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UNIVERSITY OF MIAMI

ANALYSIS OF COMPLEX I ACTIVITY WITHIN AND AMONG POPULATIONS OF *FUNDULUS HETEROCLITUS*

By

Samuel J. Loftus

A DISSERTATION

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

Coral Gables, Florida

June 2012

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ANALYSIS OF COMPLEX I ACTIVITY WITHIN AND AMONG POPULATIONS OF *FUNDULUS HETEROCLITUS*

Samuel J. Loftus

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Analysis of Complex I Activity Within and Among Populations of *Fundulus heteroclitus* (June 2012)

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This research focuses on the mitochondrial Complex I (EC. 1.6.5.3), the genes that encode this enzyme complex, the pattern of enzyme activity within and among populations, and how mRNA expression covaries with Complex I activity.

Complex I is found in nearly every organism and has diversified from a core complex of 14 subunits to the 45 subunit complex found in vertebrates. The appearance of subunits over time is characterized by a large gain of subunits by multicellular animals and only 5 vertebrate specific subunits. Subunit sequences are generally well conserved suggesting that these subunits are performing essential functions. Human disease has been associated with 21 Complex I subunits, though the function of most of the non-catalytic subunits is unclear. Correlation analysis of mRNA abundance in four taxa (humans, *Mus musculus, Drosophila melanogaster* and *Fundulus heteroclitus*) show that correlations among basal subunits (those that are found in bacteria) are shared more frequently among taxa, suggesting functional importance of these correlations.

Variation in Complex I activity in *Fundulus heteroclitus* collected along the latitudinal thermocline of its range was measured. Individuals from southern populations—*F. heteroclitus* from Georgia and *F. grandis* from Louisiana had

significantly higher Complex I activity per wet weight than *F. heteroclitus* individuals from Rhode Island or Maine. Individuals from northern populations had higher mitochondrial density, but this did not compensate entirely for the disparities in Complex I activity among populations. The increased similarity in Complex I activity between individuals from sister species relative to individuals from the same species suggests that Complex I activity is evolving by natural selection in *Fundulus*. The difference in environmental temperature between northern and southern populations, as well as the known importance of temperature on enzyme function makes it likely that temperature affects divergence among *Fundulus* populations.

The effect of temperature on Complex I activity was investigated by acclimating individuals from two populations of *F. heteroclitus* (ME, GA) and one population of *F. grandis* to three temperatures for six weeks (12°, 20° and 28°) and then assaying Complex I activity at each temperature. Acclimation to higher temperatures caused Complex I to be insensitive to acute changes in temperature and increased mitochondrial specific activity at lower assay temperatures. Individuals acclimated to 12° show the opposite response, suggesting that Complex I is more sensitive to cold temperatures.

Abundance of mRNA in five populations of *F. heteroclitus* and one population of *F. grandis* was measured using microarray methodology. One Complex I subunit, NDUFA5, showed patterns of expression consistent with directional selection. Complex I subunit mRNA abundance was regressed with Complex I activity to identify genes with expression patterns that may affect Complex I mitochondrial specific activity. Among *F. heteroclitus* populations, the genes that explained variance in Complex I activity were not

the same. Each population had a different model for Complex I activity and mRNA abundance, suggesting that the effect of variation in mRNA expression on Complex I activity is population specific.

For my family

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Chapter 1 Introduction

1.1 Background

The central dogma of molecular biology, DNA→mRNA→ Protein, ~~permits and~~ encourages the assumption that mRNA abundance is directly associated with protein abundance; that variation in gene expression should lead to variation in protein phenotype. However, there is much variation in the relationship between mRNA abundance and protein abundance(Ghazalpour, Bennett et al. 2011) Stability of mRNA, variation in protein folding, pre and post translational modifications all affect the relationship between mRNA and protein abundance (Leigh and Jeff 1997). In general, for *Fundulus heteroclitus,* there is a statistically significant relationship between mRNA expression and amount of the related protein (Rees, Andacht et al. 2011). However, the mRNA:protein ratios may vary significantly between individuals (Ghaemmaghami, Huh et al. 2003) and the role of gene expression and regulation of post-transcriptional mechanisms may be more complex for enzymes with multiple heterologous subunits (i.e., the active enzyme requires more than one protein encoded by more than one gene or locus).

Gene expression varies significantly between individuals within a population in humans, *Fundulus heteroclitus,* flies and yeast (Wei Jin 2001; Brem, Yvert et al. 2002; Ghaemmaghami, Huh et al. 2003; Morley, Molony et al. 2004; Oleksiak, Roach et al. 2004). Variation in gene expression is associated with important phenotypic change (Gibson 2003). For example, in *Fundulus heteroclitus* 94% of genes vary significantly between individuals within a population and this variation is related to differences in cardiac metabolism (Oleksiak, Roach et al. 2005). Furthermore, the genes that explain

the variance in the same metabolic processes differ among individuals. That is, among subgroups of individuals, the use of metabolic substrates is related to distinct gene expression patterns and these sets of genes are different in different subgroups (Oleksiak, Roach et al. 2005) Thus, variation in gene expression is relevant to fitness and surprisingly manifests in unique patterns even within a species. This research seeks to identify patterns of gene expression among the Complex I subunit genes that affect Complex I activity. However, this question is confounded by the poor annotation of Complex I subunits. The existence of multiple naming schemes among model organisms and the annotation of the Complex I subunits ranges from non-existent to abysmal. For example, gene sequences annotated as *Caenorhabditis elegans* prove to be *Balanus glandula* sequences upon closer inspection. One of the outcomes of this research is the proposal of a more uniform annotation scheme for Complex I subunits.

The major objective of this research was to investigate of the effect variation in mRNA expression on functional phenotype: mitochondrial Complex I enzyme activity. Complex I is essential for the production of hydrogen gradient used to synthesize ATP, and mutations in this enzyme complex are the chief cause of metabolic disorders (Smeitink and van den Heuvel 1999; Wallace 1999). The quantification of 45 genes in Complex I is ideal; it is neither too many (e.g., 100s of genes in a metabolic pathways) nor too few (one or two proteins). By comparing Complex I enzyme function and mRNA expression among many individuals, this research provides insight into whether species-level mRNA expression affects phenotypic differences.

1.2 Gene expression

In both *Fundulus heteroclitus* and *Drosophila melanogaster,* there is a large amount of variation in mRNA expression: 18% of genes have significant variation in expression among individuals within in a population and 68% of genes are significantly different among 40 inbred strains of *Drosophila melanogaster* (Oleksiak, Churchill et al. 2002; Ayroles, Carbone et al. 2009). For *F. heteroclitus* the variation among individuals explains up to 81% of the variation in cardiac substrate specific metabolisms (Oleksiak, Roach et al. 2004) and *D. melanogaster* mRNA abundance data is highly correlated within groups of genes with similar function (Ayroles, Carbone et al. 2009). Both the association of metabolic substrate utilization with mRNA variance in *F. heteroclitus* and the evidence of co-expression of metabolic mRNAs in *D. melanogaster* suggest that variation in mRNA expression affects physiological processes. The effect of variation in mRNA abundance on protein function should be especially apparent within in a heteromeric enzyme complex due to the required level of interaction among subunits. To describe whether the variation in mRNA abundance affects protein function, Complex I activity and subunit mRNA expression was assayed in livers of *Fundulus* from two species and six populations.

1.3 Complex I Activity in Liver

Oxidative phosphorylation in liver was chosen as the system to study the effects of variation in gene expression on metabolic function. Oxidative phosphorylation (OxPhos), is the culmination of oxygen-dependent metabolic processes and thus, is likely to be relevant to whole organism fitness. The liver is the functional center of metabolism and is critical to whole organism metabolism. In terms of whole organism metabolic

processes, the liver has a central role as the liver is the first stop for molecules absorbed in the digestive system and functions as the main regulator of nutrients available to other organ systems. Hepatocytes are able to quickly modulate metabolic enzymes to adjust to changing dietary states and liver enzymes are turned over at a rate up to five times faster than the enzymes in other systems (Nelson and Cox 2005). If variation in Complex I is biologically important, it should be observed in the liver. Complex I was examined because it is the first enzyme in the OxPhos and the enzyme activity is readily quantified.

All cellular aerobic metabolic processes take place within the mitochondria: fatty acid oxidation, pyruvate dehydrogenation, the citric acid cycle, the oxidation of amino acids, and ATP synthesis. Only glycolysis occurs outside the mitochondria.

These aerobic metabolic pathways perform the catabolism of lipids, carbohydrates and proteins which produces reduced electron carriers (NADH, $FADH_2$). These products supply the electron motive force that drives the reduction of O_2 and the production of CO_2, H_2O and ultimately, ATP. A chain of five enzyme complexes, the electron transport chain, is imbedded in the mitochondrial inner membrane, and is the final destination for the high-energy electron carriers. Electrons enter the chain of enzyme complexes and are shuttled through prosthetic groups and lipid soluble shuttling molecules to the final electron acceptor, oxygen. Complexes I, III and IV pump protons across the inner mitochondrial membrane into the inter-membrane space. ATP is formed through the chemiosmotic phosphorylation of ADP by ATP-synthase, Complex V, driven by a proton gradient is built across the inner mitochondrial membrane by sequential electron transport through Complexes I-IV.

The sequence of electrons through the electron transport chain is as follows. From NADH, electrons enter Complex I from the matrix and are transferred to Ubiquinone, a small lipophilic molecule. The energy obtained from the transfer of two electrons to Ubiquinone is used to pump two H^+ across the inner mitochondrial membrane. Complex II donates electrons to Ubiquinone from the oxidation of succinate to fumarate—simultaneously participating in the Citric Acid cycle. Ubiquinone then carries the two electrons from either Complex I or II to Complex III where they are transferred to Cytochrome *c* and four protons are pumped across the membrane. Cytochrome *c* moves through the inner membrane space to Complex IV where four electrons provide the energy needed to use four protons from the matrix to produce O_2 and H_2O and simultaneously pump four additional protons into the inner membrane space.

Complex I has diversified from a bacterial Complex I that has 14 subunits (Fig. 1.2, (Efremov, Baradaran et al. 2010) to the 45 subunit enzyme complex found in vertebrates. In vertebrates, Complex I is a very large, (1MD) heteromeric complex and the subunits are encoded by both the nuclear and mitochondrial genomes: of the 45 subunits identified in cows and other vertebrates, 7 are mitochondrial and 38 are nuclear (Carroll, Fearnley et al. 2006). Complex I subunit deficiencies are the most common cause of human mitochondrial pathologies (Smeitink and van den Heuvel 1999). Recognized deficiencies for Complex I disorders in humans contribute to mild (exercise intolerance) to fatal (failure to thrive in the first year of life) phenotypes. However, only in 18-40% of cases is there a defined mutation or polymorphism (Smeitink, van den Heuvel et al. 2001; Haack, Madignier et al. 2012). The majority of Complex I pathologies are likely caused by defects in unidentified accessory or assembly proteins, deficient mitochondrial

targeting for nuclear encoded subunits, or differential expression or translation of subunit genes.

Making comparisons among studies of Complex I function and subunit mutation across species is nearly impossible because of the lack of similar annotations. One of the outcomes of this research is a more uniform annotation and evolutionary analyses of 45 Complex I subunits. This was achieved by pairwise similarity searches using *Bos taurus* proteins to query a translated nucleotide database (tBLASTn).

1.4 Fundulus heteroclitus as study organism

The teleost fish *Fundulus* was used for this research because it is a vertebrate (Complex I has 45 subunits in vertebrates) with large outbred populations (local population sizes exceed 10^5 (Adams, Lindmeier et al. 2006)) found along the east coast of the United States—the steepest thermal cline in the world. Populations of *Fundulus* are well differentiated from each other at both neutral and non-neutral loci (Fig. 1.1). *Fundulus* also displays a wide breadth of physiological traits: among individuals there is significant variation in cardiac metabolism (11, 2.5 and 2 fold differences in fatty acid, lactate-ketone-alcohol and glucose metabolism, respectively (Oleksiak, Roach et al. 2005; Crawford and Oleksiak 2007). No other model system (mice, *Drosophila, C.elegans,* or yeast) has this large of variation in cardiac metabolism. It is likely that *Fundulus* has large variation in liver metabolism also. In addition to differences in physiological traits, such as metabolic substrate utilization, among *Fundulus* there are evolutionarily adaptive differences in gene expression (Pierce and Crawford 1997; Crawford, Segal et al. 1999; Oleksiak, Churchill et al. 2002; Whitehead and Crawford 2006). This natural variation in gene expression seems to affect cardiac metabolism

(Podrabsky, Javillonar et al. 2000; Oleksiak, Roach et al. 2005) because up to 81% of the variation in glucose utilization in isolated heart ventricles is related to specific patterns of gene expression. It is not enough to say that variation in mRNA affects metabolism. The relationship is more complicated because different pathways explain substrate specific metabolism among different groups of individuals. For example, variation in oxidative-phosphorylation mRNAs explains variation in glucose metabolism for one group of individuals but expression of glucose metabolic genes explains this same metabolism in a different group of individuals. A partial explanation for these patterns of different mRNAs explaining metabolism in different groups is the existence of strong positive and negative correlations among metabolic genes, both within and between pathways. These data suggest that there is much complexity in how gene expression is related to physiological processes. These variations among individuals have important implications for studies using inbred strains: conclusions based on one individual or one strain will not necessarily reflect a generalized conclusion for a population or species.

1.5 Expectations

In order to investigate the relationship between mRNA variation and functional variation in a heteromeric protein complex, I measured both mRNA expression and Complex I activity in *F. heteroclitus* and *F. grandis.* I expected to observe variation in Complex I activity both within and among populations and that this variation would regress with the expression of a few key Complex I subunit mRNAs.

I expected to observe variation in mRNA abundance of Complex I subunit genes across taxa (using publically available datasets for human, mice and *Drosophila melanogaster)*, based on observations of high variability in *Fundulus* Complex I mRNA

expression in (Oleksiak, Roach et al. 2005). Within taxa, I expected to observe strong patterns of correlation among subunit genes, indicating co-expression, based on previous studies of expression in normal populations of *F. heteroclitus,* mice and humans (Oleksiak, Roach et al. 2005; van Waveren and Moraes 2008). Correlations between specific pairs of subunits found in more than one taxa may represent functionally important relationships and may be related to Complex I function. If the expression patterns are related Complex I activity, these mRNAs may show divergent expression patterns.

Variation in Complex I activity both within and among populations is expected due to the steep thermal cline along the range of *F. heteroclitus* as well as the documented geographic variation in mitochondrial Complex I subunit sequences (Whitehead 2009). *F. heteroclitus* individuals were collected from locations with different mean annual temperatures (9°C in Maine vs. 22° in Georgia (Commerce 1955)). Because temperature causes chemical reactions to slow down, a common compensatory mechanism is increased turnover rate (K_{cat}) or increased concentration of enzymes (Somero 1995; Hochachka and Somero 2002). I expect individuals from colder northern populations would have higher Complex I activity due to the nature of chemical reactions catalyzed by enzymes and typical compensatory mechanisms observed in ectothermic animals. In addition to the hypothesis that Complex I activity would vary along the thermal cline, I expected to find variation among individuals. In other studies of metabolic enzymes in *F. heteroclitus,* high variation among individuals in the activity of Cytochrome C and 10 glycolytic enzymes, was observed by (Fangue, Richards et al. 2009) and (Pierce and Crawford 1994).

To test whether variation in Complex I activity or expression of Complex I mRNAs is likely to be biological important, a test for adaptive divergence was applied by comparing southern individuals (*F. grandis* and *F. heteroclitus*) to northern individuals (*F. heteroclitus* only). According to the neutral theory proposed by (Kimura 1985), most mutations have no effect on the function or fitness of an organism and are thus not affected by selective pressures. If mutations have no functional consequence they may drift to fixation and the number of neutral mutations that are fixed is a function only of time and population size. Thus, according to the neutral theory, most of the variation among populations or species is merely a function of time and population size rather than selection. If only neutral processes affect differences observed in a trait, ie variation of Complex I activity among populations and species is not adaptive, then individuals from *F. heteroclitus* populations should be more similar to each other than to individuals from *F. grandis* populations.

1.6 Scope of Research

Chapter 2 describes the distribution of Complex I subunits across many taxa as well as the mRNA expression patterns of Complex I subunits in humans, *F. heteroclitus, Drosophila melanogaster* and *Mus musculus.* Previous studies of metabolic gene expression in *Fundulus heteroclitus,* have shown that most genes are differentially expressed between individuals and this variation can be used to explain metabolic variation between groups of individuals (Oleksiak, Roach et al. 2004). However, because hundreds of genes appear to affect metabolism and these genes were in many different pathways (e.g. glycolysis, TCA or oxidative phosphorylation), it is difficult to draw

specific conclusions. Patterns that are important are likely to be conserved. Thus, multiple species were examined to locate shared patterns of metabolic mRNA expression.

Chapter 3 is an analysis of Complex I activity in *Fundulus heteroclitus* and *F. grandis.* Because the ultimate goal of this research is to describe the functional effect of mRNA variation, this work must confirm that variation in both mRNA and enzyme function exists among individuals. Five populations of *F. heteroclitus* and one population of *F. grandis* were acclimated to a common temperature (20°) and enzyme assays were conducted on isolated liver mitochondria to determine the amount of variation in Complex I activity within and between species. The variation in mRNA expression had been demonstrated (Oleksiak, Roach et al. 2004) and this chapter describes the variation in Complex I activity within and among species of *Fundulus* species.

Chapter 4 investigated the effect of thermal acclimation on Complex I activity. The incredible amount of inter-individual variation in Complex I activity as well as the apparent compensatory increase in mitochondrial density among northern individuals observed in Chapter 3 prompted this investigation of the effect of temperature acclimation on Complex I activity. Because *F. heteroclitus* live along a strong thermal cline and experience high daily variation in water temperature in the tidal marsh it was hypothesized that individuals from the extremes of the range would exhibit different responses to long term and acute thermal variation.

Finally, Chapter 5 is the analysis of mRNA expression and the relationship between variation in mRNA expression and Complex I activity among individuals of *F. heteroclitus* and *F. grandis*. The same individuals used for the analysis of Complex I activity variation from Chapter 3 were used. RNA was isolated from liver homogenate and hybridized to a *Fundulus* oligonucleotide microarray.

Figure 1 1 Clinal Variation Among F. heteroclitus Populations. A. Maximum parsimony tree for RFLP in mitochondria, Ldh-B coding region, and 309 bp of Cytochrome B (Bernardi, Sordino et al. 1993). Dark circles and squares are northern populations (north of Hudson River), unfilled circles and squares are southern populations (south of Hudson River). B. Fst and log-distance relative to Maine population based on summary of 14 microsatellites (Adams, Lindmeier et al. 2006; Duvernell, Lindmeier et al. 2008). Open blue circles are northern populations and closed green circle are southern populations C. Neighbor-joining tree based on microsatellites (Adams, Lindmeier et al. 2006; Whitehead and Crawford 2006; Duvernell, Lindmeier et al. 2008). D. Population structure (k =7) based on 354 SNP, 30 individuals per population and principal component analysis (Williams, Ma et al. 2010; Williams and Oleksiak 2010).

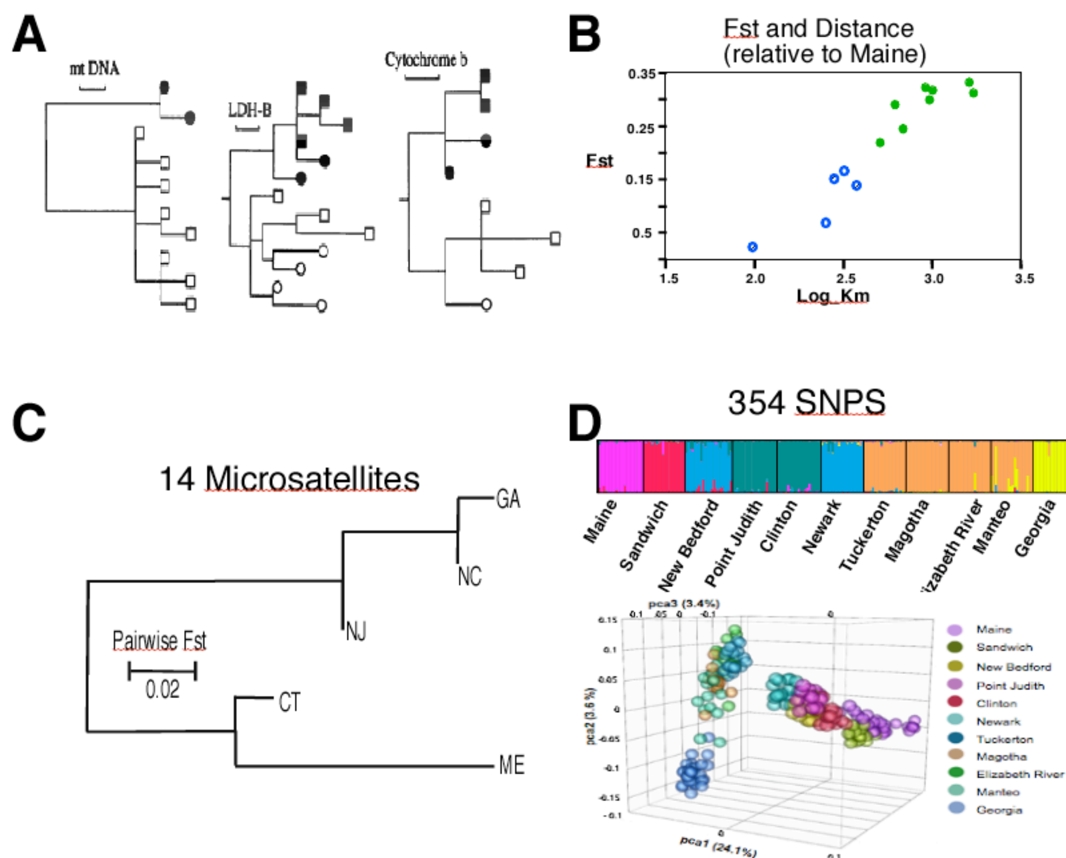
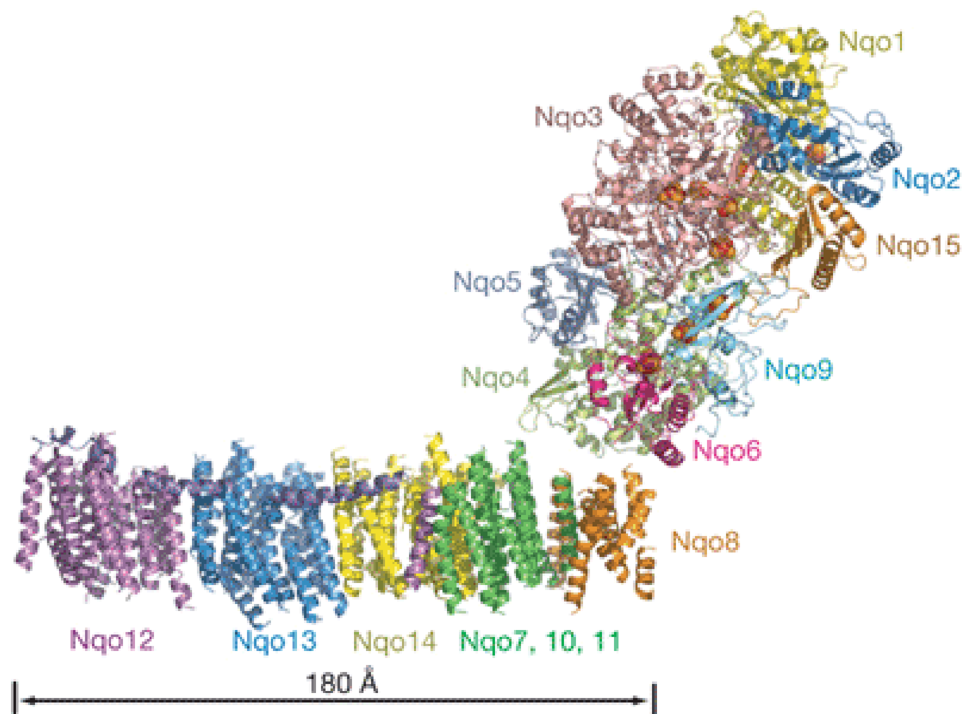


Figure 1.2 Protein Structure of Bacterial Complex I Protein structure at 4.5 Å resolution for the bacteria *Thermus thermophilus (Efremov, Baradaran et al. 2010).*



Chapter 2 Phylogenic Distribution, Annotation and Expression of Complex I Subunits

2.1 Summary

NADH: ubiquinone dehydrogenase, Complex I, is an entry point for electrons into the electron transport chain. In vertebrates this enzyme is made of 45 separate protein subunits of which 38 are encoded by the nuclear and 7 by the mitochondrial genomes. Complex I plays a crucial role in ATP synthesis and defects in the enzyme complex are implicated in 70-80 percent of mitochondrial diseases. However, in cases of identified Complex I deficiencies, only 40% of patients show mutations in any of the Complex I subunit genes. This indicates that many processes, besides protein sequence, are affecting enzyme function. Because the study of Complex I is confounded by a lack of consistent nomenclature among species, we provide a table of orthologous annotations among common model species. Subunits are remarkably well conserved. Yet, there are several subunits associated with human disease either lost or not present in some taxa. These observations suggest that all 45 Complex I subunits may not be necessary for Complex I function. The mRNA expression patterns within four species were examined; patterns of gene expression shared among species are likely to be important for Complex I function. We found a surprising amount of significant correlations among subunit mRNA expression, including both positive and negative correlations. Unlike our expectation that mRNAs would be co-expressed, there are more negative correlations among human subunits associated with Complex I than among random genes. Similar to the loss of many subunits in different taxa, the observation that there are significant negative correlations among subunits also suggests that not all subunits are necessary.

Additionally, the pairs of correlated genes are not often common among species, except for the phylogenetically oldest subunits which are often correlated with each other regardless of species. These data suggest that if the variation in mRNA expression is important for the functional variation in Complex I, it is most likely to occur in the basal subunits.

2.2 Introductory Material

The evolution of Complex I (EC 1.6.5.3, NADH:ubiquinone reductase, NADH dehydrogenase) has been the subject of several studies (Abdrakhmanova, Zickermann et al. 2004; Cardol, Vanrobaeys et al. 2004; Marques, Duarte et al. 2004; Carroll, Fearnley et al. 2006; Cardol 2011; Yip, Harbour et al. 2011). Because Complex I is positioned as the first enzyme of the oxidative phosphorylation (OxPhos) pathway it plays a critical role in ATP production and defects are associated with a majority of human mitochondrial diseases (Gautheron 1984; Smeitink and van den Heuvel 1999; Loeffen, Smeitink et al. 2000; Triepels, Heuvel et al. 2001; OMIM 2011). Complex I is a large enzyme made up of 45 subunits in both humans and cow (38 nuclear and 7 mitochondrial encoded subunits (Carroll, Fearnley et al. 2006). Human diseases are associated with single mutations in twenty-one of the Complex I subunit genes with relatively high frequency (1 per 5000) (Smeitink and van den Heuvel 1999; Wallace 1999; Schaefer, Taylor et al. 2004; Carroll, Fearnley et al. 2006; Koene and Smeitink 2009). Complex I deficiency, characterized as an inability for Complex I to assembly or function properly, can be caused by mutations in nuclear-encoded genes (Pitkanen, Feigenbaum et al. 1996; Triepels, van den Heuvel et al. 1998; Loeffen, Smeitink et al. 2000; Triepels, Heuvel et al. 2001), including NDUFA1, NDUFA2, NDUFA10, NDUFA11, NDUFA13,

NDUFS1*, NDUFS2*, NDUFS3*, NDUFS4, NDUFS6, NDUFS7*, NDUFS8*, NDUFV1*, NDUFV2* (*denotes subunits with homologues in bacteria (OMIM 2011), or mutations in any of the mitochondrial-encoded components of Complex I: ND1 ND2, ND3, ND4, ND4L, ND5 and ND6 (Carroll, Fearnley et al. 2006; OMIM 2011). Complex I deficiencies due to mutations in nuclear genes tend to manifest symptoms sooner (infancy, early childhood) than deficiencies due to mutations in mitochondrial genes (late-teens, adulthood) (Carroll, Fearnley et al. 2006). Inappropriate function can cause greater production of reactive oxygen species (ROS), prevent NADH oxidation or inhibit ATP production (Wallace 1999; Triepels, Heuvel et al. 2001; Yagi and Yagi 2003). The expectation is that genes with highly conserved sequences across taxa are more likely to provide a vital function to the enzyme. Similarly, there is an expectation that the mRNA expression of critical subunits will have consistent patterns because of the need for a stoichiometric assembly of Complex I. To provide insight into potential functional relationships between subunits we investigate subunit protein sequence similarities to define the phylogenetic distribution and then use these homologous genes to examine patterns of expression of Complex I subunit genes.

One of the difficulties with the analysis of Complex I is the lack of consistent nomenclature. For example, in the *Drosophila melanogaster* database (S. Tweedie and A. Schroeder 2009), only four nuclear subunits are annotated with any information identifying them as part of a metabolic pathway. A consequence of this dearth of annotations is that many genetic studies will not relate mutations or changes in the mRNA expression of Complex I subunits to the phenotypes being studied. There could be many studies on hypoxia, metabolism, or physiological performance which are related

to Complex I but we would be ignorant of these because of poor annotation. To resolve the conflict in the literature concerning Complex I evolution, we provide a table of annotations and the phylogenetic distribution of homologous subunits among organism with well-defined genomes.

It has been reported that Complex I had a staggered evolution—with subunits appearing in different taxa over evolutionary time (Gabaldon, Rainey et al. 2005)—and alternatively, that all eukaryotes share nearly the same complement of Complex I subunit genes (Cardol 2011). These results are re-examined here by defining homologous proteins (proteins that evolve from a common ancestral locus) based on organisms with well-defined genomes. Using sequence similarity as a measure of likely homology, the data provided here differ from studies that identify potentially-functional analogous protein subunits (Cardol 2011). Surprisingly, not all of the loci associated with human disease are conserved phylogenetically. To further our understanding of Complex I, we analyzed patterns of mRNA expression in four taxa (*Homo sapiens, Mus musculus, Fundulus heteroclitus, and Drosophila melanogaster*). Among these four taxa, there is no obvious pattern in mRNA expression among subunit genes. These data suggest although we know much about the structure of Complex I in bacteria (Efremov and Sazanov 2011), the number of protein subunits in vertebrates (Carroll, Fearnley et al. 2006) and human diseases caused by Complex I deficiencies (Smeitink and van den Heuvel 1999; Corder and Mellick 2006; Huynen, de Hollander et al. 2009), we lack an appreciation of how subunit expression is regulated to form a functional enzyme.

2.3 Materials and Methods

Phylogenetic distribution of Complex I subunits

Complex I subunits were identified in 13 species of eukaryotes and one bacteria. These include: three vertebrate classes: mammals (mouse, *Mus musculus*; cow, *Bos taurus*; and humans, *Homo sapiens)*, amphibians (*Xenopus tropicalis)*, and ray-fin fish (zebrafish, *Danio rerio*, and Atlantic salmon, *Salmo salar*); a non-vertebrate deuterostome (purple sea urchin, *(Stronglyocentrotus purpuratus)*; two triplopblastic invertebrate animals: (fruit fly, *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*)*;* sponges (*Amphimedon queenslandica*); single cell animals (collar cells, *Monsiga brevicollis*), two fungi (*Yarrowia lipolytica, Neurospora crassa),* and the bacteria *Escherichia coli.* To locate Complex I subunits in these genomes, we used pairwise alignments of *Bos taurus* and *Escherichia coli* proteins to the subject taxa's nr and est databases using the BLASTp algorithm with an E-score cut off of E^{-5}. Query (*B. taurus* and *E. coli*) proteins were compared to subject genomes individually because BLAST scores are a function of the size of the database and thus comparing a protein to a single taxon database will enhance the probability of discovery. When subunits were missing from a species, the BLAST results were double-checked by searching again using the protein from the most closely related taxa. For example, ND4L (nuoK in *E. coli*) was not found in *Y. lipolytica* using *B. taurus* or *E. coli;* yet the *N. crassa* ND4L had a significant similarity to *E. coli* ($< 10^{-10}$) and this *N.crassa* subunit was similar to a protein in *Y. lipolytica* ($e < 10^{-23}$). Thus, we suggest that there is a *Y. lipolytica* ND4L homologue. To identify pseudogenes and potential duplicated loci, tBLASTn searches were performed and potential subunit genes needed to display intron/exon structure.

tBLASTn hits that displayed high similarity to the mRNA with no intron/exon structure were classified as processed pseudogenes. Subunits with tBLASTn results with appropriate intron/exon structure at more than one locus were classified as duplicates. If subunits were not found in the genomes, the EST database was searched. Human disease annotations are based on the most recent data in OMIM (OMIM 2011).

mRNA Expression

Patterns of Complex I mRNA expression were compared in humans, mice, fish and fruit flies. Our goal was to measure "normal" patterns of expression among individuals. For human expression data we used GEO dataset (GSE7236) (Storey, Madeoy et al. 2007) which quantified mRNA expression from lymphoblast cell lines from 16 normal, healthy individuals from two populations (YORUBA, CEPH). The average of two replicates was used for all analyses. For mice, a single male from each of ten strains fed either normal or high fat diet were used (GSE10493) (Shockley, Witmer et al. 2009). For fish, mRNA expression was measured in 16 *Fundulus heteroclitus* (Oleksiak, Roach et al. 2005) individuals from two populations (Maine and Georgia). For the fruit fly *Drosophila melanogaster* we used expression data from a single male from each of the 40 inbred strains from a single North Carolina population (Ayroles, Carbone et al. 2009). Data were $\log_2$ transformed and loess normalized using SAS/JMP Genomics 4.1.

We chose hundreds of metabolic genes from each species included in the expression analysis using KEGG (www.genome.jp/kegg/pathway.htm). We choose to use only metabolic genes because they represent a diverse set of functions and because of the computational requirement for calculating pairwise correlations among genes. The

expression of all metabolic genes were sum normalized such that the expression levels for all individuals across all metabolic genes were equal to a constant.

Gene symbols and species specific accession numbers were identified for all subunits of all enzymes for human, mice and flies. Metabolic genes for *F. heteroclitus* were located as described previously (Paschall, Oleksiak et al. 2004). If an accession was missing for any organism, a tBLASTn search was performed to identify any unannotated genes that may have been included as probes in the expression data sets. The gene list generated thus was used to interrogate the expression data sets.

The correlations among genes (using individuals as replicates) were carried out using MatLab (MathWorks, Inc. Massachusetts). The number of unique informative critical correlation coefficients (i.e, excluding correlation of a gene to itself and only unique correlations [G^2 –G)/2; G is the number of Complex I genes]) that correspond to p-values < 0.01 for n-2 degrees of freedom (where n is the number of individuals) were defined from statistical tables (Sokal and Rohlf 1981) and tabulated in Matlab.

Frequency of significant correlations was determined for Complex I subunit mRNAs. Two permutation tests (1,000 permutations) were used to determine the probability of achieving more significant correlations among each of these datasets: 1) randomizing the metabolic genes among individuals and 2) randomly sampling M number of non-metabolic genes, where M is the number of metabolic genes within each species database. For the randomization of metabolic genes among individuals, gene i (G_i) for individual j is randomly assigned to another individual (that is for $G_{i,j}$ we randomize G_i among j individuals). For the permutation of non-metabolic genes, M

genes were randomly chosen among non-metabolic genes, sum normalized and the frequency of unique significant correlations among G genes ($p < 0.01$) was determined.

2.4 Results

Annotation of Complex I genes is provided in Table 2.1. Alternative annotations or gene symbols are provided for other common model organisms. Genes coding for Complex I proteins were defined in other taxa based on sequence similarity to Complex I proteins in *Bos taurus* (domestic cow) and *Escherichia coli* (Carroll, Fearnley et al. 2006). Sequence similarity—not functional similarity—is used to distinguish homologous protein from analogous proteins. Similarities are based on the E-value which indicates the number of sequence matches in the database one can expect to find by chance. The lower an E-value, or the closer it is to zero, the more likely the match reflects homologous sequences (BLAST). Genome and EST databases were searched for all species. If similarity with a *B. taurus* gene was not found within a species' database, the phylogenetically most similar protein was used to search the genome.

Distribution of Complex I Subunits

Fourteen Complex I subunits including all seven mitochondrial subunits, found in all major taxonomic groups as well as associated with human disease, are defined by their similarity to the proteins purified from *E. coli* or *B. taurus* (Fig. 2.1), are designated as basal core proteins ("b" Fig. 1A, Table 2). The similarity between *B. taurus* and *E. coli* sequences is variable, with the lowest E-values for NDUFs S2 and V1 (approx. 10^{-100} and 10^{-107}, respectively). Higher E-values ($< 10^{-20}$) are associated with three *B. taurus* mitochondrial encoded subunits (ND1, ND4, ND5) and NDUFs- S1,S7,S8 and V8.

However, 4 of the *B. taurus* mitochondrial subunits have little similarity ND2, ND3, ND6, ND4L (E-values > $10^{-5,}$ 10^{-5}, 10^{-3}, 10^{-1}; respectively)

There is a *B. taurus* subunit (NDUFAB1) with significant similarity to a protein in *E. coli* (acpP). The *E. coli* Complex I (Friedrich 1998) does not include NDUFAB1. Nor was NDUFAB1 purified from Complex I in the α-proteobacterium *Paracoccus denitrificans* (Yip, Harbour et al. 2011). NDUFAB1 was found bound to fungal Complex I (*N. crassa,* (Marques, Duarte et al. 2004). We designate NDUFAB1 as eukaryotic Complex I subunits based (“e” Fig. 1A, Table 2.2) based on both protein similarity and protein chemistry.

Among eukaryotes there are an additional 14 subunits (Figure 1A); bringing the total number of subunits found in most taxonomic groups to 28. Five of these are associated with human disease. Three of these (NDUFs A12, S4 and S6) have also recently been described in the α-protobacterial ancestor of mitochondria (Yip, Harbour et al. 2011). Thus, these subunits predate eukaryotic organisms but are not present in *E. coli*. For simplicity we designate these 14 Complex I subunits as eukaryotic (“e” Fig. 1A, Table 2).

Not all eukaryotic subunits are retained in all taxa. For example, NDUFA2, which is associated with human disease, has very low E-values between *B. taurus* and sponges (*A. queenslandica*, E-value < 10^{-34}) or *N. crassa* (E-value 10^{-18}). Yet, there is no significant similarity in sea urchins (*S. purpuratus*) or collar cells *M. brevicollis*. Similarly, NDUFs A8, AB1, B3 and S5 are not found in one or more taxa.

In addition to the 14 basal subunits and 14 eukaryotic subunits, there are six subunits found in animals (collar cells or sponges), six more in triplobastic animals and

finally, five vertebrate specific subunits. Based on protein chemistry, Complex I in *B. taurus* has 45 subunits: 38 nuclear encoded subunits and 7 mitochondrial encoded subunits (Carroll, Fearnley et al. 2006). All these are found among vertebrates (Fig. 2.1A). Among the Complex I subunits founds in animals and more complex organisms, four are associated with human diseases.

Most taxa retain all of the subunits after they have evolved. There are two notable exceptions. The *C. elegans* genome lacks significant sequence similarity for six (ND6, NDUFA4, NDUFA11, NDUFB3, NDUFB11 and NDUFC2) of the 40 subunits found in the other triploblastic animals examined, and *S. purpuratus* has lost four subunits (NDUFA2, NDUFAB1, NDUFB4 and NDUFS5). In both cases these losses include subunits associated with human diseases. The most surprising finding is the lack of similarity in the *C. elegans* genome to ND6, a mitochondrial protein. The *C. elegans* BLAST hit most similar to ND6 from *D. melanogaster* had a relatively high E-value of $>10^{-3}$. Although ND6 is not generally well conserved (high E-values across taxa); *B. taurus* ND6 is similar enough to *D. melanogaster* sequence to pass our E-value cut off. Thus, although there are many reasons to believe *C. elegans* has ND6, there is little sequence similarity to support this claim. This finding is complicated by barnacle ND6 annotated as *C. elegans* (accession number, BAD44746.1). This sequence and all the mitochondrial sequences associated with this entry (BAD####) cluster together with other arthropods, are most similar with other barnacles, and the manuscript associated with this entry is about barnacles. Finally, these BAD_ accessions are less similar to the other *C. elegans* Complex I sequences than they are to other arthropods. This may have contributed to confusion in other research.

Duplicated genes and architecture

We determined the number of duplicate loci among vertebrates (Table2. 3- green highlights). A potentially functional duplicate locus is defined as having similar or identical intron/exon structure at a different chromosomal location with a high similarity score and an e-value cut off of E^{-10}, but have not yet been verified as functional paralogues. Within the Complex I subunit genes there are several previously described paralogues. Interestingly, cow and mice have no duplicate Complex I loci. Humans have at least two functional duplicates--NDUFs A4 and A4L and A12 and A12L and three more potentially functional duplicates, identified by this search (Ogilvie, Kennaway et al. 2005). *Danio rerio* has 12 subunits with potentially functional duplicate genes: NDUFs A4, A12, AB1, B4, B5, B9, B10, V3, S4, S6, S7 and S8. These subunits are located at one or more chromosomal location with proper intron/exon structure. Functional assays are necessary to determine if they are expressed and function as Complex I subunits.

In addition to the presence of duplicate Complex I subunit genes, there are numerous processed pseudogenes. Mice have the most pseudogenes: 27 genes were identified as having pseudogenes, and the average number of pseudogenes per loci is higher than those found in other species. Sixteen of the human Complex I subunits have one or more of these processed pseudogenes; NDUFs A4 and B4 have 21 and 11, respectively. (Table 3). In comparison, fish (*D. rerio*) and cows have no pseudogenes.

Gene expression

mRNA expression levels for all metabolic genes were measured in human lymphoblast cells from 16 individuals from two populations (Storey, Madeoy et al. 2007), ten strains of male mice fed either normal or high fat diet (GSE10493) (Shockley,

Witmer et al. 2009), 16 individual teleost fish (*F. heteroclitus)* from two populations (Oleksiak, Roach et al. 2005), and a single male from each of 40 inbred strains of *D. melanogaster* from a single North Carolina population (Ayroles, Carbone et al. 2009). Correlations in mRNA abundance among all possible pairs of Complex I subunits within each species were calculated and clustered based on their shared values (Fig 2.2).

The total number of metabolic genes used for *Homo sapiens, Mus musculus, Fundulus heteroclitus* and *Drosophila melanogaster* were 119, 178, 119 and 184, respectively (Table 2.4). The percentage of correlations observed that were significant ($p < 0.01$, with rho >0.662, 0.561, 0.403, and 0.662, for human, mice, fish and flies, respectively) for all metabolic genes was: *H. sapiens,* 5.0%*; M. musculus,* 8.1% *D. melanogaster, 1.65%;* and *F. heteroclitus, 22.8%* (Table 2.4). There were 29, 39, 38 and 20 Complex I mRNAs quantified. The percentage of significant correlations among Complex I subunits was *H. sapiens,* 11.3%*; M. musculus,* 19.4% *D. melanogaster, 0.7%;* and *F. heteroclitus, 24.2%* (Table 2.4).

In order to determine whether the number of correlations observed was likely, we preformed two permutations of the data. First, we randomized metabolic genes among individuals and second, we randomly chose an equal number of non-metabolic genes and determined the frequency of significant correlations for each permutation using 1,000 iterations. For example, mice have 178 metabolic genes and of those, 39 are CI genes: 178 random non-metabolic genes were sum normalized and 39 chosen from this matrix and the frequency of significant correlations counted. The mean and variance of these 1,000 iterations provides an estimate of the p-value given the structure of the data.

For mice, flies and fish, the number of significant correlations observed among all metabolic genes, OxPhos genes and Complex I genes are significantly greater than the frequencies found when either genes or individuals were randomized 1,000 times ($p < 0.004$ for randomized genes, Table 2.4). Notice, only metabolic genes were measured in the teleost fish *Fundulus* and thus this permutation could not be performed. Humans differ from mice, flies and fish only where the frequency of significant correlations observed among all metabolic genes is not significantly high. When considering the signs of the correlations in humans, mice and flies we observe too many significant positive Complex I correlations relative to expectations based on chance alone (columns 9-11, Table 2.4).

Although the frequency of positive correlations in Complex I is greater than the random expectation, this is not true for negative correlations in mice and flies, where there are too few negative correlations observed. The frequency of negative correlations for Complex I in mice and flies is less than the lower 99% confidence level among the 1,000 permutations of randomly chosen genes.

Do some subunits have a greater frequency of correlations and are these shared among species? The frequency of significant correlations among Complex I subunits within each species varies from 3% to 36%. 28 subunits (76%) have 10% or more significant correlations (black bars, Fig. 2.3). It is reasonable to expect that less than 1% (p-value 0.01) would be significant by chance alone. The minimum frequency observed for any subunit is 2%. Two percent is not that much greater than the 1% expected, but 76% of subunits have more than ten-fold greater frequency, suggesting that these data are unlikely to be observed randomly. Five of the disease associated subunits have low

frequency (< 10%), yet five of these disease associated genes also have relatively high frequency (> 20%) of correlations to other subunits. There is no significant difference in the correlation frequency of disease associated subunits relative to other non-disease subunits ($p > 0.3$, Fisher-Exact test). In addition to total number of correlations among genes within pathways, the frequency that two or more species share a significant correlation between a specific pair of genes was calculated (gray bars, Fig. 3). That is: how often does a pair of subunits show a significant correlation in gene expression among individuals in more than one species? At a p-value of 0.01, the probability of this is 0.0001. With 35 subunits having measures from two or more species, we would expect much less than one shared correlation for any one subunit. Sixteen subunits have no shared correlations between subunits in more than one species (A1-D, A10-D, A11-D, A12-D, A13-D, A3-n, A5-n, A9-n, B11-n, B2-n, B3-n, C1-n, C2-n, S2-D, S5-n, and S6-D). Eleven of the subunits have more than one significant shared correlation (≥2, 2/35, 6%, Figure 3-gray bars). There are seven basal subunits (Table 2) measured in two or more species (there are no measures of mitochondrial subunit expression in humans, mice or flies). Five disease associated subunits (S7-D, V2-D, S8-D, S1-D, V1-D) are significantly correlated with 2, 2, 3, 4 and 6 other subunits, respectively, in two or more species. Frequently, the other half of these shared pairs of subunits are from the basal group of subunits. The probability that a majority of these shared pairs would be from the same basal phylogenetic group by chance alone is less than 0.02 (Fisher-Exact test).

2.5 Discussion

Our initial interest in the phylogenetic distribution of Complex I subunits, was to identify homologous subunits and their annotation. We provide these homologous

annotations (Table 2.1) based on protein sequence similarity for humans, cows (where the original protein biochemistry was completed (Carroll, Fearnley et al. 2006), flies, nematode worms, fungi and *E. coli* yielding a more uniform language for Complex I. We see no compelling evidence that any organisms' lifestyle imposes any special conditions on the function of Complex I, nor are many of the subunits sufficiently diverged in *H. sapiens* compared to bacterial, fungal or insect sequences to warrant multiple naming schemes. The authors would like to suggest that since there are HUGO analogs for all loci, that these abbreviations and annotations be used in all Complex I studies. It would simplify annotation, increase understanding of comparisons among phylogenetic groups and better inform us about the human condition.

Phylogenetic Distribution

For the phylogenetic analysis we assume parsimony; subunit genes evolve only once, and once evolved any missing genes in more recently diverged species must be lost because it is unlikely that the same proteins evolve more than once in separate lineages. By lost, we mean that there is too little sequence similarity to support the conclusion that the protein subunits have evolved from a common ancestor (are homologous). There are two different reasons for the lack of similarity once a protein has evolved. One is that the subunit is no longer functioning as a member of Complex I and thus has fewer functional constraints allowing it to have greater neutral drift reducing the similarity to functional subunits. The second is that the subunit has undergone extensive adaptive divergences. For adaptive divergence to reduce the similarity to a low value would require altering many of the amino acids. This is unlikely (Except in the case of ND6. See below.)

because most adaptive divergence among homologous proteins require a few amino acid changes (Hochachka and Somero 2002).

Our phylogenetic tree suggests that there are four distinct groups of subunits that appeared over evolutionary time in eukaryotes, animals, triploblastic animals and vertebrates (Fig. 2.1 and Table 2.2). A majority of the subunits of Complex I are remarkably well conserved. While it is not challenging to find well conserved subunits (NDUFs A9, S1, S8, S3, S7 and V1 as well as NDs 1 and 5); it is necessary to use more than one database when searching for less well conserved subunits. We have exhaustively searched nr, est and RefSeq genomic databases for all the Complex I subunit genes in a variety of species. When confronted with a missing subunit in an initial search, all other databases that were appropriate were examined to determine whether it was the subunit or the database that was lacking. This was followed by searches using the phylogenetically most similar subunit that was similar to either *E. coli* or *B. taurus* subunits. For instance, using *Bos taurus* NDUFC2 sequence as query against the *Stronglyocentrotus purpuratus* genome does not produce any significant BLAST results. However, this is not enough evidence to pronounce NDUFC2 absent from *S. purpuratus*. The *Drosophila melanogaster* protein, which was similar to the *B. taurus* sequence, was then used to search the *S. purpuratus* nt/nr database and a nucleotide match close to the e-score cut off requirements was found. Subsequent alignment with *D. melanogaster* NDUFC2 reveals that the BLAST result is likely to be a *S. purpuratus* homologue. In the case where the nearest neighbor's protein did not produce a significant result upon further searches, we determined that these subunits were missing. Although we conclude that the subunit is absent, it is possible that the subunit does exist but the protein has diverged

enough to make it difficult to find. However, whether the subunit gene is absent or lacks sequence similarity, the protein subunit must have little functional constraints to have so little similarity and may be unnecessary.

The phylogenetic distribution suggests that 14 subunits are essential for Complex I activity because they are found in virtually all taxa (Table 2.2, Fig. 2.1). However, three subunits NDUFs A12, S4 and S6 subunits have recently been found in in the α-protobacterial ancestor of mitochondria (Yip, Harbour et al. 2011) but are not present in *E. coli.* Because the protein structure for both *E. coli and P. denitrificans* has been described, it is likely that *E. coli* has lost these subunits. Thus, these 3 subunits may not be eukaryotic specific as suggested by phylogeny (Fig 2.1). Similarly, the loss of subunits, including subunits associated with human diseases, in *A. queenslandica, C. elegans* or *S. purpuratus* suggest that other subunits may be lost from other eukaryotes or multicellular organisms not examined here or elsewhere. Though any phylogenic analysis of Complex I is likely to change depending on which taxa are included, the more important point is that though the organisms we examined have well defined genomes, there are many subunits that are missing or lack enough sequence to be considered homologous. It is this finding that suggest that not all Complex I subunits found in mammals are necessary for functioning in the oxidative phosphorylation pathway.

Our analyses find fewer eukaryotic homologous subunits than other studies (Table 2.2; (Gabaldon, Rainey et al. 2005; Huynen, de Hollander et al. 2009; Cardol 2011). We find 28 subunits conserved among all eukaryotes, in contrast to several earlier studies, that described eukaryotes as having 37-40 subunits (using higher E-value cutoffs ≈0.01) (Gabaldon, Rainey et al. 2005; Huynen, de Hollander et al. 2009; Cardol 2011). The

phylogenetic distribution of Complex I subunits presented here includes fewer homologous subunits than the total number of purified subunits in *Neurospora crassa* or *Yarrowia lipolytica* (Abdrakhmanova, Zickermann et al. 2004; Marques, Duarte et al. 2004). In *N. crassa* 39 and in *Y. lipolytica* 37 subunits were identified in purified protein preparations. We agree that there are more protein subunits than the 28 homologous subunits we found, but our analyses suggest that the other protein subunits purified from Complex I are not homologous.

This phylogenetic distribution shows fewer subunits being common to eukaryotes compared to older multi-species data mining efforts (Gabaldon, Rainey et al. 2005; Huynen, de Hollander et al. 2009; Cardol 2011). All of the subunits we suggest are homologous across eukaryotes were found in these studies, but we find little support for up to 12 additional subunits (Table 2.2). We find NDUFs, A7 in all animals and A11, B1, B4, B5, B11 and C2 in triploblastic animals. We find A1, A3, and, C1 in vertebrates *versus* in all eukaryotes based on sequence similarities and structural properties of the subunits (Cardol 2011). For NDUFA1, a subunit associated with human disease, we find a similar subunit only among vertebrates. The closest match in *N. crassa* has an E-value of greater than 10^{-1} and for all other non-vertebrate taxa > 10^{-4}. We found no similarity for the NDUFA1 subunit listed in Cardol (Cardol 2011). For NDUFA11, also associated with human disease, we find the first homologous sequence appearing in triploblastic animals and little similarity to other non-triploblastic animals. Both Cardol and Gabaldon *et al* (Gabaldon, Rainey et al. 2005; Cardol 2011) list it as being in all eukaryotes. The best BLAST hit for NDUFA11 in *N. crassa* has only 30% protein identity for the most similar shared 109 amino acids (total of 141 amino acids) for *B.*

taurus. For NDUFA7, *Y. lipolytica* and *N. crassa* genome databases queried with *B. taurus* protein gives E-values greater than 0.01. Neither NDUFB11 nor NDUFC2 has any similarity between *B. taurus* (NP_001028792 and NP_788815 respectively) and the reported homologous subunits in *N. crassa* (NCU04753 (Marques, Duarte et al. 2004)and XP_956744 (Cardol 2011), respectively). When aligning the *N. crassa* sequence (XP_965162) reported as homologous to *B. taurus* NDUFB5 (NP_788829) a single 10 amino acid sequence with an E-score of 0.35 is returned. Of those 10 amino acids, only four are identical. This data does not support the assertion that these two sequences are homologous. For NDUFB2, the *N. crassa* sequence reported from Cardol (Cardol 2011) aligns with the *B. taurus* sequence (NP_787022) E-value 0.015 (percent quality coverage = 8%, a sequence of 9 amino acids had a max ID of 56%). NDUFB11 was suggested to be a functional analog to subunits in *Y. lipolytica* and *N. crassa* because they share similarities in shape and residue composition but not sequence identity (Abdrakhmanova, Zickermann et al. 2004). For NDUFB11 the fungal subunit is 23.4kDa in size—much larger than the 11.6 kDa *B. taurus* protein. We argue that these are analogous subunits, not homologous subunits. Thus, while other authors may argue that there are functionally similar proteins in other eukaryotes, we find too little sequence similarity to suggest that these are homologous.

One of the reasons for these differences is the acceptable E-values used to identify homologous genes. We use a more conservative E-value of $< 10^{-5}$, where others have used more lenient values of up to 0.01 (Gabaldon, Rainey et al. 2005). In addition to inappropriate labeling of homology, high E-value cutoffs may lead to spurious identification of related, but not homologous genes. For example, using an E-value

cutoff of 0.01 (per (Gabaldon, Rainey et al. 2005) to interrogate the *Homo sapiens* refseq protein database returns six proteins within the E-value cutoff that are not Complex I proteins (NDUFA10: deoxyguanosine kinase, $3E^{-5}$; NDUFA12: mimitin, 0.002; NDUFA4: normal mucosa of esophagus-specific gene 1, $1E^{-7}$; NDUFS2: F Box only protein 22, 0.008; NDUFS4: platelet derived growth factor, 0.008; NDUFS5: cytochrome c oxidase assembly protein (COX19), 0.003). If one compared human lactate dehydrogenase (LDH) to other human protein, an E-value of 0.01 would suggest homology between LDH and malate dehydrogenase (MDH). Yes, many dehydrogenases have a core conserved protein structure, and in the deepest phylogenetic evaluation, many different proteins have evolved from a smaller subset of conserved domains (Eventoff and Rossmann 1975; Doolittle 1987). Yet, saying most proteins are homologous is like saying all animals have a common ancestor. It is undeniably correct, but not very informative. We believe is more informative to provide what is still a relatively liberal similarity (E-values $< 10^{-5}$) as a measure of homology.

Other studies using purified protein data show additional subunits being part of Complex I that are not included in our phylogenetic analysis. We suggest that this indicates 1) a lack of functional constraints allow a protein to drift so it is no longer similar, or 2) the protein found in vertebrates is not homologous to the protein found in fungi or other lower taxonomic groups. Regardless of what E-value an investigator chooses, our analysis suggests little similarity in several Complex I subunits, including those associated with human disease, and thus highlights the need to define the amino acids that provide the biological function.

Of the 45 mammalian Complex I subunits 21 are related to human diseases (OMIM 2011). Eight of these disease loci are either not found in all eukaryotes or have been lost in one or more taxa. This includes NDUFV3 that is only found in vertebrates and NDUFA10 that is found only in multicellular organisms (similar to (Gabaldon, Rainey et al. 2005)). Additionally, disease associated subunits are not found in a few taxa: two of these disease associated loci have little similarity in *C. elgans* (ND6, NDUFA11) or *S. purpuratus* (NDUFA2). These data suggest that not all disease associated subunits are essential for Complex I function. Instead, disease associated subunits may perform accessory or specialized functions and when mutated in humans, the malformed protein interferes with normal function.

An unexpected finding was the differences in similarity of mitochondrial subunits across taxa. ND1, ND4 and ND5 are often well conserved, while ND4L and ND6 are not. For example, when using *E. coli* to search *N. crassa* genome, the E-value for ND6 alignment is approximately 10^{-9} where the E-value for ND4 is approximately 10^{-74}. This disparity in similarity among subunits may be because these proteins have few functional constraints and thus have greater evolution rates. Although neutral drift is the most parsimonious answer, there is evidence in *D. melanogaster* that ND6 has many amino acid substitutions affecting the hydrophobicity that may be adaptive (Montooth, Abt et al. 2009). If the reason that there is little conservation in ND6 is because of adaptive divergence, sequence differences combined with biochemical determination should inform us about the functional significance of the amino acid substitutions.

One of the biggest surprises was the lack of a similar ND6 in *C. elegans.* There is an open reading frame for a protein annotated as ND6 on the *C. elegans* mitochondrial

genome and the conservation of 13 proteins encoded by animals mitochondrial would argue for an ND6 homologue. Yet, a protein similar to the *E. coli* NUOJ, or *B. taurus* ND6 is not found in *Caenorhabditis elegans.* (Note: there are misannotated mitochondrial encoded proteins, see results). None of the other taxas' ND6 are similar to any protein in *C. elegans.* Thus, there is little sequence similarity to support a claim of a homologous ND6 in *C. elegans*. We suggest that this is most likely due to a greater evolutionary divergence in the *C. elegans* ND6 due to the lack of functional constraints or adaptive divergence.

Another subunit that shows this propensity to be less well conserved is NDUFA2, a subunit associated with human disease. There is no similar NDUFA2 in *M. brevicollis* or *S. purpuratus*. In the animals where NDUFA2 is present, it was found with high similarity to *B. taurus* protein (E-values < 10^{-20}) and it has high similarity in the fungi (E-values < 10^{-15}) suggesting a conserved protein linked to human disease. However, no similar protein was found in two animal taxa. This suggests that there are compensations in the other subunits that minimize the effect of differences in amino acids or the lack of this subunits and thus, these changes may inform us about how mutation in NDUFA2 affects human health.

Homology is necessary to understand the evolution and function of Complex I (Huynen, de Hollander et al. 2009). It is the homologous evolutionary relationships that provide information about human diseases and the role of genetic polymorphism (Bustamante, Fledel-Alon et al. 2005; Nielsen, Hellmann et al. 2007). We argue that the existence of subunits lacking sufficient similarity support our supposition that the utility of specific subunits (e.g., being necessary or contributing to disease) is not found in all

taxa regardless of whether they represent homologous or analogous evolutionary relationships. Each of these cases where a subunit appears late in phylogeny or is lost in some taxa supports the hypothesis that some Complex I subunits are accessory or required for specialized functions, though it's not clear why subunits with apparently essential functions would be lost. A deeper understanding of the biochemistry of Complex I in *C. elegans* and *Y. lipolytica* may provide insight into appropriate human medical intervention for Complex I pathologies. Additionally, those subunits that have been lost in one or more taxa should be investigated for having redundant function or tissue specific roles in Complex I assembly.

Duplicated loci

The frequency of duplicated loci and the presence of pseudogenes is quite variable among the taxa examined (Table 3). Previously identified duplicate subunits have been identified as interacting with Complex I. For example, NDUFA12L acts as a Complex I assembly factor. Mutations in NDUFA12L have been observed to cause defective Complex I assembly resulting in progressive encephalopathy (Ogilvie, Kennaway et al. 2005). More potentially duplicated loci were observed in *D. rerio* compared to the other genomes. This is not surprising in light of the whole genome duplication documented in the teleost fish lineage (Jaillon, Aury et al. 2004). The high frequency of pseudogenes observed in the mammals (*H. sapiens, M. musculus,* and *B. taurus)* is possibly related to the nature of transposons and retrotransposons in mammals relative to fish: mammals have a limited diversity of retrotransposons that are responsible for around 20% of the genomic mass, where fish have a diversity of

transposable elements 30 fold greater that is responsible for only around 2% of the genomic mass (Furano, Duvernell et al. 2004).

Complex I mRNA Expression

We examined mRNA expression in four taxa with normal patterns of gene expression (not due to disease, stress, or experimentally induced changes) to capture the variation among healthy individuals. Although the effect of physiological homeostasis in response to stress may reveal different patterns of expression; we sought to define the variation in Complex I expression among individuals which reflects normal variation that may be found in many taxa (Crawford, Pierce et al. 1999; Pritchard, Hsu et al. 2001; Oleksiak, Churchill et al. 2002; Cheung, Conlin et al. 2003; Gibson 2003; Townsend, Cavalieri et al. 2003; Lemos, Meiklejohn et al. 2005; Oleksiak, Roach et al. 2005; Crawford and Oleksiak 2007; Stranger, Nica et al. 2007; Lemos, Araripe et al. 2008). These measures of normal variation have provided valuable information on the evolution and molecular genetics affecting routine gene expression. However, finding studies with sufficient sample sizes in GEO required us to select different tissues from the four taxa analyzed. Thus, we measured lymphoblast cell lines in humans, liver tissue in mice, cardiac tissue in fish and whole bodies in flies. Additionally, in mice there were two diets that could be defined as normal. Our analyses are within a species, and thus the different tissues will not affect these interpretations. However, we also ask if there are similar patterns of expression among taxa. If there are tissue specific effects on the relationship among Complex I subunits expression (e.g., NDUFAB1 correlation to NDUFA8 only occurs in cardiac tissue), the low frequency of significant correlations suggest either a tissue specific differences or species specific affects.

It is often the case that the level of mRNA expression does not necessarily translate into an equivalent change in protein (Ghazalpour, Bennett et al.). For *Fundulus,* the patterns of divergence for many proteins are similar to their mRNAs and the population levels are often correlated both within and among populations, also suggesting that the amount of mRNA affects the amount of protein (Rees, Andacht et al. 2011). Thus, mRNA expression should be a sufficient measure of protein expression for many loci. For proper assembly and function of Complex I, not only do Complex I subunits need to be present, but one would assume that they would need to have similar expression to meet stoichiometric requirements. We examined mRNA expression to explore this. We suggest that the significant correlations observed among genes are caused by vital biological processes, in this case due to the requirement of a coordination of the amount of Complex I subunits, *visa via* the expression of mRNA.

Among Complex I subunits in humans, mice and fish the percentage of significant correlations among subunits relative to total possible unique correlations is 11%, 19,% and 24% (respectively). For humans and mice the frequency of significant Complex I correlations exceeds that found among all metabolic genes and within the OxPhos pathway (where Complex I is the first of five enzyme complexes). It is unlikely that we are missing altered expression patterns due to early lethality of the phenotype. If altered expression is lethal in mammals, there should be little variation in expression and thus few correlations among disease subunits. Yet, there are too many correlations relative to random expectations and no significant differences in the correlation frequency of disease associated subunits relative to non-disease subunits ($p > 0.3$, Fisher-Exact test), suggesting the variation in expression is not differentially affecting disease loci. For

flies, significant Complex I correlations occur with less than 50% of the frequency found among all metabolic genes or OxPhos mRNAs. The lower frequency in flies could represent a species specific effect or could be due to the use of whole body expression data, which would be affected by tissue specific effects. Regardless of the effects of sample origin in flies, there are still too many statistically significant correlations relative to random permutations. The most likely explanation for these improbable frequencies (Table 2.4) is that the variation in mRNA expression affects Complex I function.

If coordinated transcriptional regulation of Complex I subunits causes correlations among subunits, they should be predominately positive. For mice and flies, this is the case; there are too few negative correlations relative to randomly chosen genes. In humans there are too many negative correlations relative to non-metabolic genes. Negative correlations are an unexpected result among the heterologous subunits of a single enzyme complex if there must be a stoichiometric concentration of each protein. For humans at least, it is not due to the expression of duplicate loci because among the five subunits that have duplications there are negative correlations. We are unable to explain high frequency of negative correlations in humans except to suggest that it may reflect cell culture conditions which lack hormonal and other physiological inputs responsible for homeostatsis.

There are few shared pairs of subunits that are correlated in more than one species (Fig. 2.3). One might expect that if mRNA expression is important for Complex I activity or assembly, then the relationships in expression among subunits should be observed in more than one species. There is one pattern that stands out; the 14 basal subunits (found in all taxa, Fig. 2.1, Table 2.2) are more likely to have significant

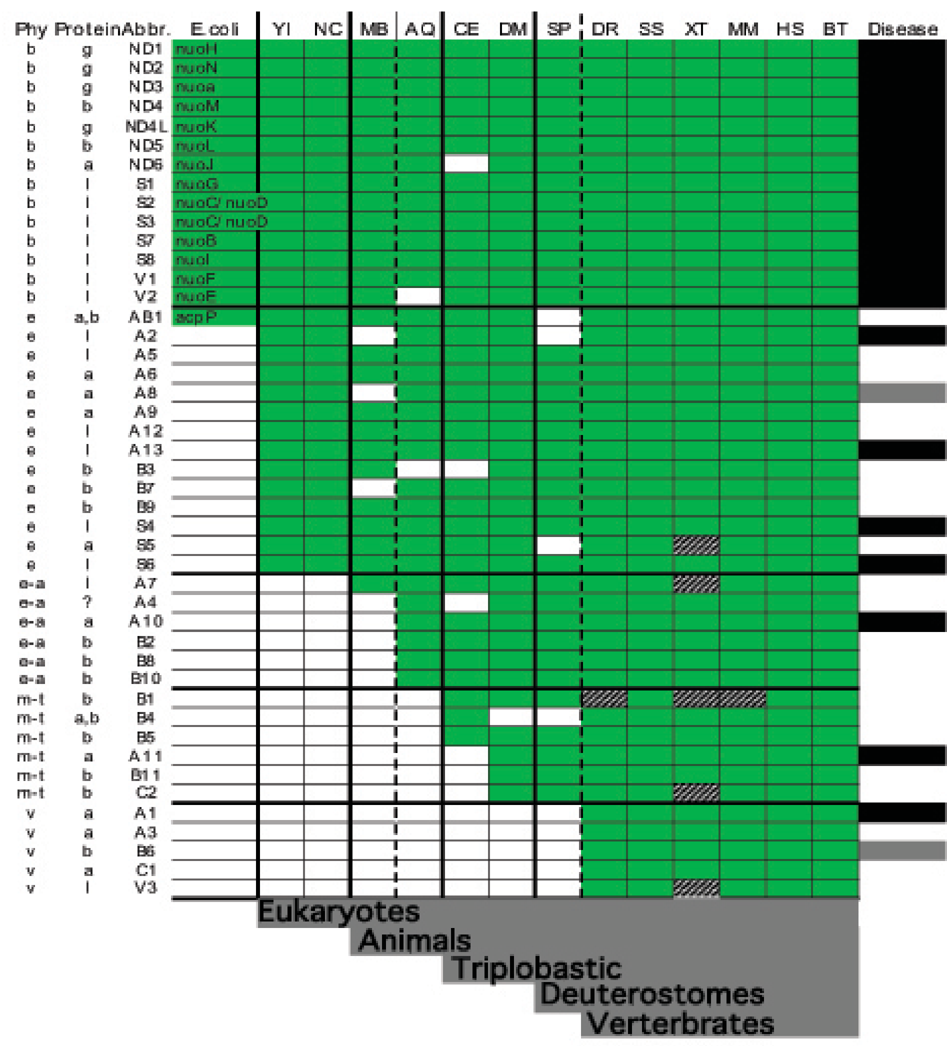
correlations than all other subunits. Notice that NDUFS1, NDUFS7 NDUFS8, NDUFV1 and NDUFV2 are all basal, associated with disease and have significant correlations with the same subunits in more than one species. However, the correlations in different species are not always the same sign (positive *versus* negative): for example, there are six subunits where NDUFV1 shares significant correlation in a different species, in two of these (NDUFV1 to B5 and B8) mice are positive and humans are negative. In general, if there is a difference in the sign it is with the basal subunits and the NDUFBx subunits, mice being positive and human being negative. We would like to emphasize that these are significant correlations (not a lack of correlations) suggesting that the increase in NDUFV1 is associated with a non-random decrease in NDUFBx subunits in humans, and we are suggesting this is unlikely if mRNA does not affect protein amount. This non-random pattern is a conundrum: either less of the NDUFBx subunit is required with more of NDUFV1 or there is increasing post-transcriptional compensation for NDUFBx (e.g., greater translation with fewer mRNAs) with more NDUFV1. Either explanation is worthy of future investigation.

If, as we have argued, the significant correlations among Complex I subunits imply a functional importance of mRNA expression; the paucity of shared correlations except for the basal subunits, suggests that the importance of mRNA expression for Complex I is different in different species and among different subunits. The observation that the phylogenetically oldest subunits (basal) have a high frequency of significant correlations to other subunits and these are more often shared with other species, suggests that variation in mRNA expression of these basal subunits is more likely to be important than variation in expression of more recently evolved subunits.

Conclusion

We used protein sequence similarity to define homology. Among the 14 taxa analyzed, conserved subunits have a high similarity (E-values < 10^{-20}). This suggests that the conserved subunits described here are affected by stabilizing selection to maintain similar sequence and presumably function. Yet, not all catalytic subunits or subunits associated with human disease occur in all taxa and some have been lost sporadically. Additionally, there are correlated patterns of mRNA expression among disease associated and other subunits, and these correlations are both positive and negative. These data suggest that although some subunits are associated with human disease not all of these are necessary in all organisms and the presence of some analogous accessory subunits may compensate for other subunits. Although these data do not clarify the utility of each subunit, they do suggest more research is required. There are clearly homologous groups of subunits that arose with the evolution of the nucleus, multicellularity, and then vertebrates. Significant correlations are most common among the phylogenetically oldest subunits and these significant correlations are shared in different species. If mRNA expression is important for the functional variation in Complex I, we suggest that it is most likely in the basal subunits.

Figure 2.1 Phylogenetic Distribution of Complex I Subunits. A) Phylogenetic distribution of Complex I subunits. In the first row, the first letter of the genus and species names are used to designate the 10 species used. These include: four vertebrate classes (right of dashed line): mammals (*Mus musculus*, and *Homo sapiens)*, amphibians (*Xenopus tropicalis)*, and ray-fin fishes (*Danio rerio*, and *Salmo salar*), One non-vertebrate deuterostome (purple sea urchin, *Stronglyocentrotus purparatus).* Four invertebrate species: (fruit fly, *Drosophila melanogaster*; the nematode *Caenorhabditis elegans;* sponge *Amphimedon queenslandica*, and the unicellular *Monosiga brevicollis*). Two fungi (*Yarrowia lipolytica, Neurospora crassa)* and the bacteria *Escherichia coli.* The first column indicates phylogenetic group (b, basal; e, eukaryotic; e-a, animals m-t multicellular triploblastic animals; v, vertebrate). Second column indicates subcomplex (a, alpha; b, beta; g, gamma; l, lambda). Green boxes represent presence of the subunit in the genome. Striped boxes represent presence of the subunit in EST database. Black boxes in the last column indicate association with human disease with allelic variants or gray boxes with molecular genetic evidence (OMIM 2011). B) Phylogenetic tree showing appearance and deletions of Complex I subunits through time. Gray boxed subunits are associated with human diseases. Black letters show presence and red letters (on upper branches) show subunits that have been lost. The seven mitochondrial subunits are abbreviated N1-N6. The official HUGO abbreviation for nuclear Complex I subunits are NDUF__, and the last letters (without NDUF) are used to designate each subunit.


Phy Protein Abbr. E.coli YI NC MB AQ CE DM SP DR SS XT MM HS BT Disease
b g ND1 nuoH
b g ND2 nuoN
b g ND3 nuoa
b b ND4 nuoM
b g ND4L nuoK
b b ND5 nuoL
b a ND6 nuoJ
b l S1 nuoG
b l S2 nuoC/ nuoD
b l S3 nuoC/ nuoD
b l S7 nuoB
b l S8 nuoI
b l V1 nuoF
b l V2 nuoE
e a,b AB1 acpP
e l A2
e l A5
e a A6
e a A8
e a A9
e l A12
e l A13
e b B3
e b B7
e b B9
e l S4
e a S5
e l S6
e-a l A7
e-a ? A4
e-a a A10
e-a b B2
e-a b B8
e-a b B10
m-t b B1
m-t a,b B4
m-t b B5
m-t a A11
m-t b B11
m-t b C2
v a A1
v a A3
v b B6
v a C1
v l V3
Eukaryotes
Animals
Triplobastic
Deuterostomes
Verterbrates

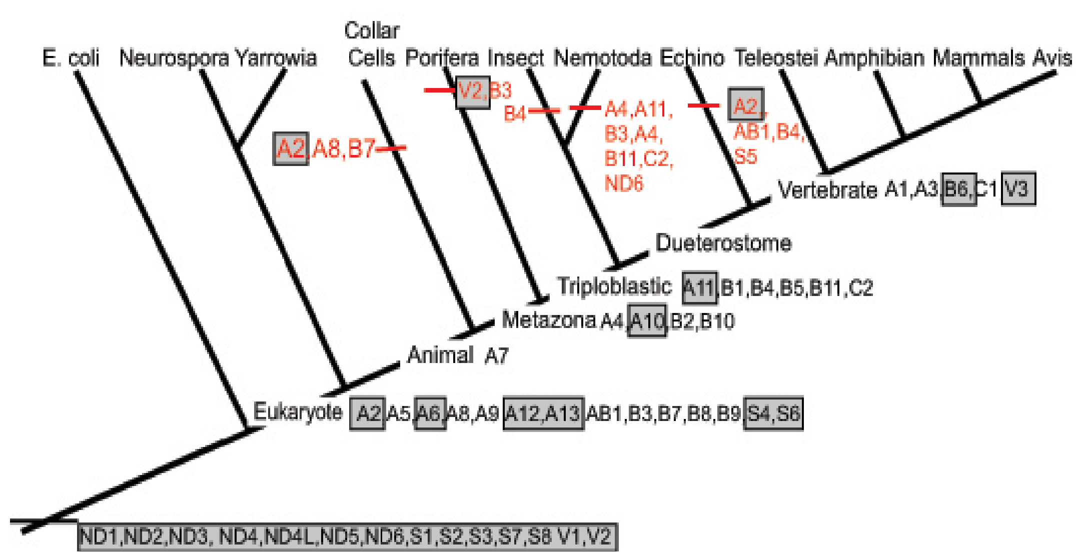

E. coli
Neurospora
Yarrowia
Collar Cells
Porifera
Insect
Nemotoda
Echino
Teleostei
Amphibian
Mammals
Avis
A2,A8,B7
V2,B3
B4
A4,A11, B3,A4, B11,C2, ND6
A2, AB1,B4, S5
Vertebrate A1,A3,B6,C1 V3
Dueterostome
Triploblastic A11,B1,B4,B5,B11,C2
Metazona A4,A10,B2,B10
Animal A7
Eukaryote A2,A5,A6,A8,A9 A12,A13,AB1,B3,B7,B8,B9,S4,S6
ND1,ND2,ND3, ND4,ND4L,ND5,ND6,S1,S2,S3,S7,S8 V1,V2

Figure 2.2 Correlation of Complex I Subunit mRNA Expression in Four Species. A symmetric matrix of correlations for mRNA expression among Complex I subunits. Positive correlations are yellow and negative correlations are blue. Color scale is for correlation coefficients (rho) from 0.6 to -0.6, where |0.623|, |0.561|, |0.433| is significant (p <0.01) for 16, 20 and 40 individuals, respectively. Loci associated with human disease are highlighted red. The four species (n-individuals) and tissues are: A. Homo sapiens (16), lymphoblast cell lines: B. Mus musculus (20), liver; C. Fundulus heteroclitus (16), heart ventricle; and D. Drosophila melanogaster (40).

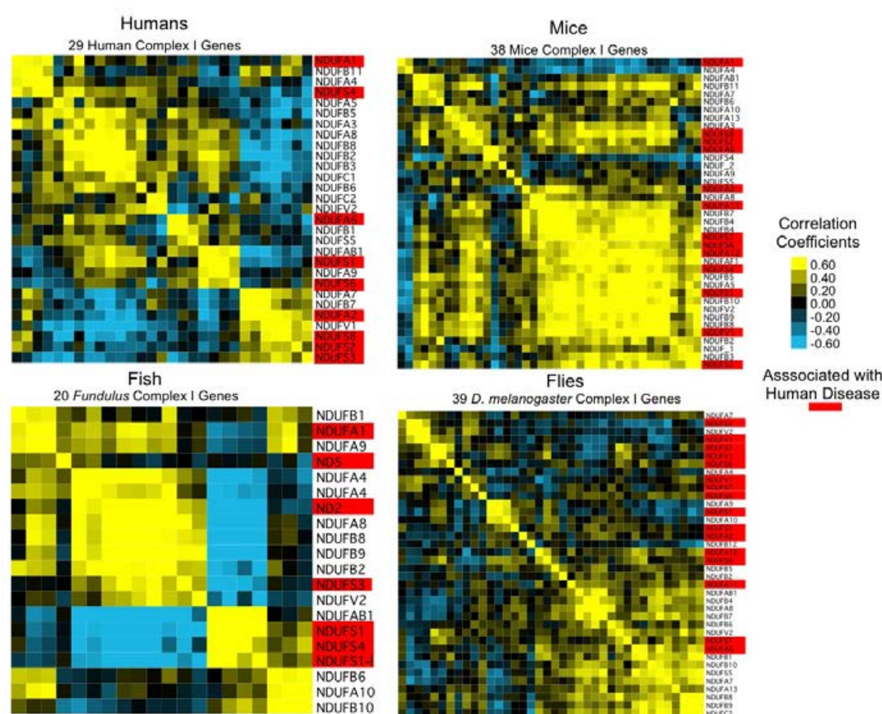


Figure 2 3 Frequency of Significant Correlations. Black bars represent total number of significant correlations for each subunit, grey bars represent correlations that occur as pairs in more than one species (i.e., subunit A is correlated significantly with subunit B in two or more species)

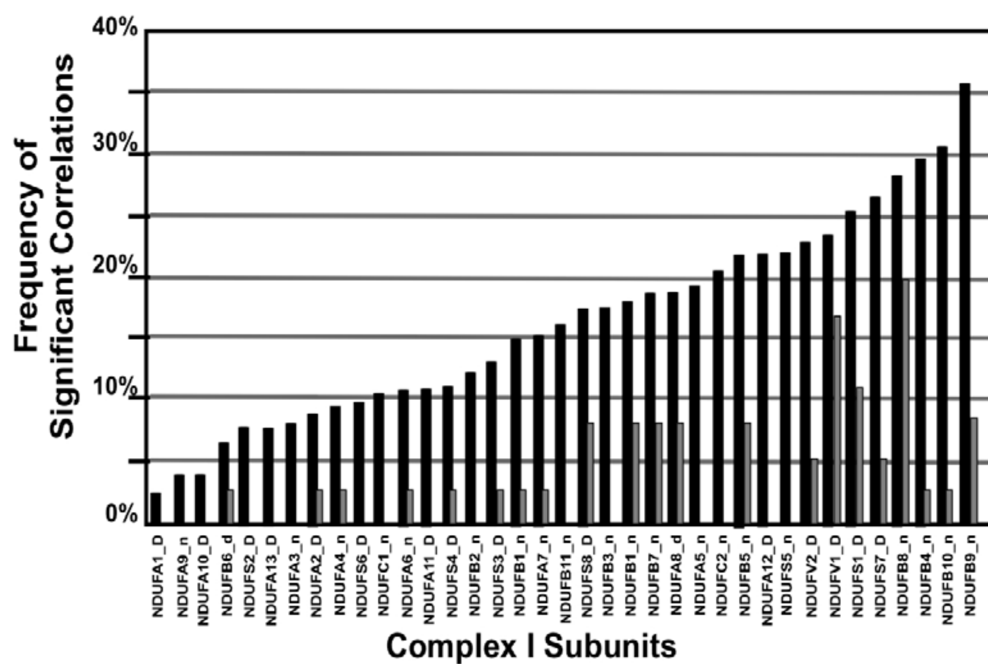


Table 2.1 Orthologous Annotations for Complex I Subunits

	D	***HUGO***	***Human KEGG***	**Description**	***Bos taurus***	***D. melanogaster***	***C. elegans***	***N. crassa***	***E. coli***	**SWISSPROT**
1	**D**	ND1	K03878	NADH dehydrogenase I subunit 1	ND1	ND1	ND1	ND1	NuoH	NU1M
2	**D**	ND2	K03879	NADH dehydrogenase I subunit 2 [EC:1.6.5.3]	ND2	ND2	ND2	ND2	NuoN	NU2M
3	**D**	ND3	K03880	NADH dehydrogenase I subunit 3	ND3	ND3	ND3	ND3	NuoA	NU3M
4	**D**	ND4	K03881	NADH dehydrogenase I subunit 4	ND4	ND4	ND4	ND4	NoM	NU4M
5	**D**	ND4L	K03882	NADH dehydrogenase I subunit 4L	ND4L	ND4L	ND4L	ND4L	NuoK	NULM
6	**D**	ND5	K03883	NADH dehydrogenase I subunit 5	ND5	ND5	ND5	ND5	NuoL	NU5M
7	**D**	ND6	K03884	NADH dehydrogenase I subunit 6	ND6	ND6	ND6	ND6	NuoJ	NU6M
8	**D**	NDUFA1	K03945	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 1	MWFE					NIMM
9	**D**	NDUFA2	K03946	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2	B8	*CG15434*	*Y63D3A.7*	10.5 kDa		NI8M
10	n	NDUFA3	K03947	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 3	B9					NI9M
11	n	NDUFA4	K03948	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4	MLRQ	*CG32230*				NUML
12	n	NDUFA5	K03949	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5	B13	CG6463	C33A12.1	29.9 kDa		NUFM

13	**n**	NDUFA6	K03950	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 6	B14	*CG7712*	*nuo-3*	14.8 kDa		NB4M
14	n	NDUFA7	K03951	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 7	B14.5A	*CG3621/CG6914*	*F45H10.3*			N4AM
15	d	NDUFA8	K03952	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 8	PGIV	*CG3683*	*Y54F10AM.5*	20.8 kDa		NUPM
16	n	NDUFA9	K03953	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9	39 kDa	*CG6020*	*Y53G8AL.2*	40 kDa		NUEM
17	n	NDUFA10	K03954	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	42 kDa	ND42	*CE01361nuo-4*			NUDM
18	**D**	NDUFA11	K03956	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11	B14.7					N5BM
19	n	NDUFA12	K11352	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 12	B17.2	*CG3214*	*Y94H6A.8*	13.4 kDa		N7BM
20	D	NDUFA13	K11353	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 13	B16.6	*CG3446*	*C34B2.8*	14 kDa		NB6M
21	n	NDUFAB1	K03955	NADH dehydrogenase (ubiquinone) 1 alpha/beta subcomplex 1	SDAP	*MTACP1*	*Y56A3A.19*	9.6 kDa ACP	acpP	ACPM
22	n	NDUFB1	K03957	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 1	MNLL	*CG18624*	*Y57G11C.5*			NINM
23	n	NDUFB2	K03958	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 2	AGGG	*CG40002*	*F44G4.2*			NIGM
24	n	NDUFB3	K03959	NADH dehydrogenase (ubiquinone) 1 beta subcomplex	B12	*CG10320*		10.6 kDa		NB2M

				3						
25	n	NDUFB4	K03960	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4	B15		*nuo-6*			NB5M
26	n	NDUFB5	K03961	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5	SGDH	L3NEO18	C25H3.9			NISM
27	d	NDUFB6	K03962	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 6	B17					NB7M
28	n	NDUFB7	K03963	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 7	B18	*CG5548*	*D2030.4*	NCU1134 8/11.3 kDa		NB8M
29	n	NDUFB8	K03964	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	ASHI	*CG3192*	*Y51H1A.3*	20.1 kDa		NAIM
30	n	NDUFB9	K03965	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9	B22	*CG9306*	*C16A3.5*			NI2M
31	n	NDUFB10	K03966	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 10	PDSW	PDSW	F59C6.5			NIDM
32	n	NDUFB11	K11351	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 11	ESSS					NESM
33	n	NDUFC1	K03967	NADH dehydrogenase (ubiquinone) 1 subcomplex unknown 1	KFYI					NIKM
34	n	NDUFC2	K03968	NADH dehydrogenase (ubiquinone) 1 subcomplex unknown 2	B14.5b	*CG12400*				N4BM
35	**D**	NDUFS1	K03934	NADH dehydrogenase (ubiquinone) Fe-S protein 1	75 kDa	ND75	nuo-5	78 kDa	NuoG	NUAM

36	**D**	NDUFS2	K03935	NADH dehydrogenase (ubiquinone) Fe-S protein 2	49 kDa	*CG11913*	*nduf-2.2*	49 kDa	NuoC/D	NUCM
37	**D**	NDUFS3	K03936	NADH dehydrogenase (ubiquinone) Fe-S protein 3	30 kDa	*CG12079*	*nuo-2*	30.4 kDa	NuoC/D	NUGM
38	**D**	NDUFS4	K03937	NADH dehydrogenase (ubiquinone) Fe-S protein 4	18 kDa (AQDQ)	*CG12203*	*lpd-5*	21 kDa		NUYM
39	n	NDUFS5	K03938	NADH dehydrogenase (ubiquinone) Fe-S protein 5	15 kDa		*nduf-5*	11.5 kDa		SIPM
40	**D**	NDUFS6	K03939	NADH dehydrogenase (ubiquinone) Fe-S protein 6	13 kDa	*CG8680*	*nduf-6*	28.7 kDa		NUMM
41	**D**	NDUFS7	K03940	NADH dehydrogenase (ubiquinone) Fe-S protein 7	PSST	*CG9172/CG2014*	*nduf-7*	19.3 kDa	NuoB	NUKM
42	**D**	NDUFS8	K03941	NADH dehydrogenase (ubiquinone) Fe-S protein 8	TYKY	ND23	T20H4.5	21.3c kDa	NuoI	NUIM
43	**D**	NDUFV1	K03942	NADH dehydrogenase (ubiquinone) flavoprotein 1	51 kDa	*CG9140/CG8102/CG11423*	*nuo-1*	51 kDa	NuoF	NUBM
44	**D**	NDUFV2	K03943	NADH dehydrogenase (ubiquinone) flavoprotein 2	24 kDa	*CG5703/CG6485*	*F53F4.10*	24 kDa	NuoE	NUHM
45	n	NDUFV3	K03944	NADH dehydrogenase (ubiquinone) flavoprotein 3	10 kDa					NUOM

Table 1.2 Major Complex I Innovations.
The most parsimonious description of subunit evolution. * denotes subunits with allelic variation implicated in human disease. ‡ denotes subunits with molecular genetic data indicating an association with human disease. Light gray box: defined as eukaryotic in (Cardol 2011). Dark gray box: defined as eukaryotic in both (Cardol 2011), and (Gabaldon, Rainey et al. 2005).

	Basal core	**Eukaryotic subunits**	**Animal Subunits**	**Triploblastic Animals subunits**	**Vertebrate subunits**
1	ND1*	A2*	A4	A11*	A1*
2	ND2*	A5	A7	B1	A3
3	ND3*	A6*	A10*	B4	V3
4	ND4*	A8**	B2	B5	B6**
5	ND4L*	A9	B8	B11	C1
6	ND5*	A12	B10	C2	V3
7	ND6*	A13*			
8	S1*	AB1			
9	S2*	B3			
10	S3*	B7			
11	S7*	B9			
12	S8*	S4*			
13	V1*	S5			
14	V2*	S6*			

Table 2.2. Psuedogenes and Possible Duplicated Genes. Pink=pseudo genes; green=candidate duplicate loci. Numbers in boxes indicate how many were found.

	Human		**Mouse**		**Cow**		***Danio***	
a1	3		28		5			
a2	1		2		2			
a3	4		3		6			
a4	21	2	6		3			3
a5	13		6		4	2		
a6			1					
a7			2					
a8						2		
a9	1							
a10								
a11			1					
a12		2	1					2
a13			1		2			
ab1	1		6		2			3
b1					1			
b2	1		1		1			
b3	4		4		2			
b4	11	2	8		2			3
b5		2						3
b6								
b7			1					
b8	4		3		1			
b9	7		2					2
b10		2						2
b11	1		3					
v1								
v2	1		1		5	2		
v3								2
c1			3		2			
c2			4					
s1								
s2			1	2				
s3			2					
s4			1					2
s5	6		3					
s6	1		1		5	2		2
s7			1					3
s8								2

Table 2.3 Significant unique correlations among metabolic pathway mRNAs.
Significant unique correlations among metabolic pathway mRNAs ($p < 0.01$) compared to 1,000 randomizations among individuals, or among non-metabolic genes (for F. heteroclitus only metabolic genes were measured). "P-value Random Individual" is probability of observing an equal or greater number of significant correlations when individuals were randomized 1,000 times (same genes as used for observed). "Random Average" is the average number of significant correlations among non-metabolic genes for 1,000 iterations. "P-value Random" is probability of observing an equal or greater number of significant correlations among non-metabolic genes based on 1,000 random iterations, only significant pvalues are reported. For all randomizations with non-metabolic genes, the number of non-metabolic genes assayed is equal to the number of genes for each species and pathway.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	# genes	# Possible Unique Corr	% unique Sig Corr	P-value Rand Indiv.	# of Sig Corr.	Rand Avg Sig	P-value Rand.	# of Sig Pos Corr.	Rand Avg Sig Pos	P-value Rand. Pos	#of Sig. Neg Corr.	Rand Avg Sig Neg	P-value Rand. Neg	Rand. lowest neg. 99%
H. sapiens														
all Metabolic	119	7021	5.07%	0	356	283	0.064	177	174	0.439	179	106	0.002	96
OxPhos	69	2346	7.03%	0	165	95	0.001	91	59	0.023	74	36	0	33
CI	29	406	11.33%	0	46	16	0	24	10	0.004	22	6	0	6
M. musculus														
all Metabolic	178	15753	8.13%	0	1280	637	0	684	357	0.003	596	281	0	186
OxPhos	104	5356	9.76%	0	523	216	0	349	121	0	174	95	0.013	78
CI	39	741	19.43%	0	144	30	0	139	17	0	5	13	0.947	12
D. melanogaster														
all Metabolic	184	16836	1.65%	0	917	421	0	640	282	0	277	139	0.001	136
OxPhos	89	3916	1.76%	0	296	98	0	227	98	0	69	66	0.008	32
CI	38	703	0.71%	0	62	18	0	57	18	0	5	12	0.461	6
F. heterociitus														
all Metabolic	119	7021	22.89%	0	1607			815			792			
OxPhos	45	990	27.98%	0	277			154			123			
CI	20	190	24.21%	0	46			26			20			

Chapter 3 Inter-individual Variation in Complex I Activity in *Fundulus heteroclitus* Along a Steep Thermocline

3.1 Summary

The first enzyme in the oxidative phosphorylation pathway is Complex I (E.C.: 1.6.5.3). Complex I is a large heteromeric enzyme complex with 45 protein subunits that translocates H^+ ions across the mitochondrial inner membrane and drives the synthesis of ATP. Among northern and southern populations of the teleost fish *Fundulus heteroclitus,* Complex I subunits have fixed amino acid substitutions. Additionally, there are differences in oxidative phosphorylation activity among populations of *F. heteroclitus.* To investigate if these differences are related to Complex I, enzyme activity was measured in 121 individuals from 5 populations of *F. heteroclitus* and its sister species *Fundulus grandis* acclimated to a constant 20°C temperature. Within each population, Complex I activity was highly variable among individuals of *F. heteroclitus* (% coefficient of variation among individual has a mean of 90% in the five *F. heteroclitus* populations), and the mean Complex I activity among populations was significantly different at the latitudinal extremes of the range. Importantly, Complex I activity was more similar between *F. heteroclitus* from the southernmost population and its sister species *F. grandis* than to the northern populations of *F. heteroclitus*; suggesting evolutionary important differences. Unexpectedly, the activity was four-fold higher in southern populations than northern populations. Increased mitochondrial density appeared to partially compensate for decreased activity in northern individuals: activity per wet weight was only two-fold higher in southern populations. We suggest that some of variation in Complex I activity is genetically based, and thus is being influenced by

directional selection. Yet, this conclusion presents a conundrum: there should not be so much variation in Complex I within a population if this variation is biologically important.

3.2 Introductory Material

Complex I (NADH dehydrogenase, also called NADH:ubiquinone oxidoreductase; EC 1.6.5.3) is a large, heteromeric enzyme complex with a central role in oxidative phosphorylation. The enzyme complex accepts electrons from NADH and pumps hydrogen ions into the inner membrane space. In vertebrates, Complex I is made up of 45 different protein subunits (Carroll, Fearnley et al. 2006). Thirty-eight of these subunits are encoded by the nuclear genome while the other 7 are encoded by mitochondrial genome (Carroll, Fearnley et al. 2006). Many Complex I subunits have been causatively linked to mitochondrial disease in humans and deficiencies in Complex I are responsible for 80% of heritable metabolic disorders (Smeitink, Sengers et al. 2001). Complex I is a well conserved respiratory enzyme; 17 of the subunits found in vertebrate Complex I have homologues in the ancestor of mitochondria, the alpha proteobacteria (Yip, Harbour et al. 2011).

Given the importance of Complex I in metabolic disorders (Smeitink and van den Heuvel 1999; Smeitink, Sengers et al. 2001) and its role in ATP production (Efremov and Sazanov 2011), one might expect constraints on enzyme activity producing low levels of inter-individual variation but adaptive differences among populations or species. Differences in enzyme activity have been measured in many species and there are many examples of divergence in enzyme activity that appears to compensate for altered environments (Somero 1995; Pierce and Crawford 1997; Crawford, Pierce et al. 1999). In *F. heteroclitus*, among ten glycolytic enzymes there is an increase in the activity of five enzymes, with southern fish having lower activity than northern fish (Pierce and Crawford 1994). Of these, three

glycolytic enzymes show patterns indicative of evolution by natural selection and are associated with increase in metabolism (Pierce and Crawford 1997; Crawford, Pierce et al. 1999; Podrabsky, Javillonar et al. 2000; Crawford and Oleksiak 2007). In addition to differences in enzyme activities among populations, northern and southern *F. heteroclitus* have many loci with strong clinal variation in allele frequencies (Powers, Lauerman et al. 1991) and amino acid differences in mitochondrial proteins (Crawford and Powers 1989; Whitehead 2009). One of the best documented allelic variants is the heart-type lactate dehydrogenase locus (*Ldh-B*), where the northern allelic isozyme has a greater K_{cat}/K_m at lower temperatures than does the southern allelic isoform (Place and Powers 1979; Place and Powers 1984). These catalytic differences affect swimming speeds (DiMichele and Powers 1982), development (DiMichele and Powers 1991) and survival (Paynter, Dimichele et al. 1991).

Fundulus heteroclitus is well established as a model species used to investigate molecular, biochemical, and physiological variation within and among populations. *Fundulus heteroclitus* has a large geographic range; they are found from Nova Scotia to Northern Florida. Along its range there is a steep thermal cline where temperatures drop 1°C for every 1° increase in latitude (Powers, Lauerman et al. 1991). This thermal gradient and the historical demography affect the divergence among populations. There is a consensus concerning the demography in *F. heteroclitus* (Smith, C. et al. 1992; Smith, Chapman et al. 1998; Adams, Lindmeier et al. 2006; Duvernell, Lindmeier et al. 2008; Williams, Ma et al. 2010): during the glaciation of the Pleistocene ~20,000 years ago there were large populations isolated north of the Hudson River and with glacial retreat the northern and southern populations eventually merged. Microsatellite analysis shows that the Hudson

River and Delaware Bay were both barriers to dispersal during this time (Duvernell, Lindmeier et al. 2008). Most of the mitochondrial diversity predates the separation of populations during the last glacial period and can be traced to the current intergrade zones at the Hudson and Delaware rivers (Gonzalez Villasenor and Powers 1990).

Differences among *F. heteroclitus* populations for SNPs associated with metabolic genes, microsatellites, enzyme activity, allelic variation and metabolic rates (Powers, Lauerman et al. 1991; Powers, Smith et al. 1993; Crawford, Pierce et al. 1999; Crawford and Oleksiak 2007; Williams and Oleksiak 2010) suggest that there could be adaptive differences in many other enzymes. Complex I is a likely candidate for adaptive differences in enzyme activity based on the role it plays in generating ATP (Efremov and Sazanov 2011), the association of many subunits to human disease (Smeitink and van den Heuvel 1999; Smeitink, Sengers et al. 2001; Haack, Madignier et al. 2012) and the number of amino acid substitutions found among *F. heteroclitus* Complex I genes (Whitehead 2009). Thus, we expect the activity of Complex I to vary significantly among and within species. Presented here is the analysis of enzyme activity among five populations of *Fundulus heteroclitus* and one population of *Fundulus grandis.* These data show considerable variation within populations and significant divergence among the geographically most extreme populations.

3.3 Materials and Methods

Collection of Fundulus

Fundulus heteroclitus from #2 Sapelo Island, GA; #3 Hayes, VA; #4 Stone Harbor, NJ; #5 Matunuck, RI and #6 Wiscasset, ME were collected in minnow traps in September 2010. *Fundulus grandis* from #1 Cocodrie, LA were collected in May 2010 (Fig. 3.1). Fish

were acclimated in the lab for a minimum of 6 weeks at 23° C and 15ppt salinity using artificial seawater with a 14h light 10h dark light schedule.

Isolation of Mitochondria

Mitochondria were isolated and measured on the same day from each of the sample groups (all six taxa) following modified protocol (Mela and Seitz 1979). Fish were sacrificed by cervical dislocation and livers were removed. Livers were rinsed, patted dry, weighted and minced in ice cold Medium A (250mM sucrose, 0.5mM Na_2EDTA, 10mM Tris, pH 7.4). Minced liver was homogenized gently in Medium A using a glass-teflon PE homogenizer. Liver homogenate was centrifuged at 500 *g* for 5 min at 4°C to remove large cellular fractions. Supernatant was centrifuged at 7,880 g for 10 minutes at 4°C and pellet resuspended by gentle pipetting in Media B (Media A with 1g/l BSA). The pellet was again resuspended and centrifuged at 7,880 g for 10 minutes at 4°C. The final pellet was rinsed with 10mM Tris (pH 7.4), brought up in Media A, then sonicated for five seconds to fracture the membranes and assayed for CI activity. An aliquot immediately removed and stored at 20°C for BCAprotein assay. Mitochondria were stored on ice until use. Total protein from this mitochondrial enriched fraction was used as a measure of total mitochondrial density.

Measurement of Complex I Activity

Complex I activity was measured by adapting the method of (Janssen, Trijbels et al. 2007). Reduced NADH was used as the electron donor, decylubiquinone (Sigma: D7911) was used as an analogue for ubiquinone and DCIP (Sigma D1878) was the final electron acceptor. The absorbance of DCIP at 600nm decreases as it is reduced. Control and experimental reactions were run side by side in a 96 well microplate (250 mM HEPES, 450 mM potassium, 3.5 g/L BSA, 60µM DCIP, 70 µM decylubiquinone, 1 µM Anti-A, 1 µM

Rotenone dissolved in ethanol). Absorbance was measured immediately after addition of NADH for five minutes using a Spectramax Plus[384] spectrophotometer (Molecular Devices, Sunnyvale, CA). Enzyme activities remained stable for greater than four hours.

All individuals' Complex I activity was measured in triplicate—three sets of control and experimental reactions. The spectrophotometer was blanked with a set of control reactions lacking isolated mitochondria. The slope of reduced DCIP concentration over time was calculated using the extinction coefficient of 19.1/mMol*cm. Individual Complex I specific activity is defined as the difference in slope between the control wells (representing total mitochondrial oxidation of NADH) and average of the three experimental wells (containing rotenone, and thus representing total NADH oxidized without Complex I specifically). In order to correct for variations in total number of mitochondria per individual as well as technical variation in mitochondrial isolation, the slopes of NADH (control) and Rotenone (experimental) reactions were divided by the total amount of protein in the mitochondrial preparation, determined with a BCA assay, in the reaction to give Complex I activity as μmol DCIP reduced min^{-1} μg total mitochondrial $protein^{-1}$.

Replicate values were excluded if the rotenone slope (experimental, without Complex I activity) exceeded the control slopes (indicating creation of NADH relative to the rotenone control). Fifteen replicate values were excluded. Additionally, a total of six individuals were excluded; one each from Maine, New Jersey, Rhode Island and Virginia populations and two from *F. grandis*. These samples had over 100-fold greater activity than other individuals and were all measured on the same day. The high Complex I activity can be attributed to instrument malfunction or incorrectly calibrated and thus these values were excluded.

Data Analyses

Data analyses were carried out using Prism (GraphPad, La Jolla, CA), Microsoft Excel and MatLab (MathWorks, Natick, MA). Analysis of variance within a population was carried out using the three technical replicates for the within mean square and n individuals (Table 1) as the among mean square (df = 2, 3*(n-1)). Analyses among populations used n individuals for the within mean square and the six taxa for the among mean square (df = n-1, n*5). Regression of mitochondrial activity and mitochondrial density was performed with the average of the three Complex I activity measurements for each individual. Mitochondrial density was derived from the total protein in mitochondrial isolate, quantified with a BCA protein assay, multiplied by total volume of mitochondrial preparation divided by wet weight of liver that was used to prepare isolated mitochondria. Activity per wet weight liver was calculated by dividing activity per mitochondria by mitochondrial density. For ANOVA analyses, Log10 transformed data was used so that variation among populations were similar.

3.4 Results

Five populations of *F. heteroclitus* and one population from its sister taxa, *F. grandis*, (n= 121, Fig. 3.1) were collected and acclimated to common conditions for a minimum of six weeks. Two individuals from each population (n = 12 per day) were assayed on the same day. Thus, assay days were distributed evenly among populations. Populations were held in the laboratory for different lengths of time; specifically, *F. heteroclitus* were held longer than the single *F. grandis* populations. However, acclimation duration (collection dates) did not have a significant effect on *F. heteroclitus* Georgia *versus F. grandis* for

Complex I enzyme activity (Complex I activity per µg of mitochondria protein; per wet weight or mitochondrial density. $p > 0.1$).

Technical Variation in Complex I Activity

Complex I mitochondrial specific activity (units/µg mitochondrial protein) was typically precise: the average coefficient of variation (CV, standard deviation divided by mean, multiplied by 100%) for the 3 replicate values was 6.58% for control wells and 7.48% for experimental wells (containing rotenone).

Inter-individual variation in Complex I Activity

The variation of Complex I mitochondrial specific activity and Complex I activity per wet weight (units per wet weight of liver) within a population were high (Table 3.1, columns 3 and 10 respectively). This variation is due to the biological variation among individuals because the variation among individuals exceeds the variation among the three technical replicates (Table 3.1, column 4 "P-value among individuals"). Body mass did not affect activity per wet weight liver or activity per mitochondria ($p = 0.0553$, $R^2 = 0.023$ and $p = 0.1971$, $R^2 = 0.010$). Mitochondrial density was calculated as total mitochondrial protein isolated divided by wet weight of liver tissue. Variation in mitochondrial density (Table 3.1, column 8), presented as the %CV among individuals within populations, ranged from 34% to 58%. The regression of mitochondrial density and Complex I mitochondrial specific activity (Fig. 3.2) was significant in four of the five *F. heteroclitus* populations considered ($p < 0.05$), though the slopes of the regressions were different ($p < 0.05$). This difference in is most apparent in the comparison between Georgia and Maine individuals (Fig. 3.2).

Complex I Activity among populations

Complex I mitochondrial specific activity was significantly different among populations (Fig 3.3A; $p = 0.002$). The mean Complex I mitochondrial specific activity for the five *F. heteroclitus* populations (Table 3.1, column 6) ranged nearly 3.7 fold from 3.2×10^{-4} µmol DCIP reduced min^{-1} µg $protein^{-1}$ in Maine (ME) to 11.7×10^{-4} µmol DCIP reduced min^{-1} µg $protein^{-1}$ in Georgia (GA, Fig. 3.3A). This difference in activity between the most northern (Maine) and most southern (Georgia) is significant (Tukey post-hoc test, $p < 0.05$).

Mitochondrial density (µg mitochondrial protein/wet weight) was significantly different among populations ($p < 0.001$, Figure 3.3B). In general northern populations had significantly greater amount of mitochondrial protein than southern populations (Tukey post hoc test; $p < 0.05$).

Both Complex I mitochondrial specific activity and mitochondrial density were significantly different among populations (Fig 3), yet they have opposite patterns. To compare the effect of these two parameters, we calculated the Complex I activity per wet weight liver. Activity per wet weight of liver was significantly different between populations ($p = 0.0321$, Figure 3.3C) and was significantly less in the extreme northern *versus* the southern *F. heteroclitus* population.

Variation within and between species

To examine the difference in Complex I activity within the neutral theory framework proposed for molecular traits (Kimura 1985), we compared the variation within northern populations (Maine and Rhode Island) to the variation among species in the southern population across both species (Georgia and *F. grandis*). The neutral expectation is that

greater evolutionary divergence among species will result in variation due to neutral drift and thus an ANOVA comparing variation within and between the Northern and Southern groups will not be significant. However, there is a significant difference in Complex I mitochondrial specific activity between Northern and Southern fish that includes both species ($p < 0.005$).

3.5 Discussion

F. heteroclitus populations are subjected to a steep thermal gradient that appears to be an important selective force responsible for the divergent adaptation of northern fish to their colder environments (Crawford and Powers 1989; Powers, Lauerman et al. 1991; Pierce and Crawford 1997; Crawford, Pierce et al. 1999; Crawford, Segal et al. 1999; Whitehead and Crawford 2006; Crawford and Oleksiak 2007). These divergences in functional sequences and proteins have evolved despite meaningful rates of migration between populations (Brown and Chapman 1991; Duvernell, Lindmeier et al. 2008; Haney, Dionne et al. 2009). Local populations of *F. heteroclitus* can exceed 100,000 individuals (Adams, Lindmeier et al. 2006; Duvernell, Lindmeier et al. 2008) and gene flow between populations, is large enough to inhibit neutral drift between individual populations (Brown and Chapman 1991; Li 1997). To overcome the effect of neutral drift, selection coefficients have to exceed $1/2N_e$ (where N_e is the effective population size). Thus, these large populations should allow natural selection to be more effective than drift in shaping population genetic structure (Kimura 1985; Li 1997). In addition to adaptive divergence among *Fundulus* populations (Crawford and Powers 1989; Powers, Lauerman et al. 1991; Pierce and Crawford 1997; Crawford, Pierce et al. 1999; Crawford, Segal et al. 1999; Whitehead and Crawford 2006; Crawford and Oleksiak 2007), there are many examples of clinal neutral divergence among allozymes (Powers, Smith et al. 1993), sequence variation in *Ldh-B,* (Bernardi, Sordino et al. 1993),

mitochondrial RFLPs (Gonzalez Villasenor and Powers 1990), mitochondrial sequences (Haney, Dionne et al. 2009; Whitehead 2009) microsatellites (Adams, Lindmeier et al. 2006; Duvernell, Lindmeier et al. 2008) and SNPs (Williams, Ma et al. 2010; Williams and Oleksiak 2010). Thus, there is an expectation that *Fundulus'* populations will be different due either to adaptive or neutral divergence. The results presented here suggest that there is likely to be adaptive divergence in Complex I activity if phenotypic differences have a genetic basis (see below: Cause and Consequence of Complex I Variation).

Inter-Individual Variation

All individuals were acclimated to constant laboratory conditions for more than six-weeks, which should remove physiologically induced acclimation affects (Pierce and Crawford 1996; Pierce and Crawford 1997). Thus, the differences among individuals after acclimation are due to genetic variation, developmental effects, and maternal effects. If the differences in Complex I activity among individuals have a genetic foundation, our results suggest a very large standing genetic variation. Within populations, individual Complex I mitochondrial specific activities were highly variable: within each of the five *F. heteroclitus* populations there is an average 29-fold difference between the lowest and highest values for *F. heteroclitus,* with an average %CV among individuals within *F. heteroclitus* populations of 91%. Similar inter-individual variation was also found for Complex I activity per wet weight (the average *F. heteroclitus* inter-individual %CV = 64%, Table 3.1, column 10). The cause of the high amounts of inter-individual variation observed in Complex I activities in all populations examined is unclear. It is not due to the technical variation because the %CV for three technical replicates was low: 6.58% and 7.48% for control and experimental reactions, respectively. Neither body nor liver mass affected Complex I mitochondrial specific activity:

Complex I mitochondrial specific activity did not regress significantly with body mass or liver mass. Thus, the observed differences were not simply due to metabolic scaling. Furthermore, mitochondrial density did not seem to be driving the inter-individual variation. Although the %CV for mitochondrial density was large, it was much smaller than the %CV for Complex I activity: average 39% versus 91%, respectively (Table 3.1). Additionally, mitochondrial density was negatively correlated with mitochondrial activity (Fig. 2). Thus, there were larger differences among individuals within a population that were not due to technical errors, difference in body mass, and were negatively related to mitochondrial density.

Though these differences among individuals within a population were high, they were comparable to other measures of mitochondrial activities. Fangue (Fangue, Richards et al. 2009) measured liver Complex IV activity (cytochrome c oxidase, COX, EC 1.9.3.1,) and mitochondrial respiration in *F. heteroclitus*. In their experiments %CV among individuals for liver COX activity was 72% and 117% for eight 15°C acclimated individuals from northern and southern populations (respectively). In the same study (Fangue, Richards et al. 2009), the authors measured mitochondrial respiration within six independent pools (with 4 to 6 individuals per pool). Assuming five individuals per pool, the CV% among pools ranged from 20% to 104% for respiration measured at the same assay and acclimation temperature. Clearly, pooling individuals should reduce the observed individual variation, yet much of this variation remains. Thus, although our measures of inter-individual differences within a population were high, other investigators find elevated inter-individual variation in *F. heteroclitus* livers.

Signatures of Natural Selection

We expected to observe significant differences between populations of *F. heteroclitus* in Complex I activity based on variation in neutral and non-neutral traits: SNP, allozymes, and amino acid substitutions in Complex I mitochondrial subunits. Significant differences in Complex I mitochondrial specific activity were observed within *F. heteroclitus* between Maine and Georgia populations but not between *F. heteroclitus* from Georgia and *F. grandis.* When southern populations from both species were grouped together (*F. heteroclitus* from Georgia and *F. grandis* from Louisiana) and compared to northern populations of *F. heteroclitus* (Maine and Rhode Island) the difference in mean Complex I activity was significant ($p < 0.01$). Thus, the variation within a species among colder northern population and among species in warmer southern populations was greater than the variation within these groups. These data suggest that the differences between northern and southern *F. heteroclitus* exceed the prediction of neutral random drift, where the differences within a species should be less than among species because there is greater length of time for taxa to drift apart. The difference in activity appears to be derived and may have evolved by natural selection.

Although the neutral hypothesis predicts that variation within species will be less than among species, it says nothing about mean values of a trait. We expected that northern populations would have greater Complex I specific activity, but we found the opposite (Fig. 3.3): there was a 3.7 fold decrease in the mitochondrial specific activity of extreme northern relative to the extreme southern populations. The lower Complex I specific activity was somewhat compensated by the increase in mitochondrial density such that there was only 2.1 fold lower activity per wet weight in the northern *versus* southern populations for Complex I

activity per wet weight. These results are unexpected based on previous studies of glycolytic and other metabolic enzymes in *Fundulus* and many other species, where cold adapted organisms to have higher activities than warm adapted organisms (Pierce and Crawford 1994; Somero 1995; Pierce and Crawford 1997; Crawford, Pierce et al. 1999; Hochachka and Somero 2002; Clarke and Portner 2010). Cold adapted species typically have faster enzymes (K_{cat}) or express more protein relative to warm adapted species (Hochachka and Somero 2002). In a comparable study of *F. heteroclitus* from New Hampshire and Georgia, mitochondrial respiration was insignificantly higher in the southern population when acclimated and measured at 15 or 20°C (Fangue, Richards et al. 2009). If our measures of Complex I activity are meaningful (e.g., not due to unknown technical difficulty that affects the northern but not southern population), we lack a clear reason for less Complex I activity in northern populations even though it appears to be adaptive.

Cause and Consequence of Complex I Variation

What is responsible for the average 29 fold difference in Complex I mitochondrial specific activity within *F. heteroclitus* populations and the nearly fourfold difference between the extreme northern and southern *F. heteroclitus* populations? Mitochondria were isolated and enzyme activity measured in two individuals per *F. heteroclitus* population and *F. grandis* each day and the technical variation is < 10% of the inter-individual variation within a population. Only one of the five populations had a significant day-effect (ANOVA with individuals as replicated, day as fixed effect, p-values ranged 0.48 to 0.01). The one significant day-effect in the Rhode Island population most likely represents random choice of two individuals with either high or low Complex I activity on one day. Thus, these differences in Complex I activities were not an obvious technical or experimental design

problem. Further, there was less Complex I activity in the northern population, which is different than expected (Pierce and Crawford 1997; Crawford, Pierce et al. 1999; Clarke and Portner 2010; Portner, Schulte et al. 2010). These observations raise questions about the biological meaning of the data. We suggest that these data are biologically meaningful; activity is a function of mitochondrial density (Fig. 3.2) and exceeds the differences expected by the neutral theory.

Differences in enzyme activity can arise due to genetic variation, physiological acclimation (reversible or irreversible), developmental effects, and maternal effects. Maternal effects are of two types: maternal genetic variation that affects maternal mRNA or traits that produce larger better provisioned eggs and non-genetic physiological traits that are passed onto offspring. It is the latter, non-genetic variation, we are most concerned with because these introduce variation that does not evolve, and yet it produces differences among offspring through transgenerational transmission. In flies, maternal temperature has a small effect on adult offspring fecundity (Huey, Wakefield et al. 1995), but transgenerational effects due to temperature had measurable effects on physiological traits (Crill, Huey et al. 1996). Transgenerational effects are also seen in humans and other animals; primarily through diet effects on birth weights or egg condition (Mousseau and Fox 1998; Einum and Fleming 1999; Drake and Walker 2004; Rasanen and Kruuk 2007). Clearly, *Fundulus* populations are found in different environments that could create maternal effects that do not contribute to evolutionary adaptation. However, within a *Fundulus* populations, where individuals are swept in and out of estuaries with each tide, it is more difficult to argue that the environmentally influenced maternal effects explain the large variation within a population. It is unlikely that individuals within a population are isolated enough to

experience vastly different environmental conditions necessary to generate such high levels of inter-individual variation. In addition to maternal effects there are developmental effects that persist through adulthood. The most notable is the effect of temperature on development in sex determination in many reptiles (Deeming, Ferguson et al. 1988). Here, we are most interested in whether the difference in environmental temperature causes an irreversible difference in metabolism. Irreversible acclimation of biochemical traits, could affect differences among populations (Kinne 1962; Zamer and Mangum 1979; Sanford, Roth et al. 2003). In *Drosophila,* developmental temperature affects body size and physiological performance (Huey, Wakefield et al. 1995; Crill, Huey et al. 1996). If a similar mechanism occurs in *Fundulus,* it is possible that the magnitude of inter-individual variation observed represents differences in the developmental environment. However, similar to the argument against maternal affects, developmental affects are less likely to be a major source of variation within a population especially in northern populations which have a single spring bout of reproduction (Kneib 1984; Kneib 1986; Able 1990; Hsiao, Limesand et al. 1996). Finally, there are no data on any non-heritable effects on any metabolic trait creating inter-individual variation of the magnitude observed here.

Much of the variation in Complex I activity could be due to non-heritable environmental effects. Yet, there are several reasons to believe that some of the variation among individuals and populations has a genetic basis and thus is subject to evolve by natural selection. For Complex I mRNA expression much of the variation is a function of genetic distance (Whitehead and Crawford 2006), and a few of the 45 Complex I loci have adaptive patterns of expression (Oleksiak, Churchill et al. 2002; Whitehead and Crawford 2006). In the mitochondrial genome between northern and southern populations there are

seven fixed substitution that alter the amino acid sequence (Whitehead 2009): two non-synonymous in each of ND1 and ND2 and three in ND5 based on four whole mitochondrial genomes. These changes in expression or amino acid substitutions could affect the overall function of the enzyme and suggest a genetic basis for the variation in Complex I activity. Further examination of the inter-individuals variation is necessary to determine causes and biological importance of Complex I activity variation in *F. heteroclitus*.

Regardless of the cause for the large variation in Complex I activity, these differences among individuals involve an enzyme that is critical for the production of ATP production and whose defects are associated with a majority of human mitochondrial diseases (Gautheron 1984; Smeitink and van den Heuvel 1999; Loeffen, Smeitink et al. 2000; Triepels, Heuvel et al. 2001; OMIM 2011). Complex I also has a significant control coefficient for the production of ATP (Davey and Clark 1996; Lemieux, Semsroth et al. 2011). However, these traits are associated with tissue or organs that have high oxygen demands (nervous and cardiac tissues). Thus, it is possible that the difference in Complex I activity has much less effect on liver physiological processes and thus, that much of differences represent excess enzyme capacity. If there is a genetic component to this variation in Complex I, as suggested above, this variation could represent a large reservoir for potential adaptation in the appropriate environment.

Conclusions

Complex I plays a major role in the production of ATP by oxidative phosphorylation and is made of 45 different subunits, making it one of the largest enzyme complexes. Due to its importance, Complex I is often involved in human disease (Smeitink and van den Heuvel 1999). Because there are many different genes that must be regulated, and many proteins

subject to post-translational modifications, there are many opportunities for individual variance to impact the function of Complex I that could be evolutionarily important. We observed variation in enzyme activity that exceeds neutral expectations among populations, suggesting that variation in enzyme activity is both biologically and evolutionarily important. Thus, we expected that the relative metabolic importance of Complex I would constrain the variance among individuals. However, in *Fundulus,* we observed a very high inter-individual variation within a population that is not due to technical error or differences among assay day. Thus, there is a conundrum: how can Complex I activity be evolutionary important yet so variable within a population? We provide no answer to this question but to suggest that within a population much of the variation in Complex I does not affect a relevant phenotypic difference, yet among populations the difference may be adaptive.

Table 3.1. Variance and Significance of Complex I Activity Within Populations. Population (column 1) refers to the location and species of individual used in the assay (*F. heteroclitus:* GA-Georgia, VA-Virginia, NJ-New Jersey, RI-Rhode Island ME-Maine; *F. grandis* GR-Florida). Column 2, "n" is the number of individuals. %CV (column 3) is CV (standard deviation/mean, multiplied by 100) among individuals for Complex I Specific activity. P-value (column 4) is the significant difference among individuals within a population. Fold difference (column 5) is highest value divided by lowest value within a population. Activity/Mito (column 6) is Complex I activity relative to amount of mitochondrial protein. Mitochondrial density (column 7) is mitochondrial protein per gram of liver tissue and population %CV for mitochondrial density (column 8). Activity/liver (column 9) is Complex I activity per gram of liver tissue. Column 9, is the CV among individuals for activity per wet liver weight.

1 Population	2 n	3 %CV among Individuals	4 P value among Individuals	5 Fold Difference	6 Activity/Mito ($*10^{-4}$)	7 Mitochondrial Density ($*10^{-3}$)	8 %CV Mitochondrial Density	9 Activity/liver ($*10^{-7}$)	10 %CV Activity/wet weight
GR	21	267.83	5.86E-41	706.2	32.01	4.30	58.21	7.59	179.92
GA	26	118.39	1.80E-24	30.4	11.74	6.97	48.74	4.66	58.09
VA	19	59.76	2.61E-05	9.63	3.59	9.93	34.08	3.18	45.71
NJ	19	72.68	1.51E-06	11.1	4.34	8.93	42.02	3.65	56.19
RI	20	108.65	2.29E-11	62.6	5.85	6.76	36.29	3.25	84.52
ME	16	97.57	1.96E-07	28.9	3.21	8.66	34.13	2.26	76.03

Figure 3.1 Collection Sites. Fish were collected from six locations. *Fundulus grandis* were collected from Cocodrie, Louisiana (1). *Fundulus heteroclitus* were collected from Sapelo Island, Georgia (2); Hayes, Virginia (3); Stone Harbor, New Jersey (4); Matunuck, Rhode Island (5) and Wiscasset, Maine (6).

Figure 3.2. Individual Complex I Activity and Mitochondrial Density. Activity per µg mitochondrial protein and mitochondrial density is negatively related in all populations. Shown here are individuals from Maine and Georgia (R^2 = 0.3293, P = 0.016 and R^2 = 0.604, P = 0.000003, respectively).

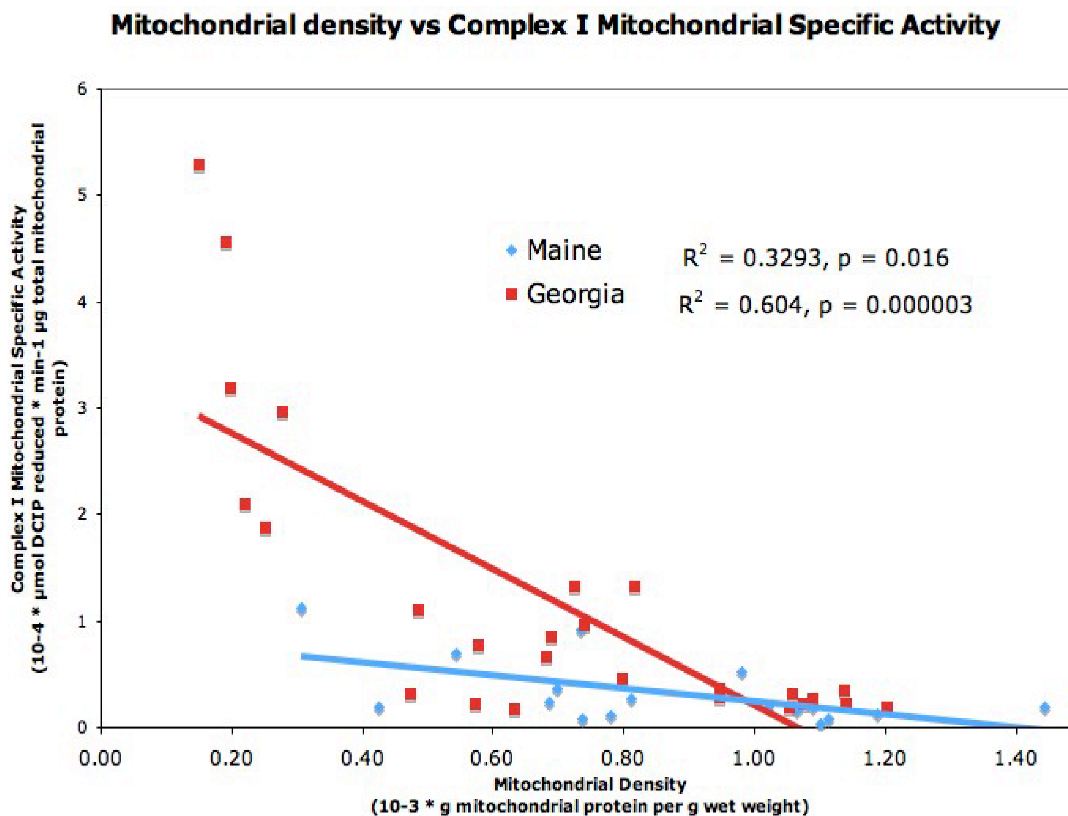
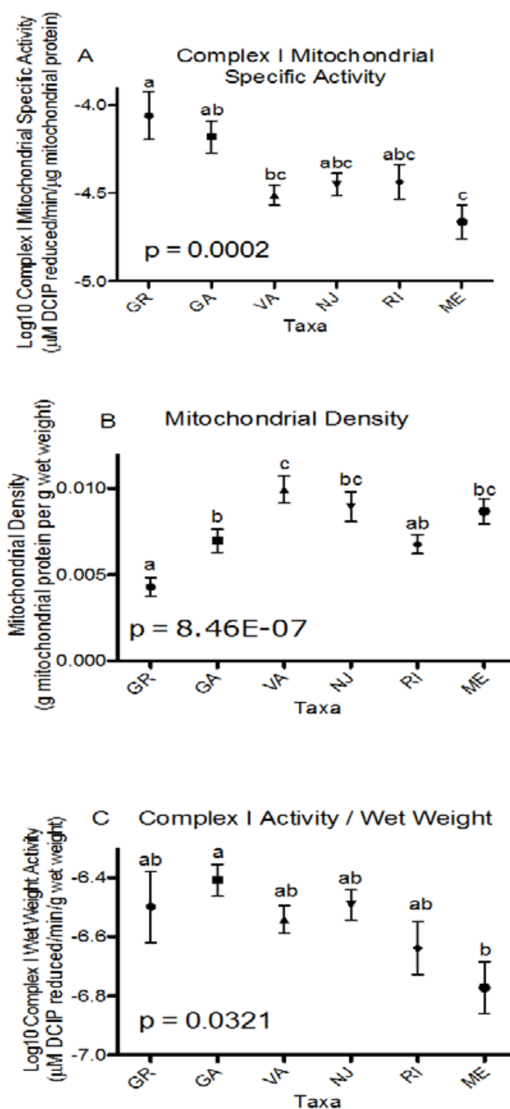


Figure 3.3. Complex I Activity and Mitochondrial Density among Populations. A) Complex I activity per microgram mitochondrial protein among populations (Log_{10} μmol DCIP reduced min^{-1} μg mitochondrial $protein^{-1}$). B) Mitochondrial density among populations (grams of mitochondrial protein isolated per gram of liver tissue). C) Complex I specific activity per wet weight among populations. (μmol DCIP reduced min^{-1} g liver $tissue^{-1}$). P-values lower left hand corner is for the ANOVA among all populations. Lower case letters show significance in Tukey's post hoc test $p < 0.05$; shared letters are not significantly different.



Chapter 4 Differential Effect of Temperature Acclimation on Complex I Activity in *Fundulus heteroclitus* and *Fundulus grandis*

4.1 Summary

Temperature shapes the distribution of species and affects nearly every biological process. To examine the response of *Fundulus heteroclitus* to acclimation, the activity of the first enzyme in the oxidative phosphorylation pathway, Complex I (NADH dehydrogenase, EC 1.6.5.3), was measured. Complex I activity is critical for oxidative metabolism and shows patterns of adaptive divergence between northern and southern populations of *F. heteroclitus* indicative of natural selection. Here we investigate the effect of three acclimation temperatures and three assay temperatures (12°C, 20°C and 28°C) on Complex I activity in the marsh killifish *Fundulus heteroclitus* and its sister species *Fundulus grandis.* Surprisingly, there were few differences among groups acclimated to different temperatures and no differences among populations of *F. heteroclitus.* The only acclimation effects occurred at the lowest assay temperature with *F. heteroclitus* having the greatest Complex I activity when individuals were acclimated to 28°C. The effect of acclimation appeared not to be due to change in enzyme expression (there are no acclimation effects at 20°C and 28°C assay temperatures) but due to the temperature insensitivity of Complex I activity at 28°C. That is, among individuals acclimated to 28°C, Complex I activity remained high regardless of assay temperature, while individuals acclimated to 12°C had less activity at the 12°C assay temperature. Finally, there were large variations among individuals that were higher at the lowest acclimation temperature but showed less inter-individual variation at high acclimation temperatures. These data suggest that individual living in colder waters may both be more sensitive to environmental temperatures and have greater phenotypic variation.

4.2 Introductory Material

Temperature affects kinetic energy, rates of diffusion, enzyme stability, interactions between proteins and other macromolecules, membrane fluidity and inter-molecular affinities (Hochachka and Somero 2002). As a result, temperature affects most physiological processes (Somero 1995; Hochachka and Somero 2002). For aerobic metabolism, temperature effects are mediated by changes in transcription, membrane composition, and altered enzyme reactions rates (Seebacher, Brand et al. 2010). Fish, as ectothermic animals, are especially susceptible to temperature fluctuations because there is limited capacity to regulate body temperature. As a result, adaptive divergences in protein sequence or expression that allow fish to inhabit warm or cold environments have been documented. (Pierce and Crawford 1994; Crawford, Pierce et al. 1999; Fangue, Richards et al. 2009; Clarke and Portner 2010).

In addition to evolved differences in enzyme activities, enzymes show plastic response to temperature: within an individual's lifetime exposure to different temperatures can cause changes, reversible or irreversible, in enzyme activities (Pierce and Crawford 1997; Hochachka and Somero 2002; Seebacher, Brand et al. 2010). These plastic responses allow individuals to have broad performance curves to maintain function in extremely variable environments through direct modifications of a single enzyme and by modulating flux through an entire pathway (Lee, Daniel et al. 2007; Ruoff, Zakhartsev et al. 2007). Acclimation responses have been observed in mitochondrial physiology. For cold acclimated individuals there is an increase in mitochondrial volume and surface area of christae (Guderley 2004); kinetic activity for oxidative-phosphorylation enzymes (Hazel 1972; Wodtke 1981; Itoi, Kinoshita et al. 2003), as well as increases in enzyme concentration

(Wodtke 1981; Guderley and Johnston 1996; Battersby and Moyes 1998; Guderley and St-Pierre 2002; Fangue, Hofmeister et al. 2006; Seebacher, Brand et al. 2010) which can maintain substrate to product conversion in colder environments.

To better understand the role of adaptive and acclimation affects, this study focuses on Complex I, (NADH dehydrogenase, also called NADH:ubiquinone oxidoreductase; EC 1.6.5.3) which is a large, heteromeric enzyme complex with a central role in oxidative phosphorylation. The enzyme complex accepts electrons from NADH and pumps hydrogen ions into the inner mitochondrial membrane space. In vertebrates, Complex I is made up of 45 different protein subunits (Carroll, Fearnley et al. 2006). Thirty-eight of these subunits are encoded by the nuclear genome while the other 7 are encoded by the mitochondrial genome (Carroll, Fearnley et al. 2006). Many Complex I subunits have been causatively linked to mitochondrial disease in humans and deficiencies in Complex I are responsible for 80% of heritable metabolic disorders (Smeitink, Sengers et al. 2001). Complex I is an especially interesting system for the study of the extent and mechanisms of thermal adaptation. It is a 'core' system—one that has a conserved function from alpha-proteobacteria to mammals, yet it has diversified from the earliest 14-17 subunits to a massive enzyme of one-million daltons with 45 subunits (Friedrich 1998; Carroll, Fearnley et al. 2006; Yip, Harbour et al. 2011). Many of these evolutionarily newer subunits are accessory—they do not participate in the catalytic core but may stabilize the enzyme or modify Complex I function (Carroll, Fearnley et al. 2003; Carroll, Fearnley et al. 2006; Carroll, Fearnley et al. 2006). The addition of accessory subunits to Complex I suggests that it has complex adaptations for buffering activity against environmental variation.

According to the Krogh principle there is an ideal organism for studying any question of physiology (Krogh 1929). This principle applies to the use of *Fundulus heteroclitus* and sister species *Fundulus grandis* for a study of the effect of temperature on Complex I activity because these species have large population sizes—increasing the probability that natural selection rather than genetic drift affects enzyme function and populations are distributed along a steep thermocline. This steep thermal cline affects enzyme activity by adaptation or acclimation (Powers, Lauerman et al. 1991; Pierce and Crawford 1997; Pierce and Crawford 1997; Crawford, Pierce et al. 1999).

In this study we compare differences among two populations of *F. heteroclitus* and one population of *F. grandis* acclimated to three temperatures (12°, 20°, and 28°) and measure Complex I from each of these nine groups at each of these three temperatures. Overall, neither acclimation temperature or assay temperature had a large effect on Complex I specific activity (units/mitochondrial protein), instead acclimation temperature effected the temperature sensitivity of Complex I.

4.3 Materials and Methods

Collection of Fundulus

Fundulus heteroclitus from Wiscasset, Maine (3) and Sapelo Island, Georgia (2)—where # refers to numbering on map, (Fig. 4.1)—were collected in minnow traps in September 2010. *Fundulus grandis* from Pensacola, FL (1) were collected in August 2010. Fish from each population were acclimated in the lab for a minimum of 6 weeks at the control temperature, 20°, and then each population was subdivided into three separate groups for additional four weeks at one of three experimental temperatures: 12°, 20° and 28°. These are temperatures that fish encounter in the field and do not elicit stress-responses from

the fish (Fangue, Hofmeister et al. 2006). The fish were maintained in 15ppt artificial seawater with a 14h light 10h dark light schedule.

Isolation of Mitochondria

Fish were sacrificed by cervical dislocation and livers were removed. Mitochondria were isolated and measured on the same day from each of the nine sample groups (three populations acclimated to all three temperatures) following modified protocol (Mela and Seitz 1979). Livers were rinsed, patted dry, weighted and minced in ice cold Medium A (250mM sucrose, 0.5mM Na_2EDTA, 10mM Tris, pH 7.4). Minced liver was homogenized gently in Media A using a glass-teflon PE homogenizer. Liver homogenate was centrifuged at 500 *g* for 5 min at 4°C to remove large cellular fractions. Supernatant was centrifuged at 7,880 g for 10 minutes at 4°C and pellet resuspended by gentle pipetting in Media B (Media A with 1g/l BSA). The pellet was again resuspended and centrifuged at 7,880 g for 10 minutes at 4°C. The final pellet was rinsed with 10mM Tris (pH 7.4) and brought up in 10mM Tris (pH 7.4). Mitochondria were then sonicated for five seconds to fracture the membranes. Mitochondria were stored on ice until use. Total protein from this mitochondrial enriched fraction was used as a measure of total mitochondrial density.

Measurement of Complex I Activity

Complex I activity was measured by adapting the method of (Janssen, Trijbels et al. 2007). Reduced NADH was used as the electron donor tot the final electron acceptor decylubiquinone (Sigma: D7911) which is used as an analogue for ubiquinone. Kinetic measures of the reduction of DCIP were accomplished by following the decrease of absorbance of DCIP at 600nm. Control and experimental reactions were run side by side in a 96 well microplate (250 mM HEPES, 450 mM potassium, 3.5 g/L BSA, 60µM DCIP, 70 µM

decylubiquinone, 1 μM Anti-A, 1 μM rotenone dissolved in ethanol). Absorbance was measured immediately after addition of NADH for five minutes using a Spectramax Plus[384] spectrophotometer.

All individuals' Complex I activity were measured in triplicate—three sets of control and experimental reactions—for each measurement temperature, giving a total of nine measurements of Complex I activity per individual. The spectrophotometer was blanked with a set of control reactions lacking isolated mitochondria. The slope of reduced DCIP concentration over time was calculated using the extinction coefficient of 19.1/mMol*cm. Individual Complex I specific activity is defined as the difference in slope between the control wells (representing total mitochondrial oxidation of NADH) and average of the three experimental wells (containing rotenone a Complex I inhibitor, and thus representing total NADH oxidized lacking Complex I activity). In order to correct for variations in total number of mitochondria per individual as well as technical variation in mitochondrial isolation, the slopes of NADH (control) and rotenone (experimental) reactions were divided by the total amount of protein in the mitochondrial preparation, determined with a BCA assay, in the reaction to give Complex I activity as μmol DCIP reduced min^{-1} μg total mitochondrial $protein^{-1}$.

Replicate values were excluded if the rotenone slope (control without Complex I activity) exceeded the experimental slopes (indicating creation of NADH relative to the rotenone control). Fifteen replicate values were excluded. Additionally, a total of 37 measurements were excluded; 17 from Florida individuals, and 10 each from Georgia and Maine individuals. These samples had values > 3*standard deviation for the population.

Data Analyses

Data analyses were carried out using Prism (GraphPad, La Jolla, CA), Microsoft Excel, and MatLab (MathWorks, Natick, MA). For ANOVA analyses, Log10 transformed data was used so that variation among populations were similar. Two sets of two-way ANOVA were performed on Complex I specific activity and Complex I activity per wet weight : one with species and acclimation temperature for each assay temperature and the second two-way ANOVA with species and assay temperature for each acclimation temperature. For difference among acclimation temperature, a Bonferroni's corrected p-value was used (0.05/9 = 0.0055) for the nine post-hoc test (3 acclimation temperatures, for each assay temperature). For the two-way ANOVA with species and assay temperature (Fig. 4.3) a Bonferroni's correction on differences between activity between assay temperatures within species (*F. grandis* y,z; *F. heteroclitus* a,b,c) was used (corrected p-value 0.05/3 = 0.017).

4.4 Results

Technical Variation and Calculation of Complex I Activity

The difference between the average of the three experimental assays and each control assay divided by total mitochondrial protein in each assay was defined as Complex I mitochondrial specific activity (μmol DCIP reduced min^{-1}/μg mitochondrial protein). The average %CV (standard deviation/mean * 100) among the replicates for Complex I specific activity values was 17.9%. Technical variation (%CV among replicates) was relatively small compared to the inter-individual variation (%CV among individuals) within each treatment: average %CV among individuals was 57% (Table 4.2).

For all assays, northern and southern populations were not significantly different and thus, analyses focused on the differences among species and the effect of temperature (assay and acclimation) on Complex I activity (Table 4.1).

Mitochondrial Density

Mitochondrial density was defined as the amount of mitochondrial protein isolated from a given amount of liver tissue. Mitochondrial density was not different among species, but was different among acclimation temperatures (Fig. 4.2, Two-way ANOVA species p-val > 0.1, acclimation temperature p-val < 0.01). Based on Bonferoni's post-hoc analyses mitochondrial density were higher at 20° and 28° acclimation temperatures relative to 12°C only for *F. grandis* individuals. Inter-individual variation (%CV among individuals) for mitochondrial density ranged from 23% to 62% (Table 4.1).

Acclimation effect on Complex I mitochondrial specific activity

Complex I specific activities for each assay and acclimation temperature are provided in Table 4.1. Complex I mitochondrial specific activity was corrected for µg mitochondrial protein in each assay and is referred to here as Complex I specific activity. Complex I specific activity was Log10 transformed to meet the ANOVA assumption of equal variance. Ignoring any species effect, both acclimation and assay temperatures had a significant effect on Complex I specific activity and no significant interactions (Two-way ANOVA; p-val $<$ 0.01 for assay and acclimation, p-val > 0.5 for the interaction). These temperature effects were different among species and among assay temperatures (Table 4.1, Fig. 4.3).

To examine the effect of acclimation temperatures and whether species had different responses, a two-way ANOVA with species and acclimation temperature at each assay

temperature was used (Table 4.3A). There were no significant interactions for any of the tests.

Acclimation effect on Complex I specific activity was dependent on the assay temperature. At 12°C assay temperature there were significant differences in Complex I specific activity among acclimation temperatures (Table 4.3A, Fig. 4.3 A-C). Specifically, at the 12°C assay temperature, there was greater mitochondrial specific activity among *F. heteroclitus* acclimated at 28°C compared to those acclimated to 12° (Fig. 4.3; Bonferoni's corrected p-val 0.0055). Acclimation temperature did not affect Complex I specific activity in *F. grandis.*

Among species there were no consistent significant differences among acclimation temperatures for any one assay temperature, (Table 4.3). However, among fish acclimated to 28°, Complex I specific activity was significant higher in *F. heteroclitus* then *F. grandis* at all assay temperatures (Bonferoni's correct p-val 0.0055; Fig. 4.3C*).*

To examine the effect of assay temperature and whether assay temperatures affected species differently a two-way ANOVA (species and assay temperature as grouping variables) for each acclimation temperature was applied (Table 4.3B). There were no significant interactions for any test. Assay temperature had a significant effect on Complex I specific activity for fish acclimated to 12° or 20°C but not for fish acclimated to 28°C (Table 4.3B, Fig 4.3). Yet, the effect of assay temperature on Complex I specific activities were only different among *F. heteroclitus* individuals acclimated to 12° and among *F. grandis* acclimated to 20°C (Fig 4.3, A and B respectively). When there was a significant difference, an increase in assay temperature was associated with an increase in Complex I specific activity.

For Complex I specific activity, the inter-individual variation (%CV among individuals) within species and acclimation temperature were high (ranging from 21% to 95%) (Table 4.2). For *F. heteroclitus*, comparing %CV among individuals when assayed at their acclimation temperature, there are larger %CV at lower temperatures. This trend was also seen among the averages (across assay temperatures or across acclimation treatments): the highest temperatures had lower %CV among individuals. However the details are a little more complex: %CV decreased with acclimation temperature only for 12° and 20°C assay temperatures in *F. heteroclitus*. When assayed at 28°C, *F. heteroclitus* acclimated to 20°C had the greatest %CV. Additionally, when *F. heteroclitus* was acclimated to 28°C the lowest %CV was at the 12°C assay temperature. Thus, in *F. heteroclitus* the trend for lower %CV at higher temperature is less obvious when fish are acclimated to 28°C or assayed at 28°C. In *F. grandis*, different patterns were more prevalent: intermediate acclimation temperature (20°C) had the highest %CV when assayed at 12°C and 20°C. When assayed at 28°C, *F. grandis* acclimated to the intermediate temperature had the lowest %CV.

Acclimation effect on Complex I activity per wet weight

To correct for variation in mitochondrial density among individuals, we multiplied Complex I activity per unit mitochondrial protein (Complex I specific activity) by mitochondrial density to obtain a value for Complex I activity per unit liver wet weight (µmol DCIP reduced min^{-1} g liver $tissue^{-1}$). Complex I activities per wet weight are provided in Table 4.1. These data were Log10 transformed so that the variance among groups was similar. Ignoring differences among species, both acclimation and assay temperature had a significant effect (Two-way ANOVA, acclimation temperature p-val <0.01; assay

temperature $p < 0.05$). These differences depended on the assay and acclimation temperature and were dissimilar among species (Table 4.1, Fig. 4.3).

To examine whether species had different response to acclimation, a two-way ANOVA (with species and acclimation temperature as fixed effects) for each assay temperature was used (Table 4.3C). Acclimation temperatures affected *F. heteroclitus* Complex I activity per wet weight at 12° assay temperatures (Table 4.3C, Fig 4.3 D-F). When assayed at 12°C for *F. heteroclitus* there was greater Complex I activity for fish acclimated to 28°C compared to those acclimated to 12° (Bonferoni's correct p-val = 0.0055, Fig. 3). Thus, acclimation effects were species and assay temperature specific, though there were no consistent differences among species at any assay temperature. However, at 20°C acclimation, *F. grandis* has greater Complex I activity per wet weight than *F. heteroclitus* at all assay temperatures (Fig. 4.3E).

To examine whether assay temperatures affected species differently a two-way ANOVA (species and assay temperature as grouping variables) for each acclimation temperature was applied (Table 4.3D). Assay temperature had a significant effect on Complex I activity per wet weight for *F. heteroclitus* acclimated to 12° and *F. grandis* 20°C but not for either species when acclimated to 28°C (Table 4.3, Fig. 4.3D-F). At both 12° and 20°C acclimation temperatures, an increase in assay temperature was associated with an increase in Complex I specific activity.

Similar to Complex I specific activity, the inter-individual variation in Complex I activity per wet weight for *F. heteroclitus* was highest at the 12° acclimation temperature for each assay temperature. The %CV was the lowest when *F. heteroclitus* was acclimated to 28°C and assayed at 28°C. The effect of acclimation temperature had the opposite effect in

F. grandis: higher %CV among individuals occurs when fish were acclimated to 28°C for each assay temperature. The %CV among *F. grandis* tended to be lower at higher assay temperatures.

4.5 Discussion

In general, one expects more enzyme activity among ectotherms adapted to or acclimated to lower temperatures to compensate for the effect of lower temperatures on chemical reactions (Crawford and Powers 1989; Somero 1995; Pierce and Crawford 1997; Pierce and Crawford 1997; Crawford, Pierce et al. 1999; Hochachka and Somero 2002). Maximal enzyme activity is often measured to investigate these compensatory changes because only two mechanisms affect it: change in the concentration of enzyme or the catalytic rate constant (k_{cat}) (Lehninger 1975). Organisms adapted to different thermal environments may alter enzyme activity by changing the level of enzyme expression or the evolution of an enzyme with different biochemical kinetic characteristics (Powers, Lauerman et al. 1991; Crawford, Pierce et al. 1999; Hochachka and Somero 2002). Physiological acclimation may also alter enzyme concentration or, for some enzymes, modify the kinetic constant by allosteric or covalent modifiers (Crawford, Pierce et al. 1999; Hochachka and Somero 2002). When comparing the same samples at different assay temperatures, only the kinetic constant can change. Within the normal range of temperatures, where an enzyme is not being denatured, increasing assay temperatures should increase enzyme activity base on the thermodynamic properties (Fersht 1999). Because Complex I is an essential enzyme complex that has measurable control of metabolic flux (Davey and Clark 1996; Lemieux, Semsroth et al. 2011) there was an expectation that acclimation to different temperatures would affect the enzyme complex. Yet, for Complex I activity measured per mitochondria or

per wet weight, there were few differences among acclimation temperatures and no differences between *F. heteroclitus* populations. If acclimation temperature had an effect it was seen at low assay temperatures and there was more Complex I activity at higher acclimation temperature *versus* the lowest acclimation temperature. That is, acclimation did not compensate for lower temperatures but may enhance thermal effects when an organisms experiences low temperatures. Even more stunning, assay temperature only affected Complex I activity 44% of the time (4 out of 9 comparisons) and Complex I was insensitive to assay temperature when either species was acclimated to the highest temperatures (28°C). Specifically, altering the assay temperature by 14°C did not affect Complex I activity in either species when they were acclimated to 28°C. Importantly, the effect of assay temperature on Complex I specific activity was both species and acclimation temperature dependent: assay temperature had a significant effect only at 12°C acclimation for *F. heteroclitus* and at 20°C acclimation for *F. grandis*.

Complex I is the first enzyme in the Oxidative Phosphorylation pathway and is responsible for the vast majority of aerobic ATP production. The production of ATP requires the displacement of H^+ into the inter-membrane space by Complex I, III and IV with the reduction of O_2 to H_2O by Complex IV and the use of the H^+ gradient by Complex V to produce ATP. Our determinations suggest that at higher temperature this process will be insensitive to environmental temperature but different between acclimation temperatures at low assay temperatures (Fig. 4.3). Measurement of whole animal oxygen consumption (Healy and Schulte 2012) and isolated mitochondria (State 3) (Fangue, Hofmeister et al. 2006) in *F. heteroclitus* support our finding. Routine and maximal metabolic rates and aerobic scopes had the largest differences at 25°C and 30°C acclimation temperatures. When

acclimated to a single temperature (15°C), metabolic rates were lower at lower assay temperatures, and the effect of assay temperature was more dramatic at lower but not higher acclimation temperatures (Healy and Schulte 2012). For mitochondrial specific metabolic rates (State 3), assay temperatures between 10°C and 25°C had the greatest affect when southern *F. heteroclitus* were acclimated to low temperatures (Fangue, Hofmeister et al. 2006). This pattern was less obvious in northern populations of *F. heteroclitus* (Fangue, Hofmeister et al. 2006). Thus, our data on Complex I activity supports separate and independent studies on *F. heteroclitus* aerobic metabolism.

What are the physiological implications of these patterns? For *Fundulus* living in cold water, a sudden change environmental temperatures enhances the effect of temperature (*i.e.,* when acclimated to lower temperatures, a change in temperature effects a change in Complex I activity). This sensitivity to environmental temperature disappears if the organism is acclimated to high temperature. This results in a difference among environmental temperatures only when *F. heteroclitus* is acclimated to low temperatures. In *Fundulus* accustomed to high temperature, the daily variation in temperature expected with tidal flow will not cause a change in Complex I activity. Thus, in the summer in Georgia where mean monthly temperatures are 28-29°C (Commerce 1955), exposure to low environmental temperature due to flooding, rain or cold-front, will have little effect on Complex I activity. For northern populations of *F. heteroclitus* where average summer monthly temperatures range from 13-16°C (Commerce 1955), a rapid change in the environment is more likely to alter the Complex I activity. Thus, ignoring any genetic divergences among *F. heteroclitus,* southern populations should be less sensitive to daily

temperature variation during the peak months of reproduction and growth (Kneib 1984; Morin and F. 1984; Kneib 1993).

The insensitivity to temperature at higher acclimation temperatures most likely involves the interactions among the 45 protein subunits of Complex I with the mitochondrial bilayer. Lipids membranes in general and mitochondrial membranes specifically are more fluid at lower acclimation temperatures due to increased frequency of unsaturated lipids (Hazel and Radin 1977; Wodtke 1981; Crockett and Hazel 1995; Logue, de Vries et al. 2000; Grim, Miles et al. 2010). These changes could affect Complex I sensitivity to rapid temperature change. Thus, when *Fundulus* are acclimated to low temperatures the changes in lipid composition would make Complex I more sensitive to environmental temperatures than when *Fundulus* individuals are acclimated to higher temperatures. Notice, that this hypothesized effect is not solely due to fluidity because *F. heteroclitus* acclimated 12°C and assayed at 28° C (which should have the most fluid membrane) were not different from *F. heteroclitus* acclimated to 28°C and assayed at 12°C (which should have the least fluid membranes).

This difference in temperature sensitivity was also reflected in the inter-individual variation: there was greater variation among individuals acclimated to lower temperature. There is a large variation in Complex I specific activity among individuals (%CV among individuals Table 4.2). In general, inter-individual variation decreased with increasing acclimation temperature for the 12°C and 20°C assay temperatures. The high inter-individual variation at the 12°C assay temperature could represent a technical problem, yet at when individuals were acclimated to 28°C, this assay temperature has the lowest variation. This seems to discount a technical challenge at low assay temperatures.

We suggest that the inter-individual variation reflects the effect of acclimation temperature on Complex I activity: when acclimated to 28°C, Complex I had little temperature sensitivity compared to 12°C acclimated fish. Similarly, fish acclimated to 28°C had less inter-individual variation. When acclimated to 12°C the differences among individuals may became more apparent because the variation Complex I for each individual is exposed. What we are suggesting is that Complex I activity becomes canalized or more robust (Wagner 1996; Gibson and Wagner 2000; Wagner 2003; Gibson and Dworkin 2004; Oleksiak and Crawford Douglas 2012) at 28°C which would hide any underlying genetic variation that contributes to Complex I activity. This is speculative because we have no measure of genetic differences for Complex I, but if there is a genetic basis for acclimation, membrane lipid composition or enzyme activity; then the smaller inter-variation when acclimated to 28°C would hide this variation. Further, because northern populations are more likely to be acclimated to lower temperatures, there may be greater phenotypic variation among individuals. If there is any genetic basis for this, then northern individuals would express greater standing genetic variation that could be acted on by natural selection.

Acclimation response among Fundulus

The lack of difference between the two *F. heteroclitus* populations was different from a previous study that examined five *F. heteroclitus* populations and a single *F. grandis* population acclimated to 20°C. (Chapter 3). In the previous study, northern populations had significantly less Complex I specific activity than southern populations, including *F. grandis.* The observation that there were no difference between species from southern latitudes, but there were differences within species, is indicative of natural selection. The lack of a similar finding here most likely reflects a difference in the statistical power of the studies.

The two *Fundulus* species had different acclimation responses. Mitochondrial density appeared to be more important for maintaining Complex I activity in *F. grandis* than in *F. heteroclitus*. *F. heteroclitus* had an increase in Complex I activity when acclimated to 28°C at 12°C assay temperature but no compensatory increase in mitochondrial density. Yet in *F. grandis* there was nearly a doubling of mitochondrial density at 20°C acclimation compared to 12°C and, although there was no significant acclimation effect, this increase in mitochondrial density enhanced the differences between species. The same enzyme having different acclimation responses has been observed in different species (Shaklee, Christiansen et al. 1977; Crawford and Powers 1989). Cytochrome C oxidase (CIV) shows no up-regulation of quantity or binding capacity in response to acclimation temperature but it does have increased activity at low temperatures which is likely mediated by modulation of the phospholipids in the lipid bilayer (Wodtke 1981).

The different acclimation responses observed could be due to differences in the range of temperatures naturally encountered by these two *Fundulus* species. The acclimation temperatures used were within the range of temperatures encountered by all individuals of *F. heteroclitus*, but not *F. grandis* used in this experiment. (Fangue, Richards et al. 2009). Additionally, it has been recently reported that thermal acclimation is unnecessary in *F.* heteroclitus due to their adaptation to estuarine habitats with widely variable temperatures (Healy and Schulte 2012).

Conclusion

Surprisingly, Complex I activity was relatively insensitive to temperature. The differences between acclimation temperature in *F. heteroclitus* was most likely not due to change in enzyme concentration (there was not an effect of acclimation on Complex I activity at the 20°C and 28°C assay temperature) but due the change in the sensitivity of Complex I to low assay temperatures: at 12°C assay temperature, there is less activity compared to warmer assay temperatures when acclimated to 12°C, but not difference among assay temperature when acclimated to 28°C). Thus, the differences among acclimation temperatures were likely due to the sensitivity of the enzyme to assay temperature and not to a change in the expression of Complex I. There was also greater inter-individual variation in 12°C acclimated individuals *versus* 28C acclimated individuals. Thus, *Fundulus* living in lower temperatures would have greater inter-individual variation.

Table 4.1 Summary of Complex I Mitochondrial Specific Activity and Activity per Wet Weight Liver

Mitochondrial density (Column 3) reported as value * $1E^{3}$ Complex I mitochondrial specific activity (columns 4-6) is reported as values * $1\ E^{5}$ and Complex I activity per gram of wet weight (columns 7-9) reported as values * $1\ E^{7}$.

Species	Acclimation temp	n	Mito Density	%CV Mito Density	CI Mito Specific Activity			CI Activity/wet weight		
					Assay Temperature					
					12	**20**	**28**	**12**	**20**	**28**
F.heteroclitus	***12***	19	6.91	90%	6.01	10.39	10.59	3.62	6.18	6.1
	20	19	7.07	43%	4.44	5.87	7.28	2.79	3.95	4.6
	28	11	6.72	32%	9.41	9.25	11.26	6.32	6.19	7.27
F. grandis	**12**	9	5.06	24%	5.28	6.91	7.81	2.65	3.39	3.98
	20	9	9.12	21%	5.17	7	8.07	4.28	6.32	7.27
	28	8	10.34	62%	7.13	5.17	8.04	7.49	6.32	8.14

Table 4.2 Variation Among Individuals CV% for each acclimated group at each assay temperature**.** P values are reported for Bartlett's test of group variances.

Species	Acclimation temp	%CV CI Specific Activity				%CV CI Activity/wet weight			
		Assay Temperature							
		12	**20**	**28**	**Avg.**	**12**	**20**	**28**	**Avg.**
F.heteroclitus	12	77%	95%	34%	69%	127%	157%	88%	124%
	20	54%	66%	68%	63%	44%	58%	51%	51%
	28	28%	30%	34%	31%	49%	45%	41%	45%
	Avg.	53%	64%	45%	54%	73%	87%	60%	73%
P_val for variance		**0.0278**	**0.0001**	0.1002		**0.0001**	**0.0005**	0.2305	
F. grandis	12	64%	34%	69%	56%	60%	30%	39%	43%
	20	68%	38%	34%	47%	63%	35%	32%	45%
	28	45%	21%	45%	37%	68%	88%	62%	73%
	Avg.	59%	31%	49%	46%	64%	53%	44%	54%
P_val for variance		0.7304	0.2255	0.6847		**0.0043**	0.6149	0.5217	

Table 4.3. Two-Way ANOVA results Probabilities associated with two way ANOVA table for effect of acclimation temperature and population on Complex I activity per total weight liver at each measurement temperature.

Complex I Specific Activity

A: Species x Acclimation Temp

Assay Temp.	Acclimation	Species
12	**0.0322**	0.679
20	0.5185	0.4173
28	0.3229	0.5628

B: Species x Assay Temp

Acclimation Temp.	Assay Temp	Species
12	**0.0211**	0.7891
20	**0.0122**	0.4813
28	0.1185	**0.0005**

Complex I Activity/ wet weight

C: Species x Acclimation Temp

Assay Temp.	Acclimation	Species
12	**0.0024**	0.7633
20	0.2931	0.6533
28	0.0661	0.4778

D: Species x Assay Temp

Acclimation Temp.	Assay Temp	Species
12	**0.0132**	0.4743
20	**0.0067**	**0.0062**
28	0.5116	0.7606

Figure 4.1. Map of Collection Sites

Fundulus heteroclitus were collected from sites 2 Sapelo Island, GA and 3 Wiscasset, ME in September 2010. *Fundulus grandis* were collected from site 1 Pensacola, FL in August 2010.

Figure 4.2. Mitochondrial Density

Mitochondrial density measured as total protein in isolated mitochondrial preparation per per wet weight liver. Lower case letters show significance of differences among acclimation temperatures in Bonferroni's post-hoc testing.

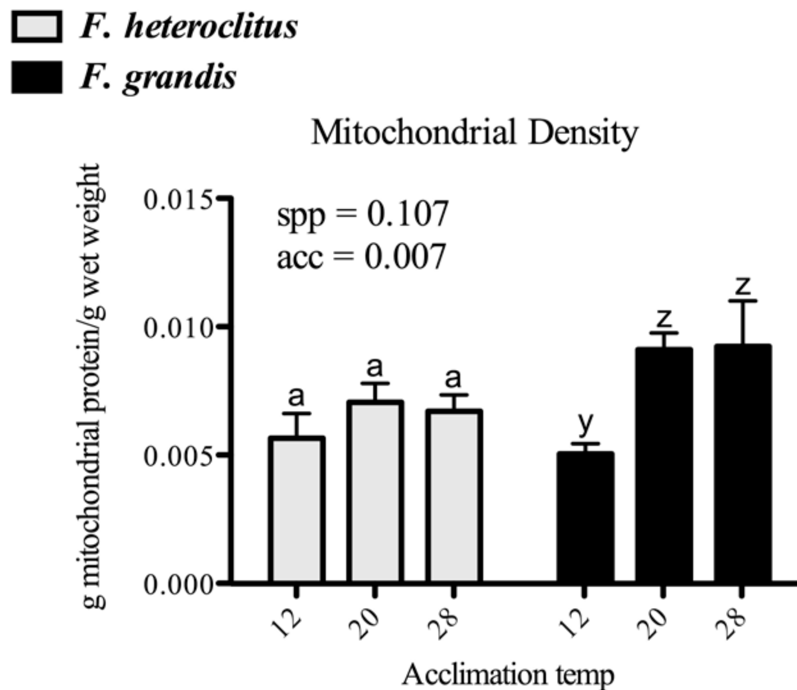
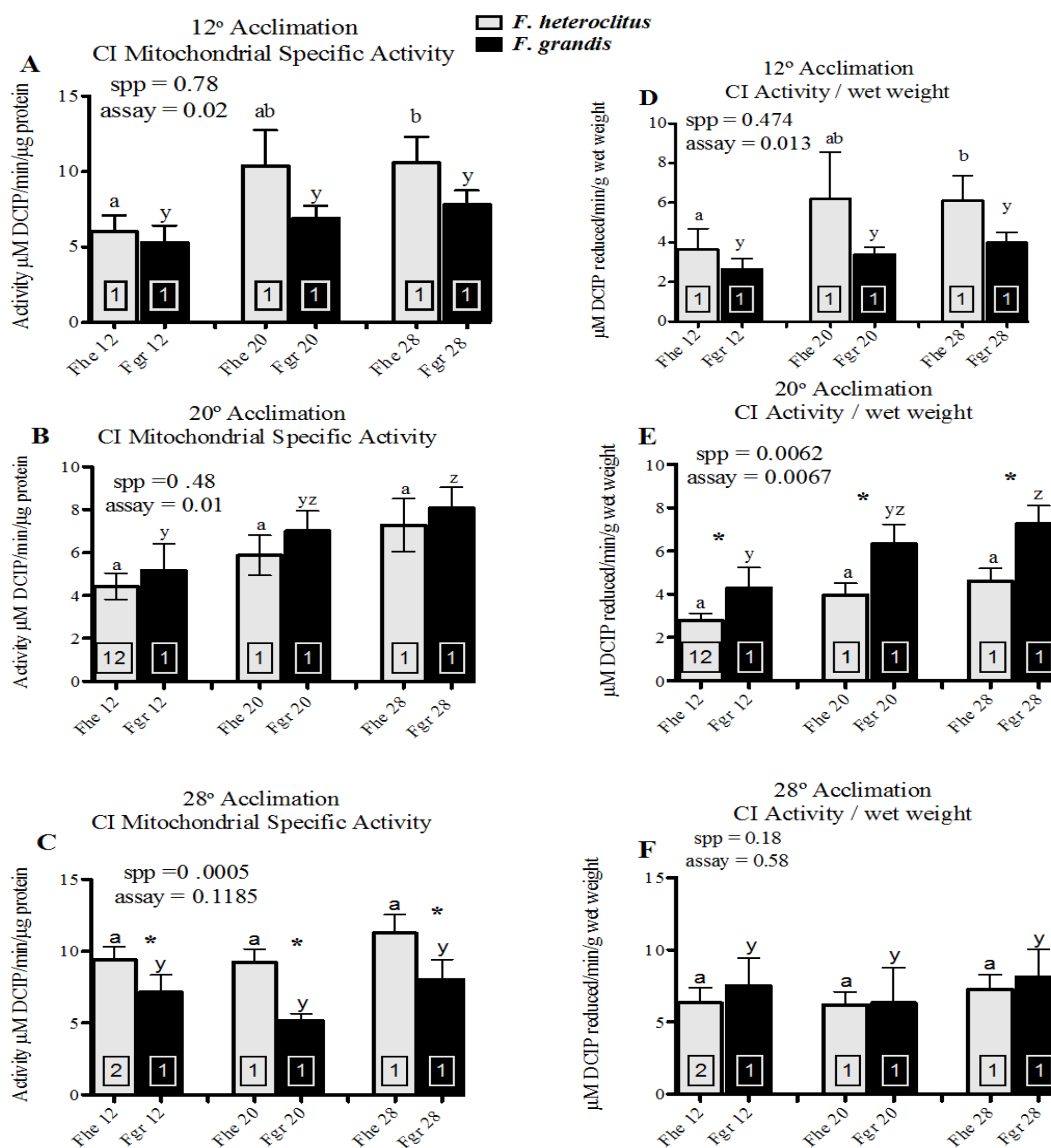


Figure 4.3. Effect of Acclimation Temperature and Measurement Temperature on Complex I Activity Among Populations
The left-hand column (A) show effect of three assay temperatures (12°, 20°, 28°) on Complex I mitochondrial specific activity for three different acclimation groups (one figure per acclimation group). The right-hand column (B) shows effect of assay temperature (12°, 20°, 28°) on Complex I activity per wet weight liver tissue. Lower case letters show significance of post-hoc Bonferroni testing on differences between activity between assay temperatures within species (*F. grandis* y,z; *F. heteroclitus* a,b,c). Asterisks * denote differences between species within assay temperatures. Numbers within bars are denote significant differences among acclimation groups for each assay temperature. P-values for two-way ANOVA considering assay temperature and species are given in the top left corner of each figure.



Chapter 5 Expression of Complex I Subunit mRNAs and Enzyme Activity of *Fundulus*

5.1 Summary

Deficiencies in Complex I (EC. 1.6.5.3) are implicated in human mitochondrial diseases with a wide variety of symptoms that can begin in infancy (e.g., fatal heart disease and respiratory failure) or appear later from childhood to adulthood (e.g., exercise intolerance, muscle weakness, hearing loss, lactic acid buildup, dementia and sensory neuropathy). Complex I also has a large control coefficient over flux through the oxidative phosphorylation pathway. Importantly, variation in Complex I activity in *Fundulus heteroclitus* varies significantly among populations along the species' geographic range in a pattern that suggests evolution by natural selection. Yet, the amount of inter-individual variation in Complex I activity is higher than has been observed for other enzyme activities in *Fundulus.* Unlike the variation among populations, this large variation within a population suggests that the differences among individuals represents random neutral variation. The large variation among individuals may reflect the molecular processes required to synthesize, transport and assemble Complex I in the mitochondrial inner-membrane which are many and present both a large target for mutations that affect function, as well as a significant challenge for coordination of molecular mechanisms. To investigate whether mRNA expression is co-regulated in *Fundulus,* and to determine if variation in mRNA abundance affects the variation of Complex I activity, expression of Complex I subunit mRNAs was quantified and compared among five populations of *F. heteroclitus* and one population of *F.*

grandis. One subunit, mitochondrial ND2 showed patterns of expression that show signatures of evolution by natural selection. However, the data suggest Complex I subunit mRNAs were not co-regulated similarly in all populations. Instead, the Complex I subunit mRNAs that explained Complex I subunit activity within populations were different and were not shared among populations. These data suggest that if mRNA expression is important in defining Complex I enzyme activity, the genes whose expression effect a change in Complex I differ in different populations.

5.2 Introductory Material

Complex I, composed of 45 separate gene products that are transcribed from both the mitochondrial and nuclear genomes, has a significant control over the flux through the oxidative phosphorylation pathway (Lemieux, Semsroth et al. 2011). There are many molecular processes necessary to construct Complex I: transcription of nuclear and mitochondrial genes, transcription and transport of nuclear mRNA or protein into the mitochondria and assembly of Complex I in the inner-mitochondrial membrane (Koopman, Nijtmans et al. 2010). If the cost of synthesizing and maintaining such a complicated system is relevant to the fitness of an organism, there may be adaptive divergence or functional constraint on the steps necessary to construct Complex I. There is much evidence to suggest that proper function of Complex I is relevant to fitness (Smeitink and van den Heuvel 1999; Janssen, Nijtmans et al. 2006; Haack, Madignier et al. 2012). Because Complex I is crucial for ATP synthesis, one would predict that the genes which encode subunits crucial for Complex I activity should be co-regulated. Here the emphasis is on describing the expression of Complex I subunit mRNAs in two different species and in relation to Complex I activity

Mitochondrial transcription may be of particular import for Complex I variation. Of the 13 protein coding genes in the mitochondrial genome, seven are Complex I subunits. All seven of the mitochondrial Complex I subunits are part of the catalytic core of the enzyme (Carroll, Fearnley et al. 2006). Transcription of mitochondrial DNA is polycystronic—two RNAs are produced from the mitochondria, one from the heavy strand and one from the light strand. Twelve of the mitochondrial mRNAs, including six Complex I mRNAs, are transcribed on the heavy strand and the seventh Complex I mitochondrial protein, ND6, is found on the light strand (Asin-Cayuela and Gustafsson 2007). Physiological induction of mRNA occurs in both mitochondrial and nuclear genomes when individuals experience hypoxia (Piruat and López-Barneo 2005; Brouwer, Brown-Peterson et al. 2008), exercise (Murakami, Shimomura et al. 1994; Asin-Cayuela and Gustafsson 2007), changing nutritional status (Huang, Eriksson et al. 1999; Joseph, Pilegaard et al. 2006; Baltzer, Tiefenbock et al. 2009; Flueck 2009; Malmgren, Nicholls et al. 2009) and development (Falkenberg, Larsson et al. 2007). Importantly, the 13 mitochondrial-encoded Oxidative Phosphorylation (OxPhos) mRNAs are differentially affected by these physiologically induced changes in expression. For instance, hypoxia differentially induces the mitochondrial COX genes: COX-I increases by 2.6-fold yet COX-III expression is unaffected in glass shrimp (Brouwer, Brown-Peterson et al. 2008). Similarly, induction of OxPhos genes differs among mitochondrial-encoded mRNAs in rats cell exposed to hypoxia (Piruat and López-Barneo 2005) and in human skeletal muscles exposed to insulin (Huang, Eriksson et al. 1999).

Among and within *Fundulus* populations there is often differential expression of the seven mitochondrial encoded Complex I mRNAs (ND1, ND2, ND3, ND4, ND4L,

ND5 and ND6). For example, 2 of the 7 Complex I mitochondrial subunits, ND2 and ND5, have significant differences in expression among individuals, and two of these are different among populations in a pattern indicative of evolution by natural selection (Oleksiak, Churchill et al. 2002). The expression of ND2 mRNA is significantly different among northern and southern individuals of *F. heteroclitus* (Whitehead and Crawford 2006). Finally, the variation in some, but not all, of these mitochondrial genes statistically explains the variation in cardiac metabolism (Oleksiak, Roach et al. 2005). The important point is that there are differences among individuals and populations in the expression of mitochondrial-encoded Complex I mRNAs and these differences appear to be biologically important.

Unlike mitochondrial encoded mRNAs, the differential regulation of nuclear encoded Complex I mRNAs can be explained by the activation of separate transcription factors that affect one or more nuclear loci (Scarpulla 2006; Cannino, Di Liegro et al. 2007; Scarpulla 2008). However, among Complex I subunits there are no clear patterns of shared transcription factor binding sites (van Waveren and Moraes 2008), suggesting that the coordination of Complex I mRNA is subject to fine-scale regulation involving many interacting proteins, rather than one or a few very important trans-acting regulatory factors.

In *Fundulus,* nuclear encoded Complex I genes are differentially expressed along the steep thermal cline and among populations exposed to pollution and statistically explain metabolic variation (Oleksiak, Churchill et al. 2002; Oleksiak, Roach et al. 2005; Whitehead and Crawford 2006; Whitehead and Crawford 2006; Crawford and Oleksiak 2007; Fisher and Oleksiak 2007; Oleksiak 2008). In this way, *Fundulus* is an ideal genus

for the study of Complex I mRNA expression because it exists in divergent populations along a steep thermocline. Inter-individual variation in Complex I activity is high (Chapter 3). Results of acclimation experiments (Chapter 4) suggest that the effect of acute temperature change on Complex I activity is a function of long-term acclimation temperature. Variation in expression of Complex I subunits may be related to this effect.

To better understand the biological importance of mRNA expression, Complex I mRNA expression and enzyme activity were measured in individuals from 6 populations. Complex I is an ideal system for this task due to its size and complexity—45 separate proteins make up the mature enzyme in vertebrates—and the critical role Complex I plays in metabolism. The expression patterns of the subunits of Complex I are known to be highly variable among individuals and also show complex patterns of correlation in mRNA abundance in humans, mice, *Drosophila melanogaster* and *F. heteroclitus* (Chapter 2). Complex I specific activity (NADH reduction min^{-1} μg total $protein^{-1)}$ is significantly different between northern and southern populations of *F. heteroclitus* (Chapter 3). Mean enzyme activity decreases with increasing latitude. Inter-individual variation in Complex I activity in these populations is extremely high; despite being subjected to a prolonged laboratory acclimation period (6 weeks, 20°) before Complex I assays were performed. In order to determine if variation among individuals in Complex I activity is related to variation in mRNA abundance of Complex I subunit genes, mRNA was quantified with microarrays for individuals from five populations of *F. heteroclitus* and one population of *F. grandis* (n=49).

5.3 Methods

Fundulus heteroclitus from #2 Sapelo Island, GA; #3 Hayes, VA; #4 Stone Harbor, NJ; #5 Matunuck, RI and #6 Wiscasset, ME (Fig. 3.1) were collected in minnow traps in September 2010. *Fundulus grandis* from #1 Cocodrie, LA were collected in May 2010 (Fig. 3.1). Fish were acclimated in the lab for a minimum of 6 weeks at 23° C and 15ppt salinity using artificial seawater with a 14h light 10h dark light schedule.

Total RNA from individuals (n = 49) was isolated by homogenizing liver tissue (0.01 to 0.1 g) in chaotrophic buffer (4.5M guanidinium thiocyanate, 2% N-lauroylsarcosine, 50mM EDTA, 25mM Tris-HCL, pH 7.5, 0.1M β-mercaptoethanol, 0.2% antifoam A (Sigma) followed by choloroform:phenol extraction and ethanol precipitation. Total RNA was quality checked spectrophotomically using a NanoDrop 1000. RNA with 260/280 ratios > 1.8 were diluted to 200µg/µl and 1 µg was amplified using the Amino Allyl message Amp II kit from Ambion, following manufacturers protocols. Resulting aaRNA was quality checked using Agilent RNA6000 Nano bioanalyzer chip kit following manufacturer's protocols. aaRNA with total concentrations > 10µg and rRNA ratios >1 were labeled. 4µg of aaRNA was labeled with $1/25^{th}$ of a CyDye pack (GE Healthcare) resuspended in high quality DMSO. With the exception of the dye resuspension volume, all other manufacturers' protocols were observed. Concentration of aaRNA was determined using the NanoDrop 1000 and sample pairs were combined and concentration was adjusted to 20pmol labeled aaRNA in hybridization buffer (5x SSPE, 1% SDS, 50% formamide, 1µg/µl sheered herring sperm). Samples were hybridized in a loop design (Oleksiak, Churchill et al. 2002). Slides were

scanned using a ScanArray Express with 5μm resolution at half speed. Images were quantitated using IMagene software.

Microarray Analysis

mRNAs with raw expression averages greater than the lowest 10% were analyzed. Of the 6912 probes on the *Fundulus* array, 6219 were used for analysis, including 39 Complex I subunits. Data was $\log_2$ transformed and sum normalized using Microsoft Excel 2008. Loess normalization was performed using JMP Genomics 5.1 to remove spatial variability in cy3 and cy5 expression data. To determine the mean expression value for each mRNA, one way Analysis of Variance (ANOVA) with dye and array as fixed effects was performed (resulting in least squared means for each mRNA, LSmean).

ANOVA was performed on individual LSmeans for both the total data (6219 mRNAs) and Complex I mRNA data (39 mRNAs), using population as the fixed effect to identify mRNAs that were differentially expressed among populations. To examine the relationship between Complex I mRNA expression and enzyme activity, individual LSmeans were then used to calculate stepwise regressions with mean Complex I mitochondrial specific activity within each population (JMP Genomics 5.1)**.** Stepwise regression was done in the forward direction, variables were stepped in at a $p = 0.25$ and out at $p = 0.10$.

Correlations of mRNA abundance among individuals were calculated for 39 Complex I subunit genes using Matlab and the same approach as in Chapter 2. Hierarchical clustering of the rho values was achieved with Eisen's Cluster and Treeview (Eisen et al. 1998 de Hoon et al. 2004) (Figure 5.2, Table 5.1). In order to determine

significance of observed number of correlations in the Complex I subunit dataset, the same number of genes (39) were randomly chosen from the Lsmeans of non-Complex I mRNAs. This was repeated 1,000 times within each population as well among all *F. heteroclitus* individuals to generate an empirical distribution to define the probability of randomly achieving a similar frequency of significant correlations.

5.4 Results

Genes with significant differences among populations

Significantly different mRNA expression for all 6219 genes among populations were identified. Among non-Complex I mRNAs, 214 (3.4%) had significantly different expression among all populations of *F. heteroclitus* ($p < 0.05$). This was not greater than the 311 genes expected to be significant due to Type I error at $p = 0.05$ (Crawford, Pierce et al. 1999). The expression of one Complex I subunit mRNA was significantly different among different *F. heteroclitus* populations (ND1, $p = 0.003$).

To evaluate if there were patterns of expression that exceeded neutral expectation, the mRNA expression in the northern *F. heteroclitus* population (Maine) was compared to the expression in both southern *F. heteroclitus* (Georgia) and *F. grandis* populations. A significant difference is unexpected because the northern and southern *F. heteroclitus* populations are genetically more similar to each other than either is to *F. grandis*. Between these two groups of northern and southern individuals, one Complex I mRNA was differentially expressed (NDUFA5, $p = 0.0126$). The frequency of significantly different mRNAs in both comparisons—between all *F. heteroclitus* populations and between northern and southern individuals—is 2.6%. With a critical p-value of 0.025, we expect to observe 0.98 mRNAs with significantly different expression.

Patterns of Complex I mRNA expression in *Fundulus* populations differed among populations (Fig 5.1), but there was no clear segregation of populations reflecting the lack of significant differences. Mean expression did not vary among populations. To illustrate the amount of inter-individual variation in Complex I subunit mRNA expression among individuals within populations, fold –change was calculated as the greatest individual Lsmean divided by the smallest individual Lsmean for each Complex I mRNA within each population. Average fold-change among all Complex I subunit mRNAs ranged from 1.08 in Virginia individuals to 1.19 in Georgia individuals (GR = 1.18, GA = 1.19, VA = 1.08, NJ = 1.21, RI = 1.16, ME = 1.17). The mRNA with the greatest range in expression was ND6 which had an average fold-change of 1.42. The fold-change for ND6 mRNA expression among individuals was greater than the population average fold change in all populations except Virginia.

Stepwise regression of Complex I mRNA expression with Complex I activity

To determine if the variation in Complex I mRNA expression statistically explained the variation in Complex I activity among *F. heteroclitus* (Chapter 3) a stepwise regression was used. Among all individuals across all *F. heteroclitus* populations, there was a significant relationship between the expression of 16 Complex I mRNAs and Complex I activity (Adjusted $R^2 = 0.84$, $p < 0.0001$ Table 5.1). To further explore these data, the relationships between mRNA and enzyme activity for Complex I was examined within each population. Within each population the stepwise regression model of mRNA expression of Complex I subunits explained > 99% (Adjusted $R^2 > 0.99$) of the variance in individual Complex I activity. The mRNAs that explained variation in Complex I activity are not the same in each population (Table 5.1). Among

Complex I subunits, NDUFs A4, A5, A11, B8, S1, V2 and ND2, ND5 appeared in more than one model, but no subunit appeared in all population regressions.

Correlations among Complex I subunit genes

Significance of correlations among Complex I subunit mRNA expression within populations was determined with a critical Pearson's rho corresponding to a p-value = 0.01 with n-2 degrees of freedom. The pair-wise correlation among mRNA Complex I subunits use individuals as replicates. Thus, a significant positive correlation among subunits indicates that if an individual expressed more of one subunit, this individual also expressed more of the correlated subunit. Among all *F. heteroclitus* individuals there were 35 significant correlations out of a possible 741 (4.7%). The number of significant correlations observed among all Complex I subunits was not significantly different than the number of significant correlations observed among randomly selected genes (5.1%, p = 0.611). To examine if similar patterns exist in each population, the correlation between pairs of Complex I mRNA was examined (Fig. 5.2). For *F. heteroclitus* populations from Georgia and Virginia the critical rho was 0.798. For Maine and New Jersey the critical rho was 0.834 and for Rhode Island the critical rho was 0.874. In Georgia, Virginia, New Jersey, Rhode Island and Maine 9, 23, 11, 9 and 6 significant correlations were observed in respectively. Similar to observations among all *F. heteroclitus* individuals, the number of significant correlations observed among Complex I mRNAs in each population was not significantly different than the number observed when random genes are chosen and correlated ($p > 0.05$). Significant correlations observed among Complex I mRNAs differed among populations (Table 5.2). No pairs of subunits were significantly correlated in more than one population. Six pairs of subunits (A11-B6, A13-V2, B5-B2, C2-A8, ND3-A13, ND4-B9) were correlated both among all *F.*

heteroclitus individuals and within one population. However, none of these shared pair of significantly correlated subunits was found in more than one population in addition to the total set of *F. heteroclitus* individuals.

5.5 Discussion

Complex I enzyme activity differs among populations and the change in activity appears to be adaptive (Chapters 2 & 3). That is, the variation between populations is greater than the variation among *Fundulus* species and thus exceeds variation expected due to neutral processes only. Similarly, Complex I subunit NDUFA5 mRNA expression showed an adaptive pattern of expression. ND1 expression differed among populations of *F. heteroclitus*. The difference in expression of ND1 among northern and southern fish, however, appeared to be driven by the large difference in expression between *F. grandis* and individuals from Maine (Fig. 5.1), suggesting that this difference is not likely to represent adaptive divergence within *F. heteroclitus* populations. Expression of ND1 and NDUFA1 was higher in northern individuals relative to southern individuals. NDUFA1 was identified as differentially expressed in cardiac tissue, and had greater abundance in northern individuals(Oleksiak, Roach et al. 2005). In these two other studies of mRNA expression in natural populations of *Fundulus* many Complex I subunit mRNAs are differentially expressed among individuals (Oleksiak, Roach et al. 2005; Whitehead and Crawford 2006). In Oleksiak *et al.* (Oleksiak, Roach et al. 2005) ,19 Complex I mRNAs were measured in cardiac tissue among 16 individuals and two Complex I mRNAs were differentially expressed between populations, NDUFB6 and NDUFA1 (Oleksiak, Roach et al. 2005). In a separate study (Whitehead and Crawford

2006) 26 Complex I mRNAs were measured in livers of 25 post reproductive males, and 13 showed signatures of stabilizing selection, suggesting that mRNA expression is biologically relevant and constrained. Five Complex I subunit mRNAs regressed with the phylogenetic distance—ND2, NDUFs A2, A4, and A8. This difference could represent random neutral changes among populations. However, two Complex I genes (NDUFs A2,andA8) have patterns of expression that regressed significantly with temperature after removing the effect of phylogeny and thus, these two Complex I subunits have patterns of mRNA expression that appears to be evolving by natural selection.

In this study of liver mRNA expression (the same tissue used in (Whitehead and Crawford 2006)) Complex I subunit NDUFA5 showed an adaptive pattern of expression. Notice that the frequency of statistically significant differentially expressed Complex I mRNA (1/39, 2.6%) is not greater than the number of mRNAs expected to be differentially expressed with a 2.5% p-value (0.975/39) by chance alone. In addition to a lack of significantly different expression among Complex I subunits, there was also less variation in mRNA expression among individuals (reported approximately as fold-change between highest and lowest expression value within populations) than expected based on previous studies of mRNA expression in *F. heteroclitus* (Oleksiak, Churchill et al. 2002; Oleksiak, Roach et al. 2005).

Previous studies of *F. heteroclitus* mRNA expression were able to distinguish differences among individuals because of the large number of technical replicates. In a previous study that measured 19 Complex I mRNAs in cardiac tissue among 16 individuals with 16 fold replication (Oleksiak, Roach et al. 2005) all 19 Complex I

mRNAs were significantly different among individuals within a population (Oleksiak, Roach et al. 2005). In the Whitehead and Crawford study of liver mRNA expression (Whitehead and Crawford 2006), they used 8 replicates per individual and 22 of the 26 Complex I mRNAs were differentially expressed among individuals. Although, there were too few technical replicates in this study to statistically test for difference in mRNA expression among individuals, in the Chapter 3 there was large variation among individuals Complex I enzyme activity (30 fold with a %CV among individuals exceeded 90%). These data on the variation among individuals for Complex I enzyme activity despite apparently small variation in mRNA expression raise the question on whether the variation in the expression of only a few Complex I subunit mRNAs are responsible for the variation in enzyme activity?

Regression of mRNA expression and enzyme activity

To identify if the expression of any mRNAs affect Complex I function, enzyme activity and mRNA expression were regressed using a stepwise model. In stepwise regression, independent variables are entered into the model sequentially. At the end of each step, correlation coefficients are determined and if the R^2 does not decrease, the variable is kept in the model, or removed if the R^2 decreases. Once the R^2 remains stable and all variables have been entered (or removed) the resulting model gives the variables for explaining the variation in the dependent variable.

A significant amount of variance in Complex I activity was explained by variation in mRNA abundance. Stepwise regression models were fit to the combined data set of all *F. heteroclitus* individuals and within each population, separately. Considering all *F. heteroclitus* individuals together, the expression of 16 Complex I mRNAs explained 84%

of the variation in enzyme activity (Table 5.2). What was surprising, was the regression analysis within each population using fewer individuals (and less statistical power) explained >0.99% of the variance in Complex I enzyme activity. Each population had a unique set of mRNAs that explained virtually all the variation in Complex I enzyme activity. Several Complex I subunits appeared in more than one population, 47% (8/17) of subunits significant in stepwise regressions within populations were shared in more than one population. NDUFs A5, V2, ND2 and ND5 were found in the stepwise regression models of three separate populations and NDUFs A4, A11, B8 and S1 were found in the stepwise regression models of two populations. NDUFA5 showed adaptive expression patterns.

The presence of different subunits included in the stepwise regression model suggests that variation in the expression of Complex I mRNAs was related to variation in Complex I enzyme activity, but that it was not likely to be governed by a single transcription factor or mechanism shared among *Fundulus*. The observation that different groups of individuals had different mRNAs related to the variance in a physiological trait is not unique to this study. Different sets of genes explain metabolic substrate use in different subgroups of individuals of *Fundulus heteroclitus* (Oleksiak, Roach et al. 2004).

The regression results suggest that the genes that affect Complex I enzyme are different in different populations. Supporting this supposition is the correlation among Complex I mRNAs. *F. heteroclitus* individuals from Georgia, had 9 pairs of significant correlations and only one is shared in other populations (Table 5.2). Similar results are found in the other 4 populations. Among all *F. heteroclitus* individuals (ignoring

populations) a greater number of significant correlations were observed (35 total significant correlations of 741). This most likely reflects the greater number of individuals and thus greater statistical power. Yet, most of the correlated pairs within populations appeared to be population-specific—these pairs were not significantly correlated when all individuals were considered (Table 5.2).

These data suggest that mRNA variation is related to variation in Complex I activity in all populations, but the regulatory pathways appear to be different. Other analyses of Complex I subunit mRNA expression have shown that different Complex I mRNAs are co-expressed across many tissues and that the expression of subunits is organized into separate groups which is not characteristic of one or a few global regulators of Complex I regulation (van Waveren and Moraes 2008; Garbian, Ovadia et al. 2010). These data suggest that there are many mechanisms that control the regulation of Complex I subunits, but these studies have not looked at expression among populations. In this study, the mRNAs included in the stepwise models and the mRNAs with significant correlations varied among populations. The apparent necessity for fine-scale regulation of Complex I subunits is thus extended to population specific regulation of Complex I subunits. This effect—where different transcripts explain the variance in Complex I activity in different populations—is similar to a prediction made by the Metabolic Control Theory (Kacser, Burns et al. 1973; Heinrich and Rapoport 1974) which suggests that many different enzymes have sway over flux through metabolic pathways. Just like there is probably not a classical rate-limiting enzyme for every metabolic pathway (Pierce and Crawford 1997), there is probably not a level of expression of one or few Complex I mRNAs that explains Complex I activity, and so

regulation of these mRNAs is likely to be variable. The lack of shared pairs of correlated genes also supports this.

Conclusion

Here we have examined nearly all the Complex I subunits in 6 populations, and mitochondrial and nuclear Complex I mRNAs have similar patterns of expression among populations (Figure 5.1A). One Complex I mRNA NDUFA5 had a pattern of gene expression suggest of adaptive divergence between northern and southern *F. heteroclitus.* Overall though, there was a lack of divergence in mRNA expression among populations of *F. heteroclitus.* This may be due to a difference among populations in the Complex I subunits that affect Complex I activity. The high inter-individual variation in Complex I activity combined with the incredibly high R^2 for the stepwise regressions within populations suggests that Complex I activity is affected by very small changes in the expression of key Complex I subunits. However, the mRNA levels were not significantly different among *F. heteroclitus* populations because their importance in regulating enzyme expression appears to be population specific.. Thus, population specific divergence in Complex I expression patterns *per se* may not be necessary to cause the high inter-individual variation observed in Complex I activity (Chapters 3 and 4). The mRNAs that affect these differences are not the same in each population, so statistical tests do not identify them.

Additionally, the fact that expression of subunit mRNA affected the activity of Complex I was not surprising. These results were expected for a system as large and

complex as Complex I. However, what is surprising is that the mechanisms that apparently fine-tune the transcription of Complex I subunits do not appear to be shared among populations.

Figure 5.1 Abundance of Complex I mRNA Among Populations. A. Expression of Complex I subunits among populations. None of the differences in mRNA abundance among *F. heteroclitus* populations are significant. Brightest colors show 1.15 fold difference among popuations. One mRNA is differentially expressed among populatinos (ND1, ANOVA, p = 0.003) **B**. Abundance of mRNA for Complex I genes among Northern and Southern individuals. Expression patterns that are similar between southern populations (GA, GR) that diverge from expression in the northern population (ME) are likely to be biologically important. Colors show a 1.15 fold difference among geographic groups. One mRNA is differentially epressed (NDUFA5, ANOVA p = 0.0126)

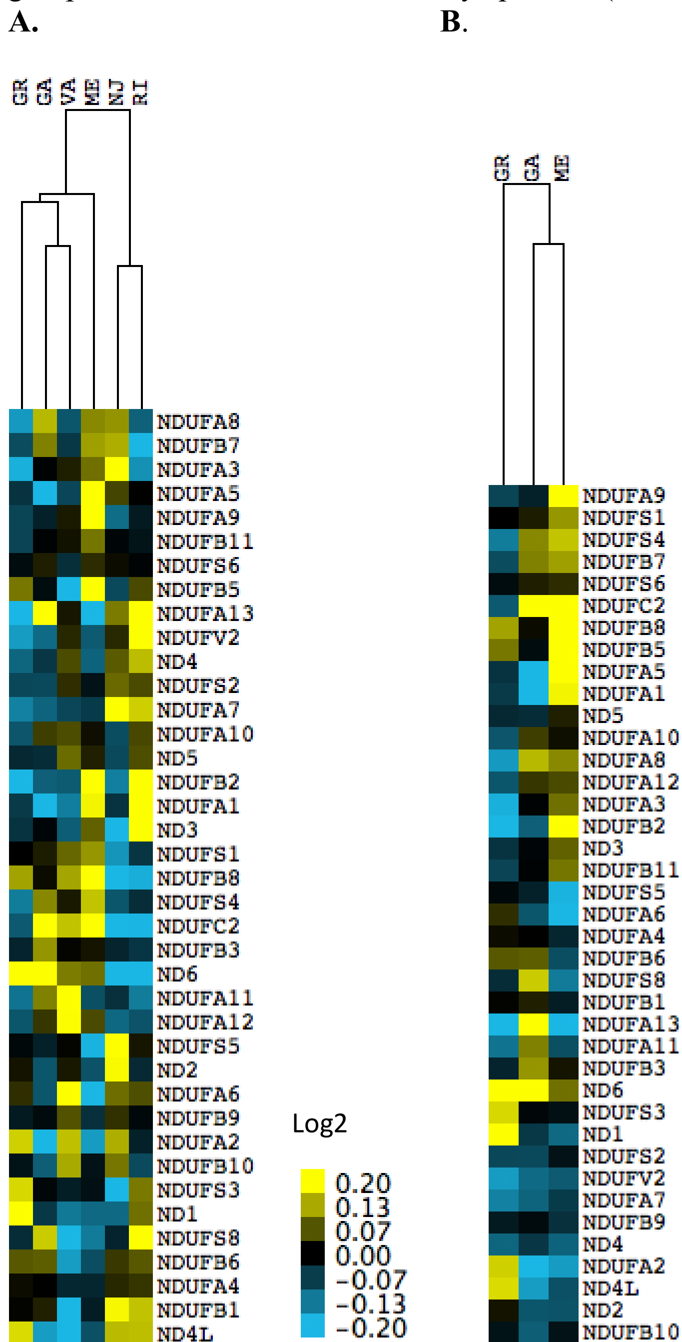
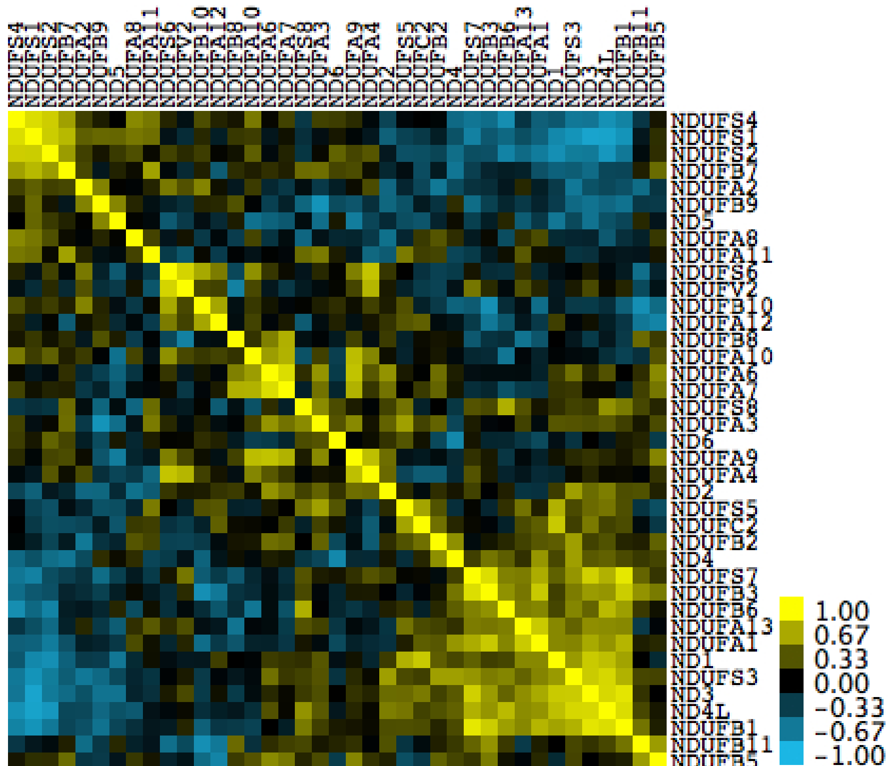


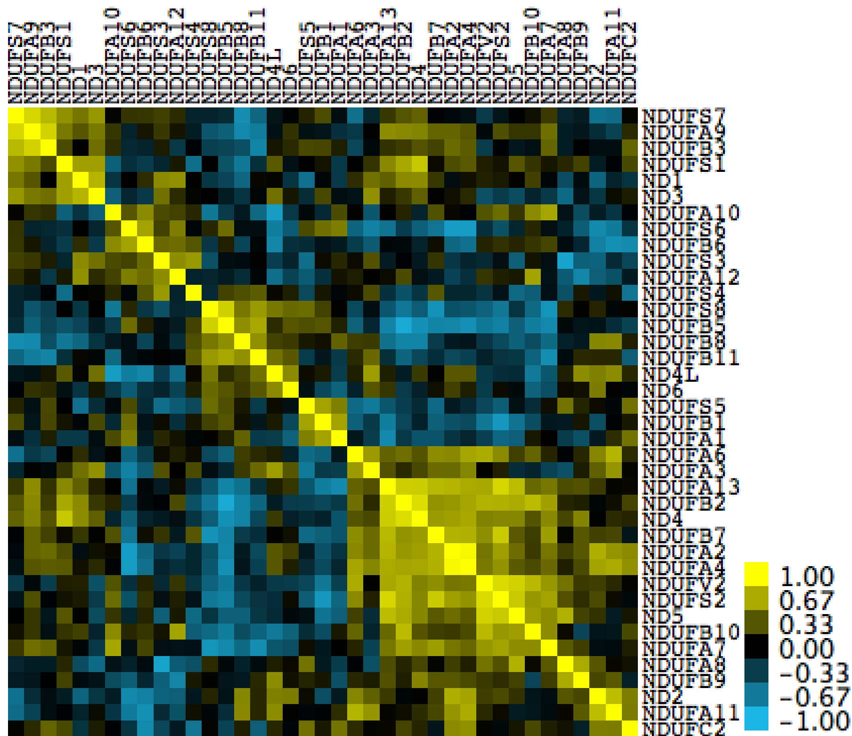
Figure 5.2 Correlations Among Complex I Subunit mRNAs. Correlation coefficients correspond to color bar. Significant rho (p = 0.01) for populations are: **A.** Georgia 0.798; **B.** Virginia,0.798; **C.** New Jersey 0.834; **D.** Rhode Island 0.874 and **E.** Maine 0.834. Color scale corresponds to rho for each correlation and is the same for all images.

A. Georgia

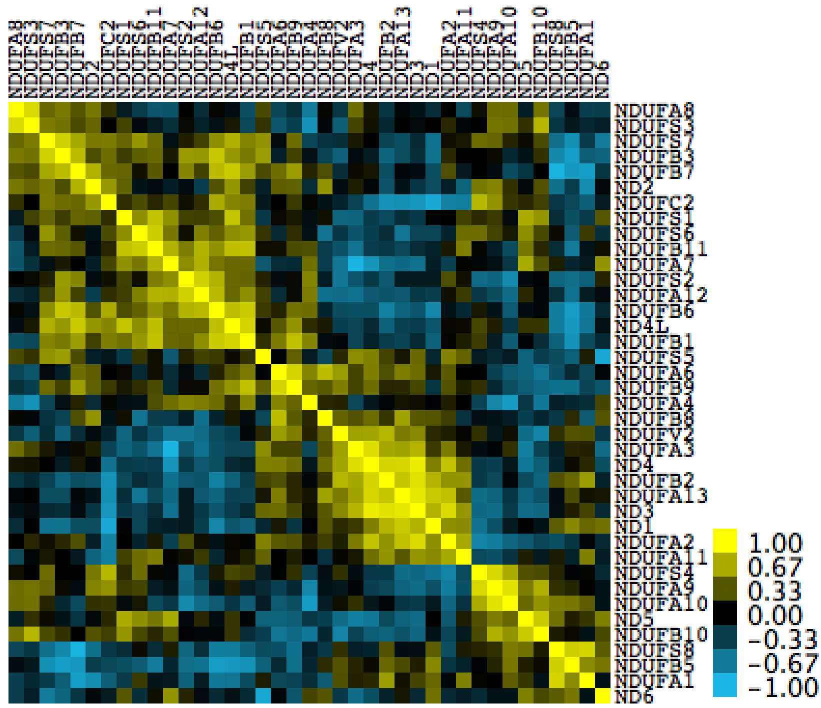
B. Virginia



C. New Jersey



D. Rhode Island



E. Maine

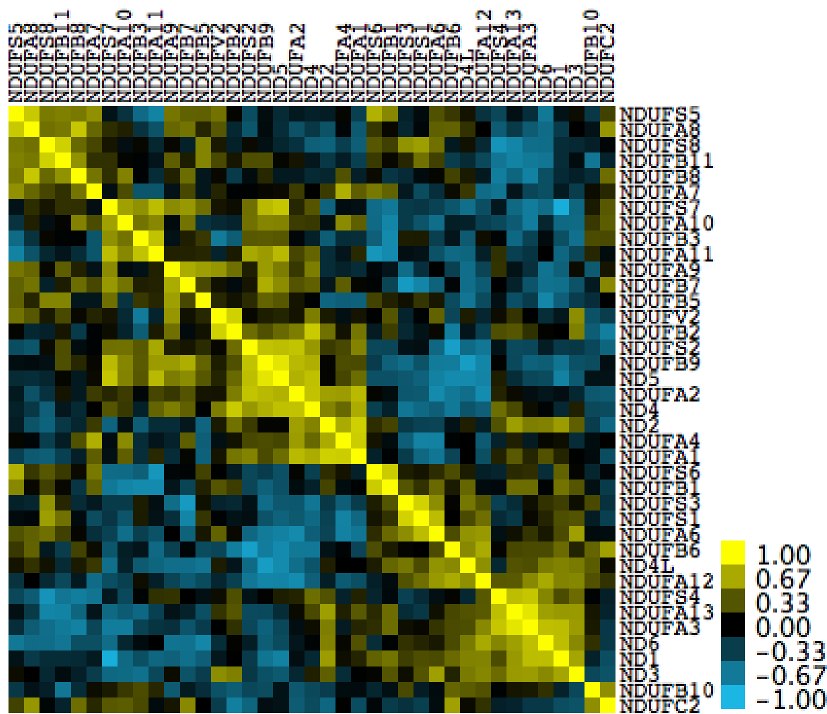


Table 5.1 Stepwise Regression of Complex I Activity and Complex I Subunit mRNA Expression. Complex I mRNA expression values for *F. heteroclitus* individuals within and among populations were fit to stepwise regression model with individual Complex I mitochondrial specific activity. Bold gene names indicate mRNAs singificant in stepwise models of more than one population. For each population, adjusted R2 > 0.99, p value for stepwise models <0.005.
* signify mRNAs with a negative regression.

F. heteroclitus	
mRNA	**pval**
NDUFS6	0.0003
NDUFA5*	0.0001
NDUFA8	0.0002
NDUFS8*	0.0283
NDUFV2	0.0001
NDUFA9*	0.0001
NDUFS2	0.0001
NDUFB2	0.0001
NDUFB3	0.0219
NDUFB6*	0.043
NDUFA2	0.0003
ND1	0.0062
ND4*	0.0001
NDUFA1*	0.0001
NDUFB10	0.0058
NDUFB5	0.0027

GA		VA	
mRNA	**pval**	**mRNA**	**pval**
NDUFA5*		**NDUFB3**	0.0059
NDUFV2		**ND2***	0.0021
NDUFB2		**ND5***	0.0016
NDUFB8*		**NDUFA4***	0.0269
NDUFB3		**NDUFB5***	0.0022
ND2		**NDUFA11***	0.0072
ND3			

NJ		RI		ME	
mRNA	**pval**	**mRNA**	**pval**	**mRNA**	**pval**
NDUFA5*	0.0002	**NDUFA5***	0.0002	**NDUFV2***	0.0005
NDUFB8	0.0286	**NDUFV2**	0.0008	**NDUFS1**	0.0004
NDUFA6*	0.0043	**NDUFS1**	0.0004	**NDUFA2**	0.0103
NDUFB9*	0.0003	**ND2**	0.0003	**ND5***	0.0026
ND5	0.0008	**ND4***	0.0136	**NDUFA4***	0.0006
NDUFB5	0.0007	**NDUFA11***	0.0077		

Table 5.2 Significant Correlations Among Complex I mRNAs Within Populations. Bold gene pairs are population specific pairs of significantly correlated subunits. Red pairs are negatively correlated subunits. Black pairs are positively correlated subunits. Italic correlations are also seen in correlations among all *F. heteroclitus* individuals.

GA		VA		NJ		RI		ME	
A11	*B6*	**A1**	**A13**	**A13**	**S2**	**A10**	**A9**	**ND4L**	**B9**
A13	*V2*	**A3**	**B9**	**A2**	**S6**	**A7**	**A3**	**A3**	**A13**
A2	**B8**	**A7**	**A6**	**A4**	**S6**	**B7**	**S8**	**B6**	**S2**
A6	**A8**	**B1**	**S1**	**A4**	**A2**	**C2**	**ND1**	**B7**	**S3**
C2	*A8*	**B1**	**S7**	**A9**	**S7**	**ND3**	**B2**	**ND1**	**S7**
C2	**B6**	**B1**	**B3**	**B3**	**A9**	*ND3*	*A13*	*ND4*	*B9*
ND2	**B2**	**B1**	**ND3**	*B5*	*B2*	**ND4**	**A3**		
ND5	**V2**	**B1**	**ND4L**	**ND4L**	**A10**	**ND4**	**ND3**		
ND6	**A13**	**B3**	**S7**	**ND4L**	**B2**	**ND6**	**S5**		
		ND3	**S1**	**S2**	**V2**				
		ND3	**S7**	**S3**	**A8**				
		ND3	**S3**						
		ND3	*ND1*						
		ND4L	**S4**						
		ND4L	**S2**						
		ND4L	**S1**						
		ND4L	**S3**						
		ND4L	**ND3**						
		S1	**S3**						
		S1	**S4**						
		S2	**S4**						
		V2	**S6**						

Chapter 6 Conclusions

The observation that many Complex I subunit mRNAs are significantly correlated among *Fundulus* individuals and that these correlations are both positive and negative was the impetus for this dissertation research. As discussed in the introduction, Complex I is a critical enzyme for oxidative phosphorylation and is the largest enzyme in the pathway. Adding to the complexity of transcribing, translating and assembling this mitochondrial protein is the bi-genomic location of Complex I subunit genes. The goals of this dissertation were to describe the phylogenetic distribution of Complex I subunit genes, the activity of Complex I within and between populations of *F. heteroclitus* and to describe the mRNA expression of Complex I subunit genes.

In order to determine if the expression patterns observed were a *Fundulus* specific phenomena or if it was conserved in several taxa, this investigation of Complex I began with a description of the phylogenetic distribution of subunits as well as the expression of Complex I mRNAs in a variety of species. This analysis of the annotation and distribution of Complex I subunits provided a groundwork for future chapters by providing a classification of subunits based on evolutionary appearance as well as a description of the homologous relationships between subunits in different species. The next step was to determine if Complex I activity varied among populations and species of *Fundulus*. Extremely high inter-individual variation in Complex I activity observed in Chapter 3 prompted the implementation of an acclimation study to determine how much variation in Complex I activity was related to differences in environmental temperature among populations. Chapter 4 is the examination of Complex I activity in response to acclimation and acute temperature change. Finally in order to determine if variation in

mRNA expression contributes to the amount of inter-individual variation observed in Chapter 2, mRNA expression was measured in *F. heteroclitus* and *F. grandis.*

Chapter 2 identified homologus Complex I subunits in a wide variety of taxa and described the expression in four species. The result was a table of orthologous annotations among common model species, as well as the identification of several subunits implicated in human disease that are lost after appearance. These results suggest that some Complex I subunits may have redundant function. Though others have also reported the phylogenetic distribution of Complex I subunit genes (Gabaldon, Rainey et al. 2005; Pagliarini, Calvo et al. 2008; Cardol 2011) this study has more conservative cutoff values for similarity searches and more stringent requirements for homology. As a result, several mis-annotated subunits were identified as well as others with little sequence similarity. In addition to a more thorough examination of the homologous relationships of Complex I subunits in eukaryotes, this study also investigated Complex I mRNA expression across several taxa. The analysis of the expression of Complex I subunit mRNAs in a variety of species (human, *Mus musculus, Homo sapiens, Drosophila melanogaster* and *Fundulus heteroclitus)* revealed that a conserved regulatory mechanism among all Complex I subunits among species is unlikely. Though many Complex I subunits are correlated, both positively and negatively, especially in *F. heteroclitus* which has many co-expressed genes in cardiac tissue, these patterns of mRNA correlation are not shared among species. The more basal subunits are more likely to be correlated with one another than with subunits from other groups (eg., subunits appearing in multicellular animals or vertebrates). This observation provided

the groundwork for Chapter 5 which examined the expression of Complex I subunits in relation to Complex I activity.

The variation observed among individuals and species in mRNA expression of Complex I subunits in Chapter 2 coupled with the apparent sensitivity of Complex I to deficiencies in subunit protein or aberrant assembly (Smeitink and van den Heuvel 1999) suggests that Complex I activity may be variable among populations and species. Many molecular processes need to be coordinated for the proper assembly of Complex I. The potential for amplification of variation from these many molecular processes through the system exists. Thus, small fluctuations at the level of transcription, translation, post-translational modification and assembly may affect enzyme function.

Among northern and southern populations of the teleost fish *Fundulus heteroclitus,* Complex I subunits have fixed amino acid substitutions (Whitehead 2009). Additionally, there are differences in cardiac metabolism among populations of *F. heteroclitus*, which could be associated with variation in Complex I activity due to its large control of flux through the electron transport chain (Ventura, Genova et al. 2002; Oleksiak, Roach et al. 2005). To investigate if these differences are caused or affected by Complex I, enzyme activity was measured in 121 individuals from 5 populations of *F. heteroclitus* and its sister species *Fundulus grandis* acclimated to a constant 20°C temperature. Complex I activity has been measured in humans (Triepels, Heuvel et al. 2001; Janssen, Trijbels et al. 2007) as a diagnostic method for mitochondrial disease and recently *Caenorhabditis elegans* has been proposed as a standard model for Complex I deficiency in humans (Chen, Thorburn et al. 2011). In *F. heteroclitus,* mitochondrial metabolism has been measured (Oleksiak, Roach et al. 2004; Fangue, Richards et al.

2009; Healy and Schulte 2012) but no direct measure of Complex I activity in natural populations of *F. heteroclitus* has been performed, though it is a particularly good model for investigating adaptive change in Complex I activity. Within each population, Complex I activity is highly variable among individuals of *F. heteroclitus* (% coefficient of variation among individual has a mean of 90% in the five *F. heteroclitus* populations and average fold difference within populations is 30), and the mean Complex I activity among populations is significantly different at the latitudinal extremes of the range. Importantly, Complex I activity is more similar between *F. heteroclitus* from the southernmost population and its sister species *F. grandis* than to the northern populations of *F. heteroclitu*s suggesting that the difference in Complex I activity is biologically important. Unexpectedly, the activity is four-fold higher in southern populations than northern populations. The effect of lower acclimation temperature on enzyme activity is usually a compensatory change that increases enzyme activity (Campbell and Spencer Davies 1978; Somero 1995; Hochachka and Somero 2002; Lee, Daniel et al. 2007). In *Fundulus,* mitochondrial density appears to partially compensate for decreased activity in northern individuals: activity per wet weight is two-fold higher in southern populations. We suggest that some of variation in Complex I activity is genetically based, and thus is being influenced by directional selection. Yet, this conclusion presents a conundrum: there should not be so much variation in Complex I within a population if this variation is biologically important.

Temperature shapes the distribution of species through effects on nearly every biological process. Typically, lower environmental temperatures correspond to increased enzyme activity (Somero 1995; Hochachka and Somero 2002). To examine the

sensitivity of Complex I to acclimation and assay temperature, the activity was determined at three acclimation temperatures and three assay temperatures (12°C, 20°C and 28°C) for three populations; *Fundulus heteroclitus* from Maine and Georgia and its sister species *Fundulus grandis* from Florida. At nearly every assay temperature in each acclimation group, Complex I activity was higher in Georgia *F. heteroclitus* individuals relative to individuals from Maine. In Chapter 3, where individuals were acclimated to 20°, we observed higher activity among southern individuals. In Chapter 4, we observed higher activity among *F. heteroclitus* relative to *F. grandis*. Though *F. heteroclitus* could be considered as northern relative to *F. grandis,* higher activity in *F. heteroclitus* relative to *F. grandis* does not contradict the results of Chapter 3. The lack of significant difference in Complex I mitochondrial specific activity among Georgia and Maine individuals resulted in the combination of these two populations into a single *F. heteroclitus* dataset to increase the statistical power of the comparisons among acclimated groups and assay temperatures.

Surprisingly, there were few differences among acclimation temperatures and no differences among populations of *F. heteroclitus.* Acclimation affects activity of Complex I at the lowest assay temperature. In *F. heteroclitus* the greatest Complex I activity was observed when individuals were acclimated to 28°C and measured at 12°C. There are no acclimation effects at 20°C and 28°C assay temperatures and acclimation does not appear to change enzyme expression, as is the case for other enzymes in *F. heteroclitus* (Crawford and Powers 1992; Pierce and Crawford 1997). Rather, acclimation of *F. heteroclitus* to higher temperatures results in a decrease in sensitivity of Complex I activity at 28°C. That is, among individuals acclimated to 28°C Complex I

activity remains high regardless of assay temperature, while individuals acclimated to 12°C have less activity at the 12°C assay temperature. Finally, there are large variations among individuals that are higher at the lowest acclimation temperature and lower at high acclimation temperatures. These data suggest that individuals living in colder waters may respond to environmental temperature fluctuations by modulating Complex I activity. Because the estuarine environment is extremely variable with respect to temperature, these individuals may also have greater variation in Complex I activity.

Results from Chapter 2 suggest that Complex I subunits are necessary—they are conserved after they appear and nearly half are implicated in human disease—but the high inter-individual variation in the activity of Complex I observed in Chapters 3 and 4 is paradoxical. When traits are biologically important they tend to be constrained—the rate of evolution of amino acids in active sites of proteins is slower than non-functional sites (Zuckerkandl 1976). When considering the rate of evolution of DNA sequences, the number of synonymous substitutions observed in coding and non-coding DNA is proportional to the sequence's importance to an organisms' fitness (Jukes and Kimura 1984). The prevalence of mitochondrial disease caused by deficiencies in Complex I activity indicates that it is relevant to fitness and thus I expected to see a range of activities that was somewhat constrained among healthy individuals because rates of protein and DNA evolution should be constrained. However, these data suggest that among fish acclimated to 12° and 20° the range of Complex I activity among individuals exceeds the range of activities observed for other glycolytic enzymes (Pierce and Crawford 1994).

Inter-individual variation in Complex I activity was decreased by increasing the acclimation temperature of *F. heteroclitus* individuals. This suggests that Complex I activity may be relevant to fitness only under certain environmental conditions. Higher acclimation temperatures affect the composition the lipid membrane, and this in turn may affect Complex I activity (Wodtke 1981; Hochachka and Somero 2002). It is likely that there is a minimum amount of Complex I activity that is necessary for proper function of the electron transport chain (Smeitink and van den Heuvel 1999; Ma, Zhang et al. 2011). The high inter-individual variation observed at 12° and 20° acclimation temperatures could represent a surplus of Complex I activity in some individuals. The apparent narrowing of the phenotypic range at higher acclimation temperatures may be due to a convergence on an optimum Complex I activity that is not relevant at other temperatures. This convergence could be caused in part by an increase in uncoupling proteins incorporated into the inner-mitochondrial membrane resulting in greater proton-leak back across the inner-mitochondrial membrane. In ectothermic vertebrates, uncoupling proteins are found more often in animals acclimated to warmer temperatures resulting in higher proton leak (Trzcionka, Withers et al. 2008; Mueller, Grim et al. 2011). Likewise, cold acclimation reduces proton leak. The assay I used to measure Complex I does not inform about whether proton leak increased at higher acclimation temperatures. If proton leak is decreased in *F. heteroclitus* at lower temperatures as it is in other ectothermic vertebrates, it could result in reduced constraint on Complex I activity at lower temperatures. If there is a minimal level of Complex I activity that needs to be attained for proper electron-transport function, then it may be easier to reach when protons that are pumped across the membrane tend to stay there—the whole electron transport chain

is more efficient and variation in Complex I has less consequence. The extreme range of individual Complex I activities may reflect other cellular processes rather than any requirement for Complex I activity *per se*. Regulation of other cellular structures or processes that may affect Complex I activity (such as membrane composition, protein degradation and synthesis processes, transport of proteins into the mitochondria) may be relaxed resulting in greater variation in Complex I.

The ultimate goal of this research was to describe whether the variation observed in expression of Complex I subunit genes has an effect on the function of the assembled enzyme complex. It was expected that there would be variation in the expression of Complex I subunit genes based on previous studies of Complex I expression in humans, mice, *D. melanogaster* and *F. heteroclitus* (Oleksiak, Churchill et al. 2002; Storey, Madeoy et al. 2007; Garbian, Ovadia et al. 2010). In addition to the variation in gene expression, there is evidence that differential expression of mRNA affects metabolic phenotype in *F. heteroclitus* and that the relevant mRNAs are different among individuals: different groups of *F. heteroclitus* individuals utilize metabolic substrates with different efficiencies and co-opt different genes to accomplish this (Oleksiak, Roach et al. 2005). To determine if this was also the case for Complex I—if individuals in different populations are able to fine-tune the regulation of subunit mRNAs to achieve different levels of activity— microarray analysis of Complex I subunit genes was carried out. Complex I subunit mRNA abundance was quantified and compared among five populations of *F. heteroclitus* and one population of *F. grandis*. Of 39 Complex I subunits measured, one subunit, NDUFA5, showed a pattern of expression that may be indicative of evolution by natural selection, based on the assumption that individuals

from different species should be more different in terms of a trait than individuals from the same species if only neutral processes contribute to the within and among species variance. In addition to the low divergence in mRNA expression among populations is the low fold-change among individuals within populations. The lack of divergent expression among and within populations despite high inter-individual variation in Complex I activity suggests that Complex I activity is affected by small changes in expression of only a few Complex I subunits. When mRNA abundance and Complex I activity was regressed in each population different patterns of significance emerged. That is, there are mRNAs that appear to affect Complex I function, though these vary among populations. Thus, the extremely high adjusted R^2 values for regression of mRNA and Complex I activity suggest that mRNA expression does affect variation in Complex I activity but they are not regulated similarly in all populations.

The expectation for the correlation analysis was that it would help identify subunits with patterns of co-expression across taxa and populations of *F. heteroclitus.* The results of Chapter 2 suggest that co-expression of beta subunits (catalytic and mitochondrial subunits) is relevant to Complex I function. Few subunit pairs were found in more than one taxa, but the beta subunits were overrepresented among those significantly correlated subunits. I expected to observe this pattern again in *F. heteroclitus* liver mRNA expression. However, the high numbers of significant correlations between Complex I subunits observed across several taxa in Chapter 2 was not replicated in Chapter 5. Several factors could have caused this apparent contradiction. First, Complex I subunit expression is tissue specific (Garbian, Ovadia et al. 2010) and 2) and second, Complex I subunits that affect Complex I activity may vary

across taxa. The tissues examined in Chapter 2 were lymphocyte in human, liver in mice, whole body in *D. melanogaster* and heart in *F. heteroclitus.* The differences between the correlations observed in *F. heteroclitus* expression in Chapter 2 and Chapter 5 may be tissue specific—*F. heteroclitus* expression data came from cardiac tissue in Chapter 2, in Chapter 5 it is liver tissue. However, there were more significant correlations among Complex I subunits, relative to random genes, in mouse liver tissue. The frequency of significant correlations observed among *F. heteroclitus* populations in Chapter 5 is similar to the frequency of significantly correlated Complex I subunit pairs in *D. melanogaster* whole body mRNA expression data in Chapter 2. Because the only other measure for liver mRNA expression is from mice, it is impossible to distinguish between species-specific expression patterns and tissue specific expression patterns.

How does a large heteromeric protein complex arrive at a solution to the regulation problem that is so intricate? If the high inter-individual variation in Complex I activity is produced by surplus of assembled Complex I enzyme, then it may be that Complex I mRNAs are generally overexpressed also. Abundance of mRNAs may become important for Complex I after an environmental shift or range expansion. Because no clear pattern of transcription factor binding sites could be identified in human or mouse upstream of Complex I subunit genes, there may be also large variation in the upstream regulatory regions of *F. heteroclitus* (van Waveren and Moraes 2008). High standing variation in regulatory sites coupled with range expansion following glaciations (Duvernell, Lindmeier et al. 2008) could have resulted in multiple soft sweeps (Hermisson and Pennings 2005) leaving populations with different expression programs.

The two specific expectations outlined in the introduction—that variation in mRNA would exist and would explain variation in Complex I activity—were generally confirmed, but the findings were complex. Populations of *F. heteroclitus* did have significantly different Complex I activity, and mRNA expression did regress with this variation in activity. However, large variation in mRNA expression of Complex I subunits among populations of *F. heteroclitus* was not observed and the utility of the correlation analysis of Complex I subunit expression across taxa was not realized with the expression data from Chapter 5 Furthermore, the direction of the difference in Complex I activity was unexpected—higher activity was observed in southern fish—and that the mRNAs that regressed with Complex I activity were different in different populations.

Summary

This work establishes both that Complex I activity is biologically important and that variation in the expression of Complex I subunits affects the activity of the enzyme complex. The subunits of Complex I are well conserved across taxa—from the alpha-protobacterial ancestor to humans—and the enzyme has gained complexity (from 14-17 subunits to the current maximum of 45 in vertebrates) while maintaining its core function in ATP synthesis. Within and among populations of *F. heteroclitus,* Complex I activity shows a paradoxical amount of variation. The patterns and direction of variation among populations suggests that the variation in activity is biologically relevant, yet the amount of inter-individual variation is much higher than expected for a critical metabolic enzyme. This high inter-individual variation may reflect a relaxation of constraint on Complex I function under certain environmental conditions. When acclimated to 28°C, Complex I became insensitive to acute fluctuations in temperature. Future studies should

focus on identifying population specific mechanisms for Complex I regulation by examining the promoter regions of Complex I subunit genes and the trans-acting factors that regulate differential expression among populations. Additionally, a further exploration of the insensitivity to temperature fluctuation as an adaptive trait in southern fish could help explain the consequences of the apparent population specific regulation of Complex I subunit expression.

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