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The effects of mitochondrial genotype on hypoxic survival and gene expression in a hybrid population of the killifish, *Fundulus heteroclitus*

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Abstract

The physiological link between oxygen availability and mitochondrial function is well established. However, whether or not fitness variation is associated with mitochondrial genotypes in the field remains a contested topic in evolutionary biology. In this study we draw on a population of the teleost fish, *Fundulus heteroclitus*, where functionally distinct subspecies hybridize, likely as a result of past glacial events. We had two specific aims: 1) to determine the effect of mtDNA genotype on survivorship of male and female fish under hypoxic stress; 2) to determine the effect of hypoxic stress, sex and mtDNA genotype on gene expression. We found an unexpected and highly significant effect of sex on survivorship under hypoxic conditions, but no significant effect of mtDNA genotype. Gene expression analyses revealed hundreds of transcripts differentially regulated by sex and hypoxia. Mitochondrial transcripts and other predicted pathways were among those influenced by hypoxic stress, and a transcript corresponding to the mtDNA control region was the most highly suppressed transcript under conditions of hypoxia. An RT-PCR experiment on the control region was consistent with microarray results. Effects of mtDNA sequence variation on genome expression were limited, however a potentially important epistasis between mtDNA sequence and expression of a nuclear-encoded mitochondrial translation protein was discovered. Overall, these results confirm that mitochondrial regulation is a major component of hypoxia tolerance and further suggest that purifying selection has been the predominant selective force on mitochondrial genomes in these two subspecies.

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Supporting Information: Supplement 1: Figure S1.

Supplement 2: Supporting tables including: information on fish used in survival analysis; raw data for microarray analysis; results of mixed models with statistics; enriched GO terms for hypoxia and sex terms; hybridization differences due to SNPs in the probes. The first tab of the spreadsheet is a key to other tabs.

Data Accessibility: DNA Sequences: Genbank accessions JF895674-JF895753

Survivorship Analysis Data: Uploaded as online supporting information.

Gene Expression Data: Uploaded as online supporting information. Array data has also been deposited at the EMBL-EBI ArrayExpress database.

Keywords

Mito-nuclear interactions; Ecological genomics; mtDNA phylogeography; Oxygen availability; microarray

Introduction

The increasing magnitude and spatial extent of environmental hypoxia has serious implications for coastal ecosystems and commercially important fisheries (Wu 2002; Diaz & Rosenberg 2008). Despite a suite of behavioral and physiological compensatory mechanisms, oxygen availability is known to influence fish survivorship and reproduction, especially in cases of repeated and prolonged exposures (Wu et al. 2003; Landry et al. 2007; Martinovic et al. 2009; reviewed in Wu 2009). The consequences of local low oxygen availability can be offset by plastic physiological responses and evolutionary adaptation in fish species (Love & Rees 2002; Chapman et al. 2000; Timmerman & Chapman 2004; Everett & Crawford 2010). In fact, in the phylogeny of fishes, increased likelihood of hypoxic exposure is correlated with instances of evolved tolerance (Farwell et al. 2007; Mandic et al. 2009); and across fish lineages there are remarkably diverse hypoxia tolerance adaptations (Hochachka & Somero 2002). Understanding the genetic basis underlying adaptive responses to oxygen availability displayed by some fish species, but not others, has become a critical issue in environmental physiology.

Direct study of the effects of mitochondrial genetic variation on fitness traits under hypoxia is a logical step toward better understanding resilience to hypoxic stress, but has not, in our knowledge, been attempted in fish species. Since mitochondrial processes consume greater than 95% of oxygen available to fish when dissolved oxygen is not limiting, one compensatory strategy for hypoxia-stressed fish is the suppression of mitochondrial function (Richards 2009). Furthermore, the physiological connection between oxygen availability and mitochondria makes hypoxia a logical candidate for an abiotic stress that may reveal fitness differences among mitochondrial genotypes (Dowling et al. 2008). Typically, aerobic organisms confronted with severe hypoxic conditions suppress mitochondrial biogenesis and decrease expression of genes with mitochondrial functions. These genes are encoded on both nuclear and mitochondrial genomes, and there is evidence for fitness consequences when coordination between the genomes breaks down (reviewed in Ballard & Rand 2005; Dowling et al. 2008). Recent literature suggests more attention needs to be paid to the consequences of mitochondrial non-neutrality, both functionally and in the context of phylogeographic inferences made from mtDNA sequence (Ballard & Melvin 2010; Wares 2010).

In addition to suppression of mitochondrial function, reduction in overall metabolic rate and organismal activity is a general approach for tolerating hypoxic conditions (Richards 2009). These mechanisms can be coordinated through gene expression changes in response to hypoxic stress (Nikinmaa & Rees 2005; Zhang et al. 2009). Several important gene pathways, including those activated through the transcription factor hypoxia-inducible factor (HIF), contribute to the physiological response to low oxygen exposure (Wang & Semenza 1993; Hochachka & Somero 2002; Nikinmaa & Rees 2005). Thus, transcriptomic approaches are an appropriate tool for investigating hypoxia tolerance mechanisms, and variation among lineages of fish in their ability to respond to hypoxia.

To test hypotheses related to gene expression and the role of mitochondria in hypoxia tolerance, we take advantage of a population of the Cyprinodontiform, *Fundulus heteroclitus*, composed of hybrids of two subspecies that differ in physiological traits, such

as thermal optima (Crawford & Powers 1992; Powers & Schulte 1998; Fangue et al. 2006, 2009). *F. heteroclitus* distributions have been strongly influenced by past glaciations resulting in a secondary hybrid zone near the mouth of the Hudson River (Ropson et al. 1990; Adams et al. 2006; Duvernell et al. 2008). Coastal populations on either side of this boundary are genetically and morphologically distinct subspecies, with *F. h. heteroclitus* to the south and *F. h. macrolepidotus* to the north (Able & Felley 1986), and adaptive divergence in gene expression has been detected among subspecies (Whitehead & Crawford 2006; Oleksiak et al. 2002). Our focal population for this analysis is located approximately 70 kilometers south of the Hudson River, at the center of the mtDNA cline, which has equal representation of northern and southern mitochondrial genomes (Gonzalez-Vilasenor & Powers 1990; Haney et al. 2009). However, microsatellite-based Bayesian clustering by Duvernell et al. (2008) shows that our focal population has approximately 80% admixture proportion for southern nuclear alleles. As a result, the north and south mtDNA genotypes segregate on a mixed nuclear genetic background that is predominantly southern alleles (Flight & Rand, unpublished data).

Metabolic physiology differs between subspecies of *F. heteroclitus*, including differences in mitochondrial properties (Fangue et al. 2009). However, specific contributions of mtDNA encoded mitochondrial proteins are difficult to infer from mitochondria isolated from distinct northern and southern populations. This is because the majority of the proteins involved in mitochondrial function are encoded by the nuclear genome, and clinal variation in many nuclear loci is well established in this pair of subspecies. Therefore, isolated mitochondria from northern and southern populations also differ in nuclear proteins due to allelic variation along the latitudinal cline. By selecting fish from the hybrid zone population we sought to control for the nuclear gene differences present in a north versus south comparison - and can test for this in the future using nuclear markers. Furthermore, because the mtDNA cline is shifted south of the nuclear cline midpoint, the fish that we have collected for these experiments should have a predominantly southern complement of nuclear alleles. Accordingly, under the mito-nuclear co-evolution hypothesis, we predict that individual fish with southern mtDNA types will have greater fitness than fish with northern mtDNA types (Rand 2001; Rand et al. 2004). We specifically test the hypotheses that genetic variation in the mitochondrial genome contributes to survival phenotypes and that mitochondrial genotypes influence gene expression differences under hypoxic stress.

Materials and Methods

Animal Collection and Care

All animals used in these experiments were collected using seine nets in Northern Barnegat Bay, Mantoloking, New Jersey (40° 02' 26.67" N 74° 03' 55.08" W) in the summer and fall of 2007. This site is in close proximity to the Holiday City locality (TOMNJ) recorded in Duvernell et al. (2008). Fish were transported to the U.S. EPA Atlantic Ecology Division (AED) laboratory in Narragansett, Rhode Island, USA, where they were held at ambient temperatures in flowing seawater aquaria for at least 3 months prior to any exposure to minimize environmental effects. During their time in the laboratory fish were held on a 12:12 light cycle and fed *Artemia* and tetra flakes. There was minimal mortality during the acclimation period. At least one month prior to low dissolved oxygen exposures water temperature was increased to 20°C at a rate of 2°C/week. Animal care was in accordance the standard practices for the U.S. EPA Atlantic Ecology Division.

Genotyping and Survival Analysis

To evaluate survivorship under conditions of hypoxic stress fish were exposed to acute levels of hypoxia at the U.S. EPA AED laboratory. Assays were run in the winter of 2008.

Fish were not fed on the day of exposure. Male and female *F. heteroclitus* were selected from the holding aquaria and placed in individual, clear, 180 ml polycarbonate cylinders, which received flowing seawater at a mean of approximately 120 ml/min. All flows were generated from 5 bins receiving computer controlled syringe injections of fully oxygenated and anoxic seawater. Each bin provided hypoxic water to 8 specimens, with cylinders housed in 4 clear glass tanks (4 tanks per bin, 2 specimens per tank). Anoxic water was produced by vacuum-degassing with the addition of CO₂ via bubbling to maintain pH (for description of the system see: Miller et al. 1994; Coiro et al. 2000). For the survival analysis fish were exposed to a mean value of 0.645 mg/L ($\sigma = 0.027$) dissolved oxygen generated from the 5 mixing bins, which was confirmed by the average of Winkler titrations (Clesceri et al. 1998) in triplicate for each mixing bin. Four to 8 control fish were also placed in cylinders receiving seawater with standard dissolved oxygen levels (mean = 6.68 mg/L, $\sigma = 0.247$) generated from a single mixing bin in each exposure. Four replicate blocks of 40 fish were run with 20 males and 20 females each plus controls. Seawater temperature in the exposure experiment averaged 21.6°C ($\sigma = 0.443$). Mortality in the clear cylinders was observed each hour for 25 consecutive hours, at which point survivors were euthanized with tricaine methanesulfonate (Finquel, Argent Laboratories, Redmond, Washington, USA). For a portion of the samples, those run on the first day, mortality was not monitored until 15 hours after the start of treatment. For this reason, interval regression with left and right censoring was used in statistical analyses. Following mortality events, masses and standard lengths of each fish were recorded and fish were frozen at -80° C for subsequent genetic analysis.

To determine the mtDNA genotype of each fish, DNA was extracted from the caudal peduncle using phenol-chloroform extraction (Sambrook et al. 1989). PCR amplification of approximately 408 base pairs of the control region of the mtDNA was carried out following (Haney et al. 2009). The primers FHCRF1 (5' CCC CAC CCC TAG CTC CCA AAG C 3') and FHCRR1 (5' GTC CCT GAC CAT CAA TAA ACG TAT TCA 3') were used to amplify the mtDNA control region. PCR reactions were done in 25 μ l volumes with 0.5 μ l of 10 μ M primer stocks, 2 μ l of 5 μ M dNTPs, 0.5 to 1 units of Choice™ Taq (Denville Scientific) and 2.5 μ l of the provided 10X buffer. Thermal cycling conditions consisted of 3 minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. The final extension was 5-10 minutes at 72°C. Northern and southern genotypes of mtDNA were distinguished based on RFLP analysis of a cut site polymorphism in the control region identified from the data in Haney et al. (2009). A fixed polymorphism between northern and southern types disrupts an ACGT HpyCH4IV cut site present on the reverse strand of the full *Fundulus* mitochondrial genomes from Whitehead (2009). Southern fish are fixed for a cytosine 76 base pairs from the 3' end of the amplicon, whereas northern fish are fixed for thymine. For digestion of control region amplicons 1.2 units of HpyCH4IV (New England BioLabs, Ipswich, Massachusetts, USA) and 1.5 μ l of the provided buffer were added to the PCR amplicon to make a total volume of 15 μ l. Digestions were carried out for at least 3 hours at 37 °C. All 15 μ l of the resulting digestion were run on 3% agarose gels to resolve mitochondrial type. The resulting banding pattern of the HpyCH4IV digested northern mtDNA type was two bands at approximately 333 base pairs and 76 base pairs. The southern mtDNA type was cut twice resulting in bands at approximately 333, 60 and 17 base pairs. Sequencing of 40 individuals for ND2 and ND5, and subsequent digests of ND2 with HindIII confirmed that the cut site diagnostic was 100% successful at resolving northern and southern haplotypes in fish from this collection site. Thus fish were divided into four groups: northern males, southern males, northern females, southern females.

Gene Expression Exposure and Genotyping

Preparation of tissues for gene expression analyses followed a similar protocol to exposure of fish for survival analysis. Assays were run in the winter and spring of 2008. Fish used in this analysis were exposed to two levels of dissolved oxygen. Normoxic conditions had a mean of 7.054 mg/L ($\sigma = 0.055$); hypoxic conditions had a mean of 0.655 mg/L ($\sigma = 0.018$), which was selected based on the prior hypoxia survival trial. Exposures for gene expression analyses were carried out at a mean of 21.3°C ($\sigma = 0.945$). Equal numbers of females and males were exposed in each of four replicate blocks used in this analysis and were weighed and measured prior to treatment. Following five hours of exposure, which was predicted to be non-lethal based on the hypoxia survival trial, fish were removed from the polycarbonate cylinders and snap frozen in liquid nitrogen. Fish livers were immediately removed and placed in RNeasy (Applied Biosystems, Foster City, California, USA). Tissues were at room temperature in RNeasy for no longer than 12 hours, 4°C for no longer than 4 days, and at -20°C for long-term storage.

As with survival analysis, preparation of fish for gene expression was done blind with respect to mtDNA genotype. Following exposure, fish were genotyped at the diagnostic site of the mtDNA control region using the same protocol described for survival analysis. The mtDNA genotype of fish used in the microarray hybridization was confirmed via sequencing of 1119 base pairs of the ND2 locus and 1128 base pairs of the ND5 locus, both of which are located on the mitochondrial genome. Primers for ND2 and ND5 amplifications were designed based on the aligned sequences of Whitehead (2009) (FhetND2F 5' CCA TAC CCC GAA CAT GTT GG 3'; FhetND2R 5' GAA GGC TCT TGG TCT GAG TG 3'; FhetND5F 5' CTC CAA GTA GCA GCT ATG CAT C 3'; FhetND5R 5' CCA GAT GGT GTA TAC CGC CTA 3'). PCR reaction volumes were the same as described for the control region. Cycling conditions were slightly different as both reactions had extension steps for 1.5 minutes. The annealing temperatures for ND2 and ND5 were 63 and 56°C, respectively. The resulting ND2 and ND5 products were sequenced in both directions.

Sequence Analysis

Mitochondrial sequences were inspected for quality and aligned in Sequencher v.4.5 (Gene Codes, Ann Arbor, Michigan, USA). The resulting alignments were 965 base pairs for ND2 and 882 base pairs for ND5 and have been deposited in Genbank (JF895674-JF895753). Summary statistics of DNA sequence polymorphism were calculated in DnaSP v.5.1 (Librado & Rozas 2009). A neighbor-joining tree of haplotypes was made in MEGA v.4.0.2 (Tamura et al. 2007) with 500 bootstrap replicates under a Tamura-Nei model. The sequence alignment file concatenated both proteins and included *F. grandis* and *F. diaphanus* sequences from Whitehead (2009) as outgroups. Dating of the age of the mitochondrial split was done in BEAST v 1.5.4 (Drummond & Rambaut 2007) (Supporting Information).

RNA Preparation and Microarray Hybridization

RNA was extracted from fish livers using Qiagen RNeasy extraction kits (Valencia, California, USA) with modifications. Liver tissue was removed from RNeasy and placed in a 1.5ml tube with 500µl of Trizol reagent (Invitrogen, Carlsbad, California, USA) and a single sterile bead. Tissue lysis was done in a Qiagen TissueLyser set to 30Hz for 2 minutes. The resulting homogenate was pipetted onto Phase Lock Gel tubes (5prime, Gaithersburg, Maryland, USA) and 100µl each of chloroform and DEPC water were added. Phase Lock tubes were centrifuged at 12,000g for 15 minutes at 4°C. The resulting supernatant was mixed with an equal volume of 70% ACS grade ethanol and centrifuged at 12,000g for 15 seconds in an RNeasy spin column. The use of Phase Lock tubes with Trizol allows for more efficient separation of aqueous and organic phase and facilitates isolation of higher quality RNA (Flight, unpublished observation). The remaining steps for RNA extraction

followed manufacturer's instructions for the RNeasy kits, beginning with the addition of buffer RW1.

Total RNA extractions were quantified using a NanoDrop ND1000 spectrophotometer (ThermoScientific, Wilmington, Delaware, USA). Approximately 150 ng of RNA from each sample was run in an Agilent Bioanalyzer (Santa Clara, California, USA) to check for evidence of RNA degradation. All samples used in the hybridizations were from high quality RNA preparations. When possible 2µg of total RNA was used for transcriptome amplification. Two of 40 samples were not at high enough concentration for 2µg to be used. Starting material for both these samples was greater than 1.6µg and sufficient final yield was obtained. Transcriptome amplification was done using MessageAmp II kits from Ambion (Austin, Texas, USA) with modification. Purification of single stranded DNA and the resulting aRNA amplification was improved when a Qiagen MinElute kit was substituted for the spin columns provided with the MessageAmp II kit. Final aRNA concentration was quantified using a NanoDrop and rerun on the Bioanalyzer to determine quality. As before, only high quality amplifications were used for hybridization.

Purified aRNA was coupled with Alexa fluor dyes (Alexa fluor 555 and 647; Molecular Probes, Inc.) and competitively hybridized to custom oligonucleotide microarrays (Agilent eArray Design ID 021434 (Whitehead et al. 2010)) in a loop design. Microarray probes were designed using OligoWiz software (Wernersson et al. 2007) from *F. heteroclitus* ESTs (Paschall et al. 2004) and additional *F. heteroclitus* protein sequences available from the National Center for Biotechnology Information (NCBI), with preference for probe position toward the 3' end of target sequences. Targets included all *F. heteroclitus* protein sequences in NCBI, all EST sequences that annotate with significant (e threshold 10^{-6}) sequence similarity following BlastX in SwissProt, and additional ESTs lacking annotation but with contig length greater than 300 bases. The custom microarray included probes for 6,800 elements, each printed in duplicate, plus control elements. Five biological replicate fish were hybridized per treatment, including a dye-swap for each replicate sample. Hybridized slides were incubated at 60°C for 18 hrs in a SureHyb microarray hybridization chamber (Agilent Technologies, Santa Clara, CA). Slide images were captured using the Packard Bioscience ScanArray Express (PerkinElmer Inc., Waltham, MA) and images processed in Imagene (Biodiscovery Inc., El Segundo, CA).

Statistical Analysis

Length, mass and Fulton's condition factor (K), calculated as $K = \text{Mass} / \text{Standard Length}^3$ following Love & Rees (2002), were compared among the individual fish based on sex and mtDNA genotype using type I ANOVA. Median lethal time estimates were calculated with the package "survival" (Therneau & Lumley 2009) in R. Survivorship curves were plotted using R software and interval censored data. Significance testing for sex, mtDNA genotype, and size was done with interval regression in the "survival" package. The day of exposure was included as a factor in the model and tested for significance. A Weibull distribution was used to model the survival function. Likelihood ratio tests were done to assess significance of terms in nested models. Additional analyses with only right censoring were run with the first block excluded.

For the microarray analysis 40 *F. heteroclitus* livers were drawn from an orthogonal combination of sex (male or female), oxygen levels (normoxia or hypoxia), and mtDNA genotypes (northern or southern), resulting in 8 categories with 5 biological replicates each. Hybridization was done in a balanced loop design with a nested dye swap so that each individual was hybridized with both dyes. Median spot intensities on the two color arrays were $\log_2$ transformed and lowess-normalized using JMP-Genomics (SAS Institute Inc., Cary, North Carolina, USA). Linear mixed model normalization was run to account for

variation associated with dye and array effects, which were treated as fixed and random, respectively.

Normalized data were analyzed using a mixed model approach in JMP genomics. Sex, oxygen level, mtDNA genotypes and the interactions between these terms were included as fixed effects in the model. The additional factors of block, array, dye, and technical replicate nested within biological replicate, were included as random effects. Q-values were calculated to estimate the false discovery rate (Storey 2002; Dabney et al. 2009).

Transcripts used to construct the microarray were compared to Swiss-Prot and the non-redundant protein databases (*e* threshold 0.001) with BLASTX, and curated by hand (Supplementary Table 2). Conversion of different annotation formats was done using the online application DAVID (<http://david.abcc.ncifcrf.gov/>; Dennis et al. 2003; Huang et al. 2009). Transcripts with a known gene symbol, but an organismal identification that was not recognized by DAVID, were changed to homologous genes in a recognized species for ontology analysis. All converted data, along with BLAST sequences and statistics are provided in the supporting information and have been deposited in the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI) ArrayExpress database. Ontology analyses of genes that were regulated by hypoxia at a *q*-value of 0.1 were done using the three gene ontology (GO) categories: biological process, molecular function, and cellular component. Analyses were conducted using BiNGO v.2.44 (Maere et al. 2005) implemented in Cytoscape v.2.8.0 (Shannon et al. 2003). Testing for significant enrichment of GO categories was done with a hypergeometric distribution test in BiNGO with a cutoff value of *P*=0.01. Standardized log₂ expression values for relevant genes were visualized with heatmaps created in R, using the “gplots” package (Warnes et al. 2008).

RT-PCR Analysis of the mtDNA Control Region

Confirmation of microarray results for the control region of mtDNA was done using the qScript™ One-Step SYBR® Green qRT-PCR Kit, ROX™ (Quanta Biosciences, Gaithersburg, MD) in an Applied Biosystems 7300 RT-PCR system. Primers for the control region amplification were CR_Fhet_F: TTC CTG GCA TTT GGT TCC T; CR_Fhet_R: TTG GTG GGT AAC GAG GAG TA. We used 18s rRNA as a reference gene following Whitehead et al. (2011). Reactions were done only in males since they showed smaller responses than females, and provided a conservative confirmation of the microarray data. For control region 2 ng of total RNA was used in a 20 µl reaction. Given the high concentration of 18s, samples were further diluted to 20 pg total RNA for these reactions. All reactions were run in triplicate and additional controls were run for both loci without the addition of the reverse transcriptase enzyme to test for genomic DNA contamination. Significance was assessed using the $\Delta\Delta C_t$ method and a mixed-model approach in R (Pinheiro et al. 2008)

Results

Survival Analysis

The models using sex, mtDNA genotype and their interaction to predict size characteristics were not significant for length (*P*=0.8589), mass (*P*=0.8703), or *K* (mass/length³) (*P*=0.7556) for fish used in the survival analysis. The median lethal time for 156 fish of known sex and genotype was 962 minutes (95% CI: 886-1057) (all survival data is available as Supporting Information), and there was no mortality among control fish. Sample size, mean length and mass, median lethal time, and confidence limits for the four sex-by-mtDNA genotype combinations, without accounting for day of exposure, are given in Table 1. Survival was independent of size, but nested likelihood ratio tests showed that the addition

of a sex term to the model significantly improved the fit ($\chi^2=9.36$, $df=1$, $P=0.0022$). Survivorship curves for each of the four sex-by-mitochondrion groups are shown in Figure 1, although no significant effect of mtDNA genotype was observed (Table 2). Right-censored regression ($\chi^2=12.9$, $df=1$, $P<0.001$), and Cox proportional hazard models ($\chi^2=13.4$, $df=1$, $P<0.001$), which excluded the samples from the first day, showed qualitatively the same results of a highly significant effect of sex, but no genotype effect.

Population Genetics

Phylogeny construction resulted in well-supported, reciprocally monophyletic northern and southern clades, with bootstrap support of 100 (Fig. 2). Nucleotide diversity (π) was 0.0134 for ND2 across all sequences (π : northern=0.00249; southern=0.00254) and the Jukes-Cantor estimate of N_{ST} was 0.896 (Lynch & Crease 1990). For ND5, π was 0.0155 across all sequences (π : northern=0.00077; southern=0.00135), and Jukes-Cantor N_{ST} was 0.964. The estimated time to most recent common ancestor for the two clades was 7.05×10^5 years (95% highest posterior density $5.14\text{--}9.06 \times 10^5$ years). These results confirm that our sample of fish contained clearly distinct northern and southern mtDNA haplotypes.

Gene expression in livers

Fish used in the treatment groups were of similar size. There was a marginally significant effect of the sex by mitochondrial type interaction only on length ($P=0.038$) for the treated fish, but no significant effects on mass (N/S=north/south, M/F=male/female, H/O=hypoxia/normal oxygen, mass(g)/length(cm); NFH = 2.59/4.98, SFH = 2.13/4.48, NMH = 2.08/4.47, SMH = 2.29/5.01, NFO = 2.33/4.64, SFO = 2.29/4.55, NMO = 2.05/4.59, SFO = 1.92/4.67). Mixed model analysis of gene expression at a q -value threshold of 0.1 resulted in 1160, 623, and 8 transcripts differentially regulated by the main effects of sex, hypoxia, and mitochondrial genotype, respectively (Supporting Information). These values do not include transcripts that are also significant at $q<0.1$ in corresponding interaction terms. Volcano plots for the main effects are shown in Fig. 3. Gene ontology categories significantly influenced by sex are given in the supporting information. Among the genes differentially regulated by oxygen level at $q<0.1$, 56% were suppressed by low dissolved oxygen. Furthermore, there was a significant overrepresentation of transcripts with no strong BLAST homology among genes induced by hypoxia relative to those suppressed ($P=8.22 \times 10^{-7}$) based on a Fisher exact test. For genes suppressed by hypoxia, predicted biological functions including oxygen binding and NADH dehydrogenase activity were significantly overrepresented GO categories (Table 3; Fig. 4). A smaller number of proteins involved in mitochondrial function and electron transport appeared to be induced by hypoxia and were generally encoded by the nuclear genome (Fig. 4); however, mitochondrial processes were not among those significantly enriched by hypoxia-induced genes. Several of the GO categories induced by hypoxia regulate the cell cycle consistent with previous studies (Gracey et al. 2001; Ton et al. 2003).

The transcript most suppressed by hypoxic exposure was a portion of the mitochondrial control region with blast homology to the D-loop (hereafter, control region), which is the origin of replication and transcription for mitochondria ($P=9.40 \times 10^{-8}$; $q=5.25 \times 10^{-5}$) (Fig. 4; Fig. 5). To confirm that this was not due to genomic DNA in the RNA samples an RT-PCR experiment was conducted. The RT-PCR reaction was done in three males (with three technical replicates per male) from each mitochondrial type and hypoxia treatment. Males were chosen because they were the more conservative of the two sexes based on the microarray result. One outlier was excluded from results based on a lower than anticipated amount of total RNA. We found a mean of 400-fold increased amplification when reverse transcriptase was added to the RT-PCR reaction when compared to the no RT control, confirming that the microarray result is not due to genomic DNA. We also found 1.4-fold

lower expression of control region transcription under hypoxia using the $\Delta\Delta C_t$ method, consistent with the microarray results. Although this change was not as great as observed in the microarray, it was marginally significant with a mixed-effect model (Table 4).

A total of 8 genes were differentially regulated by mitochondrial genotype based on the q -value cutoff of 0.1. Five of these 8 genes are encoded by the mitochondrial genome. For these loci the difference in hybridization intensity appears to be linked to nucleotide polymorphisms that differ between the sequences used to construct the array and the mtDNA genotype of the specimens. Arrays were generated using transcripts from both northern and southern *F. heteroclitus*. We observe here that in 4 of 5 cases fish with northern and southern mtDNA types are hybridizing with greater efficiency to probes that share greater sequence homology to the northern and southern type, respectively (Supporting Information). Two of the remaining three differentially expressed genes have significant BLASTX homology to nuclear encoded Troponin C ($P=2\times 10^{-11}$, $q=4.019\times 10^{-8}$) and fatty aldehyde dehydrogenase (FALDH) ($P=5.2\times 10^{-8}$, $q=6.97\times 10^{-5}$) (Fig. 4). The final differentially expressed gene is an un-annotated transcript ($P=6.9\times 10^{-7}$, $q=5.55\times 10^{-4}$).

Among interaction terms, two genes showed a significant mtDNA genotype-dependent transcriptional response to hypoxia. These transcripts include mitochondrial elongation factor Ts (EF-Ts_{mt}) ($P=1.3\times 10^{-5}$, $q=0.034$) (Fig. 4; Fig. 5), and an N-acetylserotonin O-methyltransferase-like protein (ASMTL) ($P=1.9\times 10^{-5}$, $q=0.034$) (Fig. 4). EF-Ts shows a striking reversal of expression pattern when comparing southern vs. north mtDNA in both sexes (Fig 5). An additional unannotated transcript showed significant sex-dependent transcriptional response to hypoxia ($P=2\times 10^{-5}$, $q=0.072$). These are nuclear encoded genes and their differential expression is not associated with known nucleotide polymorphism.

Discussion

mtDNA Variation and Survival Analysis

In *F. heteroclitus*, the midpoint of the nuclear genome cline is north of the midpoint of the mtDNA cline (Gonzalez-Vilasenor & Powers 1990; Duvernell et al 2008; Haney et al 2009), which provides an opportunity to test for dysfunction between mismatched mitochondrial and nuclear genomes. If intergenomic coadaptation has already occurred in these populations through the purging of deleterious alleles then fitness effects of mtDNA types may be minimal. However, the mitochondrial cline may be a tension zone where the mitochondrial types move in by migration and are purged by selection (Barton & Hewitt 1985). One hypothesis tested here was that fish with northern mtDNA genotypes would show reduced survival compared to southern types in response to hypoxia, consistent with the tension zone prediction. Our results suggest that mtDNA type did not influence mortality rates, and the effects of genotype were small compared to the large and highly significant effect of sex. However, this does not preclude the possibility that mtDNA genotype may significantly affect survivorship in the presence of other stresses such as temperature or salinity changes, or combinations thereof. *F. heteroclitus* is a hypoxia tolerant fish and frequently encounters DO levels in the field as extreme as the conditions in this experiment (Nordlie 2006). However, longer exposure to less acute hypoxic stress may reveal mitochondrion genotype-dependent fitness effects not detected here.

While we did not detect a significant effect of mtDNA genotype on survival, assays of fish under acute hypoxic stress revealed an unexpected and highly significant effect of sex, with females living longer than males (Table 1; Table 2). There are several potential explanations for this result. We considered that this could be due to individual size, but there were no significant differences among the treatment groups with respect to standard length, mass, or condition factor. Furthermore, addition of size terms did not significantly improve the fit of

the survival models. Therefore, it seems unlikely that the observed sex difference was related to size. Another potential explanation is that variation in reproductive state could influence survival. Some component of female reproductive state may contribute to increased survivorship if females are able to reallocate reproductive resources to survival, but this remains speculative. Previous work in livebearing poeciliid fishes suggests the oxygen demand of gestating females is greater than the demand during reproductive quiescence (Timmerman & Chapman 2004). *F. heteroclitus*, as an external fertilizer, is not directly comparable to a livebearer, but reproductive state could potentially alter oxygen demand. Rees et al. (2001) observed that female zebrafish survived acute hypoxic stress longer than males, consistent with our findings. However, they also noted that males displayed greater survival if a period of acclimation was introduced. Notably, in their findings, gonadosomatic index and size did not influence female survivorship. Finally, there may be an innate difference between sexes in hypoxic survival based on basal metabolic rates. However, previous work in *Gambusia* did not find evidence for metabolic rate differences between the sexes, despite an observation of increased male mortality under hypoxia (Cech et al. 1985). Further work needs to be done to determine the true cause of this effect.

Gene expression in livers

The transcriptomic response to hypoxia in fish is highly tissue-specific (Gracey et al. 2001; Ju et al. 2007; Richards 2009). In our study liver tissue was selected for microarray analysis because it is known to be among the most critical for facilitating hypoxia acclimation in fish (Fraser et al. 2006). In *F. heteroclitus* and congeners the enzymes necessary for glycolysis and gluconeogenesis are induced in livers during hypoxic events, but the specific genes can vary (Kraemer & Schulte 2004; Martínez et al. 2006; Richards et al. 2008). Furthermore, a number of microarray based analyses of liver transcriptome responses to hypoxia have been conducted in fish (Gracey et al. 2001; Ju et al. 2007; Fraser et al. 2006; Boswell et al. 2009; Zhang et al. 2009) and are useful for comparison. In this analysis, we selected a q -value cutoff of 0.1. This corresponded to 623 transcripts differentially expressed by hypoxia, of which approximately 62 are expected to be the false positives. However, this false discovery rate was considered acceptable given our goals of identifying general trends in gene expression responses. Over-represented ontology categories were only considered if they were significant at $P < 0.01$ based on the hypergeometric test implemented in BiNGO (Table 3; Supporting Information).

Previous work has identified several categories of genes commonly influenced by hypoxic exposure in fish livers. The observed responses include regulation of genes in the HIF pathway, induction of glycolytic enzymes, and induction of heme and amino acid metabolism proteins (Gracey et al. 2001; Ju et al. 2007; Richards 2009). By contrast, genes in the pathway of aerobic metabolism, and those that initiate cell proliferation are generally suppressed (Richards 2009). Here, functional annotation analysis revealed predicted gene ontologies among those significantly influenced by hypoxic stress based on previous reports (Table 3). However, some differences with previous reports were observed as well. The most commonly observed biological processes in hypoxia regulation studies, and the corresponding results in this experiment, are explained in more detail below.

HIF-1 is generally recognized as the master regulatory element for the hypoxia response in gene expression. Two HIF transcripts are present on this array platform, but the best BLAST hits are not to HIF-1. The physiological roles of all members of the HIF gene family are unknown so predicted responses are not clear (Wu 2002; Nikinmaa & Rees 2005). HIF-2 α (Powell & Hahn 2002) is one of the represented transcripts. Although it is expressed at slightly higher concentrations in the hypoxia treated fish, the results are not significant ($P = 0.6287$, $q = 0.4889$) (Fig. 4). This is consistent with previous findings in Atlantic croaker

(*Micropogonias undulatus*), where HIF-2 α did not increase in expression until fish had been exposed to hypoxia for one week (Rahman & Thomas 2007). Work in *F. heteroclitus* also shows that expression differences in HIF-2 α are not observed at the transcript level (Rees et al. 2009). The homology of the second HIF transcript on the array is not clear, but the top BLAST hit is to HIF-4 α in the teleost, *Epinephelus coioides* (GenBank accession: AAW29028). The transcript shows 61% identity at the amino acid level over 157 residues. Lower levels of sequence similarity are observed for a number of other teleost HIF transcripts. Expression of this transcript is significantly influenced by hypoxia treatment at a q -value of 0.018 ($P=9\times 10^{-4}$) (Fig. 4). However, this transcript appears to be inhibited by hypoxic stress rather than induced, with expression 1.7 fold greater in fish with normal dissolved oxygen levels.

The activities of the insulin-like growth factors (IGF) are linked to HIF and are differentially regulated by hypoxia in fish (Gracey et al. 2001; Ren et al. 2010). The most well characterized response is an increase in IGFBP-1 when oxygen is limited, which can inhibit development in zebrafish embryos (Kajimura et al. 2005); however, IGFBP-1 is not present on this array. There are two components of the insulin-like growth factor-binding protein complex: the acid labile subunit (ALS, $P=0.0057$, $q=0.0481$), and a transcript with homology to growth factor binding protein 5 (IGFBP-5, $P=0.0010$, $q=0.0192$) that are suppressed by hypoxia (1.6 fold and 2 fold greater under normal oxygen conditions, respectively) (Fig 4). An additional IGFBP, (IF2B2, $P=0.0136$, $q=0.0744$) is also significantly suppressed by hypoxia, although only slightly (1.2 fold greater expression under normal oxygen conditions). As with HIF, the exact roles of these paralogs are unclear, but they are significantly influenced by hypoxic stress. Duan and Xu (2005) suggest in a review that IGFBP-5 may increase IGF-1 activity, which could explain why it is expressed in higher levels in fish under normal oxygen levels, but this is speculative. An additional transcription factor known to be upregulated by hypoxia is *jun B* (Zhang et al. 2009). In this analysis we find it among the most upregulated genes under hypoxic conditions with greater than 2 fold higher expression in fish under hypoxic stress ($P=3\times 10^{-4}$, $q=0.0102$) (Fig. 4).

Respiratory pigment regulation is an important component of the gene expression response to hypoxia. However, previous work is conflicting on the expression of heme proteins in the presence of hypoxia, with evidence suggesting both repression and induction by hypoxic stress are possible, although suppression appears tied to conditions where diffusion can effectively deliver oxygen (Richards 2009). We observe a significant excess of transcripts involved in heme binding among those suppressed by hypoxia at $q<0.1$ (Table 3). By contrast, an additional beta hemoglobin subunit appears to be induced by hypoxic stress ($P=0.0039$, $q=0.0383$; Fig. 4). Several cytochrome P450 genes are suppressed by hypoxic exposure, consistent with other studies and general predictions about the influence of diminished oxygen (Fig. 4) (Shang et al. 2006; Landry et al. 2007; Wu 2009).

Another predicted physiological response to decreased oxygen availability is the upregulation of genes involved in glycolysis and gluconeogenesis. When aerobic metabolism is no longer an effective way to produce ATP the organism shifts to primarily anaerobic energy production through the glycolytic pathway. Induction of glycolytic enzymes at the transcript and protein levels has previously been observed in the genus *Fundulus* (Kraemer & Schulte 2004; Martínez et al 2006), although in other species the results are tissue-specific (Gracey et al. 2001; Webster 2003). In this analysis, transcripts with homology to the glycolytic and gluconeogenic genes aldolase A ($q=0.0762$, $P=0.0144$), glucokinase ($q=0.0301$, $P=0.0024$), and glucose phosphate isomerase ($q=0.0234$, $P=0.0014$) are induced by hypoxic stress (Fig. 4). However, glucose-6-phosphatase ($q=0.02$, $P=0.0011$), and 2 pyruvate kinase ($q=0.0702$, $P=0.012$) are suppressed by hypoxia (Fig. 4). Catabolism of proteins is an additional mechanism to generate substrates necessary for

gluconeogenesis (Gracey et al. 2001). Here we observe suppression of genes regulating endopeptidase activity (consistent with Gracey et al. 2001; Boswell et al. 2009; Ju et al. 2006 observe downregulation of protein synthesis), which suggests preparation for increased glycolysis (Table 3).

As organisms transition to anaerobic respiration, expression of genes associated with oxidative phosphorylation is expected to decrease. Of the 21 genes with greater than two-fold suppression under hypoxic stress in this analysis, 4 are homologous to transcripts of NADH subunits (Fig. 4), consistent with predictions. In total, 37 non-redundant transcripts with “mitochondrion” as an SP-PIR keyword appeared to be significantly influenced by hypoxic exposure at a q -value threshold of 0.1 (Fig. 4). These results indicate that the experimental design successfully isolated an important temporal component of the hypoxia response, specifically the regulation of mitochondrial function.

Interestingly, the transcript which displays the greatest suppression during hypoxic stress is the D-loop portion of the control region of the mtDNA (>3.8 fold higher expression under normal oxygen conditions; Fig. 5). This directionality of this result was supported with RT-PCR, although of the magnitude of the change was smaller. The mtDNA control region contains the origin of replication and transcription mtDNA (reviewed in Fernández-Silva et al. 2003). Therefore, suppression of a control region transcript may indicate suppression of mitochondrial biogenesis and transcription, a major prediction of this study. To our knowledge, previous studies using DNA microarrays have not identified the mitochondrial control region as a down-regulated transcript under hypoxic conditions. This may be due to the fact that control region sequences are non-coding and would not appear to have significant homology to a known protein. However, previous work demonstrates that the control region is transcribed, and adenylated in mammals (Sbisà et al. 1990; Vijaysarathy et al. 1995; Camasamudram et al. 2003). Furthermore, control region expression may be related to rates of transcription for mtDNA encoded genes; however, this was a unique case of a transgenic mouse line (Freyer et al. 2010). This control region transcript is also significantly down-regulated in gill tissues during hypo-osmotic acclimation in *F. heteroclitus*, during which mitochondria-rich cells are remodeled in the gill epithelium (Whitehead et al. 2011; Whitehead et al., unpublished data). Re-analysis of unannotated transcripts in previous studies investigating hypoxic response could reveal the non-coding control region as an important candidate for regulation by hypoxia or other environmental stressors.

As predicted, we find hundreds of genes (approximately 29%) differentially regulated by sex. Among the differentially expressed transcripts, many have higher expression in females, resulting in the observed skew in the volcano plot (Fig. 3). Significant over expression of gene transcripts in females may be indicative of proteins linked to reproductive status and egg development. Three vitellogenin homologs, which are critical for egg development, are expressed at levels more than 5 fold greater in females (Fig. 4). One of these transcripts is the most enriched of any transcript in females, at 22.6 fold increased expression ($q=7.826\times10^{-13}$, $P=7.0\times10^{-16}$; Fig.4). In zebrafish hypoxia can create male-skewed sex ratios in populations (Shang et al. 2006) and result in differential gene expression in gonads (Martinovic et al. 2009). However, despite the hundreds of genes differentially regulated by sex and hypoxia, there is only a single transcript, with no known homology, which shows a sex-dependent hypoxia response (significant sex-by-hypoxia interaction). This suggests that the hypoxia response pathways are similar across the sexes despite the greater observed resilience of females in the face of hypoxic stress, at least for a short exposure.

In contrast to sex and hypoxia treatments, which are both linked to the expression of hundreds of genes, mtDNA genotype effects on gene expression were limited. The non-mitochondrially encoded genes that were differentially expressed at $q < 0.1$ include Troponin C, fatty aldehyde dehydrogenase (FALDH), and a transcript with no known homology (Fig. 4). FALDH has been tied to oxidative stress protection in rat livers (Demozay et al. 2004). In this case FALDH is upregulated in northern mtDNA fish of both sexes and at both oxygen levels. If northern mtDNA fish generally produce more oxidative stress through inefficient aerobic respiration, FALDH may provide protection from oxidative damage. This possibility is interesting for two reasons. First, oxygen levels may influence the biological response to free radicals in fish species (GO categories observed in Ju et al. 2006; Lushchak & Bagnyukova 2006); and second, reactive oxygen species produced by mitochondria under hypoxic conditions may be a method for retrograde signaling between the genomes (Poyton et al. 2009). In the current study two superoxide dismutase transcripts, important antioxidant proteins, are differentially regulated by hypoxic stress – with one induced and one suppressed (Fig. 4). Additionally, glutathione peroxidase was induced by hypoxic stress (Fig. 4). However, even at a P -value threshold of 0.05, only 180 transcripts differentially regulated by mitochondrial genotype are observed, less than the approximately 201 predicted by chance.

Interaction terms in the mixed model analysis also yielded few significantly differentially regulated genes. However, mitochondrial elongation factor (EF-Ts_{mt}), which plays a role in elongation during mitochondrial protein synthesis, does show differential expression in hypoxic environments dependent on mitochondrial genotype (Fig. 5). The expression patterns of this transcript are consistent with induction under hypoxic conditions for fish with northern mtDNA, and suppression for fish with southern mtDNA; and the pattern is consistent across sexes. For individuals attempting to shut down mitochondrial function one prediction is that EF-Ts_{mt} should be suppressed by hypoxic conditions. In this case, it is the putatively mismatched fish, those with northern mitochondrial genomes on a predominately southern nuclear background, which display induction. Although follow-up work on any potential epistasis between mtDNA and EF-Ts_{mt} will be necessary, this gene represents an important candidate for physiological differences tied to mitochondrial genetic variation, and suggests that genetic variation that affects mitochondrial translation may be an important under-studied aspect of selection on mtDNA.

Implications for the selective contributions of mtDNA

We report here, for the first time, a study of the effects of mtDNA variation on survival and gene expression under hypoxic stress in a fish species. *F. heteroclitus* affords a unique opportunity for this kind of study because of its postglacial population history, a relict of which is a hybrid zone with offset mitochondrial and nuclear genotype clines. The level of precision in controlling the nuclear genetic background of fish used in this study is not comparable to controlled laboratory crosses of stocks. Therefore, we are unable to test for potential asymmetric effects in mtDNA/nuclear combinations. Furthermore, our ability to detect selective effects of mtDNA may be compromised if coadaptation has already eliminated deleterious alleles from the nuclear genome in the hybrid population, or if linkage between specific nuclear and mitochondrial alleles is maintaining mitochondrial function. However, analyses of four microsatellites do not show evidence of cytonuclear disequilibrium, which suggests random mating of mtDNA types (Flight & Rand, unpublished data), and there would be significant selective load associated with the maintenance of linkage between the genomes. Additional high-throughput linkage analysis of nuclear and mitochondrial markers may be an effective and informative way to address the purging of deleterious variants and linkage. Other possible explanations for the existence of the offset clines invoke ongoing selection. The population in question may simply not

have reached equilibrium and certain alleles are still undergoing selective sweeps; or, the hybrid population may be a tension zone formed by migration into selective sink.

With respect to survival under hypoxic stress, we find that female *F. heteroclitus* survive for longer periods of time than males at oxygen levels below the critical threshold. This effect is consistent across mtDNA types and there was no significant effect of body size or mtDNA variation on survivorship. Gene expression analyses revealed hundreds of genes differentially regulated by sex and hypoxia. Among the genes regulated by hypoxic stress were a number of mitochondrially-encoded transcripts and nuclear encoded genes with mitochondrial function. This result confirms one major prediction of the study, that mitochondrial function would be a primary target of hypoxic stress. The identification of the mtDNA control region as the most suppressed transcript is particularly interesting, because it functions as a master regulator of all mitochondrial transcription and biogenesis. However, we recognize that because it is non-coding it may have been overlooked in previous studies with non-genome enabled species that rely on BLASTX homology to identify targets.

Interactions in our model explained a significant portion of gene expression for a small number of transcripts. However, one potentially important epistatic interaction was an mtDNA genotype-dependent expression of a mitochondrial elongation factor. Ultimately, the effects of mtDNA variation on survival and gene expression observed here are small. Although regulation of mitochondrial function is clearly an important component of hypoxia resistance, the mitochondrial clades sampled here perform equally well in terms of survivorship and, interesting candidate genes aside, differ little in physiological response at the level of gene expression. The debate over the importance of mitochondrial “neutrality” with respect to fitness differences is important in the context of evolutionary questions and the utility of mtDNA for population genetic analyses. The non-neutrality of mtDNA sequence evolution is well documented, because mitochondrial genomes consistently deviate from neutrality in a pattern consistent with strong purifying selection (Rand & Kann 1996; Whitehead 2009). What remains controversial is the potential role of adaptive evolution on mitochondrial genomes (Bazin et al. 2006; Meiklejohn et al. 2007; Dowling et al. 2008; Ballard & Melvin 2010). When purifying selection on rare variants is the main evolutionary force in a population functional tests lack power to detect fitness differences because the weak genotypes are, by definition, rare. Here we again find that the fitness effects of mitochondrial variation within a population are small, even when substantial molecular divergence is observed. The fact that a population with such divergent mtDNA genotypes exists may be taken as evidence that fitness consequences associated with these genotypes are small. As outlined in Montooth et al. (2010), with several exceptions (including *Tigriopus*: Edmands & Burton 1999; Ellison & Burton 2006; *Drosophila*: Rand et al. 2006; *Nasonia*: Ellison et al. 2008; yeast: Lee et al. 2008; and seed beetle: Arnqvist et al. 2010), mapping fitness to mito-nuclear interactions is not common, and may be unique to species with high mitochondrial mutation rates or low effective population sizes that weaken purifying selection. In the case of *F. heteroclitus* hybrids, the isolation of northern and southern subspecies may not have led to fixation of adaptive mutations in mtDNA, or linked nuclear variants, in either the northern or southern population. If this is the case the differences observed at the nucleotide level here are likely a result of the fixation of neutral alleles through drift, and the presence of deleterious variants at low frequency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Survival curves for *F. heteroclitus* under acute hypoxic stress based on a model fit with interval regression. The four lines refer to the four categories of fish tested (Female=Black; Male=Gray; Southern mtDNA=Solid; Northern mtDNA=Dashed). These curves do not include day of exposure.

Figure 2.

Sliding window and phylogenetic analysis of mitochondrial types in *F. heteroclitus*. (A) Nucleotide diversity (π) as a function of mitochondrial position across the whole genome. The gray line shows the result of a sliding window and the black line is a line of best fit. The regions sequenced for phylogenetic analysis in this study, ND2 and ND5, are shown between the vertical black lines. Complete reference sequences were taken from Whitehead (2009). (B) A neighbor-joining phylogeny of mitochondrial types based on a concatenated alignment of ND2 and ND5 in 20 northern and 20 southern *F. heteroclitus*, and two outgroup species. Outgroup sequences come from Whitehead (2009).

Figure 3.

Volcano plots for main effects of the gene expression model. The $-\log_{10} P\text{-value}$ is shown as a function of the $\log_2$ difference in expression for the three main effect terms. The dashed horizontal line shows a $P\text{-value}$ cutoff of 0.05 and the solid horizontal line show a $q\text{-value}$ cutoff of 0.1. Filled circles are transcripts with greater than two-fold difference between treatments.

Figure 4.

Heatmaps of gene expression in each of the 8 categories. Three letter codes refer to mtDNA type (North/South), sex (Male/Female), and oxygen level (Hypoxic/Oxygenated), respectively (e.g. NFO are northern females at saturated oxygen). Color key shows the scale for standardized $\log_2$ expression values and dendrograms link similar genes based on expression patterns. The specific gene ID's used to construct the heatmaps are given in parenthesis next to the gene symbol. All data can be found in the Supporting Information. (A) Heatmap of all non-redundant genes regulated by hypoxia with SP-PIR keyword "mitochondrion." (B) Heatmap of genes that show at least 2-fold difference in expression due to hypoxia and other specific genes mentioned in the text. If more than one transcript was observed for a gene and displayed a similar response, values were averaged. Gene symbols are as follows: NDUB6 - NADH dehydrogenase 1 beta subcomplex subunit 6; IDH3A - Isocitric dehydrogenase subunit alpha; NDUBB - NADH dehydrogenase 1 beta subcomplex subunit 11; NDUA3 - NADH dehydrogenase 1 alpha subcomplex subunit 3; S2535 - Solute carrier family 25 member 35; COX3 - Cytochrome c oxidase subunit 3; NDUAD - NADH dehydrogenase 1 alpha subcomplex subunit 13; ORN - Oligoribonuclease; PPCA - Propionyl-CoA carboxylase alpha chain; SODM - Superoxide dismutase; RT24 - 28S ribosomal protein S24; D-Loop - *F. heteroclitus* control region; NU5M - NADH-ubiquinone oxidoreductase chain 5; THIM - 3-ketoacyl-CoA thiolase; ATPA - ATP synthase subunit alpha; ECH1 - Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase; CX6A2 - Cytochrome c oxidase subunit 6A; ARG12 - Arginase-2; NLTP - Non-specific lipid-transfer protein; HEM1 - 5-aminolevulinic synthase; PCKGM - Phosphoenolpyruvate carboxykinase; ACADL - Long-chain specific acyl-CoA dehydrogenase; BR44L - Brain protein 44-like protein; ODO2 - 2-oxoglutarate dehydrogenase complex component; SCOT1 - 3-oxoacid-CoA transferase 1; QCR2 - Cytochrome b-c1 complex subunit 2; NU1M -

NADH-ubiquinone oxidoreductase chain 1; MECR - Trans-2-enoyl-CoA reductase; NU4M - NADH-ubiquinone oxidoreductase chain 4; RM19 - 39S ribosomal protein L19; IVD - Isovaleryl-CoA dehydrogenase; SODM - Superoxide dismutase; S2533 - Solute carrier family 25 member 33; PRLD1 - PRELI domain-containing protein 1; NU2M - NADH-ubiquinone oxidoreductase chain 2; SCM2A - Small calcium-binding mitochondrial carrier protein 2-A; CO7 - Complement component C7; ALS - Insulin-like growth factor-binding protein complex acid labile subunit; IBP5 - Insulin-like growth factor-binding protein 5; CDO1 - Cysteine dioxygenase type 1; KPYK - Pyruvate kinase muscle isozyme; MOG - Myelin-oligodendrocyte glycoprotein; HBB - Hemoglobin subunit beta; HBAB - Hemoglobin subunit alpha-B; HEP2 - Heparin cofactor 2; CP1A2 - Cytochrome P450 3A56; CATA - Catalase; CP1B1 - Cytochrome P450 1B1; G6PC - Glucose-6-phosphatase; SFR35 - Splicing factor, arginine/serine-rich 5; T230B - Tryptophan 2,3-dioxygenase B; COPA1 - Collagen alpha-1(XXV) chain; HIF4 - Hypoxia inducible factor 4; CP2E1 - Cytochrome P450 2K1; CP2J2 - Cytochrome P450 2J2; EFTS - Elongation Factor Ts; ASML - N-acetylserotonin O-methyltransferase-like protein; HIF2A - Hypoxia inducible factor 2 alpha; ALDOA - Muscle-type aldolase; G6PI - Glucose-6-phosphate isomerase; IOD2 - Type II iodothyronine deiodinase; HXK4 - Glucokinase; JUNB - Transcription factor jun-B; YG005 - Putative uncharacterized protein FLJ40852; ART2 - Uncharacterized protein ART2; VITA2 - Vitellogenin-A2; Q3T7B3 - Vitellogenin-2; Q8JH36 - Vitellogenin; AL3A2 - Fatty aldehyde dehydrogenase; TNNC2 - Troponin C; GPX1 - Glutathione peroxidase 1.

Figure 5. Norms of reaction for the gene expression of (A) mtDNA control region, and (B) elongation factor Ts_{mt} . Lines connect standardized $\log_2$ expression value for sex and genotype combinations across the two oxygen levels. Females are in black and males are in gray. Northern genotypes are shown with hatched lines.

Table 1

Median lethal time and confidence intervals for males and females of both mitochondrial groups exposed to acute hypoxic stress. Table 2 shows statistical significance of terms.

mtDNA	Males	Females
Northern		
N	27	40
Mean Length	3.538	3.561
Mean Mass	0.963	0.988
Median lethal time	883	1017
95% confidence limits	838-966	886-1188
Southern		
N	51	38
Mean Length	3.579	3.588
Mean Mass	0.982	1.003
Median lethal time	897	1143
95% confidence limits	761-1073	982-1243

Table 2

Interval survival regression using a Weibull distribution. *P*-values were generated based on likelihood ratio tests of the nested models sequentially in the order they are listed. The “Block” term refers to the day of exposure.

Factor	Deviance	DF	<i>P</i> -value
Sex	9.375	1	0.0022
Mitochondrial Type	0.848	1	0.3572
Condition Factor	0.005	1	0.9418
Block	4.334	3	0.2276

Gene ontology analysis of transcripts influenced by hypoxic exposure at $q < 0.1$. GO terms were considered significantly enriched if the P -value from the hypergeometric test was less than 0.01. The “N” column refers to the number of transcripts in that GO category that were significantly influenced by hypoxia. “Total” is the total number of transcripts in the GO category on the array platform. “Enrichment” refers to the fold overrepresentation of the GO category relative to random chance. To limit size, some redundant categories have been removed. The full table is available in the supporting information.

Table 3

Regulation	GO Terms	Description	P-value	N	Total	Enrichment
Suppressed	9611	response to wounding	1.26E-08	28	85	3.079
	50817	coagulation	3.14E-07	14	29	4.512
	7599	hemostasis	8.81E-07	14	31	4.221
	4867	serine-type endopeptidase inhibitor activity	4.04E-06	11	22	4.673
	50878	regulation of body fluid levels	5.19E-06	14	35	3.739
	9605	response to external stimulus	6.25E-06	28	111	2.358
	61135	endopeptidase regulator activity	3.19E-05	15	45	3.115
	61134	peptidase regulator activity	7.52E-05	15	48	2.921
	5576	extracellular region	1.41E-04	45	250	1.682
	4857	enzyme inhibitor activity	1.56E-04	17	62	2.563
	6950	response to stress	3.03E-04	38	206	1.724
	6954	inflammatory response	7.39E-04	14	52	2.516
	65008	regulation of biological quality	9.80E-04	36	203	1.658
	15807	L-amino acid transport	1.21E-03	3	3	9.346
	32501	multicellular organismal process	1.40E-03	50	315	1.484
	19825	oxygen binding	2.15E-03	5	10	4.673
	50896	response to stimulus	2.16E-03	51	329	1.449
	45087	innate immune response	2.30E-03	9	29	2.901
	51605	protein maturation by peptide bond cleavage	4.28E-03	8	26	2.876
	15171	amino acid transmembrane transporter activity	4.46E-03	3	4	7.010
	6956	complement activation	4.57E-03	7	21	3.115
	15980	energy derivation by oxidation of organic compounds	5.30E-03	14	63	2.077
	51604	protein maturation	5.53E-03	8	27	2.769
	20037	heme binding	7.54E-03	10	40	2.337
	15931	nucleobase, nucleoside, nucleotide and nucleic acid transport	8.40E-03	5	13	3.595
	34754	cellular hormone metabolic process	8.40E-03	5	13	3.595

Regulation	GO Terms	Description	P-value	N	Total	Enrichment
	3954	NADH dehydrogenase activity	8.86E-03	8	29	2.578
	6952	defense response	9.67E-03	15	74	1.895
Induced	6310	DNA recombination	1.16E-03	4	9	7.517
	16772	transferase activity, transferring phosphorus-containing groups	1.38E-03	13	87	2.527
	90277	positive regulation of peptide hormone secretion	1.85E-03	3	5	10.148
	22402	cell cycle process	2.18E-03	8	41	3.300
	10647	positive regulation of cell communication	2.65E-03	6	25	4.059
	23056	positive regulation of signaling process	2.65E-03	6	25	4.059
	279	M phase	3.07E-03	5	18	4.698
	10740	positive regulation of intracellular protein kinase cascade	3.07E-03	5	18	4.698
	9617	response to bacterium	3.28E-03	6	26	3.903
	16301	kinase activity	4.21E-03	11	76	2.448
	32553	ribonucleotide binding	4.40E-03	24	236	1.720
	10627	regulation of intracellular protein kinase cascade	5.04E-03	5	20	4.228
	19902	phosphatase binding	5.93E-03	3	7	7.248
	166	nucleotide binding	6.72E-03	30	327	1.552
	51707	response to other organism	6.96E-03	6	30	3.383
	44427	chromosomal part	6.96E-03	6	30	3.383
	9898	internal side of plasma membrane	7.75E-03	7	40	2.960
	7010	cytoskeleton organization	8.22E-03	6	31	3.273
	22411	cellular component disassembly	9.08E-03	3	8	6.342
	51716	cellular response to stimulus	9.30E-03	9	62	2.455
	51050	positive regulation of transport	9.55E-03	4	15	4.510

Mixed-model analysis of RT-PCR data for mitochondrial control region. The Δ Ct values between the control region transcript and 18s rRNA were modeled as a function of mitochondrial type and hypoxia in three individuals per treatment with three technical replicates each. Technical replicate was treated as a random effect.

Table 4

Dependent Variable	Fixed Effect	num df	den df	F-value	P-value	Random effect	σ^2
Delta Ct	Intercept	1	22	5174.12	<0.0001	Technical Replicate	0.1041
	mtDNA type	1	8	23.02	0.0014		
	Hypoxia	1	8	5.62	0.0452		