Hypoxic Stress Induced Gene Modulation in Medaka

(*Oryzias latipes*) and *Xiphophorus maculatus*

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Abstract

Hypoxic zones in aquatic systems are becoming an increasing worldwide problem due to the stress these hypoxic zones place on economically important fishery resources. Evaluating gene modulation responses in fishes exposed to hypoxic conditions may provide the tools to better understand the physiological adaptations made by fish to survive environmental stress. In addition, shifts in gene regulation upon exposure to hypoxia may lead to the establishment of transcriptional profiles or identification of genetic biomarkers associated with the extent and duration of hypoxic conditions. Such profiles or biomarkers would be useful in regulation of anthropogenic factors that are known to produce hypoxia. The studies detailed herein are an attempt to identify genes that show robust changes in transcriptional expression when two different fish are exposed to hypoxia. Genes showing a robust and consistent response to hypoxia in these two divergent fish may be utilized as biomarkers to profile hypoxia. In this study, we employed quantitative Real-Time PCR (qRT-PCR) to assess modulated gene expression in four tissues (brain, liver, gill, and fin) of *Oryzias latipes* and *Xiphophorus maculatus.* We analyzed six potential gene targets that had previously been shown in microarray studies to exhibit robust (> 5.0 fold) changes in mRNA abundance when medaka were exposed hypoxic conditions. Herein we present results of the qRT-PCR studies to assess their potential utility as hypoxia biomarkers.

Introduction

I. Hypoxia in Aquatic Ecosystems: Causes and Environmental Implications

Different organisms utilize various metabolic pathways to produce energy needed for survival. Primary producers, or plants, use carbon dioxide, water, and sunlight combined with other nutrients to make glucose via photosynthesis. The glucose produced by plants eventually enters the food chain and is broken down by secondary consumers for energy. Secondary consumers can be subdivided into two broad categories based on how they metabolize glucose. A few organisms can perform anaerobic respiration, and are able to metabolize glucose in the absence of oxygen via glycolysis. However, most organisms break down glucose using oxygen via aerobic respiration. In aerobic respiration, oxygen serves as the final electron acceptor during oxidative phosphorylation. This process occurs in the mitochondria and ultimately results in the formation of adenosine triphosphate (ATP), used as energy (Voet et. al., 1995).

Hypoxia is the occurrence of lowered oxygen in an environment. Aquatic ecosystems are termed hypoxic when the dissolved oxygen (DO) concentration in the water drops below 2 $mg \cdot l^{-1}$. When the DO drops below 1 $mg \cdot l^{-1}$, the water is said to be anoxic, or void of oxygen altogether (Diaz 2001, Paerl 2004). Both hypoxic

and anoxic conditions may have detrimental effects on fish species. The reduction in available molecular oxygen not only prevents oxidative phosphorylation from occurring, but also has direct effects on many other cellular processes (Nikinmaa 2002). However, some fish are able to adapt to the environmental stress and thereby avoid the negative effects on metabolic processes. For example, some species of hypoxia-tolerant fish are able to modify the structure of hemoglobin, a protein that delivers oxygen to systemic tissues, to become more efficient in oxygen delivery (Weber 1982, Nikinmaa 1997). However, most fish are not able to adapt to lowered oxygen concentrations quickly and thus either exit hypoxic areas or suffer metabolic depression that may lead to mortality.

Hypoxia may be a naturally occurring event caused by the combination of several physical factors. Many hypoxic regions are commonly found in estuaries, where freshwater inflow from rivers enters the higher density salt water of Gulfs, seas, or oceans. In the summer, when temperatures rise in shallower waters, stratification, or vertical layering of the different densities of the water, may occur. Stratification leads to oxygen rich waters at the surface that are unable to mix with lower benthic waters that are low in DO (Paerl 2004). In stratified conditions, increased primary production in the surface waters contributes to hypoxia by creating more organic matter in the form of phytoplankton that may form “algae blooms”. In such situations, fish populations may initially increase due to the excess food supply. However, if the increase in organic matter exceeds the limits of downstream consumers to use it, the excess organic matter will settle to the lower benthic waters, where decay of the organic matter by microorganisms requires a large amount of molecular oxygen, further depleting benthic level oxygen leading to hypoxia or anoxia (Diaz 2001).

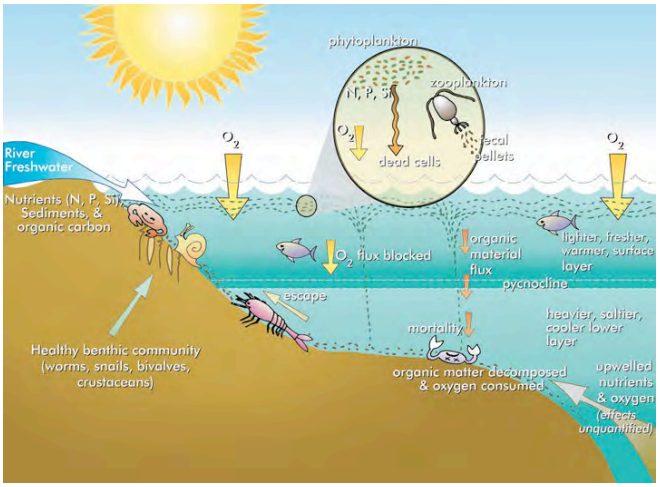

Figure 1. An artist’s interpretation of eutrophication, or nutrient loading into rivers, and the problems it causes in terms of hypoxia. Figure was taken from the Environmental Protection Agency website at www.epa.gov/msbasin/marb.htm

An increase in primary production may occur naturally with coastal upwelling (Diaz 2001), when coastal winds and currents bring bottom waters containing nutrient rich sediment to the surface. The nutrients aid the growth of phytoplankton in an algal bloom. The most common example of upwelling induced algal blooms is the red tide in the coastal region of the Sea of Japan (Pearl 2004, Wyrtki 1981). Increased primary production and algal blooms can also be anthropogenic actions induced through the process of eutrophication. Eutrophication is the loading of nutrients in the form of nitrogen and phosphorus into rivers that eventually flow into larger bodies of water (lakes, Gulfs, oceans, etc.) (Figure 1). Primary producers (phytoplankton) are

commonly limited by the availability of nitrogen and phosphorus the water. When excess nutrients are introduced into estuaries where the physical conditions for hypoxia are in place, a rapid overproduction of primary producers (and consequently their decay) may dramatically decrease DO concentrations at benthic levels.

Although hypoxia may occur naturally, an increase in the number and size of hypoxic aquatic areas has followed the increase in human population. Eutrophication is especially common at the outflow of rivers where urbanization and overuse of fertilizers in agricultural activities result in increased outflows of primary nutrients. The first hypoxic regions not associated with coastal upwelling were observed in the 1960s and 1970s, approximately 10 years after nitrogen fertilizers were first produced on an industrial scale (Galloway 2008). These hypoxic regions are commonly referred to as "dead zones," as little life can survive in the areas exhibiting decreased oxygen levels. Since the 1970s, dead zones have been documented all over the world. Some of the most notable dead zones are the Baltic, Kattegat, Black, and East China Seas (Diaz 2008). The two largest dead zones in the United States are located in the Chesapeake Bay (Boesch 2001) and in the Gulf of Mexico (Diaz 2008). Figure 2 shows a world map with known global dead zones marked (Diaz 2008). All of these regions serve as important fisheries, and hypoxia in these regions not only disrupts complex ecological webs, but also causes economic calamity.

The Gulf of Mexico dead zone is one of the largest and best understood regions of hypoxia. Water from the Mississippi-Atchafalaya River Basin (MARB), which covers over 1,245,000 square miles of land and drains water from 31 states, flows out of the Mississippi river delta and into the Gulf of Mexico. The size of the dead zone changes each year with variations in temperature and rainfall; hypoxia tends to be at its worst in the summer with warm temperatures and when the water in the gulf stratifies. The size of this dead zone is also dependent on spring algal blooms brought about by the levels of rain received in the upper MARB leading to agriculture nutrient flow into the Gulf of Mexico. At its largest, the Gulf of Mexico

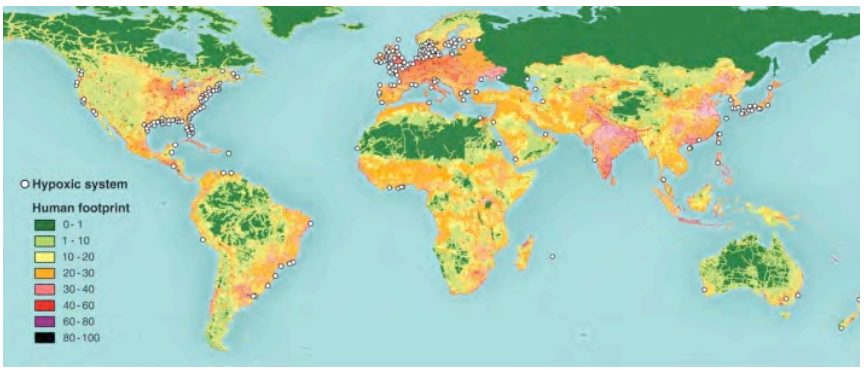

Figure 2. A global view of hypoxic "dead zones" around the world, adapted from Diaz and Rosenberg, 2008. The white spots along the coastal regions indicate eutrophication-induced hypoxic regions. As shown here, the incidence of dead zones increases in places where the human footprint is greatest.

dead zone has reached a size of greater than 15,000 km^2 (www.epa.gov/msbasin/marb.htm). The Mississippi River Gulf of Mexico Watershed Nutrient Task Force has been created to address the problems of eutrophication. Similar organizations have been created to investigate hypoxia elsewhere. For example, an organization named EUCON was created in Sweden in 1995 (Skei 2000) to study the combined effects of eutrophication and other contaminants on aquatic ecosystems. A less formal program was created in the Chesapeake Bay area with intentions to initiate plans to reduce nitrogen and phosphorus levels in rivers by limiting agricultural sources of fertilizer and by creating advanced waste-water treatment (Boesch 2001). However, if we are to better understand where we should focus our future resources in reducing eutrophication, it is important to be able to measure dissolved oxygen concentrations in real time and over a large areas. Being able to accurately detect the biological effects of lowered oxygen concentrations will enable us to make better connections between human activity and events happening at sea. Early detection and intervention may be necessary to protect natural ecosystems and provide fisheries for our increasing population. Oxygen concentrations in aquatic ecosystems are constantly changing, and to continually monitor oxygen levels over large areas is difficult and costly.

II. Application of Molecular Biology to Hypoxia

Molecular biology may present solutions to monitoring problems in large areas that experience cyclic hypoxia and formation of dead-zones. Previous toxicological studies have used genetic biomarkers as a means to measure biological responses to environmental stress. Organisms respond to environmental stressors by many mechanisms; but many of these mechanisms may be initially observed by assessing transcriptional regulation of select genes whose regulation is modulated by environmental interactions. The process of modulating transcription rates may take place quickly to ensure adaptation to a new environmental stress and thus survival of the organism. By measuring transcription levels in tissue or cells that have been exposed to an environmental stress, one may identify genes that are up-regulated or down-regulated in response to that stress in that tissue. Once a particular gene is known to respond to a specific stress, then measuring its transcription level will serve as an indicator of whether or not the organism has encountered the stress leading to an altered transcription rate. Such a gene can then serve as a bioindicator or biomarker of the stress encountered by that organism. If the functions of biomarkers are understood, they serve as both an indicator of physical changes in the environment and the biological effects the changes may have on organisms or populations. Biomarkers of hypoxic exposure in aquatic species may potentially indicate when dissolved oxygen concentrations are

reaching low level, be used to assess the duration of the reduction on DO, and provide clues into the biological effects of the hypoxic event.

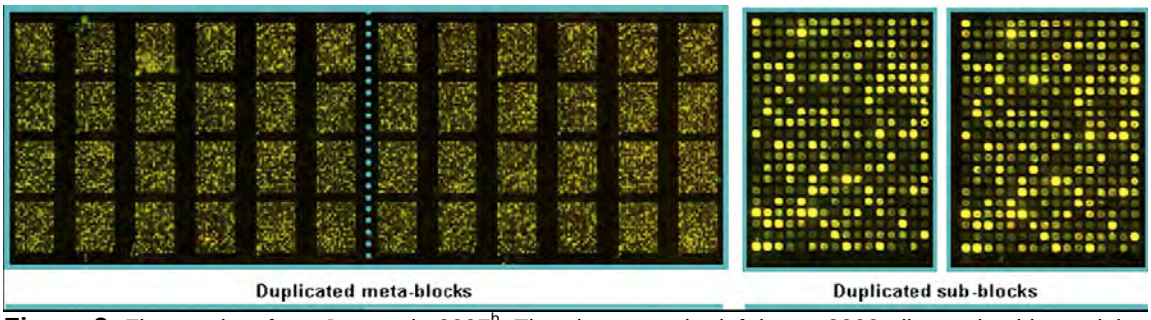

Figure 3. Figure taken from Ju, et. al., 2007[b]. The picture on the left is our 8000 oligonucleotide medaka microarray. The features are organized into sub-blocks that are duplicated on either side of the array. The picture on the right shows two duplicated sub-blocks up close. Each spot represents a different gene, and the fluorescence level indicates the amount cDNA from control and hypoxic treated tissue has hybridized to each spot.

Before an experiment to identify potential hypoxia biomarkers can be conducted, appropriate models must be considered. Attributes one may consider include small size, relatively rapid maturity, and affordability to maintain and experimentally treat. Access to able to access genomic and transcriptomic information on the experimental species is necessary in order to perform a genetic profiling. *Oryzias latipes*, commonly known as the Japanese medaka, possesses all these attributes and thus has been used in many studies aimed at determination of genetic modulation brought about by hypoxia exposure.

The goal of these studies is to identify potential biomarkers of hypoxic stress in aquatic animals, and eliminate those genes that have modulated expression patterns due to a general stress response. If the gene in question shows the same response to hypoxia across species, it further supports the gene as a hypoxia specific response gene. For this reason, *Xiphophorus maculatus* is also used in this study to provide a comparison across species in gene expression levels.

The first step to finding potential hypoxic biomarkers is to profile the expression level of many genes. Microarray technology is a high throughput tool that allows gene expression levels of thousands of genes to be measured at one time. My host laboratory developed an 8,000 oligonucleotide microarray for medaka (Figure 3) that has been used to analyze gene modulation in response to hypoxic stress in several different tissues (brain, fin, liver, and gill). Although microarrays are useful in terms of identifying large numbers of genes that respond to a particular stress, they have their limitations. Many factors can influence gene expression levels, such as sex, age, and physiological development (Ju, et. al., 2007[b]). Using microarray data to identify genes that have a strong response to hypoxic treatment does not ensure that the genes indicated are not general stress response genes. Due to the complex kinetics of microarray hybridization, they are time consuming,

costly, and exhibit a high degree of data scatter between experimental repeats. However, microarray data may be used to identify gene candidates that are then used in further studies to confirm or refute these candidates as ideal biomarkers.

A second method commonly used to measure gene expression levels is quantitative real-time polymerase chain reaction (qRT-PCR). QRT-PCR is quantitatively more precise in its measurements than microarray data, but it can only be utilized to measure the response of a few genes at a time. With qRT-PCR, the amplification process is measured through its exponential phase (Wong 2005) by measuring the release of fluorescent dyes liberated at each cycle of amplification. The number of amplification cycles needed to meet a threshold level is determined by the initial copy number of the target genes. Thus, the cycle number at which the reaction reaches exponential growth is correlated to the starting amount of parental cDNA. In this study, qRT-PCR is used to calculate the relative quantity of cDNA in a subset of six gene targets that had previously been shown to have robust (> 5.0 fold) changes in hypoxia treated medaka microarray studies.

Materials and Methods

I. Selection of Genes Used in Study

In previous microarray experiments, we exposed medaka to hypoxic conditions (<2.0 mg $DO \cdot l^{-1}$) for 72 hours and isolated RNA from different tissues. We also isolated RNA from control medaka (compressed air bubbled into tank, 8.3 $mg \cdot l^{-1}$ DO). The RNAs from each tissue were combined and used to hybridize the 8K oligonucleotide microarray (Figure 1) to assess gene regulation on a genomic scale. For detailed methods on microarray hybridizations, see previous publications (Ju, et. al. 2007[a,b]). Four different tissues from these fish (brain, liver, fin, and gill) were used in these microarray hybridizations. The data were narrowed down to 15 features (features are spots on the microarray that represent a certain gene) based upon robust fold changes (> 5.0) in at least one tissue (brain, liver, fin, gill). The medaka gene transcripts corresponding to these 15 features were compared to *Xiphophorus maculatus* sequences in a *Xiphophorus* EST database developed in my host laboratory. The sequence alignments were searched at an e-value cut off of e^{-5}. The Blast program compares similar sequences in a gapped base-pair alignment, and the e-value is a statistic that compares a particular alignment between sequences to one that could be generated by random chance. The e-value is based on the length of the region of homology, the extent of homology, and how many gaps are present. Thus, the lower the e-value the better the "hit" or more homologous the two sequences being compared. This search was done to identify sequences that might be used in cross-species comparisons of the 15 potential hypoxia biomarkers. Ten of the 15 medaka features retrieved *Xiphophorus* hits. Four of these 10

Table 1. Medaka sequences were blasted against *Xiphophorus* ESTs. The *Xiphophorus* EST retrieving The best hit is shown below with its e-value. The features highlighted in bold font indicate the sequences used in this study.

Medaka EST	*Xiphophorus* EST	e-value
AB064320	**hek002N20.r**	**5e-23**
AB111386	Contig45141	3e-16
AU167396	**XFish_subtr2 9C-G11.g**	**8e-76**
AU167844	Contig11598	4e-07
AU169680	**Contig03571**	**1e-73**
AU311099	Contig39686	1e-09
BJ514046	Contig04619	2e-18
BJ728005	**Contig44256**	**3e-43**

remaining features were eliminated because the e-values, while better than the proposed restrictions, were far greater than the e-values for the remaining 6 features that were selected for further study (Table 1). Ontologies were assessed for the 6 remaining full length medaka EST sequences using OMIM.org and

Table 2. Gene description (NCBI) and biological process information (GeneOntology.org and OMIM.org) are shown for the six medaka sequences used in the real-time PCR study. The species whose sequences were found to display high homology are denoted in brackets.

EST	Gene Description	e-value	Biological Process
AB064320	vitellogenin *[Danio rerio]*	2.10E-295	Response to Estradiol synthesis.
AU167396	Lactotransferrin *[Xenopus tropicalis]*	5.70E-64	Iron transfer into cells via receptor mediated endocytosis *[Homo sapiens]*
AU169680	enolase 1, (alpha) *[Danio rerio]*	2.50E-137	Negative regulation of cell growth; glycolysis *[Rattus norvegicus]*
BJ728005	Protein similar to keratin *[Danio rerio]*	2.00E-83	Cell migration involved in gastrulation.
BJ728238	DNA-damage-inducible-transcript *[Rattus norvegicus]*	1.00E-44	Apoptosis; response to hypoxia
BJ744248	Parvalbumin isoform 1c *[Danio rerio]*	8.00E-40	calcium ion-binding protein found in fast-contracting muscles

AmiGo at GeneOntology.org. The different biological processes as well a description of the genes that showed the highest homology with the medaka sequences (obtained from NCBI blast) are shown in Table 2.

PCR primer sequences for medaka and *Xiphophorus* sequences for each gene target were created using Primer3, an online primer design tool (Rozen 1998). All primers were designed with a melting temperature (Tm) of 60.0°C +/- 1.0, an amplicon region of 150-250 bp, and primer size of 18-22 bp. Medaka and *Xiphophorus* primer sequences are shown in Tables 3 and 4, respectively.

II. Hypoxia Exposure and RNA Extraction

Medakas were obtained from the Aquatic Biotechnology and Environmental Laboratory at the University of Georgia (Athens, GA). The *Xiphophorus maculatus* were raised by the *Xiphophorus* Genetic Stock Center at Texas State University (San Marcos, TX). Prior to exposure, the medaka were approximately 3 months in age and were held in 5 gallon aquaria at a maximum density of 10 fish per tank. The

Table 3. Primer Sequences from medaka (5' to 3')

EST	Forward primer	Reverse primer
18S rRNA	TCTCGATTCTGTGGGTGGT	TAACCAGACAAATCGCTCCA
AB064320	TGTCCATGGTCAAGACTCCA	GGCAAGGTGAGCCTCTGTAG
AU167396	CCAGAGTTCCAGCTCATGCT	ATGAGCACCCAGGTACAGGA
AU169680	AGTTCATGATCCTGCCTGTTG	AGTGTAGCCGGCTTTAGCAA
BJ728005	CAGTAAGATCATGGCGGAGG	TTGCAGCGTTGAGGTTTG
BJ728238	GTGGCCAAAAATCCAAGGAC	GCATTCCTCAATGAGCAGGT
BJ744248	TGGCCAAAAATCCAAGGAC	GCATTCCTCAATGAGCAGGT

Table 4. Primer Sequences from *Xiphophorus maculatus* (5' to 3')

EST	Forward primer	Reverse primer
X18s rRNA	CGGAAAGGATTGACAGATTGA	CTCAATCTCGTGTGGCTGAA
XAB064320	AGCACTTGGTGTTGCAGTTG	TCATGGTGACGATTCCACAT
XAU167396	GTGGCGTCTTTGCCATATTT	CGATGTCATCCTCCCTGTG
XAU169680	GCGTCTTTGCCATATTTCTCCT	TGGTGAAGAAGGGAACCAAC
XBJ728005	GCTGTACCTGCTCTGCGTTT	GCAAAGACCTGGACAACTGG

Xiphophorus were approximately 24 months of age maintained in a 20 gallon aquarium at a maximum density of 20 fish per tank.

This hypoxic treatment exposed fish for 72 hrs. Both medaka and *Xiphophorus* were co-exposed in two separate 20 gallon aquaria (one for hypoxia treatment and one control) at density of 21 fish per aquarium. The fish were sexed, and an equal ratio of male/female fish were used for the hypoxic and control fish. An OxyCycler System (Biospherix, NY, USA) was used to control and monitor the DO concentrations in the aquaria and the headspace (between the surface of the water and the aquarium cover). This OxyCycler system was specifically adapted by the manufacturer for aquarium use, and maintains a set dissolved oxygen concentration by bubbling nitrogen gas and compressed air into the water as needed. In the control tank, compressed air was continually bubbled into the tank to saturate the water at 8.0 mg $O_2 \cdot l^{-1}$. The hypoxia exposure aquaria were gradually stepped down to 2.0 mg $O_2 \cdot l^{-1}$ over 5 days and then held there for 72 hrs, at which point all fish were sacrificed and the fin, liver, gill, and brain tissues were dissected and flash frozen in a dry-ice/ethanol bath. For the medaka, tissues of 5 fish were pooled into a 1.5 mL microcentrifuge tube. For *Xiphophorus*, tissues of 2 fish were pooled (*Xiphophorus* are larger than medaka). Total RNA was extracted from tissues using TRI Reagent (Sigma-Aldrich, St. Louis, MO) and purified to remove trace amounts of phenol and DNA with an RNeasy Kit (Qiagen, Valencia, CA), according to the manufacturers' instructions. The eluate was quantified using a Nanodrop1000 spectrophotometer and the integrity of the RNA validated by running 1 µg of RNA on a 1% (w/v) agarose gel.

III. Quantitative Real-Time PCR

Medaka and *Xiphophorus* cDNA samples were made by reverse transcription of 1 µg of RNA with MMLV reverse transcriptase (Invitrogen). The quality of the cDNA samples was validated by performing PCR to amplify a region of the 18s rRNA gene using Taq polymerase (Invitrogen), and the products were run on a 2% (w/v) agarose gel with a 1 Kb ladder (Invitrogen) to visualize bands at approximately 200 bp. Quantitative real-time PCR (qRT-PCR) was performed using SYBR-Green reagent (Applied Biosystems) on an Applied Biosystems Prism 7500-fast real-time PCR system. All experiments were repeated with two biological replicates and four technical replications. The comparative threshold (C_T) method for relative quantification was used to calculate the relative concentrations of the target sequences. Derivation of the equations utilized to calculate copy numbers may be found in the Applied Biosystems User Bulletin #2 (P/N 4303859). This method involves normalizing the C_T (threshold cycle) of the target genes to the C_T of the 18s rRNA endogenous control to get C_T; where C_T = Target C_T – 18s C_T. Genes that code for ribosomal RNA are commonly used as housekeeping genes because their expression level is fairly consistent in all tissues and under most types of stress (Schmittgen 2000). The purpose of measuring the transcriptional level of a housekeeping gene is to give a baseline for constitutive expression upon which the target genes may be normalized. The C_T is then calculated by subtracting the

C_T of the calibrator from the C_T of the target gene, where $C_T = C_{T,\ target} - C_{T,\ calibrator}$. The calibrator is arbitrary, and can be any one of the target C_T values. The C_T calculation makes it so the standard deviation of the C_T and the C_T values are the same, and eliminates the need for a standard curve. When PCR efficiency is maximal, you can estimate the number of copies of target produced by the equation 2^n, where n is equal to the number of reactions that occur (the copy number rises exponentially for every reaction). In real-time PCR, this is simulated by the equation $RQ = 2^{-CT}$, where RQ is the relative quantity of the target gene. The lower the C_T number, the higher the initial amount of cDNA in solution (the negative sign placed in front of the C_T accounts for this). The C_T for the arbitrary calibrator will be zero, since the calibrator is subtracted from itself. Therefore the RQ of the calibrator will always equal one ($x^0=1$), and all other relative quantities are reported as being relative to the calibrator. The average RQ of biological replicates is used to determine the fold change of the target gene when compared in hypoxic tissue versus control tissue. The statistical program GraphPad Prism (GraphPad Software, Inc.) was employed to determine the significance of the differences between control and hypoxic samples by means of a 2-tailed student t-test at 90% confidence level ($p<0.05$). The averages of the biological replicates for control and hypoxic samples were used to calculate the fold changes.

Results

Two species of fish (*Oryzias latipes* and *Xiphophorus maculatus*) were exposed to hypoxic conditions and qRT-PCR was used to measure the transcriptional response of 6 genes in four tissues (brain, fin, liver, and gill). The transcription levels of the 6 genes in hypoxic tissues were compared to the transcription levels in fish kept under normoxic conditions. The fold changes for each target gene are represented in Figure 4, and are classified by tissue (A. brain, B. fin, C. liver, and D. gill). Results are also shown in Table 5. A positive fold change indicates that the target gene in question is up-regulated in the hypoxic tissue when compared to the control, and a negative fold change indicates that the gene was down-regulated in the hypoxic tissue. Fold changes that showed a significant difference between the control and hypoxic samples ($p<0.05$) are denoted with an asterisk. It should be noted that all medaka microarray data shown are statistically significant.

I. Brain

In the microarray results, the target genes AU169680 and BJ728238 both showed a negative fold change of 1.4. For the target gene AU169689, both the medaka and *Xiphophorus* qRT-PCR results showed a positive fold change (2.3 and 1.7, respectively), but neither of the fold changes were statistically significant. The medaka qRT-PCR fold change of -1.5 for the target gene BJ728238 agreeing with the microarray data, but the change was not significant. The *Xiphophorus* qRT-PCR results for BJ 728238 disagreed with the microarray and medaka qRT-PCR data and showed a statistically significant fold change of 2.3.

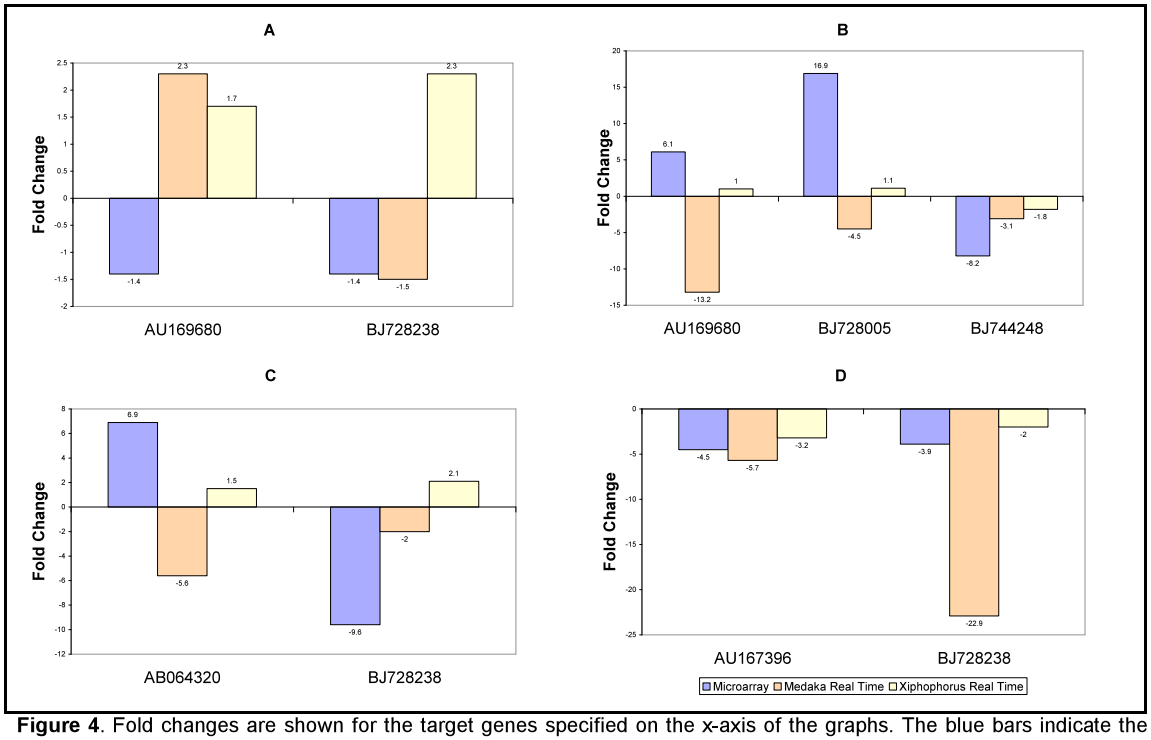

Figure 4. Fold changes are shown for the target genes specified on the x-axis of the graphs. The blue bars indicate the fold change of the microarray data, the orange the fold changes of the medaka qRT-PCR results, and the yellow of the *Xiphophorus*qRT-PCR results. The data is separated by tissue: **A.** Brain, **B.** Fin, **C.** Liver, and **D**. Gill.

II. Fin

Three target genes were tested in fin tissue: AU169680, BJ728005, and BJ744248. Both AU169680 and BJ728005 showed large positive fold changes in the microarray data (6.1 and 16.9, respectively) that were not reflected in the medaka qRT-PCR results (-13.2 and -4.5, both of which were statistically significant). The *Xiphophorus* qRT-PCR results for these two genes showed further discrepancy with positive fold changes of 1 and 1.1, which were not significant. The last target gene, BJ744248, showed consistent down regulation in the microarray results and both the medaka and *Xiphophorus* qRT-PCR results (-8.2, -3.1, and -1.8, respectively).

III. Liver

The response of two target genes was tested in liver tissue: AB064320, and BJ728238. Both genes showed inconsistency amongst the three different studies and none of the qRT-PCR results were statistically significant. The first target gene, AB164320, showed a fold change of 6.9 in the microarray data, a -5.6 fold change with the medaka qRT-PCR, and 1.5 with *Xiphophorus* qRT-PCR. BJ728238 showed a fold change of -9.6 with microarray, -2.0 with medaka qRT-PCR, and 2.1 with *Xiphophorus* qRT-PCR.

Table 5. The fold changes from the microarray data, medaka qRT-PCR, and Xiphophorus qRT-PCR are shown. A negative fold change represents down regulation of that gene in response to hypoxia, and vice versa for a positive fold change. qRT-PCR fold changes that were statistically significant ($p<0.05$) are indicated by an asterisk. All microarray results shown are statistically significant.

	Medaka microarray				**Medaka qRT-PCR**				***Xiphophorus qRT-PCR***			
Target gene	**B**	**F**	**L**	**G**	**B**	**F**	**L**	**G**	**B**	**F**	**L**	**G**
AB064320			6.9				-5.6				1.5	
AU167396				-4.5				-5.7*				-3.2
AU169680	-1.4	6.1			2.3	-13.2*			1.7	1.0		
BJ728005		16.9				-4.5*				1.1		
BJ728238	-1.4		-9.6	-3.9	-1.5		-2.0	-22.9*	2.3*		2.1	-2.0
BJ744248		-8.2				-3.1				-1.8		

IV. Gill

Two target genes, AU167396 and BJ728238, were analyzed in gill tissue. Results for all techniques (microarray and qRT-PCR) and both species (medaka and *Xiphophorus*) showed negative fold changes. The differences in hypoxic versus control for both genes in medaka qRT-PCR were statistically significant. The fold changes for the microarray data, medaka qRT-PCR, and *Xiphophorus* qRT-PCR in AU167396 were -4.5, -5.7, and -3.2. The fold changes for BJ728238 in the same order were -3.9, -22.9, and -2.0.

Discussion

I. Brain

The genes tested in brain tissue (AU169680 and BJ728238) showed a minimal fold change of -1.4 in the microarray data. The qRT-PCR fold changes in AU169680 were positive in both species, showing inconsistency with the microarray data and the qRT-PCR data. The medaka qRT-PCR results for BJ728238 showed down-regulation in hypoxic tissue, which is consistent with the microarray results. However, *Xiphophorus* showed the opposite response and BJ728238 was up-regulated. The microarray fold changes of -1.4 combined with inconsistent responses across species and techniques eliminate AU169680 and BJ728238 as possible biomarkers in brain.

II. Fin

Large transcriptional responses were seen in several genes in fin tissue by microarray analyses, indicating fin may be a viable tissue for assessing hypoxia. The target gene AU169680 showed a fold change of 6.1 in the microarray data. The gene that had the highest homology to AU169680 in the NCBI blast search was

identified as -enolase in *Danio rerio* (zebrafish), an enzyme involved in an important step in the conversion of glucose to energy in the glycolytic pathway (Pancholi 2001). Studies have proposed that -enolase is up-regulated in response to hypoxic stress to increase anaerobic respiration (Aaronson 1995). This was shown in the microarray data, but was not, however, mirrored in the qRT-PCR results. Even though -enolase does show strong responses to hypoxia, its inconsistency across species and techniques renders it useless as a hypoxic biomarker.

The gene BJ728005 showed a similar expression pattern AU169680. The gene with the highest homology to the BJ728005 sequence in the NCBI Blast was identified as a hypothetical protein, possibly keratin, in *Danio rerio*. Much like AU169680, the keratin gene BJ728005 showed an extremely high fold change in the microarray data (16.9), but was inconsistent in the qRT-PCR study across species and techniques.

The third target gene tested in fin was BJ744248, which showed consistent down regulation in the microarray data, the medaka qRT-PCR results, and the *Xiphophorus* qRT-PCR results. The gene with the highest homology to BJ744248 was identified as a parvalbumin isoform in *Danio rerio,* which has shown down regulation in response to hypoxia in zebra fish (Ton 2003). Parvalbumin is a calcium binding protein commonly found in the skeletal muscle of vertebrates, where it increases activity by shortening the recovery period between contractions (Ushio 1994). A decrease in parvalbumin and the subsequent decrease in muscle contraction could serve as an energy saving strategy for organisms facing lowered oxygen conditions.

III. Liver

Both of the target genes tested in liver, AB064320 and BJ728238, showed inconsistent results through the microarray, medaka qRT-PCR, and *Xiphophorus* qRT-PCR results. AB064320, a gene related to vitellogenin, was up-regulated in the microarray results by 6.9. Vitellogenin is a precursor for egg-yolk in fish and is a commonly known biomarker for endocrine disrupting chemicals (Tyler 1996). Endocrine disrupting chemicals mimic female hormones, and the response to estrogen is expected to be different between males and females. It is likely that vitellogenin is modulated in response to several stress factors in addition to hypoxia (Wu 2003), rendering it unusable as a hypoxia biomarker.

The other target gene tested in liver, BJ728238, showed down-regulation in the microarray data (-9.6) which was mirrored in the medaka qRT-PCR results (-2.0). The *Xiphophorus* results, however, showed the opposite response from the medaka results with an up-regulation of 2.1. The target gene BJ728238 highly homologous to a gene associated with apoptosis in response to hypoxic stress. The difference in regulation of this gene across species in liver renders this gene useless as a general hypoxia biomarker. However, it could be used in a species specific study.

IV. Gill

Gill tissue showed complete agreement among the medaka microarray data, the medaka qRT-PCR results, and the *Xiphophorus* qRT-PCR results for the two target genes tested. Both of the target genes in gill also showed statistically significant qRT-PCR results in medaka. Gill tissue is easy to harvest and requires little dissection which is less stressful on the animal, which may explain why the genetic response in gill showed less variance among biological replicates than other tissues. The gills in fish are the site at which oxygen is first absorbed into the blood stream from the water, and would logically serve as a first line of defense when fish are exposed to hypoxic conditions. Gas exchange primarily occurs through the walls of the secondary lamellae (comparable in structure with mammalian alveoli) where small capillaries are located near the surface. According to previous studies, there are two physiological ways in which fish can increase the oxygen supply in the blood: increase the water volume entering the gills through “burst” swimming, or increase the surface area of the secondary lamellae (Randall 1982).

The target gene AU167396 was down regulated in the microarray data with a fold change of -4.5, and down regulation was consistent in both qRT-PCR results. In the blast search, the gene with the highest homology to AU167396 was lactotransferrin. Lactotransferrin is a protein that has a high affinity for molecular iron, and facilitates the uptake of iron into cells where it assists the immune system against microbial and viral infections (Valenti 2006). An increase in lactotransferrin would increase the amount of iron taken up by host cells. However, in the case of hypoxia, lactotransferrin is decreased, blocking iron uptake into cells and leading to an accumulation of iron in the blood and extracellular space. An accumulation of iron in the blood would be beneficial to an organism experiencing hypoxic stress, because the binding of iron to hemoglobin increases hemoglobin’s affinity for oxygen (Voet 1995). If the expression of lactotransferrin in the gills is decreased in order to enhance the binding affinity of hemoglobin to oxygen, it might be hypothesized that lactotransferrin could also be increased in systemic tissues (such as muscle) in response to hypoxia. This would cause a more efficient release of oxygen from hemoglobin to tissues where aerobic metabolism takes place. A qRT-PCR experiment studying the modulation of AU167396 in a systemic tissue such as muscle could corroborate the results of this study and further support AU167396 as a viable biomarker for hypoxic stress.

The other target gene that was significantly down regulated in gill tissue was BJ728328. The sequence with the highest homology to BJ728328 is associated with apoptosis, or programmed cell death, in response to hypoxia in *Rattus norvegicus* (the common rat). The gills in fish are not only a means by which oxygen is diffused from the water through tissues, but are also involved in ion transfer and water loss/gain. An increase in the surface area of gills will increase the uptake of oxygen into the blood vessels through the lamellae, but it will also cause a loss of ions that may upset the osmotic balance of the organism. Therefore, a careful balance in lamellae surface area must be maintained. In a study conducted by Jorund Sollid (2003) on the crucian carp (*Carassius carassius*), apoptosis was increased and

cellular proliferation, or growth, was decreased in gill tissue under hypoxic stress. The crucian carp is extremely resistant to hypoxic conditions, and can survive in waters with less than 1 $mg \cdot l^{-1}$ DO. In normoxic conditions, the area between the secondary lamellae of the crucian carp is filled with an interlamellar cell mass, decreasing the surface area of the lamellae exposed to water flowing through the gills. Under hypoxic conditions, apoptosis increases in the interlamellar cell mass, morphologically changing the gills to expose more surface area of the secondary lamellae. When the fish were returned to normoxic conditions, the process was reversible, and proliferation increased in the interlamellar cell mass. However, the target gene BJ728328 (related to apoptosis) was decreased in medaka and *Xiphophorus* in response to hypoxic stress. One possibility for this discrepancy could be that BJ728328 is indirectly involved in apoptosis by regulating apoptotic genes. If BJ728328 is a negative regulator of apoptotic genes, then a decrease in its expression level would result in an increase in apoptosis, causing similar changes in the lamellae observed by Sollid, et. al (2003). Another possibility is that BJ728328 directly controls apoptosis, but medaka and *Xiphophorus* do not have interlamellar cell masses, and the decrease in apoptosis is directly linked to secondary lamellae tissue. In either case, further characterization of BJ728328 would require a histological examination of the secondary lamellae in medaka and *Xiphophorus*.

V. Potential Hypoxia Biomarkers Identified for Further Study

Three target genes were identified as potential hypoxic biomarkers in aquatic species. The parvalbumin response in fin (BJ744248), and the lactotransferrin (AU167396) and apoptosis (BJ728328) gene responses in gill all showed consistency in down-regulation through microarray data, medaka qRT-PCR, and *Xiphophorus* qRT-PCR results. The uniform agreement in gene response in these three genes among different techniques as well as across species renders these genes reliable indicators of lowered oxygen conditions. If these biomarkers are to be used in further studies to measure hypoxic dead zones, the experiment would need to be repeated with a model native to the region to ensure a similar response before testing the genes in vivo (for example, *Fundulus* is native to the Gulf of Mexico and could be used as an indicator species for the region).

References

Aaronson, R. M., K. K. Graven, M. Tucci, R. J. McDonald, H. W. Farber (1995). Non neuronal Enolase is an endothelial hypoxic stress protein. *J. Biol. Chem. 270*: 27752-27757.

Bishop, M. J., Powers, S. P., Porter, H. J., Peterson, C. H., 2006. Benthic biological effects of seasonal hypoxia in a eutrophic estuary predate rapid coastal development. *Estuarine, Coastal and Shelf Science xx*, 1-8.

Boesch, D.F., Brinsfield, R.B., Magnien, R.E., 2001. Chesapeake Bay eutrophication: scientific understanding, ecosystem restoration, and challenges for agriculture. *J. Environ. Qual. 30*: 303-320.

Chittur, S., 2004. DNA microarrays: tools for the 21st century. *Combinatorial Chemistry and High Throughput Screening 7*: 531-537.

Diaz, R. J. 2001. Overview of Hypoxia around the World. *Journal of Environmental Quality 30*: 275-281.

Diaz, R. J., R. Rosenberg, 2008. Spreading Dead Zones and Consequences for Marine Ecosystems. *Science 321*: 926-929.

Galloway, J. N., 2008. Transformation of the nitrogen cycle: recent trends, questions, and possible solutions. *Science: 320*: 889-892.

Ju, Z., M.C. Wells, S.J. Heater, R.B. Walter, 2007[a]. Multiple tissue gene expression analyses in Japanese medaka (*Oryzias latipes*) exposed to hypoxia. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 145*: 134-144.

Ju, Z., M.C. Wells, R.B. Walter, 2007[b]. DNA microarray technology in toxicogenomics of aquatic models: methods and applications. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 145*: 5-14.

Nikinmaa, M., 1997. Oxygen and carbon dioxide transport in vertebrate erythrocytes: an evolutionary change in the role of membrane transport. *J. Exp. Biol. 200*: 369-380.

Nikinmaa, M., 2002. Oxygen-dependent cellular functions- why fishes and their aquatic environment are a prime choice of study. *Comparative Biochemistry and Physiology Part A 133*: 1-16.

Paerl, H.W. 2004. Estuarine eutrophication, hypoxia and anoxia dynamics: causes, consequences and controls, pp. 35-56. In, Rupp, G.L. and M.D. White. Proceedings of the 7th International Symposium on Fish Physiology, Toxicology, and Water Quality. May 12-15, 2003, Tallin, Estonia. U.S. Environmental Protection Agency Office of Research and Development, Ecosystems Research Division, Athens, Georgia, USA. EPA/600/R-04/049.

Pancholi, V. 2001. Multifunctional -enolase: its role in diseases. *Cell. Mol. Life Sci. 58*: 902-920.

Randall, D., 1982.The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol. 100*: 275-288.

Rosen, S., H.J. Skaletsky (1998). Primer3. *Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.*

Schmittgen, T., B. Zakrajsek, 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J. Biochem. Biophys. Methods 46:* 69-81.

Skei, J., P. Larsson, R. Rosenberrg, P. Jonsson, 2000. M. Olsson, and D. Broman. Eutrophication and contaminants in aquatic ecosystems. *Ambio 29:* 184-194.

Sollid, J., P. De Angelis, K. Gundersen, G. Nilsson, 2003. Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol. 206*: 3667-3673.

Tyler, C. R. B. van der Eerden, S. Jobling, G. Panter, J. P. Sumpter, 1996. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. *J. Comp. Physiol. B 166:* 418-426.

Ushio, H., S. Watabe, 1994. Carp parvalbumin binds to and directly interacts with the sarcoplasmic reticulum for Ca^{2+} translocation. *Biochemical and Biophysical Research Communication 199:* 56-62.

Valenti P, Antonini G (2006). Lactoferrin: an important host defence against microbial and viral attack. *Cell. Mol. Life Sci. 62* (22): 2576–2587.

Voet, D., Voet, J. G., 1995. Biochemistry, 2nd ed. Wiley and Sons, New York, pp. 563-569.

Weber, R.E., 1982. Intraspecific adaptation of hemoglobin function in fish to oxygen availability. In: Addink, A.D.F., Spronk, N. (Eds.), Exogeneous and Endogeneous Influences on Metabolic and Neural Control. Pergamon Press, Oxford, pp. 87-102.

Wong, M., J. Medrano, 2005. Real-time PCR for mRNA quantification. *Biotechniques 39:* 1-11.

Wu., R.S.S., B. Zhou, D. Randall, N. Woo, P. Lam 2003. Aquatic hypoxia is an endocrine disrupts and impairs fish reproduction. *Environ. Sci. Technol. 37*: 1137-1141.

Wyrtki, K., 1981. An Estimate of Equatorial Upwelling in the Pacific. *Journal of Physical Oceanography 11*: 1205-1214.