RESEARCH ARTICLE

# The Effect of Short-Term Hypoxic Exposure on Metabolic Gene Expression

MEREDITH V. EVERETT, CORINA E. ANTAL, AND DOUGLAS L. CRAWFORD*
Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, Florida

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## ABSTRACT

The long-term effect of hypoxia is to decrease both the production and use of ATP and thus decrease the reliance on mitochondrial oxidative energy production. Yet, recent studies include more immediate affects of hypoxia on gene expression and these data suggest the maintenance of mitochondrial function. To better understand the short-term physiological response to hypoxia, we quantified metabolic mRNA expression in the heart ventricles and livers of the teleost fish *Fundulus grandis* exposed to partial oxygen pressure of 2.8 kPa (~13.5% air saturation). Twenty-eight individuals from a single population were exposed to hypoxia for 0, 4, 8, 12, 24, 48, and 96 hr. Liver and cardiac tissues were sampled from the same individuals at 0–48 hr. At 96 hr, only cardiac tissue was assayed. Gene expression was significantly different (ANOVA, $P<0.05$) for 17 of 226 metabolic genes (7.5%) in cardiac tissue and for 20 of 256 (7.8%) metabolic genes in hepatic tissue. For the two tissues examined in this study, the maximum response occurred at different times. For cardiac tissue, using Dunnett's post hoc test, most of these significant differences occurred at 96 hr of exposure. For liver, all but one significant difference occurred at 4 hr. Surprisingly, too many (relative to random expectations) of the genes with significant increase in mRNA are involved in the oxidative phosphorylation pathway: 44% of the significant genes at 96 hr in the heart and 33% of the significant genes at 4 hr in the liver are involved in the oxidative phosphorylation pathway. These data indicate that there are tissue-specific differences in the timing of the response to hypoxia, yet both cardiac and hepatic tissues have increases in mRNA that code for enzyme in the oxidative phosphorylation pathway. If these changes in mRNA produce a similar change in protein, then these data suggest that the initial response to hypoxia involves an increase in the oxidative pathway potentially as a mechanism to maintain ATP production. *J. Exp. Zool. 317:9–23, 2012.* © 2011 Wiley Periodicals, Inc.

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The teleost fish *Fundulus grandis* lives in estuaries along the Gulf of Mexico that experience periodic hypoxia to varying degrees: between Anclote Key, FL and Rio Grande, TX, 5.9–29.3% of estuaries surveyed were affected by hypoxia (Engle et al., '99). The differences in the frequency and extent of hypoxia among locations are associated with physiological differences within a species. Specifically, after acclimation to common conditions, populations of *F. grandis*from these different environments have significantly different metabolic rates with short-term (4 hr) exposure to low oxygen partial pressure (Everett and Crawford, 2010). Although the effect of hypoxia may be conserved among

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*Correspondence to: Douglas L. Crawford, Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098.
E-mail: dcrawford@rsmas.miami.edu

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species (Hochachka et al., '96; Hochachka and Somero, 2002; Nikinmaa and Rees, 2005), these data suggest that there are microevolutionary differences among these populations that are subjected to hypoxic events of variable frequency and extent (Everett and Crawford, 2010).

In general, the response to hypoxia has two aspects: defense and rescue: (Hochachka et al., '96). The early defense stage is achieved by reducing energy utilization and the dependence on aerobic metabolism (Hochachka et al., '96). Specifically, to save energy protein synthesis, protein breakdown, gluconeogenesis and $Na^+$ pump activity can be decreased by 90%. For the teleost *F. grandis*, data from long-term exposure (28 days) agree with these general principles: glycolytic enzyme expression increase in the liver, brain, and heart but there is a larger and broader suppression of glycolytic enzyme expression in muscle tissue (Martinez et al., 2006). Similar findings of increase reliance on anaerobic genes were also seen using a microarray approach on long-jawed mudsucker *Gillichthys mirabilis*: a rapid shut-down of skeletal muscle energy-requiring processes and, after 24 hr a induction of mRNA in the liver for genes needed for anaerobic ATP production. In both studies, there are differences among tissue with cardiac tissue having fewer or smaller response than liver or muscle. Part of this difference could be due to differential circulation (Martinez et al., 2006), and tissue-specific roles in homeostasis (Hochachka et al., '96; Gracey et al., 2001). Yet, although there is general agreement about the physiological response to acclimation to hypoxia (Hochachka and Somero, 2002; Nikinmaa and Rees, 2005), there are data indicating that the immediate short-term response may involve maintenance of or an upregulation of mitochondrial metabolism. In mammals, moderate exposure to hypoxia (vs. anoxia) enhances mitochondrial respiration via altered transcription (Essop, 2007). In tilapia, exposure to 8 hr of hypoxia caused a depression of whole animal oxygen consumption rate, in heart rate, cardiac output, and cardiac power output, reflecting a decrease in ATP demand, yet there was no change in fatty acid oxidation (Speers-Roesch et al., 2010). Finally, in cardiac tissue of *G. mirabilis* there was a greater than twofold increase in cytochrome b and cytochrome oxidase I (protein in the oxidative phosphorylation pathway), and an even larger increase in skeletal muscle (Gracey et al., 2001). These data suggest that the response to short-term exposure to hypoxia may be more complex than just reducing ATP demand and an increase in anaerobic ATP production.

To better understand the genes involved in short-term exposure to hypoxia in the teleost fish *F. grandis*, we measure mRNA expression for 384 metabolic gene among individuals from a single population exposed to oxygen partial pressure below, the critical oxygen tension ($PO_{crit}$) for this species (Cochran and Burnett, '96; Virani and Rees, 2000; Everett and Crawford, 2010). Although the variation in mRNA does not necessarily alter enzymes, a comparison of transcriptomics and proteomics for *Fundulus* suggest that the amount of many enzymes is a function of their mRNA (Rees et al., 2011). Additionally, published data suggested that exposure to hypoxia affects gene expression within the first 24 hr and that these data provide novel insights into the molecular basis for physiological homeostasis (Gracey et al., 2001; Ton et al., 2003; Bosworth et al., 2005; van der Meer et al., 2005; Martinez et al., 2006; Rocha, 2007; Boswell et al., 2009). The data we presented here suggest that there is a rapid change in hepatocyte mRNA expression, followed by slower cardiac response. Surprisingly, there is a statistically disproportionate increase in oxidative phosphorylation genes. These data suggest that short-term response (4–96 hr) may be different from long-term acclimation responses where there is typically an increase in anaerobic gene expression (Nikinmaa and Rees, 2005; Martinez et al., 2006).

## METHODS

### Animals and Hypoxic Exposure

*Fundulus grandis* were collected from a single population at Pass Aux Herons, AL. Fish were caught using minnow traps, transported back to the lab, and maintained in recirculating aquarium systems with a single shared water supply. Each population was kept in a separate tank, with shared water being circulated through all tanks via a central sump. The system was maintained at a salinity of 15 ppt in artificial seawater, made using Instant Ocean Sea Salt and municipal water dechlorinated via reverse osmosis. All fish were put through a pseudo-winter cycle: water temperature maintained at 8°C with a 10:14-hr light/dark cycle. After 6 weeks of pseudo-winter, temperatures were slowly increased to 24°C, and the lighting changed 14:10-hr light/dark cycle and the fish were allowed to spawn. Fish were fed OSI Marine Flake ad libitum once daily in the evening. Fish were moved to the test tank the evening, before beginning hypoxic exposure and allowed to settle overnight. Exposure to hypoxia was carried out in a 45-gallon glass aquarium. Water in the aquarium was maintained at 22°C and a salinity of 15 ppt and circulated using two Eheim 1046 centrifugal water pumps. Ammonia levels in the test tank were measured twice daily. Any rise in ammonia was controlled with Amquel.

Oxygen concentration in the test tank was controlled via a system designed by LoligoSystems ApS (Hobro, Denmark). Oxygen levels in the aquarium were continuously monitored and controlled using Mini-DO galvanic oxygen probe (OxyGuard International A/S, Birkerød, Denmark, measuring range 0–200% air saturation) and a solenoid valve connected to the computer control system operated by a Dell Latitude 110L PC laptop computer utilizing LoliResp Software. This setup automatically held oxygen at the set point by bubbling $N_2$ gas into the back of the aquarium until the desired partial pressure was reached. The oxygen probe was calibrated via the manufacturer's instructions. The probe was calibrated to 100% air saturation, made by

vigorously bubbling air through 100 mL of water for 20 min. The probe was calibrated at the start of the experiment, and checked for drift daily. The surface of the water was covered completely in bubble wrap to prevent aquatic surface respiration and reoxygenation. Before the initiation of hypoxia, four individuals were sampled as normoxic controls. Subsequently, oxygen in the system was dropped to $2.8 \pm 0.3$ kPa over the course of 30 min. This concentration was held for 96 hr, and four individuals were sampled at each time point: 4, 8, 12, 24, 48, and 96 hr. Fish were sacrificed via cervical dislocation and heart and liver tissues were collected and stored in RNAlater (Ambion), according to the manufacturer's instructions.

### RNA Preparation

RNA was extracted from individuals from tissue homogenate in a chaotropic buffer using phenol/cholorform/isoamyl alcohol. All reagents were from Sigma unless otherwise noted. Tissues were removed from RNAlater, blotted dry, and homogenized in 400 mL of chaotropic buffer (4.5 M Guanidinium thiocyanate, 2% *N*-laroylsarcosine, 50 mM EDTA pH 8.0, 25 mM Tris-HCl pH 7.5, 0.1 M b-Mercaptoethanol, 0.2% Antifoam A). A volume of 40 mL of 2 M sodium acetate was added to each sample followed by 400 mL of acidic phenol (pH 4.4), and 200 mL of a chloroform/isoamyl alcohol mixture (23:1). The mixture was kept at 4°C for 10 min then centrifuged at 4°C at 16,000 g for 20 min. Supernatant was removed and combined with 400 µL isopropanol, stored at −20°C for 30 min, then centrifuged at 4°C at 16,000 g for 30 min. The remaining RNA pellet was rinsed with 400 µL of 70% ethanol and was further purified using RNAClean (Agencourt, Beverly, MA) following the manufacturer's protocol. Purified RNA was quantified spectrophotometrically, and RNA quality was assessed using the Agilent 2100 Bioanalyzer. RNA was stored in 1/10 volumes 2 M sodium acetate and 2.5 volumes 100% ethanol at −20°C.

RNA was prepared for hybridization by amplification using a modified Eberwine protocol (Eberwine, '96) using an Amino Allyl MessageAmp II-96 kit (Ambion, Austin, TX). Briefly, this method amplifies the signal by using T7 RNA polymersase to synthesize many copies of RNA from cDNA made from each sample of mRNA. Amino-allyl UTPs are incorporated during transcription. Cy3 and Cy5 dyes (GE Lifesciences, Piscataway, NJ) were then coupled to the amino-allyl labeled RNA.

### Microarray

The amount of gene-specific mRNA expression was measured using microarrays with three spatially separated replicates per gene on each array. Microarrays were printed using 384 *Fundulus heteroclitus* cDNAs that included 329 cDNAs that encode essential enzymes for cellular metabolism (Table 1; Paschall et al., 2004). Average lengths of cDNAs were 1.5 Kb with a majority including the N-terminal methionine. Amplified cDNA were spotted onto epoxide slides (Corning, Lowell MA) using an inkjet printer (Aj100, ArrayJet, Scotland). Although the array was constructed using *F. heteroclitus* cDNAs, we do not expect any effect on our results hybridizing *F. grandis*. First, all comparisons will be made among groups of *F. grandis*, thus any sequence dissimilarity would not affect the comparison between groups. Second, *F. heteroclitus* from Georgia and *F. grandis* from Florida have been demonstrated to be more similar in their patterns of mRNA expression to one another than either are to *F. heteroclitus* from Maine, suggesting that potential sequence differences do not have a large effect (Oleksiak et al., 2002).

**Table 1.** Gene and pathways for cDNA printed on microarray used in this study.

| Pathway | Number of cDNAs |
|---|---|
| Amino acid metabolism | 28 |
| ATP synthesis | 27 |
| Blood group glycolipid biosynthesis | 3 |
| Channel | 3 |
| Citrate cycle (TCA cycle) | 24 |
| Fatty acid metabolism/transport | 36 |
| Fructose and mannose metabolism | 4 |
| Galactose metabolism | 2 |
| Glutamate metabolism | 7 |
| Glutathione metabolism | 10 |
| Glycerolipid metabolism | 7 |
| Glycolysis/Gluconeogenesis | 27 |
| Inositol phosphate metabolism | 14 |
| Ox-Phos-ATPsyn | 64 |
| Pentose phosphate pathway | 6 |
| Purine & Pyrimidine metabolism | 9 |
| Pyruvate metabolism | 2 |
| Signaling Pathway | 10 |
| Starch and sucrose metabolism | 2 |
| Sterol biosynthesis | 8 |
| Synthesis and degrad. of ketone bodies | 4 |
| Tetrachloroethene degradation | 3 |
| Secondary | 27 |
| Other | 47 |
| Total metabolic genes | 374 |

### Hybridization

Twenty picomoles of Cy3- and Cy5-labeled aliquots was vacuum dried together and resuspended in 10 µL of hybridization buffer (5 × SSPE, 1% SDS, 50% formamide, 1 mg/mL polyA, 1 mg/mL sheared herring sperm carrier DNA, and 1 mg/mL BSA) for a final concentration 2 pmol/µL for each sample. Immediately before use, slides were blocked in a solution consisting of 5% ethanolamine, 100 mM Tris (pH 7.8), and 0.1%SDS for 30 min. The slides were then washed in 50°C 4 × SSC, 0.1% SDS solution

for 1 hr, and rinsed with autoclaved water. Finally, slides were boiled for 2 min, given a final rinse in autoclaved water, and spun dry (800 rpm for 3 min). Label RNAs (2pmol/ul each of Cy3 and Cy5) in hybridization buffer were heated to 92°C for 2 min, quickly cooled to 42°C, and applied to the slide, and a cover slip was gently placed over the zone. Each hybridization zone is 198 $mm^2$. Slides were placed in an air-tight chamber humidified with a paper soaked in 4 × SSC to prevent hybridization from drying out. Samples were hybridized for approximately 48 hr at 42°C. Following hybridization, slides were washed in decreasing concentrations of SSC and SDS (4 × +0.1% SDS-0.1 × SSC no SDS) and then spun dry (800 rpm for 3 min). Slides were scanned using the Packard Bioscience ScanArray Express microarray scanner (PerkinElmer Life Sciences, Waltham, MA), with laser wavelengths set to 633 and 543 nm. Resulting.tiff images were imported into spot grids built in ImaGene (Biodiscovery, El Segundo, CA) for each array, and spot signals were collected as fluorescence intensities for each dye channel.

### Hybridization Design

For heart ventricles, four individuals were sample at each of seven time points (0, 4, 8, 12, 24, 48, and 96). For livers, three individuals were sampled at six time points (0, 4, 8, 12, 24, and 48). Tissues from different time points were hybridized together in a loop design (Kerr et al., 2000; Oleksiak et al., 2002). A "loop design" does not rely on a reference, rather two different experimental samples labeled with Cy3 or Cy5 fluorescent dyes are hybridized together, and each individual is measured on two slides, once with Cy3 and once with Cy5. For the heart ventricle, this creates a loop of Cy3$\rightarrow$Cy5 with 28 arrays (time with individual ♯ as subscript): $0_2^{\rightarrow}$ $4_6^{\rightarrow}$ $8_{11}^{\rightarrow}$ $12_{16}^{\rightarrow}$ $24_{21}^{\rightarrow}$ $48_{26}^{\rightarrow}$ $96_{33}^{\rightarrow}$ $4_7^{\rightarrow}$ $12_{17}^{\rightarrow}$ $48_{27}^{\rightarrow}$ $0_3^{\rightarrow}$ $8_{12}^{\rightarrow}$ $24_{22}^{\rightarrow}$ $96_{34}^{\rightarrow}$ $8_{13}^{\rightarrow}$ $48_{28}^{\rightarrow}$ $4_8^{\rightarrow}$ $24_{23}^{\rightarrow}$ $0_5^{\rightarrow}$ $12_{19}^{\rightarrow}$ $96_{35}^{\rightarrow}$ $48_{29}^{\rightarrow}$ $12_{20}^{\rightarrow}$ $8_{14}^{\rightarrow}$ $0_1^{\rightarrow}$ $24_{25}^{\rightarrow}$ $4_9^{\rightarrow}$ $96_{32}^{\rightarrow}$ first sample ($0_2$). For liver this creates a loop of Cy3$\rightarrow$Cy5 with 18 arrays (time with individual ♯ as subscript): $0_1^{\rightarrow}$ $4_2^{\rightarrow}$ $8_3^{\rightarrow}$ $12_3^{\rightarrow}$ $24_2^{\rightarrow}$ $48_2^{\rightarrow}$ $0_3^{\rightarrow}$ $8_1^{\rightarrow}$ $24_3^{\rightarrow}$ $4_1^{\rightarrow}$ $48_3^{\rightarrow}$ $8_2^{\rightarrow}$ $24_1^{\rightarrow}$ $0_2^{\rightarrow}$ $12_2^{\rightarrow}$ $4_3^{\rightarrow}$ $48_1^{\rightarrow}$ $12_1^{\rightarrow}$ first sample ($0_1$).

Each slide had six arrays, and each array had two individuals hybridized, for 12 individuals per slide.

### Data Processing and Statistical Analysis

For all analyses across time points, the mean of six replicates for each individual (see below) was used. Thus, individuals and not technical replicate were used to determine the degree of freedom.

All data processing was carried out in Microsoft Excel and SAS JMP Genomics 3.0. The microarray is printed with control spots of ctenophore DNA sequence, which do not bind to *Fundulus* sequence. Genes with average fluorescent values less than the mean of the negative control plus two standard deviations were removed from all individuals and were not analyzed. Because the photo multiplying tube (PMT) has a saturation fluorescence value of 65,535 genes whose values were within this range across most individuals and treatments in the loop were also removed. The hypoxic treatment could cause large-scale gene induction, so genes with saturated values for a few individuals at one or a few time points were kept in the data set. If only a single replicate within an array was saturated these were presumed to be from an auto-fluorescing spot. Thus, the values form this spot were converted to a missing data value in both Cy3 and Cy5 individuals.

For each gene, there are a total of six measures (three replicates per array, two arrays) for each individual. For heart ventricles, there are four individuals per time point and seven time points. For livers, there are three individuals per time point and six time points. The analyses for livers were carried out separately after the heart ventricles because the heart ventricle data suggested a delayed tissue-specific affect. Thus, we followed up heart analysis by analyzing gene expression in the liver in same individuals. Unfortunately, livers were not available for all individuals at all time points.

For all analyses, raw fluorescence values were $\log_2$ transformed, and then spatial variation was smoothed using the LOESS normalization in SAS JMP Genomics 3.0. These transformed values were used for all subsequent statistical analysis. The Mixed model analysis in SAS JMP Genomics 3.0 was used to carry out Analysis of Variance (ANOVA) for each gene. To examine the differences among individuals within each of the time treatments, we used the following ANOVA model:

$$y_{ijk} = \mu + A_i + D_j + I_k + e_{ijk}$$

where μ is the overall average signal, $A_i$ is the effect of the $i$th array (one of eight or six arrays within a time point), $D_j$ is the effect of the $j$th dye (with one of two dyes: Cy3 or Cy5), $I_k$ is the fixed effect of the Individual (one of three or four individuals within each time point), and $e_{ijk}$ is the error term. $A_i$, and $D_j$ are random terms. In this case, variance of the random terms is estimated by restricted maximum likelihood. To examine the variance among individuals across all genes, the mixed model described above was used for all 28 individuals for heart ventricles or 21 individuals for livers across all time points. The least square means from this model across all treatment with just array and dyes as affect were used for the correlation analyses.

To examine the effect of hypoxic exposure, an ANOVA was carried out on each gene across all treatments, using the following model:

$$y_{ijkm} = \mu + A_i + D_j + T_m + I_k(T_m) + e_{ijkm}$$

where μ is the overall average signal, $A_i$ is the effect of the $i$th array (one of twenty-eight arrays), $D_j$ is the effect of the $j$th dye (with one of two dyes: Cy3 or Cy5), $T_m$ is the fixed effect of time for the $m$th time point (one of six or seven time points), $I_k(T_m)$ is the effect of individuals within time point (one of three or four individual per time point), and $e_{ijk}$ is the error term. $A_i$, $D_j$, and $I_k(T_m)$ are random terms.

Hierarchical clustering of gene expression uses Macintosh's version (de Hoon et al., 2004) of Eisen's Cluster and Treeview (Eisen et al., '98). In JMP 7, a Fisher's exact test was used to determine the statistical significance of gene pathway distribution between treatments. Dunnette's post hoc test (JMP 7) was used to determine which time points differ from the zero time point.

## RESULTS

After accounting for negative controls, 374 potential genes remained for further analysis. In cardiac tissue, 148 of the 374 informative genes had fluorescent signals either lower than the negative control or saturated across all treatments and were removed. Thus, 226 genes were included in the analysis of short-term hypoxia exposure for heart ventricles. For livers, of the 374 genes, 118 were not used because the signal was below negative control values or near saturation. Thus, 256 genes were used in the analysis of liver mRNA expression. This frequency of quantifiable metabolic genes is similar to previous studies (Oleksiak et al., 2002, 2005; Whitehead and Crawford, 2005; Oleksiak and Crawford, 2006; Whitehead and Crawford, 2006a,b; Scott et al., 2009a,b; Oleksiak, 2010)

### Cardiac

There are seven time points, each with four individuals. With dyes, arrays and individuals within each time as random factors there are 6 and 21 degrees of freedom (seven time points with four individual per time point). Among all seven-time points, 17 genes of the 226 (7.5%) had significant changes ($P<0.01$) in mRNA expression (Table 2, Fig. 1A and C). Most significant differences have a small change of less than 1.5 fold ($\log_2<0.6$; Fig. 1A and C). Comparison among means for each time point relative to the zero-hour-control used Dunnett's post hoc test. The probabilities from Dunnett's test were used in the volcano plots (Fig. 1C). Among the 17 genes with significant differences in mRNA expression, 11 have a significant difference relative the zero time point (Dunnett's test, $P<0.05$; Table 2).

For cardiac tissue, of the 11 genes that are significant with Dunnett's test, nine are different at 96 hr of exposure (Table 2, Fig. 1C). The 48-hr exposure had four significant differences and three of these were also different at 96 hr. The 4-, 12-, and 24-hr time exposure had three, three, and two genes (respectively) with significant difference in mRNAs (Table 2, Fig. 1C). At the 8-hr time point, no genes had a significant difference in expression. The lack of significance at 8 hr may reflect the greater variation among individuals (see below). Across all the early exposure (4–24 hr), all but one of the significant genes was also significant at 48 and 96 hr. Examining the volcano plots (Fig. 1C), most of the changes become larger and more significant with time. Of the nine genes significantly different after 96 hr of exposure, four (44%) encode genes in the oxidative phosphorylation pathway. With 22 of the 226 measured genes from the oxidative phosphorylation pathway, four oxidative genes of nine total genes with a difference in expression represent a statistically significant over representation of oxidative phosphorylation genes (Fisher-Exact test, $P<0.01$).

### Liver

There are six time points, each with three individuals. With dyes, arrays, and individuals within each time as random factors, there are 5 and 12 degrees of freedom (six time points, with three individuals per time point). Of 256 genes quantified from the liver, 20 (7.8%) had significantly different mRNA expression among the six time points ($P<0.05$, ANOVA, Fig 1B and D, Table 3). Among the 20 genes with a significant difference in mRNA expression, 12 were significantly different with Dunnett's post hoc test that contrasts the 4 to 48-hr exposure relative to the zero-hour-control ($P<0.05$). All these 12 genes had a significant difference at the 4-hr exposure and one of these also was different at the 8-hr exposure. Four of these 12 genes (33%) with a significant difference in expression are in the oxidative phosphorylation pathway and all four have greater expression at the 4-hr exposure relative to the zero hour exposure. There were 26 oxidative phosphorylation gene among the 256 measured genes, and four oxidative genes of 12 total genes with a difference in expression represent a statistically significant over representation of oxidative phosphorylation genes (Fisher-Exact test, $P<0.01$).

Most changes in liver mRNA expression are small, less than 1.5-fold ($\log_2<0.6$, Fig. 1D). Interestingly, the magnitude of the difference ($\log_2$ difference from zero exposure) does not predict if there is statistical significance. For example, SCL1 and ZP3 at 12 hr or ALDOB at 4 hr (Fig 1B and D) has equally large fold changes, but they are not statistically significant ($P>0.5$). This most likely reflects a greater variation among individuals, because the statistical difference is based on the ratio of variation among treatment relative the variation among individuals within a treatment.

### Correlation Among Genes

Correlations among genes (with individuals as replicates) provide a quantitative measure of the similarity of expression across all time points. We determined the correlation in mRNA expression among the 17 significant heart genes (correlations compared the expression of one gene in 28 individuals with the expression of a second gene in the same 28 individuals; $r=0.37$ has $P<0.05$) or 20 significant liver genes (where correlations compared the expression in the 18 individuals; $r=0.47$ has $P<0.05$). These correlations used the least square means for each individual from the mix-model ANOVA (with array and dyes as factors). There are two patterns in both liver and hearts (Fig. 2): genes with positive or negative correlations. For cardiac mRNA expression (Fig. 2A), the upper left genes show significantly greater expression at 48 and 96 hr (Fig. 1A). These include

Table 2. List of cardiac genes with significant difference in mRNA expression among exposure times (ANOVA $P<0.05$).

| # | Accession # | Abbr. | Name | Function | P-val-Time | P-Val 4 hr | P-Val 8 hr | P-Val 12 hr | P-Val 24 hr | P-Val 0–48 hr | P-Val 96 hr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CN985218 | **DBI** | Acyl_CoA_binding | PPAR signaling | 2.E-04 | 0.33 | 0.99 | 0.68 | 0.55 | 0.11 | **0.01** |
| 2 | CN985001 | **COX6C2** | Cytochrome_c_oxidase_ _VIc_2 | Oxidative phosphorylation | 2.E-03 | 0.25 | 0.96 | 0.82 | 0.78 | 0.05 | **0.00** |
| 3 | CN985092 | **COX7A2** | Cytochrome_c_oxidase_ _VIIa | Oxidative phosphorylation | 2.E-02 | 0.88 | 0.99 | 0.96 | 0.98 | 0.58 | **0.02** |
| 4 | CN985221 | **SLC37A4** | Glucose_6_phosphate_translocase | Glycogen Storage | 9.E-04 | **0.01** | 0.44 | **0.03** | 0.22 | **0.01** | **0.00** |
| 5 | CN985002 | **UQCRC1** | Ubiquinol_cytochrome_C_reductase | Oxidative phosphorylation | 3.E-02 | 0.06 | 0.86 | 0.51 | 0.91 | 0.29 | **0.03** |
| 6 | CN985076 | **AMBP** | Alpha_1_microglobulin_Inter_alpha_trypsin_inhibitor_light_chain | Glycoprotein | 1.E-02 | 0.06 | 0.72 | 0.68 | 0.99 | **0.00** | **0.03** |
| 7 | CN984992 | **NA_29** | NA_29 | Unknown | 4.E-02 | **0.00** | 0.10 | **0.04** | 0.21 | **0.00** | **0.01** |
| 8 | CN985074 | **NDUFA2** | NADH_ubiquinone_oxidoreductase_B8 | Oxidative phosphorylation | 4.E-02 | 0.42 | 0.37 | 0.61 | 1.00 | 0.12 | **0.01** |
| 9 | CN985202 | **CALM** | Calmodulin | Calcium signaling | 3.E-02 | 0.90 | 0.22 | 0.06 | **0.01** | 0.74 | 0.92 |
| 10 | CN985143 | **NA_3** | NA_3 | Unknown | 4.E-03 | 0.95 | 0.47 | **0.04** | **0.02** | **0.04** | 0.99 |
| 11 | CN985196 | **CYP3A56** | Cytochrome_P450_3A56 | Steriod-metabolism | 6.E-03 | **0.04** | 0.63 | 0.53 | 1.00 | 0.99 | **0.02** |
| 12 | CN985198 | BHMT | Betaine_homocysteine_S_methyltransferase | Glycine, serine and threonine metabolism | 2.E-02 | 1.00 | 1.00 | 0.98 | 1.00 | 0.76 | 0.99 |
| 13 | CN985209 | SOD1 | Superoxide_dismutase_Cu_Zn | Antioxidant | 2.E-02 | 0.75 | 0.99 | 1.00 | 1.00 | 0.83 | 0.12 |
| 14 | CN985293 | ATP1 | ATP_synthase_alphar | ATP synthesis | 4.E-02 | 0.29 | 0.12 | 0.87 | 1.00 | 1.00 | 0.97 |
| 15 | DR109342 | ATP1A1 | Sodium_potassium_transporting_ATPase | Membrane transport | 3.E-02 | 0.46 | 1.00 | 0.98 | 0.91 | 0.34 | 0.18 |
| 16 | CN985213 | GA | Glutaminase_liver_isoformr | Glutamate metabolism | 3.E-02 | 0.51 | 0.30 | 0.95 | 0.67 | 0.36 | 0.33 |
| 17 | CN985069 | PIK4CB | Phosphatidylinositol_4_kinase_beta | Inositol signally | 5.E-02 | 1.00 | 0.99 | 0.65 | 0.88 | 0.93 | 0.95 |

#-refers to order in Figure 1A. *P*-val are probabilities associated with ANOVA, or for each time point Dunnett's test (**bold** are significant).

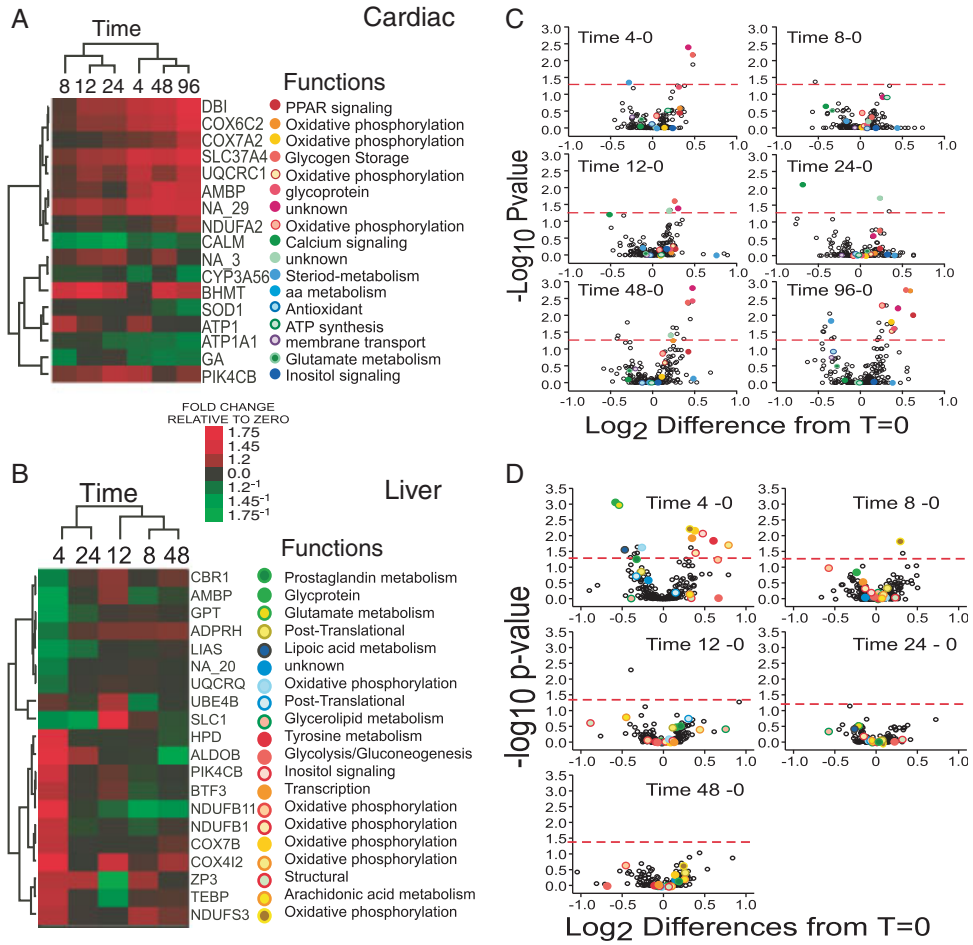


Figure 1. Hypoxia gene expression. **(A, B)** Cardiac and liver hierarchical cluster of mRNA expression relative to zero time exposure for genes with significant difference in expression among exposure time to hypoxia (2.8 Kpa) for 0,4,8,12,24, 48, and 96 hr. Color scale for changes in mRNA expression 1.75 ($\log_2 = 0.8$) to 1/1.75 ($\log_2 = -0.8$). **(C, D)**: Cardiac and liver plots of $\log_2$ difference from zero time point and negative $\log_{10}$ *P*-values from Dunnett's post hoc test. Colored spot are significantly different from zero time point based on ANOVA and Dunnett's test. Colors refer to specific genes in A and B.

all the oxidative phosphorylation genes. Notice the expression of genes upregulated at 48 and 96 hr tend to be negatively correlated with SOD, Cytochrome p450-3A56 (CYP3A56), and Na-$K^+$-ATPase (ATP1A1).

For the liver, a similar pattern exists (Fig. 2B). Genes upregulated at the 4-hr exposure (upper left) tend to be positively correlated (Fig. 1B). Similarly to cardiac tissue, this includes four of the five oxidative phosphorylation genes. The genes upregulated at the 4-hr exposure have very strong negative correlation to genes suppressed at this same time.

### Maximum Gene Expression

To examine patterns of expression beyond the 17 or 20 significant genes, we sorted the maximum expression levels at each time point using the standardize least square means for each time point for all 226 or 256 genes (Fig. 3; where the standardize least square mean of mRNA expression for each gene has a $\log_2$ mean of zero and a variance of one across all time points). Even though there are only a few significant differences in gene expression and most of this significant difference in expression occurs at one or a few time points, there are waves of maximum expression (Fig. 3). This pattern mirrors the number of significantly expressed genes (Tables 2 and 3), with the highest number of genes having maximum expression after 96 hr of hypoxia exposure for cardiac tissue, or at 4 hr for liver tissues.

There are distinct patters of gene expression within the distribution of maximized gene expression over time. To determine whether the maximum peak of expression is disproportionally associated with a specific pathway, we applied

Table 3. List of liver genes with significant difference in mRNA expression among exposure times (ANOVA $P < 0.05$).

| # | Accession # | Gene Abbrev. | Name | Function | P-val Time | P-Val 4 hr | P-Val 8 hr | P-Val 12 hr | P-Val 24 hr | P-Val 0–48 hr |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CN985183 | CBR1 | Carbonyl_reductase_NADPH_1 | Prostaglandin metabolism | 7.E−03 | 0.055 | 1.000 | 0.241 | 0.914 | 0.583 |
| 2 | CN985076 | **AMBP** | Alpha_1_microglobulin_Inter_alpha_trypsin_inhibitor_light_chain | Glycoprotein | 8.E−03 | **0.001** | 0.134 | 0.277 | 0.899 | 0.736 |
| 3 | CN985197 | **GPT** | Alanine_aminotransferase | Glutamate metabolism | 5.E−03 | **0.001** | 1.000 | 1.000 | 0.299 | 0.999 |
| 4 | CN985275 | ADPRH | ADP_ribosylarginine_hydrolase | Post-translational | 1.E−02 | 0.131 | 0.450 | 0.469 | 0.839 | 0.369 |
| 5 | CN985219 | **LIAS** | Lipoic_acid_synthetase | Lipoic acid metabolism | 2.E−02 | **0.026** | 0.950 | 1.000 | 0.316 | 0.996 |
| 6 | CN985165 | **NA_20** | NA_20 | Unknown | 2.E−02 | **0.023** | 0.970 | 1.000 | 1.000 | 0.995 |
| 7 | CN985013 | **UQCRQ** | Ubiquinol_cytochrome_C_reductase | Oxidative phosphorylation | 4.E−02 | **0.024** | 0.972 | 0.789 | 0.997 | 0.998 |
| 8 | CN985303 | UBE4B | Ubiquitin_conjugation_factor_E4_B | Post-translational | 2.E−02 | 0.622 | 0.198 | 0.177 | 0.990 | 0.998 |
| 9 | CO436097 | SLC1 | 1_acyl_sn_glycerol_3_phosphate_acyltransferase | Glycerolipid metabolism | 4.E−02 | 0.970 | 1.000 | 0.366 | 0.477 | 0.964 |
| 10 | CN985146 | **HPD** | 4_hydroxyphenylpyruvate_dioxygenase | Tyrosine metabolism | 3.E−03 | **0.014** | 0.993 | 0.998 | 1.000 | 0.878 |
| 11 | CN992491 | ALDOB | Fructose_bisphosphate_aldolase_B | Glycolysis | 2.E−02 | 0.962 | 0.982 | 0.999 | 0.905 | 0.972 |
| 12 | CN985069 | **PIK4CB** | Phosphatidylinositol_4_kinase_beta | Inositol signaling | 2.E−03 | **0.008** | 0.623 | 0.793 | 1.000 | 0.998 |
| 13 | CN984997 | **BTF3** | Transcription_factor_BTF3 | Transcription | 1.E−02 | **0.012** | 0.284 | 0.895 | 0.997 | 0.952 |
| 14 | CN985243 | NDUFB11 | NADH_ubiquinone_oxidoreductase_ESSS | Oxidative phosphorylation | 4.E−02 | 0.056 | 0.100 | 0.806 | 0.997 | 0.235 |
| 15 | CN985128 | **NDUFB1** | NADH_ubiquinone_oxidoreductase_MNLL | Oxidative phosphorylation | 1.E−02 | **0.032** | 0.428 | 0.982 | 0.779 | 0.972 |
| 16 | DR109356 | **COX7B** | Cytochrome_c_oxidase_VIIb | Oxidative phosphorylation | 2.E−02 | **0.007** | 1.000 | 0.999 | 1.000 | 0.424 |
| 17 | CO436094 | **COX4I2** | Cytochrome_c_oxidase_subunit_IV_isoform_2 | Oxidative phosphorylation | 5.E−02 | **0.020** | 0.667 | 0.407 | 0.998 | 0.588 |
| 18 | CN976443 | ZP3 | Zona_pellucida_3 | Structural | 1.E−02 | 0.949 | 0.855 | 0.241 | 0.830 | 0.996 |
| 19 | CN985280 | TEBP | Telomerase_p23 | Arachidonic acid metabolism | 3.E−02 | 0.670 | 0.980 | 0.150 | 0.957 | 0.962 |
| 20 | CN985109 | **NDUFS3** | NADH_ubiquinone_oxidoreductase_30_kDa | Oxidative phosphorylation | 3.E−02 | **0.006** | **0.014** | 1.000 | 0.971 | 0.228 |

#-refers to order in Figure 1B. *P*-val are probabilities associated with ANOVA, or for each time point Dunnett's test (**bold** are significant).

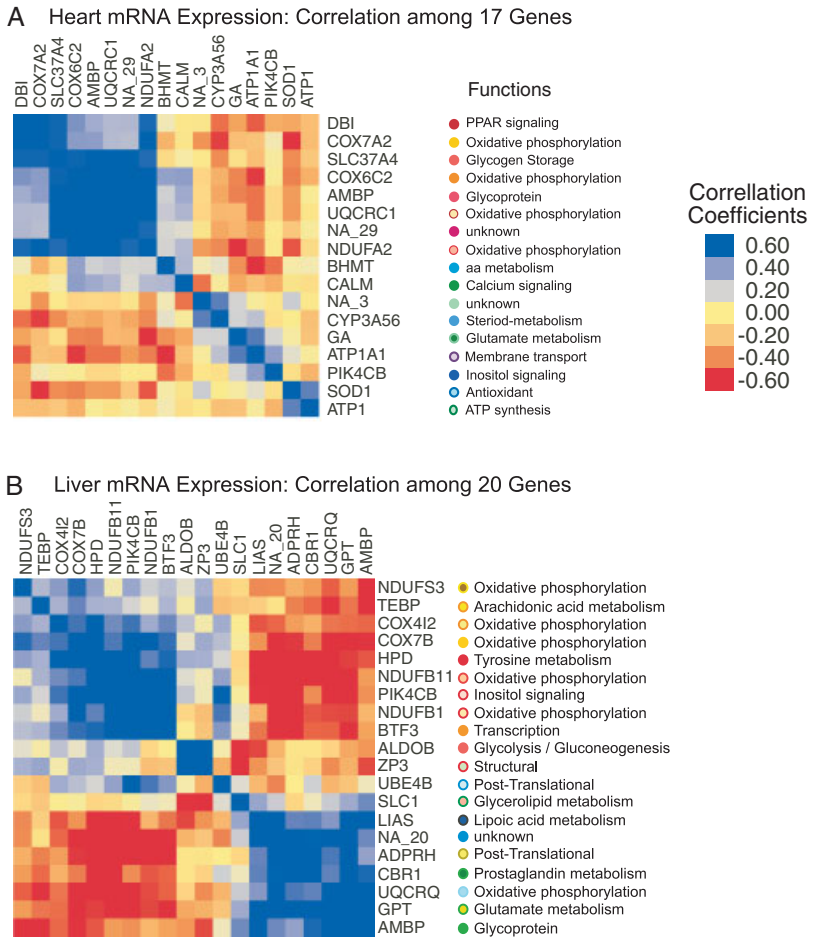


Figure 2. Correlations among gene expressions. Correlations of mRNA expressions between pairs of genes with significant hypoxia effect. **(A)** Correlations among 17 significant cardiac genes. Correlation between genes used the 28 individual subjected to seven different exposure times. Correlation coefficients > 0.48 or > 0.37 or are significant at *P* value of 0.01 or 0.05, respectively. **(B)** Correlations among 20 significant liver genes. Correlations between genes used the 18 individual subjected to six different exposure times. Correlation coefficients > 0.59 or > 0.47 are significant at *P* value of 0.01 or 0.05. Function and color spots are defined in Figure 1.

a Fisher exact test. The specific hypothesis is whether the genes that are maximized at any time point contain too many or too few genes from a specific pathway. The results of a Fisher exact test demonstrate that in cardiac tissue oxidative phosphorylation genes have significantly more maximally expressed genes at 96 hr ($P < 0.02$). For liver at 4 hr, there are 14 oxidative phosphorylation genes with maximal expression (out of 63), but this is not a significant over representation ($P > 0.10$).

## DISCUSSION

In the teleost fish *Fundulus grandis*, 17 genes in heart ventricle (7.5%) and 20 genes in liver (7.8%) had significant differences ($P < 0.05$) in mRNA expression across all time treatments (Tables 2 and 3). This is proportionally similar to the findings in other studies (Gracey et al., 2001; Ton et al., 2002, 2003; Marques et al., 2008). However, the timing of the changes in gene expression was different between the tissues. For cardiac tissue, most of the significant changes in mRNA expression occurred at 96 hr (82% of the genes with significant Dunnett's test). For liver, most of the significant changes in mRNA expression occurred at 4 hr (100% of the genes with significant Dunnett's test). In liver, these change were transitory, only one other time point (8 hr) had a significant change. A possible explanation for the difference in the temporal pattern of gene expression between liver and heart is that during hypoxia blood flow is redirected to preferentially oxygenate vital organs, such as the heart and the brain (Cohen et al., '67; Axelsson and Fritsche, '91; Kuwahira et al., '93; Nilsson et al., '94; Hicks and Farrell, 2000; Stecyk et al., 2004). Consequently, the liver could potentially experience hypoxia sooner than the heart. Since adequate oxygenation could not be maintained after prolonged

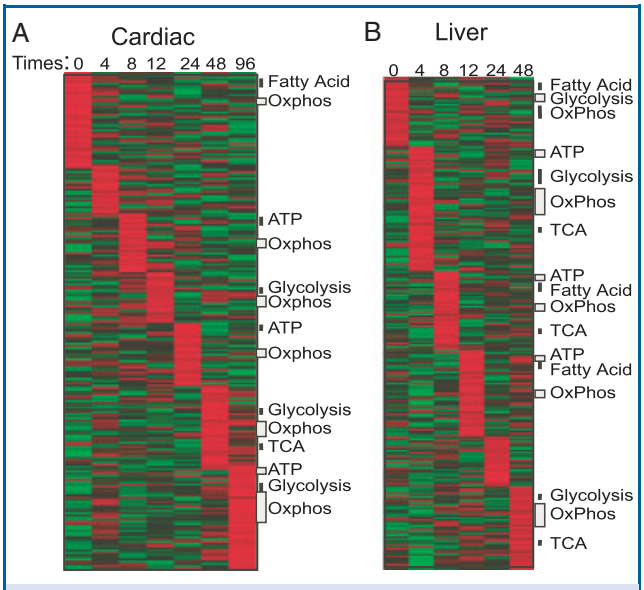


Figure 3. Maximum gene expression during exposure to hypoxia. Cluster of maximum expression at each time point using the standardize least square mean for hypoxia exposure. **(A)** Cardiac mRNA expression for 226 genes for 4, 8, 12, 24, 48 and 96 hr. **(B)** Liver mRNA expression for 256 genes for 4, 8, 12, 24, and 48 hr. Pathways are listed if three or more genes in pathway occur at the same maximum.

hypoxia, even the vital organs would have to eventually elicit a transcriptional response in order to adapt to hypoxia (Semenza, 2005; Ward et al., 2005). This temporal pattern of hypoxia-induced gene expression is similar to the expression of HIF1A protein, which reaches a maximum in 1 hr in mouse livers and returns to baseline by 4 hr (Stroka et al., 2001). However, in the brain, HIF1A requires 5 hr to reach maximum expression and returns to baseline after 12 hr (Stroka et al., 2001). This pattern is similar to the results in our study; the liver responded to hypoxia much earlier and was completed before the changes at 96 hr in the heart.

Interestingly, at 4 hr exposure for the liver or 96 hr exposure for the heart, more genes that are significant are upregulated than downregulated (7 of 12 for liver and 8 of 11 for heart, Fig. 1). This is similar to cardiac response in *Danio rerio*, where 2.5% of genes had differential expression, with two-thirds of these being upregulated, and has also been observed in the livers of mice exposed to altitudinal hypoxia gradients (Marques et al., 2008; Baze et al., 2010). This also reflects the patterns of maximal gene expression (Fig. 3) where during exposure to hypoxic conditions the greatest number of genes with maximum expression vs. other time points occurs at 4 hr for livers and 96 hr for heart ventricles.

In the heart and liver, there is an increase in mRNAs that encode proteins in the oxidative-phosphorylation pathway: the pathway that oxidizes NADH and consumes $O_2$ to produce the energy gradient that is used to create ATP. This increase includes the both statistically different patterns of expression (Fig. 1) and the peak or maximum expression (Fig. 3). That is, the time of maximum response (where there was the greatest number of significant changes relative to zero exposure, or the largest number of genes with maximal expression) includes the four upregulated mRNAs that encode protein in the oxidative phosphorylation pathway. Taking into account the frequency of oxidative-phosphorylation genes, for the heart ventricle, this is a significant over representation of oxidative phosphorylation genes at 96 hr of exposure ($P<0.01$, Fisher Exact test). For the liver, this bias in oxidative genes occurs at 4 hr where there are too many of oxidative phosphorylation genes with significant changes ($P<0.05$, Fisher Exact test). These data indicate both an increase in oxidative phosphorylation genes due to hypoxia, and among metabolic genes, a disproportionate affect on this pathway.

We suggest that in both liver and heart there is an increase in expression for genes in the oxidative phosphorylation pathway, although they occur at different times. However, these upregulated oxidative phosphorylation genes are not the same in both tissues. Both liver and hearts have increased expression of subunits for Complex 1 (NADH dehydrogenase) and complex IV (Cytochrome oxidase), but the genes that change in the liver or heart encode different subunits of these protein complexes (Tables 2 and 3). Notice, these enzyme complexes require a stoichiometric amount of each protein subunits (45 proteins for Complex 1 and 13 proteins for Complex IV). Thus, if these changes in mRNA effect a change in protein, then the other subunits are upregulated by post-transcriptional mechanisms. Alternatively, the changes in these mRNAs are not meaningful. Yet, changes in mRNA expression are often biologically meaningful. The change in mRNA expression for Ldh_B in *Fundulus* is evolving by natural selection and thus must affect a phenotype that is evolutionarily important (Pierce and Crawford, '97; Crawford et al., '99b). Similarly, there are evolutionary significant patterns of mRNA expression for many metabolic genes in *Fundulus* (Oleksiak et al., 2002; Whitehead and Crawford, 2006a,b). It is difficult to imagine that an evolutionary significant pattern in mRNA expression would not involve a similar change in the protein that these genes encode. Finally, for *Fundulus* the increase in Ldh_B mRNA is associated with an increase in enzyme for Ldh-B (Crawford and Powers, '89; Crawford and Powers, '92; Crawford et al., '99a). This significant correlations between mRNA and the protein it codes for is generally true for many other metabolic genes (Rees et al., 2011). These data suggest that in general a significant change in mRNA is associated with a similar change in protein. However, we would like to point out that these correlations between mRNA and the protein they encoded are difficult because there needs to be both significant variation in the expression of a gene and a sufficient sample size to detect a correlation. An additional complexity is that these correlations are dependent on the metabolic network;

where taking into consideration interactions within pathways enhances the predictive nature of mRNA expression (Moxley et al., 2009). Although we only have measure of oxidative phosphorylation mRNA and lack a direct measure of these proteins, we suggest that the early response to hypoxia is an increase in oxidative phosphorylation, potentially to maintain ATP production. However, the increase expression of mRNAs that code for a few oxidative phosphorylation subunits would have to be accompanied by other molecular mechanisms that would change the expression of other subunits.

The number and tissue-specific nature of changes in gene expression are comparable to other transcripomic publications. In *Gillichthys mirabilis* (another hypoxia tolerant teleost) between 8 and 144 hr of hypoxia, exposure alter the expression of 126 genes (approximately 6% of those tested) (Gracey et al., 2001). For the liver in *G. mirabilis*, the changes in gene expression that were cited as being important for hypoxia were involved in anaerobic ATP generation (Gracey et al., 2001). However, in cardiac tissue of *G. mirabilis* there was a twofold increase in cytochrome b and cytochrome oxidase I (protein in the oxidative phosphorylation pathway), and a larger increase in skeletal muscle, (Gracey et al., 2001). In *D. rerio* (zebra fish) exposed to 3 weeks of hypoxia, 376 genes (2.5%) in hearts and 367 genes (2.4%) in gills had changes in mRNA expression (van der Meer et al., 2005; Marques et al., 2008). In the cardiac tissue, none of these genes were in the oxidative pathway (Marques et al., 2008). In the gills, there was an overall reduction in oxidative phosphorylation genes: specifically 12 Complex 1 subunits were repressed, and 2 were upregulated and 2 Complex IV subunits were depressed.

Our finding of enhanced oxidative-phosphorylation mRNA expression is unusual. In general, there is an expectation of enhanced glycolytic gene expression (Hochachka and Somero, 2002; Nikinmaa and Rees, 2005; Martinez et al., 2006), yet there are no significant changes in glycolytic genes in cardiac tissue, and only one (aldolase) in livers. However, some hypoxia-tolerant vertebrates have been shown to have an enhanced aerobic enzyme activity. For instance, high altitude populations of llama, deer, and cattle have been demonstrated to have enhanced levels of enzymes involved in oxidative metabolism (Ou and Tenney, '70; Hochachka et al., '83). In sparrows, individuals from high altitude were shown to have enhanced expression of numerous genes involved in oxidative phosphorylation including subunits of cytochrome oxidase and NADH, though this study included the compounding effect of temperature (Cheviron et al., 2008). Enhanced expression of oxidative phyophorylation genes during chronic minor hypoxia has been demonstrated in mammals (Essop, 2007). There are two important differences between this study and the other studies on hypoxia in fishes: the time course and the criteria used to distinguish differences in expression. The shortest exposure time in these other studies was 8 hr in liver for *G. mirabilis* (Gracey et al., 2001), where the criteria for differential expression was a 2.5-fold change. This duration and criteria would have missed all of the change in mRNA expression we found in livers. For *D. rerio* (Marques et al., 2008), differential expression was measured after 3 weeks and required that hearts have a twofold change and $P<0.02$. If we applied similar statistical criteria, it would reduce the number of genes with differential expression from 17 to 6 and from 20 to 11 for hearts and liver respectively. Application of a fold-change of twofold would have eliminated all differences. We would like to point out that fold-changes (*X*-axis of Fig. 1B and D) have little to do with the statistical difference (*Y*-axis of Fig. 1B and D) because large changes in expression are often associated with large variation among individuals within treatments. Thus, there can be a large average difference from control, but the variation among individuals includes the control. Pooling individuals does not eliminate this variation; instead, when pooling several individuals the variation among individuals is hidden. Finally, a third explanation is that there were few oxidative phosphorylation genes annotated for these arrays. Few of *G. mirabilis* EST are annotated as oxidative genes (cytochrome oxidase, NADH dehydrogenase, Complex I-IV, etc.). We suggest that oxidative phosphorylation genes may more often be involved in the physiological response to short-term hypoxia, but this conclusion would require applying similar analyses to more species with microarrays that have probes annotated for oxidative phosphorylation genes.

### Correlations in mRNA Expression

There are significant correlations among genes in their level of expression (Fig. 2). For example, in the heart, the increase in the expression Cox 7A2 (a subunit of Complex IV, cytochrome oxidase) is significantly correlated with Cox 62C and NDUFA2 (a subunit of Complex I, NADH dehydrogenase). Similarly, the Cox 7B, Cox 4IB, NDUF1, and NDUF11 are all significantly correlated. The correlations (Fig. 2) matched with patterns of expression (Fig. 1) indicate a set of genes that are upregulated with hypoxia and another set that are downregulated. There are two explanations for these and other correlated patterns of gene expression in *Fundulus* (Crawford and Oleksiak, 2007): these genes share similar transcription factors, or there are many different transcription factors which are affected by hypoxia and each gene has a separate regulatory pathway. Given what we know about the control of hypoxic response (Nikinmaa and Rees, 2005), a shared regulatory pathway would be most parsimoniously explain the correlated patterns of gene expression.

Gene expression during hypoxia is regulated by the HIF transcription factor family (Rocha, 2007; Kenneth and Rocha, 2008). Under normal oxygen conditions, a subunit of the HIF transcription factor is hydroxylated by prolyl hydrolases, and subsequently rapidly degraded. However, during hypoxia the protein stabilizes and associates with its other subunits and enters the nucleus where it interacts with cofactors and can affect transcription (Hoogewijs et al., 2007; Rocha, 2007; Kenneth and Rocha, 2008). Although, HIF is the most well known of the

transcription factors associated with the hypoxic response, there are a number of other controls. The NF-kB, AP-1, p53, and Myc families of transcription factors are also known to affect hypoxic gene expression. Several of these factors affect transcription directly and others modulate the effects of the HIF transcription factors (Kenneth and Rocha, 2008). More recently groups of microRNAs (miRNAs) have been demonstrated to affect hypoxic gene expression (Kulshreshtha et al., 2007; Rocha, 2007; Kenneth and Rocha, 2008). If one or a few transcription factors are affecting hypoxic gene expression, we should expect that the expression of these hypoxia-induced genes to be significantly correlated (Fig. 2). Thus, although we do not measure HIF gene expression, we suggest that much of the correlated pattern of expression may be due to the post-transcriptional regulation of this factor.

### The Effect of Individual Variation

We can examine the mean variance at each time point using the variance among individuals for each significant gene as replicates: $n = 17$ and 20 significant genes for cardiac and liver, respectively. In these analyses, the $\log_2$ mean expression for each gene is equal to zero; thus, the difference in the variation reflect the variation among individuals and not the magnitude of expression.

The statistically significant changes in gene expression at 4 and 96 hr in liver and hearts reflect a synchrony among individuals: it is at these times that the individuals have similar responses. That is, it is the combination of low variation among individuals with a relatively large response to hypoxia at 4 and 96 hr that define a statistically significant change. Recall, that all the significant changes are relatively small relative to the zero-hour-control (i.e. less than twofold, Fig. 1). Thus, because the ANOVA is testing whether the variation among time points is greater than within time-point (i.e.variance among individuals) and, these relatively small fold changes in expression will only be significant if the variation among individuals is only approximately 40% as large as the variation among time points (1/2.5 when the $F$ value of 2.57 yields a $P$-value of 0.05 at 6, 21 degree of freedom). Accordingly for genes with a significant difference in expression, the variation among individuals tends to be smaller at 4 hr in the liver and at 96 hr of exposure in the heart (Fig. 4A). Notice the mean and variance reflect the mean variation among four individuals for all 17 significant genes in the heart and all 20 significant genes in the liver. We suggest that at other exposure times, the large variation in interindividual gene expression hide potentially important changes in expression. For example, this helps to explain the lack of significant genes at 8 hr: there is too much intraindividual variation with too small of change relative to the zero-hour-control. To provide support for this concept, we compare the expression of the 17 significant genes among all 28 individuals for hearts (Fig. 4B) and the expression for 20 significant genes for the 18 individuals

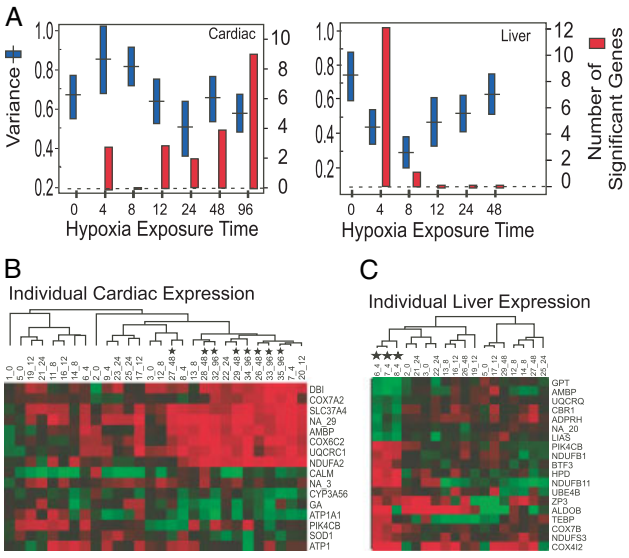


**Figure 4.** Individual patterns of expression. **(A)** The number of significant genes relative to zero exposure (Dunnett's test $P < 0.05$) and variation among individuals within each exposure. Blue bars indicate the mean variation among individuals with the 17 or 20 significant genes as replicates. The mean $\log_2$ expression of each gene is equal to zero. **(B, C)** Hierarchical cluster of individual mRNA expression. Cluster of individuals for genes (least square means of six technical replicates) with significant differences in expression among exposure times. **(B)** Cardiac mRNA expression of 28 individuals. **(C)** Liver MRNA expression for 18 individuals. Columns are labeled with individual (first) and time (second). Stars indicate the exposure time where most genes that have significant differences. Color scaling is the same as Figure 1.

for livers (Fig. 4C). For hearts, only the individuals from the 96- and 48-hr exposure time points form a cohesive cluster. For liver, only the individuals from the 4-hr exposure form a cluster. The individuals from other time points have equally as large increases or decreases (relative to the grand mean); however, they do not cluster together. For example, in the heart the expression of COX6C2 (Cytochrome C oxidase VIc) at the 4-hr exposure is not significantly different from zero exposure. Yet, two individuals (8-4 and 7-4, Fig. 4B) have very high expression, but the other two individuals have very low expression. Similarly in the liver, COX412 (Cytochrome C oxidase IV) has individuals at different time points with relatively large expression, but only the 4-hr exposure is significantly different from the zero exposure time. Thus, we are suggesting the statistically significant induction of hypoxia gene expression at 4 hr in the liver and 96 hr in the heart, reflects a conserved response among individuals, but this response may be initially induced at different times for different individuals. If these patterns are genetically based, it would mean that an inbred strain from an individual

may be different from the "norm" or at least not similar in all individuals. Thus, identifying important physiological patterns of gene expression may require examining more than one strain or inbred line.

## CONCLUSION

Hochachka et al. ('96) suggest that there is a unifying theory for hypoxia tolerance that involved the reduction of ATP utilization and gene expression within minutes to hours of exposure. Our data from liver suggest that much of the hypoxic response in gene expression happens within the first few hours and is transitory. Additionally, the correlation among genes supports the hypothesis that sensing and effecting a change in expression is an essential mechanism for hypoxia tolerance (Hochachka et al., '96). However in heart ventricles, the largest change in expression occurs much later at 96 hr than the change in the liver. This difference between tissues could represents preferential blood flow to the heart, difference in the sensitivity or physiological role of heart vs. livers. Thus, the theory presented (Hochachka et al., '96) is altered in different tissue. Surprisingly, in both tissues the induction of genes includes oxidative-phosphorylation genes. This is unexpected unless the increased expression of these subunits alter the P:O ratio, production of oxygen radical or the coupling between oxidative reduction and ATP production (Brown et al., '90; Hafner et al., '90; Gnaiger et al., '95; Gnaiger, 2007). These data do suggest that an early or short-term response may include the upregulation of mitochondrial function potentially as a mechanism to maintain ATP production

Finally, the pattern of gene expression suggests that many small changes in mRNA expression may be important. Yet, there is much variation among individuals in gene expression. This interindividual variation for hypoxia responsive genes is less apparent at 4 hr in the liver and 96 hr in the heart (the times with the maximum response). We suggest that this convergence may reflect a biologically important response. Even with this complex pattern of response, the large number of correlations among genes suggests that a few transcription factors may be responsible.

## ACKNOWLEDGMENTS

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