southern populations used in our study, F. grandis and GA F. heteroclitus, had higher respiration rates when significant differences were present at 12°C acclimation and 28°C assay temperature.

Population differences could be due to heritable (evolved), developmental or epigenetic effects. Development at one environmental temperature affects adult metabolic rates, and there is always a possibility of epigenetic changes [103,104]. In fish, there is a complex interaction between acclimation and irreversible developmental effects [104-106]. For example, developmental temperatures alter adult growth and enzyme expression in response to acclimation [104-106]. In contrast, while thermal sensitivity of swimming performance is reduced at the developmental temperature, this effect is not maintained after acclimation, suggesting that the magnitude of developmental plasticity’s effect is limited relative to thermal acclimation. Although we cannot rule out irreversible
developmental or maternal effects, we propose that many of the significant differences in OxPhos function likely have a heritable genetic basis. First, acclimation in fish reaches a steady state after 3-4 weeks [107,108], and developmental plasticity on swimming performance is largely eliminated with acclimation [105] suggesting that maintaining fish for 4 weeks to a common temperature removes most developmental effects. Second, in *Fundulus*, many physiological determinations are a function of genetic distance within and among species, especially for biochemical traits that affect metabolism [34,77-79,109,110]. Third, variations in these traits are higher when a population has greater genetic variation [111]. Importantly, variations in these traits affect changes in metabolism [35,37], suggesting that the differences in these biochemical traits manifest physiological changes. Fourth, specific DNA changes are associated with enhanced metabolic and biochemical functions [110,112]. Finally, acclimation had the largest effect on *F. grandis*, which shares a similar thermal environment with southern (GA) *F. heteroclitus*. However both *F. heteroclitus* populations were relatively insensitive to acclimation. This pattern would require complex population-specific developmental effects. These observations for *Fundulus* suggest that much of metabolism, including mitochondrial dependent respiration (OxPhos), has a strong heritable component. Assuming that many of the differences are heritable, the data suggest that *F. heteroclitus* has evolved to be less sensitive to both acclimation temperature and acute temperature than its sister taxa *F. grandis*.

The greater differences between populations at the low acclimation temperature shown here and in other *F. heteroclitus* studies [87], the small effect of acclimation to 5 or 15°C [47], combined with our data showing that ME and GA *F. heteroclitus* were insensitive to acclimation temperature, indicate that *F. heteroclitus* has evolved
mechanisms to be less sensitive to acute and acclimatory temperature changes. We speculate this is due to both change in protein sequence and expression. The greater OxPhos function in *F. grandis* versus ME *F. heteroclitus* only at the low acclimation temperature (Fig. 2.6) suggests OxPhos protein expression differences in *F. grandis*; either a quantitative difference in the expression or a change in isozymes. Yet, the largest difference between ME *F. heteroclitus* and *F. grandis* occurs at the 28°C assay temperature with little difference at the 12°C assay temperature (Fig. 2.5). The enzyme complexes and the 89 proteins making up these complexes have different sensitivities when acclimated to low or high temperatures. Unlike acclimation, these acute effects are unlikely due to changes in expression. Instead, this acute change could reflect differences in the sensitivity of the proteins: ME and GA *F. heteroclitus* are less sensitive to acute change while *F. grandis* is much more sensitive. We speculate that this could occur by altering the importance of hydrogen and ionic versus hydrophobic interactions among the proteins that make up the OxPhos enzyme complexes or changing the mitochondrial membrane where these enzymes function. At low temperatures, hydrogen or ionic bonds would have to play a more important role in maintaining protein stability and flexibility, and at higher temperatures greater thermal stability would occur with a greater reliance on hydrophobic interactions [45]. The balance of these can be altered by a few amino acid substitutions [113,114], and because a large number of proteins (approximately 89) are involved in the five OxPhos enzyme complexes, many opportunities exist to balance interactions and maintain catalysis across a broad temperature range. The differences between populations are dependent on acclimation effects. These differences reflect both a
change in OxPhos proteins and their sensitivity to acute temperature change and the effect of acclimation on the expression of these proteins.

2.6 CONCLUSION

The data presented here suggest that acclimation, while rarely a significant factor on OxPhos functions, alters the acute temperature response when measured near the acclimation temperatures. This is seen within acclimation temperatures where acute affects are dampened at temperatures similar to acclimation temperatures and across acclimation temperatures where higher acclimation temperatures are less sensitive to acute changes. Thus, these data support the supposition that acute responses provide insight into the biochemistry and biophysics of temperature effects, and acclimation tends to mitigate these responses [82,84]. These changes in mitochondrial respiration are most reliant on Complex I and to a lesser degree Complex II.
<table>
<thead>
<tr>
<th>Trait</th>
<th>Definition</th>
<th>Substrate or inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3</td>
<td>ADP stimulated respiration</td>
<td>ADP plus substrates (pyruvate, glutamate, and succinate)</td>
</tr>
<tr>
<td>RCR</td>
<td>Respiratory Control Ratio</td>
<td>State 3 (ADP + substrates) / State 2 (substrates only)</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
<td>cytochrome c / State 3 (per assay)</td>
</tr>
<tr>
<td>LEAK Ratio</td>
<td>O$_2$ consumption due to proton leak across membrane when no ADP is present</td>
<td>State2 / State 3</td>
</tr>
<tr>
<td>E State</td>
<td>Uncoupled respiration, without limitation from Complex V</td>
<td>FCCP, proton gradient uncoupler</td>
</tr>
<tr>
<td>Complex I</td>
<td>Complex I activity</td>
<td>E State activity – rotenone (CI inhibitor)</td>
</tr>
<tr>
<td>Complex II</td>
<td>Complex II activity</td>
<td>Rotenone - Malonic acid (CII inhibitor)</td>
</tr>
<tr>
<td>Complex IV</td>
<td>Complex IV activity</td>
<td>TMPD + ascorbate (electron donors)</td>
</tr>
</tbody>
</table>
Table 2.2. Population sample sizes and body masses

<table>
<thead>
<tr>
<th></th>
<th>ME</th>
<th>GA</th>
<th>Fg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample size</td>
<td>59</td>
<td>61</td>
<td>49</td>
</tr>
<tr>
<td>Sample 12°C Acc</td>
<td>33</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Sample size 28°C Acc</td>
<td>26</td>
<td>31</td>
<td>25</td>
</tr>
<tr>
<td>Average Body mass (g)</td>
<td>7.86</td>
<td>8.35</td>
<td>10.34</td>
</tr>
<tr>
<td>Std Err Mean</td>
<td>0.30</td>
<td>0.42</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Table 2.3. Three-way ANOVA for Acclimation (Acc), population (Pop) and assay temperature (Assay) and all interactions. Significant p-values are bold. Body mass effects are the slope and the significance of this slope.

<table>
<thead>
<tr>
<th>3way</th>
<th>Body Mass Effect</th>
<th>Treatment p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass Slope</td>
<td>p-val</td>
</tr>
<tr>
<td>State 3</td>
<td>0.063</td>
<td>0.096</td>
</tr>
<tr>
<td>E State</td>
<td>0.065</td>
<td>0.090</td>
</tr>
<tr>
<td>Complex I</td>
<td>0.066</td>
<td><strong>0.013</strong></td>
</tr>
<tr>
<td>Complex II</td>
<td>-0.022</td>
<td>0.882</td>
</tr>
<tr>
<td>Complex IV</td>
<td>-0.086</td>
<td>0.098</td>
</tr>
<tr>
<td>LEAK 2/3</td>
<td>0.004</td>
<td>0.178</td>
</tr>
</tbody>
</table>
Table 2.4. Population and Acclimation Effects. P-values for significant effects are bold.

A: Population significance at each acclimation temperature.

<table>
<thead>
<tr>
<th>Effect of Population</th>
<th>State 3</th>
<th>E State</th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex IV</th>
<th>LEAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop p-val at 12°C Acc</td>
<td>0.006</td>
<td>0.025</td>
<td>0.007</td>
<td>0.343</td>
<td>0.094</td>
<td>0.332</td>
</tr>
<tr>
<td>Pop p-val at 28°C Acc</td>
<td>0.771</td>
<td>0.916</td>
<td>0.872</td>
<td>0.326</td>
<td>0.566</td>
<td>0.661</td>
</tr>
</tbody>
</table>

B: Acclimation significance for each population.

<table>
<thead>
<tr>
<th>Effect of Acclimation</th>
<th>State 3</th>
<th>E State</th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex IV</th>
<th>LEAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acc p-val for ME</td>
<td>0.228</td>
<td>0.502</td>
<td>0.345</td>
<td>0.852</td>
<td>0.314</td>
<td>0.084</td>
</tr>
<tr>
<td>Acc p-val for GA</td>
<td>0.433</td>
<td>0.378</td>
<td>0.614</td>
<td>0.157</td>
<td>0.862</td>
<td>0.422</td>
</tr>
<tr>
<td>Acc p-val for Fg</td>
<td>0.017</td>
<td>0.051</td>
<td>0.017</td>
<td>0.570</td>
<td>0.882</td>
<td>0.162</td>
</tr>
</tbody>
</table>
Figure 2.1. Three *Fundulus* populations with mean annual temperatures.

*F. heteroclitus* population are from Maine (ME) and Georgia (GA). *F. grandis* (Fg) is from the Florida panhandle in the Gulf of Mexico.

Figure 2.2. Box plot of respiratory control ratio for each “group” of individuals tested.

The first number for the group label is the assay temperature, followed by population then followed by acclimation temperature. Plot includes mean, 25 and 75% quartile and range.
Figure 2.3. Complete dataset for six measured traits.

Graphs are separated by acclimation temperature. Within each panel are the population and the assay temperature of measured individuals. Grey background indicates 12°C acclimation, and white background indicates 28°C acclimation. Green lines represent F. grandis, red and blue lines represent the GA, and ME F. heteroclitus populations, respectively. Assay temperature is always significant (p<0.05). See table 3 for other p-values. Means are graphed, and error bars represent standard error. Lines are to aid in the identification of the three populations and acute effects and are not a regression.
Figure 2.4. The effect of acclimation and assay temperature on OxPhos functions.

Acclimation at 12°C is depicted by a grey dashed line. Acclimation at 28°C is depicted by a black solid line. Lower case letters on figure indicate differences between assay temperature: “a” and “b” for 12°C acclimation, and “c” and “d” for 28°C acclimation. Asterisks represent significant effect of assay temperature at each acclimation temperature (p< 0.05). Means are graphed, and error bars represent standard error. Lines are to aid in the identification of the two acclimation groups and acute effects and are not a regression.
Figure 2.5. The effect of acute temperatures on OxPhos

p-values represent significant differences between slopes, with an asterisk noting a p-value <0.05. Green lines represent the *F. grandis* population. Red and green lines represent the GA and ME *F. heteroclitus* populations, respectively. Regression is significant for all populations (p<0.01), for State 3, E State, Complex I and Complex IV. For Complex II, regression is only significant (p<0.01) for the GA and *F. grandis* populations. Regression was only significant in the GA population for LEAK (p<0.01). Means are graphed, and error bars represent standard error.
Figure 2.6. Difference in OxPhos between populations at different acclimation temperatures:

12°C (grey background) and 28°C (white background). Green represents the *F. grandis* population. Red and green represent the GA and ME *F. heteroclitus* populations, respectively. Asterisks show significant effect of population at each acclimation temperature (p<0.05). Means are graphed, and error bars represent standard error.
CHAPTER 3. GENE BY ENVIRONMENTAL INTERACTIONS AFFECTING OXIDATIVE PHOSPHORYLATION THERMAL SENSITIVITY

3.1 SUMMARY

The oxidative phosphorylation (OxPhos) pathway is responsible for most aerobic ATP production and is the only metabolic pathway with proteins encoded by both nuclear and mitochondrial genomes. In studies examining mito-nuclear interactions among distant populations within a species or across species, the interactions between these two genomes can affect metabolism, growth, and fitness, depending on the environment. However, there is little data on whether these interactions impact natural populations within a single species. In an admixed Fundulus heteroclitus population with northern and southern mitochondrial haplotypes, there are significant differences in allele frequencies associated with mitochondrial haplotype. In this study, we investigate how mitochondrial haplotype and any associated nuclear differences affect six OxPhos parameters within a population. The data demonstrate significant OxPhos functional differences between the two mitochondrial genotypes. These differences are most apparent when individuals are acclimated to high temperatures with the southern mitochondrial genotype having a large acute response and the northern mitochondrial genotype having little, if any acute response. Furthermore, acute temperature effects and the relative contribution of Complex I and II depend on acclimation temperature: when individuals are acclimated to 12°C, the relative contribution of Complex I increases with higher acute temperatures, whereas at 28°C acclimation the relative contribution of Complex I is unaffected by acute temperature change. These data demonstrate a complex gene by environmental interaction affecting the OxPhos pathway.
3.2 Introductory Remarks

The oxidative phosphorylation (OxPhos) pathway is responsible for most aerobic ATP production and is the only metabolic pathway that involves both nuclear and mitochondrial encoded proteins. The OxPhos pathway has 5 enzyme complexes with approximately 89 proteins; the mitochondrial genome encodes 13 of these proteins while the nuclear genome encodes 76. The interactions among the 89 proteins in the OxPhos pathway are likely to be sensitive to acute and chronic temperature exposures [82,84]. In Fundulus, OxPhos studies have provided insight into how exposures to acute and chronic (acclimation) temperature change alter physiology [47,48,87,115]. These studies have shown that the acclimation process influences acute temperature effects on OxPhos (4, 9). Baris et al. show that while acclimation does not always have a significant effect on OxPhos functions, it alters the acute temperature response of F. heteroclitus [115]. Specifically, when acclimated to a low temperature (12°C) the acute response was shallower at the lower range of acute test temperatures (12°C and 20°C), and when acclimated to a high temperature (28°C), the acute response was shallower at the higher range of acute test temperatures (20°C and 28°C) [115]. Overall, studies of OxPhos in Fundulus support the hypothesis that acclimation tends to mitigate acute temperature effects on this pathway’s biochemistry and biophysics [82,84].

In ectotherms, how the thermal environment affects OxPhos function depends on which population or mitochondrial genotype is assayed [48,61,62,76,82,87,91,115,116]. The interactions between the environment and genotype (nuclear or mitochondrial) influences the evolution of nucleotide substitutions among OxPhos proteins (GxE).
What is surprising, and adds another layer of complexity, is that the thermal effects are affected not only by nuclear or mitochondrial genotypes but also by significant interactions between these genomes due to mito-nuclear epistasis [60-63]. For example, substituting *Drosophila simulans* mitochondria into *D. melanogaster*’s nuclear background affects one inbred *D. melanogaster* line but not another at high temperatures, and these mito-nuclear interactions affect the acute response [62,63,118]. Similarly, in seed beetles, *Callosobruchus maculatus*, temperature dependent metabolic rates depend on the interaction between the mitochondrial and nuclear genomes [116]. These mito-nuclear interactions that affect OxPhos are biologically important because they affect fitness and survivorship [56,60-64,118-121]. In general, these data suggest that mito-nuclear interactions among species or divergent populations are likely to affect an organism’s physiology, and these interactions are environmentally dependent [120,122,123].

For northern and southern *Fundulus heteroclitus* populations, which are distributed along a steep thermal cline (approx. 1°C/degree latitude), northern populations experience temperatures more than 12°C colder than southern populations. These northern and southern populations harbor two distinct mitochondrial haplotypes (mt-haplotypes) differing by five amino acid replacements [29]. This genetic divergence between mt-haplotypes is influenced by a historical break at the Hudson River due to the last glaciation [25], allowing the evolution of the nucleotide differences between northern and southern populations [30]. Importantly, *F. heteroclitus* has large populations with low migration rates and are adapted to local environmental conditions [39,40]. These demographic and environmental features are likely to affect the nuclear and mitochondrial
divergences among \textit{Fundulus} populations [25]. In addition to mt-haplotype divergence among \textit{F. heteroclitus} populations, there are differences in the nuclear genome that alter enzymes and enzyme expression, which affect metabolism [25,31-38]. Because of the divergence among populations in both mitochondrial and nuclear genomes, it is difficult to ascribe the relative importance of mito-nuclear interaction effects on physiology. Additionally, for animals assayed from different environments/populations the importance of these mito-nuclear interactions could be masked due to developmental effects (irreversible acclimation) or epigenetic effects, which could generate non-heritable differences. Therefore we use a single population in northern New Jersey just south of the Hudson River, where individuals experience a similar environment. This population has both the “northern” mitochondrial haplotype, common in populations north of the Hudson River, and a “southern” mitochondrial haplotype, common in populations south of the Hudson River [124,125]. In this admixed population, both mt-haplotypes occur at high frequencies [125,126], and while nearly all nuclear genes are randomly associated with these mt-haplotypes, several hundred have a biased frequency: 349 nuclear SNPs (61 with 1\% FDR correction) have significantly different allele frequencies between mt-haplotypes that, in combination with the divergences in mt-haplotypes, affect cardiac OxPhos function [126]. This significant bias in nuclear genotypes based on the mt-haplotype suggests epistatic adaptive evolution, and thus differences in OxPhos functions would be expected.

In this study, we provide detailed analysis of OxPhos function among individuals with the two different mt-haplotypes from this single, admixed population. Our results suggest that the mt-haplotypes affect OxPhos function by altering the acclimation and
acute temperature effects in this admixed population. However, acclimation’s effects were
dependent on the mt-haplotype and acclimation temperature interactions. This indicates
that the physiological response (both acclimatory and acute) is dependent on the
organism’s genotype. We suggest that these G x E interactions help maintain genetic
variation by altering temperature effects.

3.3 METHODS

EXPERIMENTAL ANIMALS

Adult *F. heteroclitus* were collected during the summer months from Mantoloking, NJ (40.049427°N, -74.065087°W). All fish were kept at 20°C for 4 weeks, then
acclimated to either 12°C or 28°C for 4 weeks. During this time, they were exposed to a
14 hour light cycle, kept at 15ppt salinity and fed twice a day, 7 days a week. Housing and
protocols were in compliance with the University of Miami Institutional Animal Care and
Use Committee (IACUC) guidelines.

Fieldwork was completed within publically available lands and no permission was
required for access. *F. heteroclitus* does not have endangered or protected status, and
small marine minnows do not require collecting permits for non-commercial purposes. All
fish were captured in minnow traps with little stress and removed in less than one hour.
IACUC approved procedures were used for acclimation and non-surgical tissue sampling.
Fish were sacrificed by pithing and decapitation, using procedures approved by IACUC.

GENOTYPING MITOCHONDRIA

ND2 and cytochrome b were amplified from isolated DNA from fin clips and cut
with restriction enzymes, PleI, and BstYI, respectively, to identify the mitochondrial
haplotype. PleI cut ND2 into 2 pieces for the northern mt-haplotype, and into 3 pieces for the southern mt-haplotype. BstYI cut cytochrome b into 3 pieces for the northern mt-haplotype and 2 pieces for the southern mt-haplotype. The digests were run on a 1% agarose gel to separate out bands. Individuals from Maine and Georgia were used as controls. Both restriction enzymes yielded the same results for each individual.

**HIGH-RESOLUTION RESPIROMETRY**

**Tissue Permeabilization**

Heart ventricles were dissected, cut into halves, and one half was placed into a muscle relaxation solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl₂, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) [92]. The second half was saved for future RNA experiments. Hearts were cut anteriorly/posteriorly, and pieces were randomized into different acute temperature tests. Prior to measuring respiration, each tissue was permeabilized using 2.5mg/ml saponin solution for 15 minutes, followed by 4 washes in respiration medium for 5 minutes each [93]. Once permeabilized, tissues were immediately transferred to the respirometry chambers containing respiration medium Miro5 (5mM EGTA, 3mM MgCl₂.6H₂O, 60mM K-lactobionate, 20mM Taurine, 10mM KH₂PO₄, 20mM HEPES, 110mM Sucrose, 1% BSA pH 7.1) and substrates to reach state 2 (pyruvate, glutamate, succinate).

**OxPhos Determinations**
The acute temperature effect on mitochondrial activity was measured at three temperatures (12°C, 20°C, and 28°C) using respirometers containing respiration medium, Miro5. The pH of the solution at 20°C was 7.1, and the pH changed by approximately 0.11 pH unit at 12°C (+0.11) and 28°C (-0.11). Oxygen consumption was measured and analyzed using the Oxygraph 2-k and DatLab software (OROBOROS INSTRUMENTS, Innsbruck, Austria). Oxygen sensors were calibrated with air-saturated Miro5 and zero oxygen concentration after sodium dithionite addition before all assays. For measurements, the order of the population, acclimation temperature, and acute temperature were all randomized between the three different respirometers.

After tissue addition to the respiration chamber containing pyruvate, glutamate and succinate, State 2 [127,128] respiration rates were reached. This was followed by the addition of ADP (5 mM, State 3), cytochrome c (10 µM, to check outer mitochondrial membrane integrity), FCCP (a mitochondrial uncoupler used to reach E State; 0.5 µM was added sequentially until activity no longer increased), rotenone (0.5 µM to block Complex I), malonate (5 mM to block Complex II) and Antimycin A (2.5 µM to block Complex III). The rates obtained after inhibition of complexes I, II and III represent the residual oxygen consumption (i.e., the oxygen consumed by oxidative side reactions occurring in permeabilized fibers) and was used to correct all the respiration rates. Finally, TMPD and ascorbate (artificial substrate for Complex IV, 0.5 mM TMPD and 0.2 mM ascorbate) (Table 3.1) were added to measure Complex IV activity. Background oxygen consumption levels that could arise from TMDP and ascorbate were not measured and thus, if substantial, could mask significant effects of acclimation and mt-haplotypes for Complex IV. Background respiration would not affect the other measures of OxPhos. It
should be noted that malate, a Kreb’s cycle intermediate, was not used. When malate was added to test tissues, we observed no difference, or reduced reaction rates at any assay temperatures, and therefore excluded it from our experimental design.

LEAK ratio in this study was calculated as State 2/State 3. It is usually calculated as State 4/State 3, where state 4 is reached through inhibition of Complex V with oligomycin. However, we found that when oligomycin is used, subsequent E state measurements were always lower than State 3 [115]. State 2 is the measure of oxygen consumption without ADP, while State 4 measures respiration where Complex V is blocked. Both measure respiration when Complex V cannot phosphorylate ADP to ATP. With State 2, there may be endogenous ATPases, which would affect respiration measurements. However, due to the problems with oligomycin, we measured LEAK as State 2/State3 [94].

Cytochrome c addition tests whether the outer mitochondrial membrane was damaged during cell permeabilization and was performed for each assay. Assays in which cytochrome c caused more than 10% change in oxygen consumption were not included in the analyses. Chambers were made hyperoxic (800 nmol/ml O₂) through injection of O₂ into chambers to ensure that oxygen levels did not influence measurements.

The tissue from each chamber was recovered after respiration assays and homogenized, and total DNA was quantified from the homogenate using AccuBlue high sensitivity dsDNA quantitation solution (BIOTIUM, Hayward, CA). All activity was normalized by ng of DNA. OxPhos respiration rates are reported as pmol O₂ s⁻¹ ml⁻¹ per ng DNA. Although DNA concentration, protein concentration and cell count have a linear relationship, DNA has been found to be the consistent way of normalizing metabolomics
data across a range of cell numbers [95]. Since we had small tissue amounts that we permeabilized before assays, we recovered the tissue from the respiration chambers to accurately quantify tissue amount. We normalized OxPhos values by DNA quantity, as this is a proportional measure to total mitochondrial DNA [129].

**STATISTICAL ANALYSES**

A mixed general linear model analysis was performed with all factors: mt-haplotype, acclimation temperature, and acute temperature (JMP, SAS, Cary NC). Body mass (Table 3.2, 3.3) often affects metabolic physiology [99,100], and to correct for variation in body masses, we included body mass as a covariate in our mixed model. Linear or log-transformed body masses yielded similar results because a linear model captures the small range of body masses. There was no significant interaction of body mass with other factors. The analysis was performed on six important parameters: State 3 (substrates and ADP), E State (uncoupled mitochondrial activity with FCCP), Complex I, Complex II, Complex IV and LEAK ratio. Measurements of Complexes I-IV are OxPhos respiration rates determined by selective complex inhibition or enzyme specific substrates (Table 3.1). LEAK state reflects the number of protons that “leak” across the membrane where proton gradient creation consumes O₂ but does not produce ATP [94]. In our study LEAK ratio is measured as State 2/State3 (Table 3.1).

### 3.4 Results

The six OxPhos parameters are quantified by measuring oxygen consumption rates with substrates, uncoupler or inhibitor addition (Table 3.1): State 3 (ADP stimulated respiration), E State (uncoupled mitochondrial), Complexes I, II and IV, and LEAK ratio.
Cytochrome c addition revealed no damage to outer mitochondrial membrane (mean ratio of State3/Cyt c = 1.01, st. dev. = 0.09). A separate measure of functional coupling for OxPhos is RCR (State3/State2), which measures respiration dependency on ADP. We included all data with an RCR of 2.5 or higher (Fig. 3.1), [97,98]. The RCR for our data on 155 individuals is large relative to those in Chung et al 2015 [47]. This variation in RCR may reflect the large sample size (rare alleles), the admixture effect of two mitochondria or the potential effect of different non-specific ATPases (where small amounts of ATPases would cause a large RCR). However, the presence of non-specific ATPases and its potential effect on RCR, should not affect our analysis of OxPhos metabolic functions.

Table 3.2 provides sample sizes and body masses for the individuals used in the analysis for the mt-haplotypes. Body masses ranged from 1.1 to 12.3 grams for the northern mt-haplotype individuals and from 1.2 to 13.3 grams for the southern mt-haplotype individuals. Body mass often affects metabolic physiology [99,100]. The effect of body mass was significant for State 3, E State, Complex I and Complex II (Table 3.3). We used body mass as a covariate in our model to avoid potential biases due to body mass.

We performed a mixed general linear model analysis using acclimation temperature, population and acute temperature change and their interactions (Table 3.3). Acute temperature change had a significant effect on all measured parameters except for Complex II. Acclimation only had a significant effect on State 3 and Complex IV.
Figure 3.2 displays the general mixed model data summarized in Table 3.3. Mt-haplotype significantly affected all parameters except Complex IV and LEAK ratio. No interactions were significant.

The initial mixed general linear model reveals that mt-haplotype and acute temperature change are significant factors for a majority of the parameters tested. Although we do not see a significant interaction term between acclimation, acute temperature and mt-haplotype, in Figure 3.2, mt-haplotype and acute temperature change seems to be affected by acclimation temperature. We believe that the lack of a significant interaction is due to a lack of power, and is therefore a type II error. We wanted to understand the interaction seen in Figure 3.2 and therefore performed subsequent analyses. A mixed general linear model was run by mt-haplotype and acclimation temperature with body mass as a covariate. These subsequent analyses revealed that acute temperature effects differed between the northern and southern haplotypes and acclimation temperature (Table 3.4, asterisks represent \(p<0.05\), double asterisks represent \(p<0.0125\) for multiple test correction). At the 12°C acclimatization temperature, acute temperature change had a significant effect on Complexes I and IV and LEAK ratio for the northern mt-haplotype. For the southern mt-haplotype individuals acclimated to 12°C, acute temperature change had a significant effect on State 3, Complex IV and LEAK ratio. There was a stark contrast on the influence of acute temperature change between haplotypes at the 28°C acclimation temperature. For the northern mt-haplotype individuals acclimated to 28°C, acute temperature change never had a significant effect on any of the measured parameters. For the southern mt-haplotype individuals, acute temperature change affected nearly all measured parameters (with the exception of LEAK
ratio). When a multiple comparison correction was performed, this pattern was only significant for State 3 (p<0.0125, Table 3.4).

To understand how different OxPhos components affect overall respiration, we examined the roles of Complex I and Complex II, the two entry points for electrons. Complex I’s response to acute temperature looks similar to E-State for all four mt-haplotype/acclimations combinations (Fig. 3.2). We provide a relative measure of Complexes I's and II's contributions to OxPhos by dividing each by the total E State activity (Fig. 3.3). A mixed general linear model was run with arcsin transformed data and body mass as a covariate, to test if acute temperature change was significant for both mt-haplotype individuals at the two different acclimation temperatures. At the 12°C acclimation temperature, Complex I's contribution increases with increasing acute temperatures whereas Complex II's contribution decreases (Fig. 3.3). This trend is evident in both the northern and southern mt-haplotype individuals although it is only significant for the southern mt-haplotype individuals (p<0.006 for effect of acute temperature for CI and CII at 12°C acclimation, Fig. 3.4 ). At the 28°C acclimation temperature, no differences were observed in the relative contributions for Complexes I and II among acute temperatures for either the northern or southern mt-haplotype.

3.5 DISCUSSION

We investigated how mito-nuclear genotypes and temperature affect cardiac metabolism adaptation in an admixed *F. heteroclitus* population by examining the effect of two acclimation temperatures and three acute temperatures. Our mixed-model analysis with all factors present and mass as a covariate revealed that acclimation temperature only had a significant effect on State 3 and Complex IV. Acute temperature change had a
significant effect on all measured parameters except for Complex II, and mt-haplotype had a significant effect on State 3, E State, Complex I and Complex II. Although there was no significant interaction effects in our mixed model analysis, upon further analyses, there was an interesting pattern revealed for State 3: the acute temperature effects were dependent on mt-haplotype. Thus, the southern mt-haplotype had acute effects at both acclimation temperatures, but the northern mt-haplotype at 28°C acclimation had no acute temperature effect (p-value = 0.44, Table 3.4, Fig. 3.2) and at 12°C acclimation there was little acute temperature effect (p-value = 0.09, Table 3.4, Fig. 3.2). This pattern of the northern mt-haplotype having no significant acute temperature effect at 28°C is observed for all OxPhos parameters.

Previous studies comparing a northern *F. heteroclitus* population to a southern one have shown a significant difference in mitochondrial function at different acclimation and acute temperatures [47,87]. In one study, differences between ME and GA populations were more apparent when individuals were acclimated to low temperatures (5°C and 15°C *versus* 25°C), and assayed at temperatures above 25°C [87]. Baris *et al.* found a similar response between ME and GA, where differences between populations are restricted to low acclimation temperatures and are larger at higher acute temperatures, and activity for GA is greater than ME [115].

In this study we use a freely interbreeding, naturally occurring admixed population where both southern and northern mt-haplotypes have nearly equal frequencies [125,126]. In a separate study, this population was used to study individual survivorship under hypoxic conditions, and no mitochondrial effect was found [130]. For our measurements on OxPhos we find that mt-haplotype does significantly affect four of the six OxPhos
parameters tested (Table 3.3). Importantly, the effect of mt-haplotype is most obvious in the response to acute temperature change at the high acclimation temperature for State 3, E State, Complex I and Complex II (Fig. 3.2). While individuals with the southern mt-haplotype show a significant relationship between overall mitochondrial respiration (State 3) and acute temperature change, individuals with the northern mt-haplotype show almost no response (Table 3.4). This is consistent with results from Chung et al., who found that a northern population from Nova Scotia, acclimated to 33°C showed reduced overall State 3 and reduced acute temperature effects [47]. These results are also similar to those in Baris et al. [115] where a population from ME with the northern haplotype, and one from GA containing the southern haplotype were used to study the effects of acclimation and acute temperature change. For the ME individuals, the effect of acute temperature was weaker than those for the GA population. Specifically, when acclimated to 12°C, acute temperature effects were stronger for the GA population than the ME population. Interestingly, the differences between haplotypes (unlike northern ME and southern GA) are more apparent at high and not low acclimation temperatures because the northern mt-haplotype is less sensitive to acute temperature change.

State 3 (overall mitochondrial metabolism) and E State (not limited by ATP synthase) are highly correlated ($r^2 = 0.9$). Complexes I and II were determined after the determination of E State. Thus, to determine the relative contributions of both enzyme complexes, we calculated the percent contribution of Complexes I and II to E State (Fig. 3.3). When both haplotypes were acclimated to 12°C, increasing acute temperature reveals that Complex I's contribution increases whereas Complex II's contribution decreases (Fig. 3.3). At 28°C acclimation, the contribution of Complexes I and II are unaffected by acute
temperatures. The difference in acclimation's effect for the relative versus absolute respiration rate is because we are dividing by E State. A similar pattern was seen in goldfish muscle homogenate and isolated mitochondria in which fish acclimated to cold temperatures had much higher Complex II activity compared to those acclimated to warmer temperatures [131]. These data suggest that at lower acclimation temperatures there is greater reliance on Complex I as acute temperatures increases.

**Evolutionary Physiology: Genetic difference in the response to acute temperatures**

An interesting pattern revealed from our data is the difference between mt-haplotypes in response to acute temperature change when acclimated to 28°C. An explanation for the State 3 differences among mt-haplotypes at the different acclimation temperatures could reflect differences in thermal performance curves (TPCs, Fig. 3.4). TPCs can vary based on taxa and acclimation effects (reviewed in [82]). *F. heteroclitus* follows the typical predictions of the Arrhenius equation, which determines the shape of the TPC based on thermodynamics (reviewed in [82]). *F. heteroclitus*’ TPC was more thoroughly examined by measuring acute effects at many temperatures between 2 or 5°C and 37°C [47,87]. In general, in those and other studies in ectotherms, cold acclimation shifts the TPC curve to the left [132,133]. Here, we presented the acute effects measured at only three temperatures, yet we suggest that these data reflect a left shift, and a change in shape in TPC curves for both mt-haplotypes when acclimated to 12°C (Fig. 3.4). The difference in the acute effect between mt-haplotypes, where the northern mt-haplotype is insensitive to acute temperature change at 28°C acclimation temperature, can be explained by a more extreme right shift relative to the southern haplotype. Thus, the data
demonstrating that sensitivity to acute temperature change is affected by mt-haplotype and acclimation temperatures can be explained by a genetic difference that alters the TPC.

It is difficult to determine whether the response to acute temperature difference between northern mt-haplotype and southern mt-haplotype individuals acclimated to 28°C is necessarily beneficial for one group. One could argue that as temperature increases, so do ATP demands, and that the southern mt-haplotype is able to fulfill those demands, while individuals with the northern mt-haplotype fail to do so. This would lead to a decrease in aerobic scope at high temperatures, which could induce a decline in performance traits for northern mt-haplotype individuals [82]. Alternatively, one can argue that there may be benefits to lower respiration rates seen in the northern mt-haplotypes. A temperature increase, which results in a metabolic rate increase, also requires an increase of nutrient assimilation. A mismatch between metabolic rate and nutrient uptake may result in decreased performance. Lower respiration rates may be beneficial when faced with lower food availability, which in the long-term can lead to low substrate availability. Furthermore, increased mitochondrial respiration could result in increased reactive oxygen species (ROS) formation that can damage cellular macromolecules [82,134,135]. Chung et al [47] found that ROS production increased with assay temperature in a northern population of F. heteroclitus but do not have similar data for a southern population. We are simply hypothesizing that individuals with a northern mt-haplotype acclimated at a high temperature (28°C), may have less ROS production than their southern mt-haplotype counterparts, due to insensitivity to acute temperature change. Most likely, it is not that one mt-haplotype’s TPC is consistently superior, because a consistent difference between haplotypes in performance that affects fitness
would selectively remove one of the haplotypes. But rather, these different TPCs vary in their fitness effects depending on their environment. Of relevance, these individuals were captured together in the same estuarine creek. It is therefore unreasonable to suggest spatial variation in the environment. Instead, temporal variation is common in the *F. heteroclitus* environment. We suggest that the large temporal environmental variation associated with estuarine environments is effectively selecting for both nuclear-mitochondrial genotypes depending on the performance advantage in different environments. This balancing selection occurs even though the conditions for maintaining mitochondrial polymorphism are very restricted (depending on pleiotropy, allele effect size and consistency [14,136]).

**SPECULATION ON DIVERGENCE BETWEEN Mt-HAPLOTYPES AND THE ROLE OF POPULATION GENETICS**

In this admixed New Jersey population, the two mt-haplotypes have unexpected and significant divergence in nuclear allele frequencies at 349 loci (61 loci after 1% FDR correction), that is most parsimoniously explained by evolution due to natural selection [126].

In addition to differences in nuclear allele frequencies in the admixed NJ population, there is another surprising finding that is dependent on the mt-haplotype [126]: the differences in OxPhos metabolism between mt-haplotypes within a population are larger than the differences between northern Maine and southern Georgia populations [115]. This is unexpected because the Maine and Georgia populations have different mt-haplotypes with the same non-synonomous substitutions found within our admixed NJ population. We would have predicted that populations that are geographically separated
would be more phenotypically different than individuals from a single population subdivided by mt-haplotype. This could be attributed to a greater effectiveness of natural selection between isolated populations than within a population; natural selection will act on smaller selection coefficients between isolated populations [136,137]. We speculate that because natural selection may not act on small selection coefficients in the admixed NJ population, but can on ME and GA, potentially compensatory loci that differ between ME and GA are not different between mt-haplotypes in NJ. Thus we can speculate GxG (genome by genome) interactions as a reason for NJ individuals with the northern mt-haplotype having a different TPC than ME individuals with the same mt-haplotype. This speculation is supported by the observation that the 349 nuclear genes with significant allele frequency differences are upstream of the OxPhos pathway and that individuals with mixed nuclear backgrounds are phenotypically intermediate [126].

3.6 CONCLUSIONS

The data presented here suggests that mt-haplotype plays an important role in OxPhos function, and mt-haplotype differences for some parameters become more apparent at different acclimation temperatures. Specifically, the acute temperature effect on OxPhos is dependent on acclimation temperature and mt-haplotype. We hypothesize that the northern mt-haplotype has evolved a TPC that tempers the effects of acute temperature change on OxPhos function at higher acclimation temperatures. Additionally, we hypothesize that the mt-haplotype differences seen here are a result of the interaction between mt-haplotype and nuclear background and their environments. We hypothesize that the environment (chronic and acute) alters the interactions between the nuclear and mitochondrial genome and that these interactions result in complex thermal response
patterns. We suggest that it is these interactions in a variable environment that maintain both mt-haplotypes and these different mt-haplotypes affect the selection for changes in the nuclear genome. Thus, natural selection to variable environments maintains selectively important interactions between mitochondrial and nuclear genomes resulting in significantly different OxPhos metabolism.
Table 3.1. OxPhos function expressed as mean respiration rates in pmol O$_2$ s$^{-1}$ ml$^{-1}$ per ng DNA

<table>
<thead>
<tr>
<th>Trait</th>
<th>Definition</th>
<th>Substrate or inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3</td>
<td>ADP stimulated respiration</td>
<td>ADP plus substrates (pyruvate, glutamate, and succinate)</td>
</tr>
<tr>
<td>RCR</td>
<td>Respiratory Control Ratio</td>
<td>State 3 (ADP + substrates) / State 2 (substrates only)</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
<td>cytochrome c / State 3 (per assay)</td>
</tr>
<tr>
<td>LEAK Ratio</td>
<td>O$_2$ consumption due to proton leak across membrane when no ADP is present</td>
<td>State2 / State 3</td>
</tr>
<tr>
<td>E State</td>
<td>Uncoupled respiration, without limitation from Complex V</td>
<td>FCCP, proton gradient uncoupler</td>
</tr>
<tr>
<td>Complex I</td>
<td>Complex I activity</td>
<td>E State activity – rotenone (CI inhibitor)</td>
</tr>
<tr>
<td>Complex II</td>
<td>Complex II activity</td>
<td>Rotenone - Malonic acid (CII inhibitor)</td>
</tr>
<tr>
<td>Complex IV</td>
<td>Complex IV activity</td>
<td>TMPD + ascorbate (electron donors)</td>
</tr>
</tbody>
</table>

Table 3.2. Mt-haplotype sample sizes and body masses

<table>
<thead>
<tr>
<th></th>
<th>North mt-haplotype</th>
<th>South mt-haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample size</td>
<td>64</td>
<td>83</td>
</tr>
<tr>
<td>Sample size 12°C Acc</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>Sample size 28°C Acc</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>Average Body mass (g)</td>
<td>4.41</td>
<td>4.24</td>
</tr>
<tr>
<td>Std Err Mean</td>
<td>0.32</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 3.3. Three-way general mixed model for acclimation (Acc), mt-haplotype (mt-hap) and acute temperature (Acute) and all interactions. Significant p-values are bold. Body mass slopes and slope significances are also listed.

<table>
<thead>
<tr>
<th>Body Mass Effect</th>
<th>3way</th>
<th>Mass Slope</th>
<th>p-value</th>
<th>Treatment p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mt-hap</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acc x mt-hap x Acute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acc x mt-hap</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acc x Acute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mt-hap x Acute</td>
</tr>
<tr>
<td>State 3</td>
<td>0.185</td>
<td>0.0257</td>
<td>0.0351</td>
<td>0.0078</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.812</td>
</tr>
<tr>
<td>E State</td>
<td>0.209</td>
<td>0.0327</td>
<td>0.1487</td>
<td>0.0070</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.527</td>
</tr>
<tr>
<td>Complex I</td>
<td>0.1359</td>
<td>0.0226</td>
<td>0.0755</td>
<td>0.0179</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8822</td>
</tr>
<tr>
<td>Complex II</td>
<td>0.0924</td>
<td>0.0295</td>
<td>0.5367</td>
<td>0.0050</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1812</td>
</tr>
<tr>
<td>Complex IV</td>
<td>0.1514</td>
<td></td>
<td>0.6628</td>
<td>0.0223</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0001</td>
</tr>
<tr>
<td>LEAK 2/3</td>
<td>0.0082</td>
<td></td>
<td>0.4758</td>
<td>0.5864</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0003</td>
</tr>
</tbody>
</table>
Table 3.4. p-values for acute temperature effects by mt-haplotype and acclimation temperature

<table>
<thead>
<tr>
<th></th>
<th>North mt-haplotype</th>
<th>South mt-haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12°C</td>
<td>28°C</td>
</tr>
<tr>
<td>State 3</td>
<td>0.091</td>
<td>0.444</td>
</tr>
<tr>
<td>E State</td>
<td>0.109</td>
<td>0.627</td>
</tr>
<tr>
<td>Complex I</td>
<td>0.047*</td>
<td>0.616</td>
</tr>
<tr>
<td>Complex II</td>
<td>0.423</td>
<td>0.570</td>
</tr>
<tr>
<td>Complex IV</td>
<td>0.024*</td>
<td>0.161</td>
</tr>
<tr>
<td>LEAK 2/3</td>
<td>0.001**</td>
<td>0.274</td>
</tr>
</tbody>
</table>

Figure 3.1. Box plot of respiratory control ratio for each “group” of individuals tested. The first term for the group label is the mt-haplotype, followed by acclimation temperature, and then the acute temperature. Plot includes mean, 25% and 75% quartile and range.
Figure 3.2. Complete dataset for six measured parameters.

Graphs are separated by acclimation temperature. Within each panel are the population and the acute temperature change of measured individuals. Red and blue lines represent the southern mt-haplotype, and the northern mt-haplotype, respectively. See table 3.3 for p-values. Means are graphed, and error bars represent standard error. Lines are to aid in the identification of the two mt-haplotypes and acute effects and are not a regression.
Figure 3.3. Relative contributions of Complex I and Complex II.

Upper graph is for northern mt-haplotype and lower graph is for southern mt-haplotype. Within these graphs, the upper panel shows relative Complex I activity (CI/E State) at both acclimation temperatures. Lower panel shows the relative Complex II activity (CII/E State) at both acclimation temperatures. Statistics were performed on arcsin transformed data and p-values denote effect of acute temperature.
Figure 3.4. Hypothesized thermal performance curves for mt-haplotypes at two acclimation temperatures.

The red curve represents the 28°C acclimation temperature, and the blue curve represents the 12°C acclimation temperature. The y-axis represents mitochondrial respiration. The x-axis represents increasing acute temperature, and the three acute temperatures used are marked (12°C, 20°C, and 28°C).
CHAPTER 4. ADAPTIVE GENETIC AND PHENOTYPIC DIFFERENCES DUE TO MITOCHONDRIAL-NUCLEAR INTERACTIONS

4.1 SUMMARY

The oxidative phosphorylation (OxPhos) pathway is responsible for most aerobic ATP production and is the only pathway with both nuclear and mitochondrial encoded proteins. The importance of the interactions between these two genomes has recently received more attention because of their potential evolutionary effects and how they may affect human health and disease. In many different organisms, healthy nuclear and mitochondrial genome hybrids between species or among distant populations within a species affect fitness and OxPhos functions. However, what is less understood is whether these interactions impact individuals within a single natural population. The significance of this impact depends on the strength of selection for mito-nuclear interactions. We examined whether mito-nuclear interactions alter allele frequencies for ~11,000 nuclear SNPs within a single, natural Fundulus heteroclitus population containing two divergent mitochondrial haplotypes (mt-haplotypes). Between the two mt-haplotypes, there are significant nuclear allele frequency differences for 349 SNPs with a p-value of 1% (236 with 10% FDR). Unlike the rest of the genome, these 349 outlier SNPs form two groups associated with each mt-haplotype, with a minority of individuals having mixed ancestry. We use this mixed ancestry in combination with mt-haplotype as a polygenic factor to explain a significant fraction of the individual OxPhos variation. These data suggest that mito-nuclear interactions affect cardiac OxPhos function. The 349 outlier SNPs occur in genes involved in regulating metabolic processes but are not directly associated with the
79 nuclear OxPhos proteins. Therefore, we postulate that epistatic selection affects OxPhos function by acting upstream of OxPhos.

4.2 INTRODUCTORY REMARKS

The Oxidative Phosphorylation (OxPhos) pathway is composed of approximately 89 proteins encoded by the two genomes in animal cells: all 13 mitochondrial proteins and 76 nuclear proteins. These proteins form the five OxPhos enzyme complexes and are responsible for most cellular ATP production. Genetic defects in the OxPhos proteins affect at least 1 in 8,000 people and are the cause for the most common inherited metabolic diseases [138-140]. The interactions between mitochondrial and nuclear OxPhos proteins may be equally as important as deleterious mutations within either genome. The importance of these interactions have been demonstrated experimentally in humans, *Mus, Drosophila, Tigriopus, Callosobruchus*, and *Saccharomyce* where healthy nuclear and mitochondrial genome hybrids between species or among distant populations within a species affect fitness and OxPhos functions [58,59,61,63,119,121,141-150]. For example, hybrid breakdown due to mito-nuclear incompatibilities among *Tigriopus californicus* populations occur in F2 individuals [119] and alter ROS production [142], OxPhos enzyme activities [151], ATP production and survival [56]. These mito-nuclear interactions (GxG) are often affected by the environment as demonstrated by *Drosophila simulans* mitochondria that have pleiotropic effects at high environmental temperatures when substituted into one *D. melanogaster* genotype but not another [62,63,118]. Similarly, in seed beetles, *Callosobruchus maculatus*, temperature dependent metabolic rates rely on the interactions between the mitochondrial and nuclear genomes [116]. These mito-nuclear interactions that affect OxPhos are biologically important because they affect
fitness (egg production, survivorship, and mating success) [56,60-64,118-121]. In general, these data suggest that mito-nuclear interactions among species or divergent populations are likely to affect an organism’s physiology and these interactions are environmentally dependent [63,120,122,123,152].

Mito-nuclear interactions between different species or populations affect biological function [58,59,61,63,119,121,141-150]. However, it is less understood whether these interactions impact individuals within a single natural population. Theoretically, natural selection due to mito-nuclear interactions could alter allele frequencies when one mt-haplotype has greater fitness with a specific nuclear allele [153]. To determine if mito-nuclear interactions affect genotypes in naturally occurring populations, we examined a Fundulus heteroclitus population from a single inter-tidal estuarine creek. This population, just south of the Hudson River in Mantoloking NJ, USA, has two major mt-haplotypes with five non-synonymous substitutions: a “northern” haplotype, common in populations north of the Hudson River and a “southern” haplotype, common in populations south of the Hudson River [125]. The genetic divergence among mt-haplotypes may have been influenced by a historical break at the Hudson River due to the last glaciation [25], enhancing nucleotide pattern differences between northern and southern populations [30]. Populations with two distinct mt-haplotypes are the result of secondary-intergradation, whereby migrants meet where there was once a physical barrier [154]. Importantly, F. heteroclitus has large populations with low migration rates and is adapted to local environmental conditions [34,36,39,78,109,155,156]. Northern populations experience temperatures more than 12°C colder than southern populations and have evolved adaptations to temperature in cardiac metabolism and enzyme expression.
Because the evolutionary variation in OxPhos genes has been associated with divergence among populations in response to environmental variation [153,157-159] and has also been proposed to drive speciation [121,123,160-162], we might expect the mt-haplotype to affect the nuclear genotype and alter OxPhos function in the admixed population [163].

To explore potentially evolved epistatic interactions between nuclear and mitochondrial genomes, we addressed two questions: are allele frequencies at nuclear loci significantly different between the two specific mt-haplotypes, and if so, do these differences affect OxPhos function? To answer these questions, 155 Mantoloking, NJ F. heteroclitus individuals were genotyped at >11,000 SNPs, and their cardiac OxPhos metabolisms were measured. Individuals with southern and northern mt-haplotypes are present at a 60/40 ratio, respectively. We demonstrate significant allele frequency differences at 349 SNP loci between the two mt-haplotypes, and the different nuclear genotype and mt-haplotype combinations are associated with significant OxPhos metabolic differences.

4.3 MATERIALS AND METHOD

DATASETS

For the sake of clarity, we use two informative names for the two datasets analyzed in this manuscript. “MK-specific” refers to the SNP dataset that is solely based on the 180 individuals from New Jersey. To define admixture, a second data set (“3-population”) includes these MK individuals and individuals from Maine (ME, n = 35) and Georgia (GA, n =38). SNP discovery pipeline [164] defines SNPs that are polymorphic with
specific frequency and read depth, and thus, while many of the SNPs in the MK-specific and 3-population dataset are the same, 46% are unique.

**EXPERIMENTAL ANIMALS**

Adult *F. heteroclitus* were collected during the summer months from Mantoloking, NJ (40.049427°N, -74.065087°W), Wiscassett, ME (43° 57’ 15.10”N, 69° 43’ 13.64”W), and Sapelo Island, GA (31° 27’ 13.39”N 81° 21’47.65”W). All fish were captured in minnow traps with little stress and removed in less than one hour. Fieldwork was completed within publically available lands, and no permission was required for access. *F. heteroclitus* does not have endangered or protected status, and small marine minnows do not require collecting permits for non-commercial purposes. All fish were acclimated for 4 weeks to either 12°C or 28°C, temperatures naturally encountered in their natural environment. These two acclimation temperatures were used to explore how chronic (acclimation) and acute temperatures affect physiological functions [165]. Fish were exposed to a 14 hour light cycle, kept at 15ppt salinity and fed twice a day, 7 days a week. Housing, acclimation and non-surgical tissue sampling protocols were in compliance with and approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

**ISOLATING DNA**

DNA was isolated from fin clips and stored in 270 ul of Chaos (buffer 4.5M guanidinium thiocynate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCL pH 7.5, 0.2% antifoam, 0.1M β-mercaptoethanol) with ~ 1g of silica beads and combined with 130 ul of 10X TE (100mM Tris pH 7.8, 10mM EDTA pH 8.0). Tissue was homogenized using zirconium beads. Supernatant was removed and placed in a new tube with 200 ul of
95% EtOH and mixed. This solution was then quickly added to silica columns for DNA isolation. Loaded columns were centrifuged for 1 minute at 6,000xg, and flow through was discarded. As modified from [166], columns were washed three times with 750 ul of protein wash buffer (70 ml 96% EtOH and 26 ml binding buffer which contained 6M guanidine thiocyanate, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 4% Triton X-100) followed by centrifugation for 1 minute at 6,000xg. Then, samples were washed with 650 ul wash buffer (60% EtOH, 50mM NaCl, 10mM Tris-HCl pH 7.4, 0.5mM EDTA pH 8.0) and centrifuged for 1 minute at 16,100xg followed by another wash with 650 ul wash buffer and centrifugation for 3 minutes at 16,100xg to dry the silica column. 100 ul of 0.1XTE (10mM Tris, 0.1 mM EDTA) was added to elute the genomic DNA upon centrifugation for 1 minute at 6,000xg.

**Genotyping Mitochondria**

Mitochondrial haplotypes were defined by PleI, and BstYI restriction digest of ND2 and cytochrome b respectively. The digests were run on a 1% agarose gel to separate DNA fragments. Individuals from Maine and Georgia were used as controls. Both restriction enzymes yielded the same results for each individual. Haplotypes defined by restriction enzymes were the same as mitochondrial SNPs identified by GBS (genotyping by sequencing [71]). There were 19 mitochondrial SNPs that were in complete linkage disequilibrium, LD, (D’ 1.0). A single mitochondrial SNP was imputed for all individuals and used to determine relationships among nuclear-mitochondrial genotypes.

**Genotyping by Sequencing**

Isolated DNA quality was assessed via gel electrophoresis, and concentrations were quantified using Biotium AccuBlue™ High Sensitivity dsDNA Quantitative Solution
according to manufacturer’s instructions. After quantification, 100 ng of DNA from each sample was dried down in a 96-well plate. Samples were then hydrated overnight with 5 ul of water before AseI restriction enzyme digestion. This digest, based on in silico digest of the *F. heteroclitus* genome (NCBI accession JXMV00000000.1 [167]), should produce 523,349 fragments with 117,639 <500bp in size. Adaptors with separate barcodes for each individual (0.4 pmol/sample) were ligated to the genomic DNA after digestion with AseI. DNA samples were then pooled and purified using an equal volume of carboxyl coated magnetic beads (Fisher Scientific) in a PEG/salt solution (0.5 g beads in 100 mls of 20% PEG 8000, 2.5 M NaCl). Two bead purifications were used to select fragments between 100 and 400 bp. First, DNA less than 400 bp was separated from larger DNA which is bound to magnetic beads at low NaCl₂ concentration (0.87 M), then bead-salt solution was raised (NaCl₂ at 1.25M) so that only DNA larger than 100 bp are bound. These beads were washed with 70% EtOH, and DNA was eluted. The size range of purified products was verified using Agilent 2100 Bioanalyzer (Santa Clara, CA). A range of PCR cycles on the 100-400bp genomic fragments was used to optimize the amplification of restriction fragments using primers that anneal to the adapters. The distribution and concentration of the amplified library was verified using Agilent 2100 Bioanalyzer (Santa Clara, CA). DNA from the 18-cycle run formed the GBS library that was sequenced (Illumina Hi Seq 2500, 75bp single end reads; Elim Biopharmaceuticals, Inc., Hayward, CA).

**GBS Bioinformatics**

The Java program, TASSEL [164] used the first 64bps of single end sequences and aligned them to the *F. heteroclitus* genome to call SNPs. The *F. heteroclitus* genome (NCBI accession JXMV00000000.1), which consists of 10,180 scaffolds plus
mitochondria, was used to map sequencing reads. Two GBS datasets were produced: 1) the MK-specific, and 2) the 3-population dataset. For the MK-specific dataset, individuals were removed that had less than 50% of SNPs, reducing the number of individuals from 180 to 155. The data were filtered to remove SNPs with less than 1% minimum allele frequencies that occurred in less than 70% of individuals. Hardy-Weinberg expectation was calculated for individual loci using Arlequin v3.5.1.2 [168], and we excluded 256 SNPs where Ho>He and was significant (p<0.01). This latter filter is used to remove potential SNPs that represent differences between paralogs versus true allelic variants for a single locus [169]. For the 3-population dataset, individuals were removed that had less than 30% of SNPs, reducing the number of individuals from 257 to 234. SNPs that occurred in less than 77% of individuals were removed.

**Population Analyses**

For the MK-specific dataset, allele frequencies were defined using adegenet in R [170], and minor alleles were defined among all 155 NJ individuals. That is, a minor allele was defined across all individuals even when their frequencies >0.5 within a mt-haplotype. Two approaches were used to identify allele frequencies that had a bias relative to mt-haplotype: Fisher-Exact test and outlier-test using Arlequin v3.5.1.2 [168]. The Fisher-Exact test determines the bias in allele frequencies at each locus relative to mt-haplotype using PLINK [171]. Arlequin was used to compare the relative genetic distance between the two mt-haplotype relative to other loci. Specifically, we used an outlier test to define fixation index (F_{ST}) values that exceed the expectation based on the observed data. For comparisons between the two mt-haplotypes, we use fixation index (F_{ST}), and for clarity we use \( wF_{ST} \) (within population among mt-haplotypes). To identify SNPs with
\( \omega_{FST} \) outlier values, we used Arlequin v3.5.1.2 [168]. Outlier \( \omega_{FST} \) values are based on FDIST [172,173] as implemented in Arlequin, where coalescent simulations are used to get a null distribution and confidence intervals around the observed values and then tested to determine if observed locus-specific \( \omega_{FST} \) values can be considered as outliers conditioned on the globally observed \( \omega_{FST} \) value.

For Admixture analysis we thinned the 3-population dataset, removing SNPs closer than 100 bp (as suggested by the Admixture manual [174]). Thinning resulted in 3,700 SNPs. These 3,700 SNPs were input into Admixture v.1.3.0 [175] to infer ancestries of ME, GA, and MK individuals and provide an unbiased estimation of overall population structure.

LD was determined for MK individuals in 1) all SNPs in the MK-specific dataset, and 2) among 3.7K thinned SNPs from the 3-population dataset [164]. LDs were determined using a moving 50bp-SNP window providing \( r^2 \) (correlation coefficient), D’ and p-values associated with pairs of SNPs within and among scaffolds. The significant LD between SNP pairs and each SNP with mt-SNPs are reported as p-values <0.01 and with FDR correction [176]. FDR based on Benjamini & Hochberg [176] and were calculated in R using p.adjust [177].

Tajima’s D was calculated using VCFtools [178] with 50bp non-overlapping windows. VCFtools uses the physical distance (50bp) to calculate Tajima’s D. We used a 50bp window because nearly all SNPs within a 64 bp tag are captured by this window (i.e. SNPs occur at +10 bp in a tag-sequence). Using a 100bp window produced nearly identical results.
STRUCTURE v2.3.4 [179] was used to identify the number of ancestral populations (K) with similar allele frequencies and was also used to predict the magnitude of admixture within the single collection site. CLUMPAK [180] was used to average output from multiple STRUCTURE runs. For the 349 outlier SNPs, models allowing admixture and correlated gene frequencies were used with seven independent runs for each K-value from 1-5. Eleven thousand permutations with 11,121 initial runs (burn-in) were used for each run. The most parsimonious K was defined as that with the most likely K (largest mean Ln-likelihood) and the ΔK was based on the rate of change Ln-likelihood [181] using STRUCTURE HARVESTER [182]. We chose the most likely K if K was equal to 1, and used ΔK for K where the most likely was greater than one because ΔK can only resolve the best K with K >1.

Discrimination analysis of principal components (DAPC) was conducted in R using “adegenet” [170]. DAPC uses the principal components of allele frequencies to infer the number of clusters of genetically related individuals by partitioning into a between-group and within-group component and maximizing discrimination between groups [183].

**OxPhos Metabolism**

All individuals used for GBS analyses had their cardiac OxPhos metabolism measured as described in [184,185]. Heart ventricles were dissected, cut into halves, and half was placed into a muscle relaxation solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) [93]. The other half was saved for future RNA work. Tissues were then permeabilized using 2.5mg/ml saponin solution for 15 minutes, followed by 4 washes in relaxation solution for 5 minutes each [93]. Once
permeabilized, tissues were immediately transferred to the respirometry chambers containing a respiration medium (5mM EGTA, 3mM MgCl2.6H2O, 60mM K-lactobionate 20mM Taurine, 10mM KH2PO4, 20mM HEPES, 110mM Sucrose, 1g/l BSA).

The acute effect of temperature on mitochondrial activity was measured at three temperatures: 12°C, 20°C, and 28°C. Activity was measured and analyzed using the Oxygraph 2-k and DatLab software (OROBOROS INSTRUMENTS, Innsbruck, Austria). Population, acclimation temperature, and acute temperature changes were all randomized. All OxPhos determinations were relative to the amount of DNA in the measured tissue. Respiration rates were measured as pmol O2 s⁻¹ ml⁻¹ per ng DNA. The detailed analyses of acclimation and acute effect on OxPhos function within population are lengthy and are the subject of a separate publication [184].

After addition of the tissue to the respiration chamber, state 3 was determined. State 3 is defined as routine oxygen consumption resulting in ATP production in the presence of substrates and ADP. First, the substrates pyruvate (5 mM), glutamate (10mM), and succinate (10mM) were added, followed by ADP addition (5mM, state 3); cytochrome c (10µM) was added to check mitochondrial membrane integrity [93]. The tissue was recovered after respiration assays, and total DNA was quantified using AccuBlue high sensitivity dsDNA quantitation solution (Biotium). All activity was normalized by ng/ul of DNA. OxPhos function is represented as residuals from acclimation, assay temperature, body mass, and percent admixture from the 3-population SNP dataset. Percent admixture from Admixture v1.3.0 had no significant effect on OxPhos function [99].
4.4 RESULTS AND DISCUSSION

Two genotyping by sequencing (GBS) [71] datasets were used: 1) MK-specific (individuals from a single Mantoloking, NJ population) to assess nuclear-mitochondrial associations, and 2) 3-population dataset (Maine, MK, and Georgia individuals) to ascertain the effect of recent admixture. After filtering, 11,705 nuclear SNPs were distributed among 10,180 *F. heteroclitus* genome-scaffolds [186] for the MK-specific dataset, and 10,471 for the 3-population dataset. All SNPs and annotations were derived from the 64bp sequence tags used to call SNPs.

NUCLEAR-MITOCHONDRIAL ASSOCIATIONS

Strong selection at many nuclear loci creates a genetic load that is detrimental to a species' survival [187,188]. Therefore, it is unlikely that a population could maintain biologically important mito-nuclear interactions at many loci in a panmictic population (where migration = 0.5 of effective population size, Ne) [136,137,189]. We suggest that selection due to mito-nuclear interactions may occur if there is extensive standing genetic variation and many genes of small effect affect biological traits. To investigate whether these interactions between genomes do affect allele frequencies, we calculated F_{ST} values for each of the 11,705 SNPs in the MK-specific dataset, using the two mt-haplotypes as independent groups or populations. F_{ST} provides a statistically robust measure of the relative allelic variation between groups versus within groups. We denote this within population value as wF_{ST}. To be clear, although we are examining a single population, we use the two mt-haplotypes as artificial populations for wF_{ST} calculations. We found that 349 nuclear SNPs have wF_{ST} values that are large statistical outliers (p<0.01; Fig. 4.1).
SNPs with \(wF_{ST}\) outliers values are defined as having significantly large \(wF_{ST}\) values that are unlikely to occur relative to SNPs with similar heterozygosity (He) [168,172,190]. With 10% or 1% FDR, 236 or 72, respectively, of these 349 nuclear SNPs were significant; with a more conservative Bonferroni’s correction, 44 SNPs were significant (Fig. 4.1). Among the 349 outlier SNPs, none had significant linkage disequilibrium with each other (\(D’\) is not significant, \(p > 0.1\) and \(r^2 < 0.3\)). Differences in minor allele frequency (MiAF) could affect \(wF_{ST}\) values [191], yet outlier versus non-outlier SNPs have similar MiAF: mean MiAF=0.132 and 0.162 for 9,440 non-outlier, non-significant SNPs (this excludes SNPs that had significant \(F_{ST}\) values but not significant outliers) and 349 outlier SNPs respectively (Fig. 4.2). A separate analysis using allele counts for a Fisher Exact test revealed 229 SNPs with significantly biased allele frequencies (\(p<0.01\)). Of these 229 SNPs, 219 were also \(wF_{ST}\) outliers.

To investigate whether dividing individuals into two arbitrary groups can produce many significant \(wF_{ST}\) values, we produced a thousand random permutations for 9,440 non-significant SNPs (Fig. 4.3). None of the 1,000 permutations across 9,440 SNPs produces many \(wF_{ST}\) values as large as the 349 outlier \(wF_{ST}\) values as seen in the small overlap in their distributions (Fig. 4.3). Furthermore, the 99% upper confidence level for the arbitrary \(wF_{ST}\) values is less than the minimum \(wF_{ST}\) value for the 349 outlier SNPs (>0.002 and 0.0269, respectively, Fig. 4.3). Thus, grouping individuals into two arbitrary groups produces few SNPs with significant \(wF_{ST}\) values, indicating that the 349 outlier SNPs are statistically meaningful.

Each of the 349 outlier SNPs has a \(wF_{ST}\) value dissimilar from the genome wide \(wF_{ST}\) value (Fig. 4.1) and is unlikely to occur (Fig. 4.3). However, even though the 349
outlier $w_{F_{ST}}$ values are unlikely, the data could still suffer from type I error. We proceed with our analyses using the 349 outlier SNPs for three reasons. First, to balance type I and type II errors -- there are likely to be many more adaptive SNPs we have not discovered because of the weakness of adaptive tests [190,192]. Second, the use of different FDR values (1% -10%) yields a large range of significant SNPs (72 to 236), and it has been argued that FDR of 20% or more may be appropriate [193]. Third, and most importantly, we are asking if these 349 outlier SNPs are related to population structure and mitochondrial physiology. Including false positives (type I error) will not bias these tests except to make them less likely to find significant structure or association.

Given the evolutionary history of *F. heteroclitus* and the observation that the MK population has both mt-haplotypes, recent admixture may bias allele frequencies between individuals with northern and southern mt-haplotypes. In order to ascertain the effect of recent admixture, we used Admixture version 1.3.0 to infer ancestries from the 3-population SNP dataset (Fig. 4.4). For the Admixture analysis, we thinned the 3-population 10K SNPs so that all SNPs were >100bp apart, resulting in 3,700 thinned SNPs. Among these 3,700 SNPs, the MK population’s average admixture was 3.2% and never exceeded 14.7%. The plot of ancestry fraction (Q values) from Admixture clearly distinguishes Maine and Georgia from MK (Fig. 4.4B). These analyses indicate that the MK population is a separate and independent population from Maine and Georgia with little recent ancestral admixture and that allele frequency differences between mt-haplotypes within MK are not due to shared genealogies with mt-DNAs. Significant LDs for 3,700 thinned SNPs in the 3-population data are rare and physically close together (Fig. 4.5): 5 SNP pairs have significant LD (FDR <0.1) with the largest distance = 222 bp.
In comparison, nearly all SNP pairs within a scaffold (98%) are > 1,000 bp apart (Fig. 4.6A).

Even though there is little indication of recent admixture (Fig. 4.4), the outlier $wF_{ST}$ values could arise with the recent admixing of distinct populations resulting in SNPs with linkage disequilibrium over large distances [194,195]. Thus we might expect long distance LD for the 349 outlier SNPs with other nuclear SNPs or with mt-haplotypes. Among the 11K MK SNPs are 221,348 calculated LDs using a 50 SNP sliding window; 6,401 of these are significant with 10% FDR [196], and yet only 66 are significant and >100bp apart (Fig. 4.6B). That is, 99% of all SNPs with significant LDs are less than 100bp apart (Fig. 4.6B). This is also true for the 349 outlier SNPs. For each of the 349 outlier SNPs, there are only 229 SNPs in significant LD (FDR 10%) with any other of the 11K SNPs (Fig. 4.6C). Of these 229, six are between SNPs >100bp apart, and among these six, five are less than 200bp apart. One of the 349 outlier SNPs is in LD with another SNP greater than a million bp apart, yet this SNP lacks significant LD with many other closer SNPs.

Between each of the 11K SNPs and the mt-haplotype, none are in LD with any reasonable FDR (minimum FDR 0.57). Thus, none of the 349 outlier SNPs are in LD with the mitochondria. The lack of LD between any of the 349 outlier SNPs and the mitochondria reflects the relatively small allele frequency differences between mt-haplotypes for the 349 outliers SNPs (Fig. 4.2). Specifically, no SNP is close to fixation between mt-haplotypes (i.e., a difference close to 1). These patterns of LD among nuclear SNPs, including between the 349 outlier SNPs with mitochondrial SNPs, do not support recent admixture. Thus, based on LDs, there is little evidence that the biased allele frequencies in the 349 outlier SNPs are due to recent admixture.
We performed a Tajima’s D analysis on the 11,706 SNPs with a 50bp window using VCFtools [178]. The Tajima’s D value distributions for non-significant and outlier 349 SNPs (Fig. 4.7) are similar, and the 1% tail of these distributions have equal frequencies of both significant and non-significant SNPs (p-value > 0.2). Tajima’s D compares pair-wises differences to the number of segregating sites where linked SNPs should share large positive values when associated with balancing selection. Our Tajima’s D analysis provides no support for balancing selection. This result likely reflects how SNPs are called: SNPs are called from 64 bp sequenced tags that are typically hundreds of thousands to millions of bp apart (Fig. 4.6A), and only 0.1% of SNPs are in LD with other SNPs (Fig. 4.6B). The limited LD among SNPs within a 64bp tag, and the distance among tags suggest that significant Tajima’s D values are unlikely to occur because the assumption for Tajima’s D analyses is that sites affected by non-neutral processes will affect nearby linked sites. The rarity of SNPs in LD (Fig. 4.6) and the large MiAF (Fig. 4.2) suggest that SNPs have existed as long-term standing genetic variation; Tajima’s D analyses are unlikely to detect selection in this case [197].

To confirm and explore if there is any hidden population structure, we applied discriminant analysis of principal components (DAPC) [170]. Using the 349 outlier SNPs, DAPC identified two groups as the most parsimonious grouping: those associated with the northern or southern mt-haplotype (Fig. 4.8A-B). Using all 11K SNPs or 9,440 non-outlier, non significant SNPs, revealed a single grouping (Fig. 4.9). The observation that distinct groups are not seen with all 11K SNPs (Fig. 4.9) or the 9,440 non-outlier, non-significant SNPs but are seen with the 349 outlier SNPs lends additional evidence that
there is a single well mixed population and the outlier SNPs clearly discriminate individuals into two mt-haplotype associated groups.

STRUCTURE analyses [179] with K=1-5 using MK’s 349 outlier SNPs corroborated the DAPC results (Fig. 4.8C): K=2 was much more likely than K=1 and was the best supported K based on ΔK (the rate of change Ln-likelihood [181,182]). With K=2, individuals form two distinct clusters associated with each mt-haplotype. Larger Ks did not produce more definitive groups.

To understand the magnitude of the 349 outlier $wF_{ST}$ values, we compared these within population fixation index values to those between MK and a population 40 miles further south (Rutgers, NJ). Unexpectedly, MK $wF_{ST}$ values were larger than the between population $F_{ST}$ values (Fig. 4.10). Rutgers only has the southern mt-haplotype, and between population $F_{ST}$ values are a function of mt-haplotype: $F_{ST}$ values are smaller for comparisons of nuclear SNPs between Rutgers and just individuals in MK with southern mt-haplotypes than for comparisons of Rutgers to individuals in MK with northern mt-haplotypes (Fig. 4.10, red versus blue curve). Specifically, comparisons using MK individuals with northern mt-haplotypes are right-shifted with more loci with large $F_{ST}$ values. These data indicate that the genetic distances of the 349 outlier SNPs between mt-haplotypes are larger within the MK populations than between populations and that the genetic distances between populations for nuclear SNPs are a function of the mt-haplotype.

In summary, the MK population is a well-mixed population with a few hundred unlinked nuclear SNPs that have significant allele frequency differences between the two mt-haplotypes (Fig. 4.1). These biased allele frequencies create large $wF_{ST}$ values that are
distinct from the rest of the genome and unlikely to occur based on data permutations. The hypothesis that the MK population is well-mixed is supported by A) Admixture analysis on 3-population 3,700 nuclear SNPs (Fig. 4.4), B) the DAPC indicating a single population based on all 11K nuclear SNPs (Fig. 4.9) and C) the few significant SNPs in LD across the genome or with the mt-haplotype (Fig. 4.5 and 4.6). These data, and the observation that MK $wF_{ST}$ values among 349 outlier nuclear SNPs are larger than the $F_{ST}$ value for the same loci among populations (Fig. 4.10), suggest that that demographic effects including migration would not cause an association between the mitochondrial and nuclear genomes. These 349 outlier nuclear SNPs have an allele frequency difference between mt-haplotypes of 11.19% (95% CI = 10.69 to 11.69%), more than 3 times larger than the 3.28% (95% CI =3.22% to 3.35%) allele frequency difference for the remaining 9,440 non-outlier, non-significant SNPs (Fig. 4.2). For the 349 outlier SNPs, this allele frequency difference translates to $wF_{ST}$ values >0.054 (Fig. 4.1), compared to the majority of SNPs (9,440, 81%) where 95% of $wF_{ST}$ values are <-0.001 and have p-values >0.1 (Fig. 4.1). The evolutionary importance of these 349 outlier SNPs is suggested by $wF_{ST}$ values that are not likely to occur relative to other SNPs that share similar $He$. These differences are not due to different allele frequencies (MiAF=0.132 for the 349 outlier versus 0.162 for 9,440 remaining SNPs, Fig. 4.2) or heterozygosity (0.23 and 0.20 for 349 outlier and 9,440 non-significant SNPs, respectively). Yet for the 349 outlier SNPs, as indicated by $wF_{ST}$ values, the allele frequency differences between the two mt-haplotypes are significantly larger than the allele frequency variance within these groups; this is unusual relative to 96% of the other SNPs. These data on the 349 outlier nuclear SNPs are surprising given what we know about *F. heteroclitus* ecology and reproduction:
individuals occupy small home ranges in estuaries [155,198] and share a common reproductive strategy of laying and fertilizing eggs in the upper intertidal zone [199,200]. We tentatively conclude that these 349 outlier SNPs are most likely evolving by natural selection due to the epistatic interaction between the nuclear and mitochondrial genomes.

**OxPhos Function**

If these differences in 349 outlier SNPs are meaningful, we would expect differences in biological functions between mito-nuclear genotypes. To determine if the 394 outlier SNPs affect a biological function, cardiac OxPhos metabolism was measured as State 3 respiration (an integrative measure of ADP and substrate dependent mitochondrial respiration) and compared to mito-nuclear genotypes among the MK individuals (Fig. 4.11). In ANCOVA (with Admixture coefficients, acclimation, assay temperature and body mass as covariates), we used four mitochondrial groups as the main effect (Fig. 4.11A). The four mitochondrial groups are based on STRUCTURE analysis of the 349 outlier SNPs (Fig. 4.8C). Most individuals have >70% of nuclear alleles associated with one of the two mt-haplotypes (Fig. 4.8C). However, 21 individuals have mixed ancestry; these individuals shared at least 30% of nuclear alleles with the opposite cluster (northern 349 SNP alleles with southern mt-haplotype or southern 349 SNP alleles with northern mt-haplotype). This defines four groups: the two main structure groups (Fig. 4.8C) and two groups with mixed ancestry from each cluster (individuals with >30% of the alternative allele). Among these four mito-nuclear groups, State 3 is significantly different (Fig. 4.11A, p < 0.0194); admixture was not significant (p >0.8) while mass, acclimation and acute temperature were significant (p < 0.05). State 3 respiration was significantly lower in individuals with the northern mt-haplotype compared to those with
the southern mt-haplotype (Fig. 4.11A, Tukey post hoc test). Individuals with “mixed” nuclear backgrounds showed intermediate mitochondrial respiration. For mixed ancestry individuals with a northern mt-haplotype, having a larger number of “southern” associated nuclear alleles increased respiration rates, whereas the opposite effect was observed for individuals with a southern mt-haplotype with a larger number of “northern” associated nuclear alleles. Thus, this analysis indicates that variation in the nuclear genome modulates mt-haplotype effects on OxPhos metabolism.

A second analysis regresses State 3 respiration against the fraction of southern mt-ancestry (Fig. 4.11B) using the same covariates as above (Admixture coefficients, body, mass, acute and acclimation temperature). The fraction of southern mt-ancestry is significant ($p < 0.0055$) and explains 6% of the variance (Fig. 4.11B). These data indicate that individuals with the greater number of southern alleles with a northern mt-haplotype have greater OxPhos metabolism and individuals with more northern alleles with a southern mt-haplotype have lower OxPhos metabolism. Notice, that we form a polygenic score for each individual using the 349 outlier SNPs, which defines the fraction of ancestry related to southern mt-haplotype. Thus, because the MK 349 SNPs have significantly biased allele frequencies and their inferred ancestry of $K = 2$ reflects the two mt-haplotype, significant regression indicates a significant mito-nuclear interaction.

The potential genes that could affect State 3 mitochondrial respiration include approximately 89 nuclear and mitochondrial proteins that form the five OxPhos complexes, and approximately 1,500 other nuclear genes are involved in mitochondrial functions [201]. Using BLAST [202], we aligned the 64bp sequences containing outlier SNPs against the *F. heteroclitus* genome. Sixty-four base pairs were used because this is
the length of the sequences retained from sequencing and pipeline analysis[164].

Although all outlier sequences aligned to the genome, only 162 aligned to annotated genes. Many of these genes encode transcription factors (e.g., zinc finger proteins), are involved in signaling pathways (e.g., 1-phosphatidylinositol 4,5-bisphosphate, GTPase or receptors), or are trans-membrane proteins. Four genes were identified that likely influence OxPhos metabolism (Table 4.1). The first, acyl-coenzyme A thioesterase, which is mitochondrially localized, affects intermediates in the citric acid cycle, which forms OxPhos substrates via lipid metabolism regulation [203-205]. A second gene, adenylate kinase, regulates mitochondrial respiration by altering ADP/ATP ratios [206] and creating feedback signal communication. A third gene, NAD-dependent malic enzyme, catalyzes a reaction that forms pyruvate from malate with the reduction of NAD+ to NADH [207-209]. The mitochondrial variant acts as a regulatory enzyme, allosterically activated by fumarate and inhibited by ATP [210]. These substrates, products and allosteric regulators are all involved in OxPhos metabolism. The fourth gene, ribosomal mitochondrial protein (MRP) S35, is responsible for translating the 13 mitochondrial proteins making up the OxPhos pathway. MRPs are linked to human mitochondrial disorders such as Leigh Syndrome, multiple mitochondrial dysfunctions syndrome, Russell-Silver syndrome, Spinocerebellar ataxia with blindness and deafness, Stuve-Wiedemann syndrome, and Usher syndrome [211].

The described genes may play an important metabolic role through mitochondrial OxPhos protein regulation and translation. Surprisingly, none of the 349 outlier sequences aligned to the 76 nuclear encoded proteins that form the OxPhos complexes. There are two possible reasons for this: 1) none of the 11,000 SNP sequences include OxPhos
proteins, or 2) SNPs in these proteins are not affected by the mito-nuclear interactions. Five of the 11,000 SNPs mapped to nuclear proteins that directly participate in OxPhos: one in Complex I, two in Complex II, and two in Complex V. Yet, there was no allele frequency bias in these OxPhos proteins. Mito-nuclear interactions may still affect other nuclear OxPhos genes that were not sequenced in our study. However, given our data, we postulate that epistatic selection affects OxPhos functions and is acting upstream of the OxPhos pathway.

Although significant associations between nuclear loci and mt-haplotype point towards epistatic selection, an alternative explanation could be assortative mating. Allelic bias may occur if individuals can recognize mates with similar mt-haplotypes and accordingly, preferentially mate. However, no evidence of this has been documented in *F. heteroclitus*. Our data is more likely explained by epistatic selection for two reasons. First, there is a large overlap in 349 significant loci found by using two different methods: Arlequin [168] F<sub>ST</sub> test and Fisher Exact test. It is very unlikely that these significant associations are random. Second, Admixture using 3,700 SNP from the 3-population set (Fig. 4.4) and DAPC using all 11K SNP from the MK SNP set (Fig. 4.9) indicate little if any population structure within the MK population. Assortative mating would have to be highly selective to maintain allelic bias because the unlinked 349 outlier SNPs would come to equilibrium if only drift and incomplete isolation was responsible. Thus, assortative mating seems unlikely; instead MK seems to be a well-mixed random breeding population.
Difficult questions to answer are how two mt-haplotypes are maintained in a single population and how so many loci are potentially affected by natural selection due to GxG interactions. Theoretically, it is difficult to maintain functionally different mitochondrial haplotypes in a single population due to GxG interactions [136,137,189]. Mito-nuclear polymorphisms can be maintained with sex-linked loci under restricted conditions [52,137]. Yet, the 349 outlier $wF_{ST}$ loci are distributed over 100s of scaffolds, and thus it seems unlikely that they are limited to sex-linked chromosomes. Mutation-selection balance also seems unlikely because there is a high frequency of the minor alleles: the average heterozygosity for the 349 outlier $wF_{ST}$ loci is 0.23 and is similar to neutral loci. It is also difficult to suggest migration or other demographic effects because the allele frequency difference within MK population for the 349 outlier $wF_{ST}$ loci is larger than the allele frequency difference among populations (Fig. 4.6). Additionally, the 3-population data set indicates a well-mixed population (Fig. 4.4). The high allele frequencies for both alleles among the 349 outlier $wF_{ST}$ loci (Fig. 4.2) suggest that there is balancing selection, which might arise if the two alleles have different fitness effects in different environments. GxE interactions where allele effects have a high variance among environments could maintain selectively important polymorphisms especially if there is extensive pleiotropy [137] or unpredictable environmental variations [212]. Notice, because these individuals were captured together in the same estuarine creek, it is unreasonable to suggest spatial variation in the environment; instead temporal variation is common in the *F. heteroclitus* environment and may contribute to maintaining the observed mito-nuclear genetic variation.
What we do know is that there is environmentally dependent, adaptive divergence in OxPhos mRNA expression among populations [34,35,213,214], suggesting that GxE interactions are possible. In other species, mito-nuclear interactions have pleiotropic effects [62,63] and affect genome wide mRNA expression patterns [215,216]. Thus, although lacking data to specifically address the evolutionary genetics that maintain selectively different mito-nuclear interactions, we suggest that temporal environmental variation affects mito-nuclear polymorphisms that have pleiotropic effects. The hypothesis of pleiotropic effects is supported by the diversity of annotations associated with the 349 outlier $wF_{ST}$, which include transcription factors and signaling pathway genes that are likely to have a wide diversity of phenotypic effects.

4.5 Conclusion

Although epistatic interactions between mitochondrial and nuclear genes have been shown to affect overall organismal fitness and metabolic activity [58-60,62,63,116,118,143,217], these studies have used divergent mt-haplotypes and divergent nuclear backgrounds or fail to show an effect on allele frequencies in natural populations. The data we present show that mito-nuclear interactions influence allele frequencies in a natural, freely interbreeding population. We show that 349 outlier SNPs have greater allele frequency differences between mt-haplotypes than within a mt-haplotype, creating large, significant $wF_{ST}$ values ($wF_{ST}$ values are $F_{ST}$ values within a population between two mt-haplotypes). The distribution of $wF_{ST}$ values within and $F_{ST}$ values between populations for neutral SNPs is different from that of the 349 outlier SNPs. These 349 outlier SNPs were used to form a polygenic factor, where the individual scores affected OxPhos metabolism, supporting the hypothesis that mito-nuclear
interactions are evolutionarily important. These observations are difficult to resolve with any neutral or realistic demographic mechanisms. Thus, we tentatively conclude that the most parsimonious explanation is that epistatic selection is strong enough to alter allele frequencies for 100s of SNPs. The observation that several of these genomic SNPs are for genes that modulate OxPhos supports this hypothesis. The selection for mito-nuclear interactions that modulate OxPhos may occur if there is extensive standing genetic variation and the genes have small effects.

Table 4.1. Genes of interest that aligned to 64bp sequences.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Position</th>
<th>Gene</th>
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<td>383976</td>
<td>Acyl-coenzyme A thioesterase 9, mitochondrial</td>
<td>7E-16</td>
</tr>
<tr>
<td>9927</td>
<td>383976</td>
<td>Adenylate kinase isoenzyme</td>
<td>1E-23</td>
</tr>
<tr>
<td>9976</td>
<td>1473252</td>
<td>NAD-dependent malic enzyme, mitochondrial</td>
<td>5E-26</td>
</tr>
<tr>
<td>98</td>
<td>588044</td>
<td>Ribosomal mitochondrial protein S35</td>
<td>2E-19</td>
</tr>
</tbody>
</table>
Figure 4.1. Distribution of wFST values for nuclear SNPs calculated between the two mt-haplotypes within the single MK population

Although we are examining a single population, we use the two mt-haplotypes as artificial populations for wFST calculations. Plot contains wFST values and corresponding negative log_{10} p-values ( - log_{10}(0.01) = 2 ). Blue values are significant with a p-value < 0.01, green values are significant with a 1% FDR correction, and purple values are significant with a Bonferroni correction. Histograms show wFST and p-value distributions.
Figure 4.2. Differences in minor allele frequencies (MiAF).
Differences in MiAF between northern and southern mt-haplotypes versus overall MiAF (minor allele frequencies among all individuals). Blue dots indicate values for the 349 outlier SNPs.

Figure 4.3. Ten million random FST values versus 349 outlier wFST values.
One thousand random permutations of ten thousand SNPs with non-significant wFST values. wFST values were determined when individuals were randomly assigned to one of two groups at the same frequency as mt-haplotypes. Rarely were wFST values equal to or greater than the 349 outlier FST values.
Figure 4.4. Admixture Analysis on 3-population 3,700 SNP data set.

A: Plot of Admixture ancestry fractions (Q) from the 3-population SNP data set. 35 Maine individuals are blue, 38 Georgia individuals are red and the 161 MK New Jersey individuals are green. Georgia ancestry coefficients are on the x-axis, and Maine ancestry coefficients are on the y-axis. B: Individual ancestry fractions. Colors are the same as in A. Each individual is represented by a thin vertical line, which is partitioned into 3 colored segments that represent the individual’s estimated membership into one of the three populations.
Figure 4.5. LD for 3-population 3,700 SNPs.

Linkage disequilibrium for 3,700 SNPs among three populations: Maine, MK-NJ and Georgia. Plots are Log\textsubscript{10} distance (bp) \textit{versus} negative log\textsubscript{10} for the FDR p-value (1 = 10\% FDR). Distances are within scaffolds for 3,700 SNPs thinned so that all SNPs >100bp apart.
Figure 4.6. 11K SNPs from MK, New Jersey population.

A: Histogram of $\log_{10}$ distance in base pairs between SNPs for all 11K SNPs. B: Histogram of $\log_{10}$ distance in base pairs for SNPs with significant LDs (FDR 10%). 66 SNPs are in LD with another SNP > 100 bp away. C: Distribution of p-values for significant LD (FDR 10%) versus $\log_{10}$ distance in base pairs. Dark blue solid spots are for the 349 outlier SNPs.
Figure 4.7. Tajima’s D for significant and non-significant SNPs.

The relative frequency (density) Tajima’s D based on 50 basepair windows that include the 349 outlier SNPs (blue) or only the non-significant SNP (red). Tajima D values were calculated using VCFtools.
Figure 4.8. DAPC and STRUCTURE plots.
Population structure based on 349 outlier SNPs. A) Results of the Bayesian information criterion used to infer the number of genetic clusters when using 349 outlier SNPs. B) Discriminant analysis of principal components (DAPC) based on 349 outlier SNPs. Discriminant function separates individuals into two distinct groups, which are the two mt-haplotypes. “North” is for northern mt-haplotype. “South” is for southern mt-haplotype. C) STRUCTURE plot for 349 outlier SNPs. Plot shows probability that individuals’ nuclear genetic variation belongs to one of two clusters, which are northern and southern haplotypes. Each individual is represented by a thin vertical line, which is partitioned into two colored segments that represent the individual’s estimated membership into one or the other cluster. Twenty-one individuals have a mixed ancestry (> 30% of alternate SNP alleles).
**Figure 4.9** DAPC for 11K SNPs from the MK dataset.

A) Results of the Bayesian information criterion used to infer the number of genetic clusters. B) Discrimination of two mt-haplotypes based on all 11K SNPs. Discriminant function separates individuals into one group based on mt-haplotype. “North” is for northern mt-haplotype, “South” is for southern mt-haplotype.

**Figure 4.10** Density plot of FST values for both between mt-haplotypes within MK populations and between populations.

Areas under each curve are the same. Green line is for the 349 outlier SNP $F_{ST}$ values between the two mt-haplotypes within the MK population. The light purple line represents 9,440 non-outlier SNP $F_{ST}$ values between the two mt-haplotypes within the MK population. Two curves compare the MK population with a separate Rutgers’ population for the same 349 outlier SNPs: red is for individuals from the MK population with the southern mt-haplotype compared to the Rutgers population, and blue is for individuals from the MK population with the northern mt-haplotype compared to the Rutgers population.
State 3 OxPhos metabolism in the MK population is oxygen respiration dependent on substrate and ADP, and the data graphed are the residuals from Admixture ancestry, body mass, acclimation temperature and assay temperature [184]. A: ANCOVA (p-val = 0.0194): individuals were assigned to one of four groups based on their 34 outlier genotypes and mt-haplotypes: “North Mito North Nuclear” are individuals with >70% of alleles from the 349 outlier SNPs associated with northern mt-haplotypes, “South Mito South Nuclear” with >70% of alleles from the 349 outlier SNPs associated with southern mt-haplotypes, and the two mixed ancestral groups: individuals where >30% of nuclear genotypes are associated with the opposite mt-haplotype, as defined by STRUCTURE. Means (dot) and standard errors are displayed. N=155. Letters (“a”, “b” are significantly different based on Tukey post-hoc comparison). B: Regression of residual of State 3 versus fraction of southern ancestry (0.0 -1.0) as defined by STRUCTURE with K=2. Linear regression is significant (p <0.0055) with an R² = 0.

Figure 4.11. Mito-nuclear effects on State 3 Respiration.
CHAPTER 5. SYNTHESIS

Mitonuclear interactions between different species or populations affect biological functions [58,59,61,63,119,121,141-150]. My thesis focuses on Fundulus heteroclitus populations living along a thermal cline along the east coast of the United States where northern populations experience temperatures more than 12°C colder than southern populations [34,218]. These populations show genetic divergence along this thermal cline [219], with variation in enzymes, enzyme expression, and mitochondrial genomes [25,34,36]. F. heteroclitus populations have two major mitochondrial genomes with five non-synonymous amino acid substitutions: a “northern” haplotype, common in populations north of the Hudson River and a “southern” haplotype, common in populations south of the Hudson River [220]. This break at the Hudson River is due to isolation between northern and southern populations during the last glaciation. Post-glaciation, a secondary-intergradation event occurred just south of the Hudson River in Mantoloking, NJ, whereby the previously isolated populations created an admixed population containing both major mt-haplotypes. Because evolutionary variation in OxPhos genes has been associated with divergence among populations in response to environmental variation [153,157-159] and has also been proposed to drive speciation [121,123,160-162], we expect the mt-haplotypes and nuclear genotypes to affect OxPhos function [163].

Understanding the interaction of mitochondrial and nuclear genomes in the OxPhos pathway and its implications for fitness and evolution was the motivation of this dissertation. The OxPhos pathway is a crucial part of the cell, providing it with most of
the energy it needs. It is also unique in that it is encoded by both the mitochondrial and nuclear genomes. Mitochondria are derived from alpha-protobacteria and through their endosymbiosis with eukaryotes have significantly reduced their genome size [221,222] through both gene transfer from the mitochondria to the host genome and from gene loss [222,223]. This was the beginning of “co-evolution” between the mitochondrial and nuclear genome. Futuyma defines co-evolution as “Reciprocal genetic change in interacting species owing to natural selection” [224]. Rand et al. argues that when “species” is replaced by “genomes,” mitonuclear evolution acts in the same manner [123]. In this co-adaptive model, selection would act on both genomes to promote optimized OxPhos pathway function [1]. An alternative model, the compensatory model, argues that accumulation of mtDNA mutations occurs due to small effective population size (Ne), maternal inheritance, and haploidy of the mitochondria, and that therefore, the efficacy of selection is lower than that of the nuclear genome [1,123]. This would place higher selective pressure on the nuclear genome to compensate for mitochondrial genome mutations [1,123]. The genomic landscape of the two genomes involved in OxPhos is most probably shaped by both of these models. This compensatory model is observed when OxPhos is measured in divergent populations, and compensatory changes in nuclear genes hide potential functional differences between mt-haplotypes (Chapter 2). In contrast, when OxPhos is measured within an admixed population, functional differences are observed between the two mt-haplotypes (Chapter 3). In an admixed population with two functionally different mt-haplotypes and nuclear alleles from both ancestral populations, a conflict is created between the two genomes. Natural selection could
address this conflict by selecting for specific nuclear alleles depending on the maternal mitochondria (Chapter 4).

The mitochondrial and nuclear genomes have very different modes of evolution whereby the mitochondrial genome has a higher mutation rate than the nuclear genome, strict maternal inheritance, and no recombination [1]. This begs the question of how exactly they have managed to evolve together in a manner that optimizes fitness. We chose to explore this question in *Fundulus heteroclitus*, an ectothermic teleost. Because temperature plays a crucial role in enzyme kinetics, it is also important in shaping the evolution of enzymatic pathways, such as OxPhos [45,218,219]. First, we wanted to understand OxPhos differences in divergent populations; one that inhabits a colder environment in ME, one that inhabits a warmer environment in GA, and a sister taxa *F. grandis* in the Gulf of Mexico. Next, to understand the role that mt-haplotype plays in OxPhos activity, we focused on a *F. heteroclitus* population in Mantoloking, NJ where both mt-haplotypes exist in a mixed, panmictic population. Our physiological experiments measured cardiac metabolism in *Fundulus* hearts. Because we wanted to take into account different temperature regimes, we therefore measured OxPhos at two different acclimation temperatures (12°C and 28°C) and three different acute temperatures (12°C, 20°C, and 28°C). We measured six parameters of the OxPhos parameters: State 3, E State, Complex I, Complex II, Complex IV, and LEAK.

When OxPhos was measured in divergent populations (ME, GA, *F. grandis*), no significant differences were found between the two *F. heteroclitus* populations. Significant differences between populations were driven by *F. grandis* and were a function of both assay and acclimation temperatures. State 3, E State, and Complex I were
greater in *F. grandis* than ME *F. heteroclitus* and the GA *F. heteroclitus* population was intermediate between the other two populations. These differences only occurred at the 12°C acclimation temperature and were more pronounced at the high acute temperatures. Although populations reflected genetic distance (ME *F. heteroclitus* < GA *F. heteroclitus* < *F. grandis*) our results are surprising because we expected distant populations exposed to different environments (ME populations are on average 12°C colder than those in GA), to show much greater differences in OxPhos. This expectation was based on previous research that showed OxPhos mRNA expression differences that affected OxPhos dependent metabolites [35] and significant Complex I enzyme activity differences [225]. This suggests that there may be compensatory responses in the nuclear genome counteracting the contribution of the two mt-haplotypes. There are only 13 genes encoded by the mitochondrial genome, and selection on mt-DNA has been argued to be mostly purifying, leaving less room for non-neutral mtDNA variation to accumulate [1]. The nuclear genome has much higher standing genetic variation, and natural selection will act on smaller selection coefficients between isolated populations. We therefore speculate that this led to potentially compensatory loci between the ME and GA population, decreasing the variance in the OxPhos phenotype. This type of compensatory behavior has been shown in primate Complex I where nuclear encoded proteins show evidence of positive selection, which tends to follow changes in mtDNA amino acid sequences at a nearby site [2].

To address whether the two mitochondrial haplotypes affect OxPhos in the absence of compensatory nuclear effects, we reduced the nuclear genetic differences among individuals with different mitochondrial haplotypes by measuring OxPhos in a
single admixed population in Mantoloking, NJ (Chapter 3). In this single population, both mitochondria should be randomly associated with all nuclear allelic variation, and even if there is a bias in allele frequencies, it will have marginal effects [226-228]. Unlike the comparison between the ME and GA populations, within a single population there are significant effects of mt-haplotype for State 3, E state, Complex I and Complex II. These differences were mostly driven by the response in OxPhos to acute temperature change at the 28°C acclimation temperature. The most obvious difference was that the northern mt-haplotype was insensitive to acute temperature changes at 28°C acclimation, yet there was a significant increase in OxPhos activity with increase in acute temperature for the southern mt-haplotype. Both mt-haplotypes had significant increases in OxPhos activity with increasing acute temperature at the 12°C acclimation temperature. These data in Chapter 3 demonstrate that the mitochondrial haplotypes present in the ancestral northern and southern populations significantly affect OxPhos in a panmictic population.

It is important to note, that acclimation and acute temperature significantly affected all measured OxPhos parameters. Most notably, in the ME and GA populations we found an interesting interaction; acclimation altered the acute response shape. For state 3, E state, Complex I, and Complex II, at the low acclimation temperature, the acute response was dampened at low assay temperatures, and at the high acclimation temperature, the acute response was dampened at high assay temperatures (Fig. 5.1). This suggests that acclimation temperature can mitigate acute temperature responses [82,84], an important factor to consider when trying studying the thermal tolerance of different ectothermic species. In the NJ population although this pattern was only observed with the southern mitochondrial haplotype, there was still an important interaction of acclimation
temperature, acute temperature and mt-haplotype. When acclimated to 12°C, both mt-haplotypes had increased OxPhos activity with an increase in acute temperature. When acclimated to 28°C, the southern mt-haplotype had an increase in OxPhos activity with an increase in acute temperature, but the northern mt-haplotype showed no difference in activity across all acute temperatures. This means that when acclimated to high temperatures, the northern mt-haplotype in the NJ population is insensitive to acute temperature (Fig. 5.2). This suggests that the large temporal variation experienced by this population selects for both mt-haplotypes, which interact differently with their nuclear background. This would allow the maintenance of both of these haplotypes in this population due to different selective advantages during different environmental conditions. These data demonstrate the importance of examining environmental effects, such as chronic and acute temperature when investigating genetic effects.

Chapter 3 demonstrated that mitochondrial haplotypes affect OxPhos function, but did not address if the nuclear genome modified this effect. To test if nuclear genomes modified mitochondrial effects, OxPhos was quantified among individuals from the admixed NJ population and all individuals were genotyped to test for significant associations between nuclear genes and mt-haplotype (Chapter 4).

Mitonuclear interactions affect fitness and OxPhos functions in humans, *Mus, Drosophila, Tigriopus, Callosobruchus,* and *Saccharomyces* when healthy nuclear and mitochondrial genome hybrids (cybrids) between species or among distant populations are experimentally created [58, 59, 61, 63, 119, 121, 141-150]. For example, hybrid breakdown due to mitonuclear incompatibilities among *Tigriopus californicus* populations occur in F2 individuals [119] and alter ROS production [142], OxPhos enzyme activities [151],
ATP production and survival [56]. These mitonuclear interactions are often affected by the environment (G x G x E). Mossman et al [229] created different combinations of genotypes from *D. melanogaster* and *D. simulans* mitochondria and nuclear genomes and found that mitonuclear epistasis was dependent on the environment (diet, in this case) but affected fitness traits such as development time and egg-to-adult viability [229].

*Drosophila simulans* mitochondria have pleiotropic effects at high environmental temperatures when substituted into one *D. melanogaster* genotype but not another [62,63,145]. Similarly, in seed beetles, *Callosobruchus maculatus*, temperature dependent metabolic rates rely on the interactions between the mitochondrial and nuclear genomes [141]. These mitonuclear interactions that affect OxPhos are biologically important because they affect fitness (egg production, survivorship, and mating success) [56,60-64,119-121,145]. These published data experimentally demonstrate that the interactions between animal genomes are biologically important. The results presented in Chapter 4 are different from these studies because I examined mitonuclear interactions in a naturally occurring population *versus* experimental systems that replace native mitochondrial with divergent or mutant mitochondria. The critical difference here is that selection would have to occur in natural populations and still maintain both mitochondrial haplotypes, and this is theoretically difficult [123,136,230].

Early theoretical models suggested that mitonuclear interactions would not be able to maintain mitochondrial genome polymorphisms in a panmictic population [136,231,232]. Additionally, Clark and Lyckegaard found no evidence of mitonuclear epistasis among *D. melanogaster* lines [189]. Therefore it was thought that mitonuclear polymorphisms were quickly removed in panmictic populations but that there was some
sort of coevolution happening as epistatic interactions were observed when different populations were crossed. There were two main drawbacks to these studies. First, these studies focused on juvenile viability, which represents only a small portion of genetic variation for *D. melanogaster* fitness (~15%), and second, these results cannot be applied to outbred populations [60].

In Chapter 4, to test the theory of epistatic interaction between the nuclear and mitochondrial genome affecting OxPhos, genotyping by sequencing [71] was used to find associations between the nuclear and mitochondrial genome in an admixed *F. heteroclitus* population. This population contains individuals with two mt-haplotypes, which are freely interbreeding, therefore representing a panmictic population. We found significant associations of 349 nuclear SNPs with mt-haplotype. More importantly, these associations significantly affected the OxPhos phenotype. Individuals with the northern mt-haplotype and mostly northern nuclear SNPs (from among the 349 significant SNPs) had lower OxPhos activity than individuals with the southern mt-haplotype and mostly southern nuclear SNPs. Individuals with “mixed” nuclear backgrounds had intermediate OxPhos activity. For mixed ancestry individuals with a northern mt-haplotype, having a larger number of “southern” associated nuclear alleles increased respiration rates, whereas the opposite effect was observed for individuals with a southern mt-haplotype with a larger number of “northern” associated nuclear alleles. These data show that in natural populations with large standing genetic variation, naturally occurring healthy mitochondria are substantially different when placed in different nuclear genomes. None of the SNPs that influenced OxPhos function were present in genes that encode proteins.
directly involved in the pathway. Therefore, I conclude that epistatic selection is affecting genes upstream of OxPhos.

Finding a bias in nuclear allele frequencies that affected OxPhos was extremely surprising. In an interbreeding population, one expects complete mixing of nuclear gene mixing, despite mt-haplotype differences. The allele frequency bias among many genes in a panmictic population would require effective selection every generation. Large selection coefficients could accomplish this, but it would create an unbearable genetic load (excessive selective death, [136,187,188,233]). Although small selection coefficients among many loci relieves the cost of genetic load, it makes it unlikely that there will be significant allele frequency differences [234]. Yet, in Chapter 4 there are highly significant allele frequency differences at 349 SNPs, and these SNPs appear to be functionally important. While these results are difficult to theoretically interpret in the context of the standard neutral model, they indicate that epistatic interactions between the mitochondrial and nuclear genomes are important in shaping the OxPhos pathway (G x G, gene by gene interactions).

5.2 THEMES

Any type of physiological and genetic data needs to be properly placed in context. In the physiological data presented in this dissertation, it is very apparent that genotype by environmental interactions play an important role in affecting OxPhos function. This is most evident when comparing three populations of Fundulus at different acute temperatures. There are no differences between populations when individuals are measured at the 12°C acute temperature, but differences become apparent at the 28°C acute temperature (Fig. 5.2). Similarly, differences between mt-haplotypes are only
apparent at the high acclimation temperature, at high acute temperatures. This is an important theme to take into consideration when trying to understand how organisms can adapt to changing environments. Aerobic scope is now considered an important factor in determining how fish will fare with global climate change [235], but genetic factors should also be taken into consideration when measuring aerobic scope.

Secondly, it is important to the complexity of the genotypic-phenotypic map. Although most people are familiar with the one gene-one effect Mendelian model, phenotypic effects can be due to multiple genes (polygenic trait), or one gene can cause multiple effects (pleiotropy) (Fig. 5.3, [236]). And of course, there can always be a mix of polygenic and pleiotropic effects. Although there is a limited number of “phenotypes,” there are many ways to achieve these different phenotypes (Fig. 5.4, [237]).

5.3 Summary

This work establishes the importance of compensatory, co-adaptive and epistatic mitonuclear interactions. It also demonstrates that these interactions are closely tied to the environment that they are found in, and that when studying such processes, we must always consider all interacting factors. Many important patterns were revealed when measurements were taken at, or individuals were acclimated to different temperatures. We found that two divergent F. heteroclitus populations, with different mt-haplotypes who experience very different temporal thermal environments have minimal difference in OxPhos functional differences. In contrast, an interbreeding population with these two mt-haplotypes shows larger OxPhos differences. Acclimation temperature modulated acute temperature changes in the divergent ME and GA populations and also affected the sensitivity to acute temperature in NJ individuals. Lastly, in a panmictic population
containing two mt-haplotypes, there were significant associations of 349 nuclear SNPs between mt-haplotypes. These associations influenced OxPhos function and were found in genes upstream of the OxPhos pathway.

The combination of physiological, genomic and evolutionary analyses provides a comprehensive dataset that gives us insight into the biological consequences of nucleotide polymorphisms as well as insight into the evolution of adaptive change in two genomes: the mitochondrial and nuclear genomes. Thus, this research, focusing on the functional genetic variation in response to a thermal cline, informs us of the mechanisms by which organisms can evolve and adapt to their environments and the complex interactions between genomes and the environment.

Figure 5.1 Idealized model of acclimation influence on acute effects for ME and GA *F. heteroclitus* populations.
Figure 5.2 Idealized model of acclimation effect on mt-haplotype differences in response to acute temperature for NJ *F. heteroclitus* populations.
Figure 5.3 Polygenic and pleiotropic effects.

Figure 5.4 Genotypic-phenotypic map. Effects of pleiotropic and polygenic effects on fitness and phenotypes.


