Gene by environmental interactions affecting oxidative phosphorylation and thermal sensitivity

Tara Z. Baris,1 Pierre U. Blier,2 Nicolas Pichaud,2,3 © Douglas L. Crawford,4 and Marjorie F. Oleksiak1
1Marine Biology and Ecology, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Miami, Florida; 2Department de Biologie, Université du Québec à Rimouski, Rimouski, Quebec, Canada; and 3Department of Chemistry and Biochemistry, Université de Moncton, Moncton, New Brunswick, Canada

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Baris TZ, Blier PU, Pichaud N, Crawford DL, Oleksiak MF. Gene by environmental interactions affecting oxidative phosphorylation and thermal sensitivity. Am J Physiol Regul Integr Comp Physiol 311: R157–R165, 2016. First published May 25, 2016; doi:10.1152/ajpregu.00008.2016.—The oxidative phosphorylation (OxPhos) pathway is responsible for most aerobic ATP production and is the only metabolic pathway with proteins encoded by both nuclear and mitochondrial genomes. In studies examining mitonuclear interactions among distant populations within a species or across species, the interactions between these two genomes can affect metabolism, growth, and fitness, depending on the environment. However, there is little data on whether these interactions impact natural populations within a single species. In an admixed Fundulus heteroclitus population with northern and southern mitochondrial haplotypes, there are significant differences in allele frequencies associated with mitochondrial haplotype. In this study, we investigate how mitochondrial haplotype and any associated nuclear differences affect six OxPhos parameters within a population. The data demonstrate significant OxPhos functional differences between the two mitochondrial genotypes. These differences are most apparent when individuals are acclimated to high temperatures with the southern mitochondrial genotype having a large acute response and the northern mitochondrial genotype having little, if any acute response. Furthermore, acute temperature effects and the relative contribution of Complex I and II depend on acclimation temperature: when individuals are acclimated to 12°C, the relative contribution of Complex I increases with higher acute temperatures, whereas at 28°C acclimation, the relative contribution of Complex I is unaffected by acute temperature change. These data demonstrate a complex gene by environmental interaction affecting the OxPhos pathway.

THE OXIDATIVE PHOSPHORYLATION (OxPhos) pathway is responsible for most aerobic ATP production and is the only metabolic pathway that involves both nuclear and mitochondrial encoded proteins. The OxPhos pathway has five enzyme complexes with ~89 proteins; the mitochondrial genome encodes 13 of these proteins, while the nuclear genome encodes 76. The interactions among the 89 proteins in the OxPhos pathway are likely to be sensitive to acute and chronic temperature exposures (55, 56). In Fundulus, OxPhos studies have provided insight into how exposures to acute and chronic (acclimation) temperature change alter physiology (4, 9, 14, 20). These studies have shown that the acclimation process influences acute temperature effects on OxPhos (4, 9). Baris et al. (4) show that while acclimation does not always have a significant effect on OxPhos functions, it alters the acute temperature response of F. heteroclitus. Specifically, when acclimated to a low temperature (12°C), the acute response was shallower at the lower range of acute test temperatures (12°C and 20°C) and when acclimated to a high temperature (28°C), the acute response was shallower at the higher range of acute test temperatures (20°C and 28°C) (4). Overall, studies of OxPhos in Fundulus support the hypothesis that acclimation tends to mitigate acute temperature effects on this pathway’s biochemistry and biophysics (55, 56).

In ectotherms, how the thermal environment affects OxPhos function depends on which population or mitochondrial genotype is assayed (1, 4, 14, 15, 20, 29, 32, 46, 55). The interactions between the environment and genotype (nuclear or mitochondrial) influence the evolution of nucleotide substitutions among OxPhos proteins (GxE) (2, 17, 27, 42, 45, 51, 63–65). What is surprising, and adds another layer of complexity, is that the thermal effects are affected not only by nuclear or mitochondrial genotypes, but also by significant interactions between these genomes due to mitonuclear epistasis (15, 16, 29, 35). For example, substituting Drosophila simulans mitochondrial into D. melanogaster’s nuclear background affects one inbred D. melanogaster line but not another at high temperatures, and these mitonuclear interactions affect the acute response (29, 35, 37). Similarly, in seed beetles, Callosobruchus maculatus, temperature-dependent metabolic rates depend on the interaction between the mitochondrial and nuclear genomes (1). These mitonuclear interactions that affect OxPhos are biologically important because they affect fitness and survivorship (3, 8, 15, 16, 18, 29, 35–37, 62). In general, these data suggest that mitonuclear interactions among species or divergent populations are likely to affect an organism’s physiology, and these interactions are environmentally dependent (36, 52, 53).

For northern and southern Fundulus heteroclitus populations, which are distributed along a steep thermal cline (~1°C/degree latitude), northern populations experience temperatures more than 12°C colder than southern populations. These northern and southern populations harbor two distinct mitochondrial haplotypes (mt-haplotypes) differing by five amino acid replacements (61). This genetic divergence between mt-haplotypes is influenced by a historical break at the Hudson River due to the last glaciation (49), allowing the evolution of the nucleotide differences between northern and southern populations (22). Importantly, F. heteroclitus has large populations with low migration rates and are adapted to local environmental conditions (7, 34). These demographic and environmental features are likely to affect the nuclear and mitochondrial
divergences among Fundulus populations (49). In addition to mt-haplotype divergence among F. heteroclitus populations, there are differences in the nuclear genome that alter enzymes and enzyme expression, which affect metabolism (11–13, 40, 41, 47–50). Because of the divergence among populations in both mitochondrial and nuclear genomes, it is difficult to ascribe the relative importance of mitonuclear interaction effects on physiology. Additionally, for animals assayed from different environments/populations, the importance of these mitonuclear interactions could be masked as a result of developmental effects (irreversible acclimation) or epigenetic effects, which could generate nonheritable differences. Therefore, we use a single population in northern New Jersey just south of the Hudson River, where individuals experience a similar environment. This population has both the “northern” mitochondrial haplotype, common in populations north of the Hudson River, and a “southern” mitochondrial haplotype, common in populations south of the Hudson River (24, 26). In this admixed population, both mt-haplotypes occur at high frequencies (5, 26), and while nearly all nuclear genes are randomly associated with these mt-haplotypes, several hundred have a biased frequency: 349 nuclear SNPs (61 with 1% FDR correction) have significantly different allele frequencies between mt-haplotypes that, in combination with the divergences in mt-haplotypes, affect cardiac OxPhos function (5). This significant bias in nuclear genotypes based on the mt-haplotype suggests epistatic adaptive evolution, and thus differences in OxPhos functions would be expected.

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

METHODS

Experimental Animals

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

Fieldwork was completed within publically available lands and no permission was required for access. F. heteroclitus does not have endangered or protected status, and small marine minnows do not require collecting permits for noncommercial purposes. All fish were captured in minnow traps with little stress and removed in less than 1 h. IACUC-approved procedures were used for acclimation and non-surgical tissue sampling. Fish were killed by pithing and decapitation, using procedures approved by IACUC.

Genotyping Mitochondria

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

High-Resolution Respirometry

Tissue permeabilization. Heart ventricles were dissected, cut into halves, and one half was placed into a muscle relaxation solution (10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) (23). The second half was saved for future RNA experiments. Hearts were cut anteriorly/posteriorly, and pieces were randomized into different acute temperature tests. Prior to measuring respiration, each tissue was permeabilized using 2.5 mg/ml saponin solution for 15 min, followed by 4 washes in respiration medium for 5 min each (44). Once permeabilized, tissues were immediately transferred to the respirometry chambers containing respiration medium Miro5 (5 mM EGTA, 3 mM MgCl2-6H2O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, 1% BSA, pH 7.1) and substrates to reach State 2 (pyruvate, glutamate, succinate).

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

After tissue addition to the respiration chamber containing pyruvate, glutamate, and succinate, State 2 (39, 45) respiration rates were reached. This was followed by the addition of ADP (5 mM, State 3), cytochrome c (10 μM, to check outer mitochondrial membrane integrity), FCCP (a mitochondrial uncoupler used to reach E State; 0.5 μM was added sequentially until activity no longer increased), rotenone (0.5 μM to block Complex I), malonate (5 mM to block Complex II), and antimycin A (2.5 μM to block Complex III). The rates obtained after inhibition of Complexes I, II, and III represent the residual oxygen consumption (i.e., the oxygen consumed by oxidative side reactions occurring in permeabilized fibers) and was used to correct all the respiration rates. Finally, TMPD and ascorbate (artificial substrate for Complex IV, 0.5 mM TMPD, and 0.2 mM ascorbate) (Table 1) were added to measure Complex IV activity. Background oxygen consumption levels that could arise from TMDP and ascorbate were not measured and thus, if substantial, could mask significant effects of acclimation and mt-haplotypes for Complex IV. Background respiration would not affect the other measures of OxPhos. It should be noted that malate, a Kreb’s cycle intermediate, was not used. When malate was added to test tissues, we observed no difference, or reduced reaction rates at any assay temperatures, and, therefore, excluded it from our experimental design.

LEAK ratio (ratio of State 2 and State 3) in this study was calculated as State 2/State 3. It is usually calculated as State 4/State 3, where State 4 is reached through inhibition of Complex V with oligomycin. However, we found that when oligomycin is used, subsequent E state measurements were always lower than State 3 (4). State 2 is the measure of oxygen consumption without ADP, while State 4 measures respiration where Complex V is blocked. Both measure respiration when Complex V cannot phosphorylate ADP to
ATP. With State 2, there may be endogenous ATPases, which would affect respiration measurements. However, because of the problems with oligomycin, we measured LEAK as State 2/State 3 (25).

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

The tissue from each chamber was recovered after respiration assays and homogenized, and total DNA was quantified from the homogenate using AccuBlue high-sensitivity dsDNA quantitation solution (BIOTIUM, Hayward, CA). All activity was normalized by DNA. OxPhos respiration rates are reported as pmol O2·s⁻¹·mg⁻¹ DNA. Although DNA concentration, protein concentration, and cell count have a linear relationship, DNA has been found to be the consistent way of normalizing metabolomic data across a range of cell numbers (58). Because we had small tissue amounts that we permeabilized before assays, we recovered the tissue from the respiration chambers to accurately quantify tissue amount. We normalized OxPhos values by DNA quantity, as this is a proportional measure to total mitochondrial DNA (33).

**Statistical Analyses**

A mixed general linear model analysis was performed with all factors: mt-haplotype, acclimation temperature, and acute temperature (JMP, SAS, Cary NC). Body mass (Tables 2 and 3) often affects metabolic physiology (19, 54). The effect of body mass was significant for our data on 155 individuals is large relative to those included in a study by Chung and Schulte (9). This variation in admixture effect of two mitochondria, or the potential effect of different nonspecific ATPases (where small amounts of ATPases would cause a large RCR). However, the presence of nonspecific ATPases and its potential effect on RCR, should not affect our analysis of OxPhos metabolic functions.

Table 2 provides sample sizes and body masses for the individuals used in the analysis for the mt-haplotypes. Body masses ranged from 1.1 to 12.3 g for the northern mt-haplotype individuals and from 1.2 to 13.3 g for the southern mt-haplotype individuals. Body mass often affects metabolic physiology (19, 54). The effect of body mass was significant.

### RESULTS

The six OxPhos parameters are quantified by measuring oxygen consumption rates with substrates, uncoupler, or inhibitor addition (Table 1): State 3 (ADP stimulated respiration), E State (uncoupled mitochondrial), Complexes I, II, and IV, and the LEAK ratio.

Cytochrome c addition revealed no damage to outer mitochondrial membrane (mean ratio of State 3/Cyt c = 1.01, SD = 0.09). A separate measure of functional coupling for OxPhos is respiratory control ratio (RCR) (State 3/State 2), which measures respiration dependency on ADP. We included all data with an RCR of 2.5 or higher (Fig. 1) (6, 31). The RCR for our data on 155 individuals is large relative to those included in a study by Chung and Schulte (9). This variation in RCR may reflect the large sample size (rare alleles), the admixture effect of two mitochondria, or the potential effect of different nonspecific ATPases (where small amounts of ATPases would cause a large RCR). However, the presence of nonspecific ATPases and its potential effect on RCR, should not affect our analysis of OxPhos metabolic functions.

**Table 2. Mt-haplotype sample sizes and body masses**

<table>
<thead>
<tr>
<th></th>
<th>North mt-Haplotype</th>
<th>South mt-Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample size</td>
<td>64</td>
<td>83</td>
</tr>
<tr>
<td>Sample size 12°C Acc</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>Sample size 28°C Acc</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>Average body mass, g</td>
<td>4.41</td>
<td>4.24</td>
</tr>
<tr>
<td>SE of the Mean</td>
<td>0.32</td>
<td>0.25</td>
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</table>

Fig. 1. Box plot of respiratory control ratio for each “group” of individuals tested. The first term for the group label is the mt-haplotype, followed by acclimation temperature, and then the acute temperature. Plot includes mean, 25%, and 75% quartile and range.
for State 3, E State, Complex I, and Complex II (Table 3). We used body mass as a covariate in our model to avoid potential biases due to body mass.

We performed a mixed general linear model analysis using acclimation temperature, population, and acute temperature change and their interactions (Table 3). Acute temperature change had a significant effect on all measured parameters except for Complex II. Acclimation only had a significant effect on State 3 and Complex IV.

Figure 2 displays the general mixed-model data summarized in Table 3. Mt-haplotype significantly affected all parameters except Complex IV and LEAK ratio. No interactions were significant.

The initial mixed general linear model reveals that mt-haplotype and acute temperature change are significant factors for a majority of the parameters tested. Although we do not see a significant interaction term between acclimation, acute temperature, and mt-haplotype, in Fig. 2, mt-haplotype and acute temperature change seems to be affected by acclimation temperature. We believe that the lack of a significant interaction is due to a lack of power and is, therefore, a type II error. We wanted to understand the interaction seen in Fig. 2 and, therefore, performed subsequent analyses. A mixed general linear model was run by mt-haplotype and acclimation temperature with body mass as a covariate. These subsequent analyses revealed that acute temperature effects differed between the northern and southern haplotypes and acclimation temperature (Table 4). At the 12°C acclimation temperature, acute temperature change had a significant effect on Complexes I and IV and LEAK ratio. For the northern mt-haplotype individuals acclimated to 12°C, acute temperature change had a significant effect on State 3, Complex IV, and LEAK ratio. There was a stark contrast on the influence of acute temperature change between haplotypes at the 28°C acclimation temperature. For the northern mt-haplotype, individuals acclimated to 28°C, acute temperature change never had a significant effect on any of the measured parameters. For the southern mt-haplotype individuals, acute temperature change affected nearly all measured parameters (with the exception of LEAK ratio). When a multiple-comparison correction was performed, this pattern was only significant for State 3 ($P < 0.0125$, Table 4).

To understand how different OxPhos components affect overall respiration, we examined the roles of Complex I and Complex II, the two entry points for electrons. Complex I’s response to acute temperature looks similar to E-State for all four mt-haplotype/acclimations combinations (Fig. 2). We provide a relative measure of Complexes I’s and II’s contributions to OxPhos by dividing each by the total E State activity (Fig. 3). A mixed general linear model was run with arcsin transformed data and a body mass as a covariate, to test whether acute temperature change was significant for both mt-haplotype types at the two different acclimation temperatures. At the 12°C acclimation temperature, Complex I’s contribution increases with increasing acute temperatures, whereas Complex II’s contribution decreases (Fig. 3). This trend is evident in both the northern and southern mt-haplotype individuals, although it is only significant for the southern mt-haplotype individuals ($P < 0.006$ for effect of acute temperature for CI and CII at 12°C acclimation, Fig. 3). At the 28°C acclimation temperature, no differences were observed in the relative contributions for Complexes I and II among acute temperatures for either the northern or southern mt-haplotype.

**DISCUSSION**

We investigated how mitonuclear genotypes and temperature affect cardiac metabolism adaptation in an admixed *F. heteroclitus* population by examining the effect of two acclimation temperatures and three acute temperatures. Our mixed-model analysis with all factors present and mass as a covariate revealed that acclimation temperature only had a significant effect on State 3 and Complex IV. Acute temperature change had a significant effect on all measured parameters except for Complex II, and mt-haplotype had a significant effect on State 3, E State, Complex I, and Complex II. Although there were no significant interaction effects in our mixed-model analysis, upon further analyses, there was an interesting pattern revealed for State 3: the acute temperature effects were dependent on mt-haplotype. Thus, the southern mt-haplotype had acute effects at both acclimation temperatures, but the northern mt-haplotype at 28°C acclimation had no acute temperature effect ($P$ value = 0.44; Table 4 and Fig. 2) and at 12°C acclimation, there was little acute temperature effect ($P$ value = 0.09, Table 4, Fig. 2). This pattern of the northern mt-haplotype having no significant acute temperature effect at 28°C is observed for all OxPhos parameters.

Previous studies comparing a northern *F. heteroclitus* population to a southern one have shown a significant difference in mitochondrial function at different acclimation and acute temperatures (9, 20). In one study, differences between ME and GA populations were more apparent when individuals were acclimated to low temperatures (5°C and 15°C vs. 25°C) and assayed at temperatures above 25°C (20). Baris et al. (4) found a similar response between Maine and Georgia, where differences between populations are

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**Table 3. Three-way general mixed model for acclimation (Acc), mt-haplotype (mt-hap), and acute temperature (Acute) and all interactions**

<table>
<thead>
<tr>
<th></th>
<th>Body Mass Effect</th>
<th>Treatment P Values</th>
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<tbody>
<tr>
<td>Three-way</td>
<td>Mass Slope</td>
<td>$P$ Value</td>
</tr>
<tr>
<td>State 3</td>
<td>0.185</td>
<td>0.0257</td>
</tr>
<tr>
<td>E State</td>
<td>0.209</td>
<td>0.0327</td>
</tr>
<tr>
<td>Complex I</td>
<td>0.1359</td>
<td>0.0226</td>
</tr>
<tr>
<td>Complex II</td>
<td>0.0924</td>
<td>0.0295</td>
</tr>
<tr>
<td>Complex IV</td>
<td>−0.1514</td>
<td>0.6628</td>
</tr>
<tr>
<td>LEAK 2/3</td>
<td>−0.0082</td>
<td>0.4758</td>
</tr>
</tbody>
</table>

Significant $P$ values are in bold. Body mass slopes and slope significances are also listed.
restricted to low acclimation temperatures and are larger at higher acute temperatures, and activity for Georgia is greater than Maine.

In this study, we use a freely interbreeding, naturally occurring admixed population where both southern and northern mt-haplotypes have nearly equal frequencies (5, 26). In a separate study, this population was used to study individual survivorship under hypoxic conditions, and no mitochondrial effect was found (21). For our measurements on OxPhos, we find that mt-haplotype does significantly

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**Fig. 2.** Complete data set for six measured parameters. Graphs are separated by acclimation temperature. Within each panel are the population and the acute temperature change of measured individuals. Black and gray lines represent the southern mt-haplotype and the northern mt-haplotype, respectively. See Table 3 for P values. Means are graphed, and error bars represent standard error. Lines are to aid in the identification of the two mt-haplotypes and acute effects and are not a regression.
Affect four of the six OxPhos parameters tested (Table 3). Importantly, the effect of mt-haplotype is most obvious in the response to acute temperature change at the high acclimation temperature for State 3, E State, Complex I, and Complex II (Fig. 2). While individuals with the southern mt-haplotype show a significant relationship between overall mitochondrial respiration (State 3) and acute temperature change, individuals with the northern mt-haplotype show almost no response (Table 4). This is consistent with results from Chung and Schulte (9), who found that a northern population from Nova Scotia, acclimated to 33°C showed reduced overall State 3 and reduced acute temperature effects. These results are also similar to those in Baris et al. (4), in which a population from Maine with the northern haplotype and one from Georgia containing the southern haplotype were used to study the effects of acclimation and acute temperature change. For the Maine individuals, the effect of acute temperature was weaker than those for the Georgia population. Specifically, when acclimated to 12°C, acute temperature effects were stronger for the Georgia population than the Maine population. Interestingly, the differences between haplotypes (unlike northern Maine and southern Georgia) are more apparent at high and not low acclimation temperatures because the northern mt-haplotype is less sensitive to acute temperature change.

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

### Table 4. P values for acute temperature effects by mt-haplotype and acclimation temperature

<table>
<thead>
<tr>
<th></th>
<th>North mt-Haplotype</th>
<th>South mt-Haplotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12°C</td>
<td>28°C</td>
<td>12°C</td>
<td>28°C</td>
</tr>
<tr>
<td>State 3</td>
<td>0.091</td>
<td>0.444</td>
<td>0.017*</td>
<td>0.012**</td>
</tr>
<tr>
<td>E State</td>
<td>0.109</td>
<td>0.627</td>
<td>0.223</td>
<td>0.026*</td>
</tr>
<tr>
<td>Complex I</td>
<td>0.047*</td>
<td>0.616</td>
<td>0.237</td>
<td>0.051*</td>
</tr>
<tr>
<td>Complex II</td>
<td>0.423</td>
<td>0.570</td>
<td>0.886</td>
<td>0.019*</td>
</tr>
<tr>
<td>Complex IV</td>
<td>0.024*</td>
<td>0.161</td>
<td>0.009**</td>
<td>0.001**</td>
</tr>
<tr>
<td>LEAK 2/3</td>
<td>0.001**</td>
<td>0.274</td>
<td>0.0001**</td>
<td>0.226</td>
</tr>
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</table>

Bolded values denote significance. *P < 0.05. **P < 0.0125 for multiple test correction.

An interesting pattern revealed from our data is the difference between mt-haplotypes in response to acute temperature change when acclimated to 28°C. An explanation for the State 3 differences among mt-haplotypes at the different acclimation temperatures could reflect differences in thermal performance curves (TPCs, Fig. 4). TPCs can vary on the basis of taxa and acclimation effects (reviewed in Ref. 55). *F. heteroclitus* follows the typical predictions of the Arrhenius equation, which determines the shape of the TPC based on thermodynamics (reviewed in Ref. 55). *F. heteroclitus*' TPC was more thoroughly examined by measuring acute effects at many temperatures between 2°C and 5°C and 37°C (9, 20). In general, in those and other studies in ectotherms, cold acclimation shifts the TPC curve to the left (30, 57). Here, we presented the acute effects measured at only three temperatures, yet we suggest that these data reflect a left shift, and a change in shape in TPC curves for both mt-haplotypes when acclimated to 12°C (Fig. 4). The difference in the acute effect between mt-haplotypes, where the northern mt-haplotype is insensitive to acute temperature change at 28°C acclimation temperature, can be ex-

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**Evolutionary Physiology: Genetic Difference in the Response to Acute Temperatures**

Fig. 3. Relative contributions of Complex I and Complex II. A: northern mt-haplotype. B: southern mt-haplotype. Within these graphs, the upper panel shows relative Complex I activity (CI/E State) at both acclimation temperatures. Lower panel shows the relative Complex II activity (CII/E State) at both acclimation temperatures. Statistics were performed on arcsin-transformed data, and P values denote effect of acute temperature.
plained by a more extreme right shift relative to the southern haplotype. Thus, the data demonstrating that sensitivity to acute temperature change is affected by mt-haplotype and acclimation temperatures can be explained by a genetic difference that alters the TPC.

It is difficult to determine whether the response to acute temperature difference between northern mt-haplotype and southern mt-haplotype individuals acclimated to 28°C is necessary beneficial for one group. One could argue that as temperature increases, so do ATP demands, and that the southern mt-haplotype is able to fulfill those demands, while individuals with the northern mt-haplotype fail to do so. This would lead to a decrease in aerobic scope at high temperatures, which could induce a decline in performance traits for northern mt-haplotype individuals (55). Alternatively, one can argue that there may be benefits to lower respiration rates seen in the northern mt-haplotype. A temperature increase, which results in a metabolic rate increase, also requires an increase of nutrient assimilation. A mismatch between metabolic rate and nutrient uptake may result in decreased performance. Lower respiration rates may be beneficial when faced with lower food availability, which in the long term can lead to low substrate availability. Furthermore, increased mitochondrial respiration could result in increased reactive oxygen species (ROS) formation that can damage cellular macromolecules (38, 55, 59). Chung and Schulte (9) found that ROS production increased with assay temperature in a northern population of F. heteroclitus but do not have similar data for a southern population. We are simply hypothesizing that individuals with a northern mt-haplotype acclimated at a high temperature (28°C), may have less ROS production than their southern mt-haplotype counterparts, due to insensitivity to acute temperature change. Most likely, it is not that one mt-haplotype’s TPC is consistently superior, because a consistent difference between haplotypes in performance that affects fitness would selectively remove one of the haplotypes. But rather, these different TPCs vary in their fitness effects depending on their environment. Of relevance, these individuals were captured together in the same estuarine creek. Therefore, it is unreasonable to suggest spatial variation in the environment. Instead, temporal variation is common in the F. heteroclitus environment. We suggest that the large temporal environmental variation associated with estuarine environments is effectively selecting for both nuclear-mitochondrial genotypes depending on the performance advantage in different environments. This balancing selection occurs, even though the conditions for maintaining mitochondrial polymorphism are very restricted [depending on pleiotropy, allele effect size, and consistency (10, 51)].

Speculation on Divergence Between Mt-haplotypes and the Role of Population Genetics

In this admixed New Jersey population, the two mt-haplotypes have unexpected and significant divergence in nuclear allele frequencies at 349 loci (61 loci after 1% FDR correction), that is most parsimoniously explained by evolution due to natural selection (5).

In addition to differences in nuclear allele frequencies in the admixed New Jersey population, there is another surprising finding that is dependent on the mt-haplotype (5): the differences in OxPhos metabolism between mt-haplotypes within a population are larger than the differences between northern Maine and southern Georgia populations (4). This is unexpected because the Maine and Georgia populations have different mt-haplotypes with the same nonsynonomous substitutions found within our admixed New Jersey population. We would have predicted that populations that are geographically separated would be more phenotypically different than individuals from a single population subdivided by mt-haplotype. This could be attributed to a greater effectiveness of natural selection between isolated populations than within a population; natural selection will act on smaller selection coefficients between isolated populations (10, 60). We speculate that because natural selection may not act on small selection coefficients in the admixed New Jersey population, but can on Maine and Georgia, potentially compensatory loci that differ between Maine and Georgia are not different between mt-haplotypes in New Jersey. Thus, we can speculate GxG (genome by genome) interactions as a reason for New Jersey individuals with the northern mt-haplotype having a different TPC than Maine individuals with the same mt-haplotype. This speculation is supported by the observation that the 349 nuclear genes with significant allele frequency differences are upstream of the
OxPhos pathway and that individuals with mixed nuclear backgrounds are phenotypically intermediate (5).

**Perspectives and Significance**

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

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


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