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Fundulus grandis and the Evolutionary Response to Hypoxia

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FUNDULUS GRANDIS AND THE EVOLUTIONARY RESPONSE TO HYPOXIA

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Hypoxia in the marine environment is a growing environmental concern, and can have profound impacts on organisms. This dissertation seeks to understand the physiologically induced changes in gene expression, the relationship between gene expression and metabolism, and how these parameters vary among populations, in response to hypoxic stress. By comparing evolved intraspecific variation in gene expression and physiological parameters among populations from multiple regions in the Gulf of Mexico we seek to determine the physiologically induced changes that are essential to hypoxic survival.

First, whole body metabolism, measured as oxygen uptake, was profiled across seven decreasing oxygen concentrations. Metabolism and the critical oxygen tension (P_{O2crit}) were compared between populations from across the Gulf of Mexico. This study demonstrated a significant interaction of body mass with the hypoxic response. Additionally, populations only differed in their metabolism at the lowest oxygen concentration, 1.8 kPa. P_{O2crit} did not differ between populations, but was body mass dependant.

Next, the effects of hypoxia on gene expression were examined. These studies examined the effects of hypoxia on gene expression over time and at different hypoxic doses, utilizing a 384 gene microarray. In the first studies individuals were subjected to

0, 4, 8, 12, 24, 48, or 96 hours of hypoxia. Different genes had different times for peak gene expression, with most changes occurring after 96 hours of exposure. However, only 14 genes had significant changes in gene expression. To determine the effect of differing hypoxic dose, individuals were exposed to normoxia, 7.8 kPa O_2 (moderate hypoxia), or 1.8 kPa (severe hypoxia) for 4 or 48 hours. Sixty-nine genes had significant changes in gene expression for either dose or time. To elucidate the relationship between effect of time and dose, genes were examined for dose response within each time. The maximum number of changes occurred at 1.8 kPa after 48 hours of exposure. Interestingly different sets of genes had changes in gene expression at either 7.8 or 1.8 kPa.

Finally, to ascertain the difference among populations, for thousands of genes, individuals from six populations of *Fundulus grandis* were exposed to hypoxia (1.8 kPa) for 4 or 96 hours. Hypoxia had a significant effect on the expression of 609 genes, while population affected the expression of 355 genes. Genes with significant differences in expression among populations reflect geographic separation. For the 59 genes with significant differences in expression for both hypoxia response and population, shared hypoxic histories appears to be more important than simply the neutral patterns expected with geographic distance. The majority of significant changes for the 609 hypoxia responsive genes take place after 96 hours of hypoxia exposure.

This research demonstrates that *F. grandis* cope with hypoxia through changes in metabolism and gene expression. Overall, the response to hypoxia is dependent on an individual's size (body mass), the ambient oxygen concentration, and the duration of hypoxia exposure. Additionally, there appear to be some differences between populations with differing exposure history to hypoxia in the Gulf of Mexico.

For my family

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Chapter 1 Introduction

What is the role of gene expression in evolutionary adaptation? *Fundulus grandis* from the Gulf of Mexico provide a unique opportunity for the study of gene expression and evolutionary adaptation. *F. grandis* inhabits a wide geographic range and populations experience differential hypoxic exposure, both in frequency and severity. Across the Gulf of Mexico, severe environmental hypoxia was documented in 5.9% to 29.3% estuaries surveyed (Engle, Summers, and Macauley 1999). This dissertation seeks to understand the importance of physiologically induced changes in gene expression, the relationship between gene expression and metabolism, and how this varies among populations. By comparing evolved intraspecific variation in gene expression and physiological parameters among populations from multiple regions in the Gulf of Mexico, I seek to determine the physiologically induced changes that are essential to hypoxic survival.

1.1 Background

Environmental Hypoxia

Hypoxia in the marine environment is a growing environmental concern, as hypoxic regions increase worldwide both spatially and in severity (Rabalais and Turner 2001). Hypoxia is also a factor in numerous medical conditions from cancer to stroke, and understanding its effects on organisms and in specific tissues is of critical concern for the treatment of these disorders (Mense et al. 2006). *Fundulus grandis* from the Gulf of

Mexico provide a unique opportunity for the study of organisms' ability to cope with and adapt to hypoxia. By examining the variation among populations for the induction of gene expression with hypoxia and how it relates to metabolism, we can begin to understand the relationship between gene expression and adaptation to hypoxia.

Hypoxia is generally defined as oxygen concentrations less than or equal to 2 $ml \bullet L^{-1}$. Oxygen levels considered normoxic range from 2 $ml \bullet L^{-1}$ to 8 $ml \bullet L^{-1}$ (Diaz and Rosenberg 1995; Rabalais and Turner 2001; Breitburg 2002). The most prevalent unit for reporting oxygen concentrations in the marine environment are ml gas per L of seawater, with the amount of oxygen expressed as the volume it would occupy at standard temperature and pressure (Libes c1992). Diaz and Rosenberg (1995) report equivalencies (at salinity 20 psu, 25 ºC, and 1 atm pressure) 1 $ml \bullet L^{-1}$= 1.4 $mg \bullet L^{-1}$= 1.4 ppm = 23.9 mmHg = 23.9 torr = 3.18 kPa = 45.7 mM = 4.3 % O_2 vol. = 14% air saturation. More recently, a number of authors have suggested that the definition of hypoxia should be more accurately defined by the tolerance of the organisms it affects. Many organisms begin to experience stress or even death at oxygen concentrations above 2 ml L^{-1}, and a more relevant definition for conservation may be necessary to take this in to account (Pollock, Clarke, and Dube 2007; Vaquer-Sunyer and Duarte 2008).

Hypoxia can occur naturally and such occurrences have been recorded in habitats from Scandinavian fjords to the Gulf of Mexico. However, hypoxia appears to be significantly increasing, both in occurrence and severity, linked with increased anthropogenic eutrophication (Diaz and Rosenberg 1995; Rabalais and Turner 2001). Diaz and Rosenberg (1995) list 44 systems experiencing hypoxia globally, most of which are increasing in frequency and severity. Systems now experiencing recurrent hypoxia

include the Chesapeake Bay, the Louisiana and Texas shelf system in the Gulf of Mexico, the North Sea, the Adriatic Sea, and numerous Scandinavian fjords.

Hypoxia in the Gulf of Mexico can affect both the near shore and offshore environments. A large subsurface hypoxic zone was first reported in the 1970's, and has been recorded annually since (Rabalais and Turner 2001). This zone is located offshore, but has been documented in depths as shallow as 4 to 5 m, and covering a geographic area approaching 20,000 km^2 (Rabalais and Turner 2001). The exact size and structure of the offshore hypoxic zone varies annually in response to local conditions including weather (storms), climate (drought or flood), and nutrient input. For instance the hypoxic zone averaged 13,500 km^2 from 1985 to 2007, ranging from negligible in 1988 (a summer drought year for the Mississippi River basin) to 22,000 km^2 in 2002. Weather related variability of the size of the zone is well documented for the summer of 2005. The 2005 summer distribution of hypoxia was affected by the series of hurricanes passing through the Gulf of Mexico. In early July 2005 there was a large area of hypoxia off the southwestern Louisiana coast. In July 2005 Hurricanes Cindy and Dennis affected the Louisiana shelf, and as a result the extent of the zone was smaller than predicted (Rabalais et al. 2007). By mid-August 2005 patchy hypoxia was still present in the region. Hurricane Katrina made landfall at the end of August and disrupted hypoxia in depths less than 25 m, but it reformed in the shallow waters of the Louisiana Bight a few weeks later. A series of frontal passages and Hurricane Rita in September dissipated the hypoxia for the remainder of the year (Rabalais et al. 2007). Despite local variability in the size and structure of the hypoxic zone, the primary underlying driver behind

formation of this zone has been linked to increased nutrient loading in the Mississippi-Atchafalaya River Basin (Osterman, Poore, and Swarzenski 2008; Boesch et al. 2009).

Estuaries in the Gulf of Mexico also experience periodic hypoxia to varying degrees. Between Anclote Key, FL and Rio Grande, TX 5.9% to 29.3% of estuaries surveyed were affected by hypoxia (Engle, Summers, and Macauley 1999). Estuaries in one study were classified into two groups, degraded and undegraded. Degraded had oxygen minimums less than 2 mg L^{-1} over a 24-hour period during survey by Engle et al (1994). Estuaries classified as undegraded never recorded an oxygen minimum below 3 mg L^{-1}. A study carried out as part of an EPA estuarine survey recorded numerous estuaries with at least one oxygen minimum of less than or equal to 2 mg L^{-1} during the course of a four-year survey. However, of nine estuaries surveyed all four years, only three experienced hypoxia multiple years (Engle, Summers, and Macauley 1999). Of the estuaries included in this dissertation, Mobile Bay was sampled all four years, and hypoxia was found in multiple years. Terrebonne Bay (Leeville, LA), was sampled all four years and hypoxia was found in one year, and Aransas Bay (Port Aransas, TX) was sampled only one year, but hypoxia was detected. Table 1.1 summarizes known hypoxic and undegraded estuaries in the Gulf of Mexico used for this study. Of all the estuaries included in this study (Table 1.1), Mobile Bay has potentially the longest record of hypoxia of any Gulf of Mexico estuary, and is reported by the EPA as a "Priority Hypoxia Area" (USEPA 1999). The record of hypoxia in Mobile Bay goes back to 1821, recorded as reports of "Jubilees." In a Jubilee, hypoxic water advects onshore, pinning fleeing fish, which are easily caught by local residents (May 1973). Thus we expect that *F. grandis* sampled from Mobile Bay, AL (WB); Terrebonne Bay, LA (LV); and Aransas

Bay, TX (PA); will have evolved adaptations for coping with hypoxia to a greater extent than the relatively pristine sites, including Matagorda Bay, TX (PO), Lake Calcasieu, LA (LC), and Dauphin Island, AL (DA).

Hypoxia and Organisms

Hypoxia in the marine environment can have dramatic effects on organisms' behavior, physiology, and survival (Hochachka et al. 1996; Hochachka and Somero 2002; Wu 2002; Mandic, Todgham, and Richards 2009). In most organisms, the initial response to hypoxia is behavioral, organisms detect and avoid hypoxic regions (Keister, Houde, and Breitburg 2000; Wannamaker and Rice 2000; Burnett and Stickle 2001; Breitburg 2002; Bell, Eggleston, and Wolcott 2003b). Wannamaker and Rice (2000) performed a series of hypoxia choice experiments on seven estuarine species. All seven were able to detect hypoxia, and all but *Fundulus heteroclitus* demonstrated at least some avoidance response. Bell et al. (2003b) recorded the movements of blue crabs *Callinectes sapidus*, in response to hypoxia. During hypoxic upwelling events the crabs attempted to move to shallower oxygenated water to avoid the hypoxic zones. A recent study in largemouth bass used telemetry to monitor movement of bass overwintering under ice, while simultaneously monitoring oxygen conditions. This study demonstrated that adult bass avoided zones where oxygen levels were less than 2 $mg \bullet L^{-1}$, but do not avoid moderately hypoxic regions, supporting the idea that avoidance behavior is species specific and may have a species specific threshold (Hasler et al.).

When hypoxia avoidance is not possible, many organisms may become quiescent in an apparent attempt to minimize oxygen needs. In shallow systems that become hypoxic, such as marsh systems or tidal pools, fish may perform aquatic surface

respiration (ASR), gulping the oxygenated layer of water at the surface (Virani and Rees 2000; Wannamaker and Rice 2000; Wu 2002; Watters and Cech 2003). Avoidance and ASR can have an effect on survival, not only by compensating for hypoxia directly, but also through their potential impact on predator-prey dynamics. For instance several studies have shown that predator prey dynamics can be altered by behavioral responses to hypoxia by changing encounter rates (Breitburg 2002; Bell, Eggleston, and Wolcott 2003a). Watters and Cech (2003) found different behaviors in response to hypoxia between two closely related sculpins from two levels of the intertidal zone. The species from the upper intertidal, which would be exposed to an increased predation risk, was less inclined to perform ASR than those from the lower intertidal. While ASR can mitigate the effects of hypoxia, it does not prevent physiological effects. Stierhoff et al. (2003) measured growth rates in *Fundulus heteroclitus* with and without access to the surface. The low oxygen concentrations reduced growth rates in both groups; however, those individuals allowed access to the surface experienced a smaller reduction in growth.

Physiological Changes: Ventilation and Metabolism

Physiologically, the first response to hypoxia is often an increase in ventilation rate, increasing oxygen delivery to the blood to compensate for lowered environmental oxygen. In oxygen regulators, this increased ventilation will maintain the rate of metabolism until a critical environmental oxygen concentration, the P_{O2crit}, is reached. At oxygen concentrations less than the P_{O2crit} increased ventilation can no longer maintain metabolism. Intraspecific differences in the P_{O2crit} exist. Two studies have demonstrated lower P_{O2crit} in populations experiencing more frequent hypoxic events (Timmerman and

Chapman 2004; Mandic, Todgham, and Richards 2009), and we hypothesize that populations of *F. grandis* subjected to severe and frequent hypoxia will have a lower P_{O2crit}.

In addition to ventilatory changes, many organisms may also increase their oxygen binding capacity in the blood (Greaney 1980; Virani and Rees 2000; Burnett and Stickle 2001; Cooper et al. 2002; Wu 2002; MacCormack et al. 2003). When ventilatory compensation mechanisms cannot make up for the oxygen deficit, hypoxia can lead to shifts in metabolism (Wu 2002). These shifts can include decreases in metabolism or a switch to an anaerobic metabolic strategy. In addition to changes in metabolic rate, metabolic enzyme activity also changes, and changes in antioxidant enzyme activities have also been reported (Hochachka et al. 1996; Gracey, Troll, and Somero 2001; Webster 2003; Martinez et al. 2006; Wood et al. 2007; Lundgreen et al. 2008; Richards, Sardella, and Schulte 2008).

One of the complexities in profiling the response to hypoxia on the physiological level and determining meaningful differences between individuals or populations is the effect of body mass. Body mass affects metabolism, enzyme activities, and most other physiological functions (Hochachka and Somero 1984; Childress and Somero 1990; Pierce and Crawford 1997b; Pierce and Crawford 1997a; Burness et al. 1999; Darveau et al. 2002; Hochachka and Somero 2002); and consequently, one would expect that body mass would affect the response to hypoxia. The effect of body mass on hypoxic response is complex and not all studies agree. In the cichlid fish *Astronotus ocellatus* the enzymes associated with hypoxic response, survivorship, and P_{O2crit} scale with body mass, with larger animal being more hypoxia tolerant (Almeida-Val et al. 2000; Almeida-Val,

Gomes, and Lopes 2006; Sloman et al. 2006b). However, in sharpsnout sea bream, *Diplodus puntazzo*, the P_{O2crit} appears be independent of size, but survival was negatively correlated with body size (i.e., greater survival in smaller fish) (Cerezo and Garcia 2004). Similarly in several fish species smaller individuals used waters with lower oxygen levels than larger individual, leading the authors to suggest that the smaller individuals might be utilizing lower oxygen zones as a shelter (Wannamaker and Rice 2000; Burleson, Wilhelm, and Smatresk 2001; Robb and Abrahams 2003). The differences among these data suggest that the effect of body mass on hypoxia represent different adaptive solutions and thus evolutionary plasticity in how body mass affects hypoxic responses.

Molecular Changes

Many studies of hypoxia examine the effects of hypoxia at an ecosystem level and physiological level, but hypoxia has repercussions at the molecular level as well. Cheng et al. (2003) demonstrated an increase in HSP70 expression in barnacle larvae in response to hypoxia, proposing it as a potential biomarker for hypoxia exposure. A study in *Fundulus heteroclitus* found a homolog for the human Hypoxia inducible factor 2α (HIF-2α), and demonstrated that is involved in the hypoxia signaling response in fish (Powell and Hahn 2002).

Several studies have used microarrays to examine gene expression changes in response to hypoxia. One profiled the effects of hypoxia on the development of zebrafish, *Danio rerio*, embryos. This study profiled >4,500 genes for embryos exposed to 24 hours of hypoxia. Exposure to hypoxia induced gene expression changes and halted development in the embryos. Embryos returned to normoxic conditions resumed normal development, and gene expression returned to pre- hypoxic levels (Ton,

Stamatiou, and Liew 2003). Another study profiled the gene expression to hypoxia in adult *Gillichthys mirabilis,* over the course of 144 hours of exposure. This study characterized the differences in gene expression between hypoxia and normoxia across a range of tissues. The authors identified 126 hypoxia responsive genes, corresponding to metabolism, locomotion, protein synthesis, antigrowth and proliferation, and amino acid metabolism. Additionally, most change in gene expression occurred in liver or skeletal muscle in the first 24-72 hours of exposure (Gracey, Troll, and Somero 2001). Other studies of changes in gene expression with hypoxia exposure have been carried out in adult *Danio rerio*, and highlight the tissue specificity of hypoxic response. The first study examined changes in gene expression in gills of *D. rerio*. This study exposed *D. rerio* to hypoxic conditions for three weeks. Out of over 15,000 unique oligonucleotides, 367 differentially expressed genes were identified after 3 weeks of hypoxia exposure. Of these, 250 were repressed, and 117 were induced (van der Meer et al. 2005). A second study profiled transcriptome changes in adult *D. rerio* hearts in response to hypoxia, also over the course of three weeks, using the same progressive hypoxia set up as van der Meer et al. (2005). This study used an Affymetrix zebrafish array to profile changes in 15,000 genes, and attempted to link changes in gene expression to observed changes in cardiac physiology. The authors found 376 differentially expressed genes covering a variety of individual functions (Marques et al. 2008). Interestingly, in this case, of the 376 genes with differential expression the majority (260) were induced, while the remainder (116) were repressed, almost exactly opposite of the gill study.

1.2 Scope of the research

To determine whether populations of *F. grandis* from different regions of the Gulf of Mexico are undergoing evolution by natural selection to hypoxia, we must first characterize their response to hypoxia. The goal of Chapters 2, 3 and 4 of this dissertation is to characterize the organismal response of *F. grandis* individuals to hypoxia. Chapter 2 characterizes the metabolic response of *F. grandis* to hypoxia by measuring metabolism over declining oxygen concentrations, and establishing P_{O2crit} values for individuals from 7 populations of *F. grandis*. This chapter also addresses variability in the metabolic response between populations, and the effect of body mass on the metabolic response to hypoxia for this species. Chapter 3 of this dissertation determines the mRNA expression response to hypoxia over time. By comparing cardiac mRNA expression among individuals exposed to different durations of hypoxia, the time at which the maximum differences in gene expression occur can be determined. Chapter 4 examines the effects of variable hypoxic dose on gene expression to determine at what oxygen concentrations maximize changes in gene expression, and whether observed changes in mRNA expression are consistent at different oxygen concentrations.

None of the microarray studies described in the review above examined the variation among individuals, or compared results across populations. Without this information, the change in any molecular trait could be a common response to the stress, or represent individual variation in hypoxic response. Microarray studies profiling gene expression will allow for detection of changes that affect many biochemical pathways and provide inference on these physiological strategies (Crawford and Oleksiak 2007). Chapter 5 examines the effects of hypoxia across 170 individuals from six populations.

The goal of this chapter is to examine whether individuals from environments with differing hypoxia exposure histories are experiencing local selection for hypoxia. According to the neutral theory of evolution, much of the variability within a species is caused by random genetic drift, which is variation that is functionally and selectively equivalent (Lewontin and Hubby 1966; Kimura 1968). My approach (Whitehead and Crawford 2006) is to use the neutral genetic differences as a covariate to specifically test whether differences in induced gene expression are best explained by neutral theory or selection. Evolution by natural selection between populations will select among variations in these populations. Thus, by comparing patterns of gene expression under natural selection, we can identify the genes of biological importance to survival under hypoxic conditions (Oleksiak, Roach, and Crawford 2005; Gilad et al. 2006). Thus, Chapter 5 of this dissertation examines gene expression from thousands of genes in many individuals from 6 populations in order to determine population specific effects that most likely represent evolutionary adaptation to hypoxia.

Table 1.1. Study Sites and their record of hypoxia from the literature

Site	Record of Hypoxia	Reported by
Port Aransas/Aransas Bay, TX	First in 1988, recurring 1988-1996	Ritter and Montagna (1999); Engle et al. (1999)
Port O'Conner/Matagorda Bay, TX, mouth of the bay	No record, reported as an undegraded reference	Engle et al. (1994)
Lake Charles/Lake Calcasieu, LA	No record, reported as an undegraded reference	Engle et al. (1994)
Terrebonne-Bartaria/Leeville, LA	Offshore zone to mouth of bay, One year measurement out of four year study	Rabalais and Turner (2001); Engle et al. (1999)
Weeks Bay, AL/Mobile Bay interior	Long record of hypoxia, dating to 1820's	May (1973); US EPA, Engle et al. (1994); Engle et al. (1999).
Mobile Bay-Dauphin Island AL/Pass Aux Heron	No record found	

Chapter 2 Adaptation Versus Allometry: Population and Body Mass Effects on Hypoxic Metabolism in *Fundulus grandis*

2.1 Summary

Hypoxia has significant effects on organisms, from metabolic reduction to death, and could be an important evolutionary force affecting the variation among populations within a species. To determine intraspecific variation in hypoxic metabolism and the effect of body-mass we examine rates of oxygen consumption ($\dot{M}O_2$) at seven oxygen concentrations among seven populations of *Fundulus grandis* that inhabit a mosaic of habitats with different frequencies and intensities of hypoxia. For $\dot{M}O_2$ there is a significant interaction ($p < 0.05$) between body mass and oxygen concentrations: log-body mass: log-$\dot{M}O_2$ slopes were steeper at intermediate oxygen partial pressures than either normoxic or lowest oxygen partial pressure (ANCOVA, $p<3.9*10^{-6}$). Additionally, the P_{O2crit} (oxygen partial pressure where $\dot{M}O_2$ can no longer be maintained) was a negative function of body mass ($p<0.04$). At the lowest oxygen partial pressure (1.8 kPa) there was a significant difference in $\dot{M}O_2$ among populations: one of the populations from environments more frequently stressed by hypoxia has greater $\dot{M}O_2$ at the lowest oxygen concentrations. With few difference among population, the most important effects were how body mass affected $\dot{M}O_2$ at intermediate oxygen partial pressure and the negative relationship between body mass and P_{O2crit}. These findings suggest that an increase in body size is a useful approach to minimize the effect of hypoxia.

2.2 Introductory material

Hypoxia in the marine environment is a growing environmental concern, as hypoxic regions increase worldwide both spatially and in severity. This increase in hypoxic environments can affect organisms on a variety of biological timeframes, from rapid biochemical changes to long-term evolutionary adaptation (Hochachka et al. 1996; Gracey, Troll, and Somero 2001; Hochachka and Somero 2002; Wu 2002; Webster 2003; Mandic, Todgham, and Richards 2009).

Physiologically, the first response to hypoxia is often an increase in ventilation rate, maintaining oxygen delivery to the blood, to compensate for lowered environmental oxygen (Randall 1982; Steffensen, Lomholt, and Johansen 1982; Kramer 1987; Wannamaker and Rice 2000). In oxygen regulators, this increased ventilation will maintain the rate of metabolism until a critical environmental oxygen concentration, the P_{O2crit}, is reached. At oxygen concentrations less than the P_{O2crit}, increased ventilation can no longer maintain aerobic metabolism. In addition to increased ventilation, many organisms may also increase their oxygen binding capacity in the blood. This increased binding capacity can take place rapidly through Bohr shifts or more progressively through compositional changes in respiratory proteins such as hemoglobin or hemocyanin (Greaney 1980; Virani and Rees 2000; Burnett and Stickle 2001; Cooper et al. 2002; Wu 2002; MacCormack et al. 2003). When compensatory mechanism like ventilation or increase oxygen loading are insufficient, hypoxia can lead to decreased metabolism and growth rate (Wu 2002). These changes in metabolism reflect an organisms switching to a less efficient anaerobic metabolic strategy, or changes in metabolic enzyme activities and expression (Hochachka et al. 1996; Gracey, Troll, and Somero 2001; Webster 2003;

Martinez et al. 2006; Wood et al. 2007; Lundgreen et al. 2008; Richards, Sardella, and Schulte 2008).

Eventually, if these metabolic adjustments are insufficient, or a hypoxic event is prolonged, hypoxia should reduce fitness and will eventually cause death in many organisms (Hochachka et al. 1996; Wu 2002). Thus, hypoxia should exert significant selective pressure so that adaptive differences evolve. There are recent examples where lower P_{O2crit} are associated with populations from estuaries experiencing frequent hypoxia. This has been demonstrated in *Poecilia latipinna* (Timmerman and Chapman 2004) from estuaries with different frequencies of hypoxia, and among sculpins species that inhibit near shore environments with frequent hypoxic events (Mandic, Todgham, and Richards 2009). These patterns suggest that environmental difference in the frequency of hypoxia affect adaptive differences.

One of the complexities for profiling the response to hypoxia on the physiological or evolutionary time frame, and determining meaningful differences between individuals or populations is the effect of body mass. Body mass affects metabolism, enzyme activities and most other physiological functions (Hochachka and Somero 1984; Childress and Somero 1990; Pierce and Crawford 1997b; Pierce and Crawford 1997a; Burness et al. 1999; Darveau et al. 2002; Hochachka and Somero 2002); consequently, one would expect that body mass would affect the response to hypoxia. Yet, it has been argued that P_{O2crit} should be independent of body mass because of the scaling of gill surfaces (Nilsson and Ostlund-Nilsson 2008). However, in the cichlid fish *Astronotus ocellatus* the enzymes associated with hypoxic response, survivorship and P_{O2crit} scales with body mass with larger animal being more hypoxia tolerant (Almeida-Val et al. 2000;

Almeida-Val, Gomes, and Lopes 2006; Sloman et al. 2006b). Not all studies agree. In sharpsnout sea bream, *Diplodus puntazzo*, the P_{O2crit} appears be independent of body mass, but survival was negatively correlated with body size (i.e., greater survival in smaller fish; (Cerezo and Garcia 2004)). Similarly in several fish species smaller individuals used waters with lower oxygen levels than larger individual, leading the authors to suggest that the smaller individuals might be utilizing lower oxygen zones as a shelter (Wannamaker and Rice 2000; Burleson, Wilhelm, and Smatresk 2001; Robb and Abrahams 2003). The differences among these data suggest that the effect of body mass on hypoxia represent different adaptive solutions and thus evolutionary plasticity in how body mass affects hypoxic responses.

To investigate the influence of body size and population differences to hypoxia, we examine metabolic rates ($\dot{M}O_2$) at seven oxygen concentrations among seven populations of the teleost fish *Fundulus grandis* that inhabit a mosaic of habitats with different frequencies and intensities of hypoxia. *F. grandis* from the Gulf of Mexico provide a unique opportunity to study size specific responses to hypoxia and the variation among populations because this species inhabit a wide geographic range, dispersal is limited, and populations experience differential hypoxic exposure, both in frequency and severity (Engle, Summers, and Gaston 1994; Williams, Brown, and Crawford 2008). Specifically, populations from Weeks Bay, AL, Terrebonne Bay, LA, and to a lesser extent Aransas Bay, TX, live in degraded habitats (oxygen minimums less than 2 $mg \bullet L^{-1}$ over a 24-hour period (Engle, Summers, and Gaston 1994; Engle, Summers, and Macauley 1999)). Thus we expect that *F. grandis* sampled from Mobile Bay, AL, and Terrebonne Bay, LA, will have evolved adaptations for coping with hypoxia to a greater

extent than those sites with only rare periodic hypoxia such as Aransas Bay, TX, or relatively pristine sites, including the mouth of Matagorda Bay, TX, Pass Aux Heron, AL and Calcasieu Lake, LA. The results from this study demonstrate body mass and oxygen concentration has complex and significant interactions which effect metabolic rates and that body mass may be of greater importance than the divergence among populations.

2.3 Materials and methods

Animals

Fundulus grandis were collected from seven locations along the Gulf of Mexico with variable histories of hypoxic conditions in February 2006. From west to east the sites include: 1) Aransas Bay, TX (PA); 2) Matagorda Bay, TX (PO); 3) Calcasieu Lake, LA (LC); 4) Terrebonne Bay, LA (LV); 5) Mississippi Sound, MS (MS); 6) Pass Aux Herons, AL (DA); and 7) Weeks Bay, AL (WB) (Figure 2.1). A second collection from Pass Aux Herons was carried out a year later. Fish were either trapped using minnow traps or purchased from local bait shops if the site of collection by the shop could be confirmed. Fish were transported back to the lab and maintained in recirculating aquarium systems. Each population was kept in a separate tank, with 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 six weeks of pseudo-winter, temperatures were slowly increased to 24° C, and the lighting changed 14:10 light/dark cycle and the fish were allowed to spawn. Fish were fed OSI Marine

Flake *ad libitum* once daily in the evening. Oxygen consumption measurements were begun in the morning, thus they were unfed for at least 12 hours before experiments, though feeding status was not quantified. Individuals to be assayed on any given morning, were separated from the whole population, and kept in a subdivided tank in the same recirculating system. Each individual fish was identified by mass and fin clips. To minimize potential sex effects only male individuals were assayed.

Metabolism Measurements

All oxygen consumption measurements were carried out via automated intermittent flow-through respirometry, in a system designed by LoligoSystems ApS (Hobro, Denmark). The system consists of a glass respirometer chamber, with pumps connected to a central control unit and operated by a Dell Latitude 110L PC laptop computer utilizing LoliResp Software, a program for automated respirometry. The respirometer chamber was submerged in a 20-gallon aquarium, covered with opaque paper, with a small viewing port, to prevent disturbance of fish. A heated/refrigerated circulator maintained the temperature of the water at 20°C. Salinity was maintained at 15 ppt. Two Eheim 1046 centrifugal water pumps moved water through the respirometer chamber; one flushing the chamber with aquarium water, and one that recirculated water within the chamber. Water from the tank was flushed through the chamber, back into the aquarium; at this point the respirometer was open. At a given interval, the flush pump was shut off, and a second pump recirculated water within the chamber, past the oxygen probe, at this stage the respirometer was closed. The Oxygen in the chamber was measured using a Microx TX3 fiber-optic oxygen probe (PreSense GmbH, Regensburg, Germany, detection limit 0.041 kPa or 0.2% air saturation). Oxygen levels in the ambient

aquarium were continuously monitored and controlled using Mini-DO galvanic oxygen probe (OxyGuard International A/S, Birkerød, Denmark, measuring range 0-200% air saturation), connected with a solenoid to the computer control system. 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 probes were calibrated via manufactures instructions. The fiber-optic probe was calibrated with oxygen free water, made with 1 g Na_2SO_3 dissolved in 100 ml water and with 100% air saturated water, made by vigorously bubbling air through 100 ml water for 20 minutes. The galvanic probe was calibrated to 100% air saturated water, made the same way as for the fiber optic probe. At the end of each day, the respirometer chamber was cleaned in fresh water and allowed to dry to help prevent bacterial growth on surfaces.

Five to nine fish were measured per population (Table 2.1). The order of populations and selection of individual fish were random to avoid introducing any sequential or circadian bias. At the start of each metabolic determination the fish was weighed (wet weight) and placed in the respirometer. After placement in the respirometer the fish would initially swim vigorously for a few minutes, and then settle down. Preliminary data on five fish explored the time course to minimize handling stress and obtain minimal standard metabolic rates. These preliminary data showed greater metabolic rates attributed to handling stress within the first 70 minutes. Thus only measurements taken after the first 70 minutes in the respirometer were used in analyses. The cycle for each individual measurement was 10 minutes, consisting of 4 minutes of a flushing period, 1 minute of a waiting period, as the respirometer switched to closed respirometry allowing the signal to stabilize, and 5 minutes of a measurement period.

Thus there is one measurement of oxygen uptake ($\dot{M}O_2$) every ten minutes, and a minimum of 3 replicate measurements was taken at each oxygen treatment. While behavior was not quantified in this analysis, fish were monitored throughout the experiment for unusual behavior or loss of equilibrium. Metabolism was calculated as:

$$\dot{M}O_2\ [mgO_2*hr^{-1}] = 3600*O_2\ \text{solubility}\ [mgO_2*l^{-1}*kPa^{-1}]*\text{slope}\ [kPa*s^{-1}]$$
$$*\text{respirometer volume}\ [l]$$

where slope is calculated as the difference in O_2 at the start and end of the measurement period divided by the measurement time. At least three rounds of metabolic determination on different days were carried out for each individual.

Metabolism was measured at different oxygen concentrations. $\dot{M}O_2$ was first measured under normoxic conditions in triplicate as described above. In subsequent tests the partial pressure of oxygen (P_{O2}) in the water in the experimental tank was decreased stepwise from normoxia (20.5kPa) to 13.8, 10.8, 7.8, 4.8, 2.8, and 1.8 kPa by bubbling N_2 gas into the tank, while maintaining tank circulation at a low level to ensure even mixing. The new oxygen levels were introduced into the respirometer during the flush period and three replicate metabolic rate determinations were taken at each oxygen setting, thus each individual spent 30 min at each P_{O2}. Total experimental determination time for measure metabolism for seven oxygen partial pressures was 3.5 hours. Three replicate sets of these hypoxic measurements were taken on different days for each individual, for a total of 9 measurements per P_{O2}. Background respiration without fish was measured, but found to be negligible.

Data Analysis

Data analyses were carried out using GraphPad Prism, Microsoft Excel and Matlab. Metabolic rates were determined by quantifying the consumption of oxygen three times each day on three different days for all individuals and all oxygen concentrations. The average of each of these nine determinations per oxygen concentration were used for all other statistical analyses. To correct for the effect of body mass, $\log_{10} \dot{M}O_2$ was regressed against $\log_{10}$ body mass and the residuals were used for all other analyses (Packard and Boardman 1999). Regression analysis, analysis of variance (ANOVA) and Analysis of Covariance (ANCOVA) were performed in Matlab and SAS JMP 7.

The critical oxygen tension (P_{O2crit}) was calculated using methods similar to Yeager and Ultsch (Yeager and Ultsch 1989) using the residuals of the $\log_{10} \dot{M}O_2$- $\log_{10}$ body mass regression. The P_{O2crit} is the point where $\dot{M}O_2$ can no longer be maintained and thus, there is an expectation of change in slope of the regression of $\dot{M}O_2$ and P_{O2} at oxygen tensions below this critical point. This change in slope that defines P_{O2crit} was determined as the intersection of the two slopes of $\dot{M}O_2$ versus P_{O2} across the entire P_{O2} gradient using segmented linear regression fitted to the data using the non-linear regression tools in GraphPad. A single P_{O2crit} was calculated for each individual.

2.4 Results

Over the course of all measurements, only two fish lost equilibrium at the lowest oxygen concentration, in one set of measurements each. The overall run for each of these fish was included, with eight measurements, rather than nine at the lowest oxygen

concentration. Of the two fish that lost equilibrium, one was large; the other small, so no size relationship could be inferred. ANOVA on mass indicates a significant difference in mass between collections sites; with post hoc test determining this difference is limited to the 2006 Pass Aux Heron collection (Tukey-Kramer). The 2006 Pass Aux Herons fish were significantly larger than all other collection sites, with lower variation in size (all the fish were large), however, initial data analysis indicate no change of other parameters when the 2006 and 2007 collections from Pass Aux Heron (6) are removed or combined, rather than treated as separate populations (data not shown). The only exception is in the analysis of normoxic $\dot{M}O_2$. If the two collections are treated as separate populations, there is a significant difference between populations in normoxic $\dot{M}O_2$ (ANOVA $p<0.02$, degrees of freedom 7, 45). A Tukey Kramer post hoc test demonstrates that this difference is limited to the larger 2006 Pass Aux Heron individuals. The results above suggest that any difference due to body mass is limited, thus the two collections are treated as a single group for all analysis.

Normoxic oxygen consumption, $\dot{M}O_2$, was significantly affected by body size ($R^2 = 0.77$, $p=6.7*10^{-18}$; Table 2.2; Figure 2.2). The significant relationship of log metabolic rate and log body mass remains across all hypoxic oxygen treatments (Table 2.2; Figure 2.2; $R^2= 0.37$, $p<2.65*10^{-29}$). However, there is significant interaction between oxygen treatment and log body mass ($p < 0.05$). This interaction is due to significantly different log body mass: log $\dot{M}O_2$ slopes at different oxygen treatments (ANCOVA $p<3.9*10^{-6}$, degrees of freedom 6, 266, Table 2.3). Post-hoc analysis demonstrated the differences in the slopes for log body mass: log $\dot{M}O_2$ were between normoxia and the next five lower P_{O2} treatments (13.8-2.8 kPa), and 13.8 kPa versus 4.8-2.8 kPa. The slope of regression

of log $\dot{M}O_2$ and log body mass across all treatments is not significantly different among populations (ANCOVA $p<0.33$, degrees of freedom 6, 266).

Due to the significant interaction of body-mass and treatment described above, the effect of body mass on $\dot{M}O_2$ was examined within each treatment, followed by an analysis of variance between populations within each treatment. There is a significant difference for $\dot{M}O_2$ among populations at the lowest oxygen partial pressure (1.8 kPa; Figure 2.3; ANOVA, $p < 0.03$, degrees of freedom 6, 33, Table 2.4.). Tukey-Kramer post-hoc test indicates that this difference is due to the greater metabolic rate for Terrebonne Bay, LA (Fig 3). For all other oxygen partial pressure there are no differences among populations (ANOVA p-values range from $p<0.33$ to $p<0.90$; degrees of freedom 6, 33, Figure 2.3).

The P_{O2crit} is defined by the strong inflection in the non-linear regression (Figure 2.4). P_{O2crit} values varied among individuals, ranging from 2.4 to 7.8 kPa. The P_{O2crit} are smaller for larger fish: across all populations P_{O2crit} had a significant negative regression with log body mass ($p<0.04$; Figure 2.5). There were no significant differences among populations in P_{O2crit}, and no significant differences in the slope of log body mass: P_{O2crit} between populations (ANCOVA $p<0.20$ for populations, $p<0.51$ for population*body mass, degrees of freedom 6, 26, Table 2.5).

2.5 Discussion

There are two important findings from this research, which can be broadly described as the differences among populations and the effect of body mass on metabolic responses to hypoxia. Of these, the interactions between the environmental oxygen

concentration and the effect of body mass on metabolic rate may be of broader interest because it suggests a more general solution to the debate about mass specific hypoxic effects.

Differences among Populations

Our initial hypothesis was that there would be significant difference in the response to hypoxia among *F. grandis* populations because a few of these populations suffer from frequent hypoxic exposure in their native environments (Engle, Summers, and Gaston 1994; Engle, Summers, and Macauley 1999). Hypoxia in the Gulf of Mexico affects both the near shore and offshore environments, and is associated with a large subsurface hypoxic zone. First reported in the 1970's, it has been recorded annually since then (Rabalais and Turner 2001). Between Anclote Key, FL and Rio Grande, TX; 5.9% to 29.3% of estuaries surveyed were affected by hypoxia (Engle, Summers, and Macauley 1999). These locations are classified as "degraded" estuaries, and experience oxygen concentrations less than 2 mg L^{-1} (4.6 kPa at 20°C and 1 standard atmosphere) over a 24-hour period (Engle, Summers, and Gaston 1994). Undegraded estuaries never recorded an oxygen minimum below 3 mg L^{-1} (6.8 kPa at 20°C and 1 standard atmosphere). In this study, three populations (Aransas Bay, TX; Terrebone Bay, LA and Weeks Bay, AL) were considered degraded. Weeks Bay, part of Mobile Bay, has potentially the longest record of hypoxia of any Gulf of Mexico estuary, and is reported by the EPA as a "Priority Hypoxia Area" (USEPA 1999). The record of hypoxia in Mobile Bay goes back to 1821, recorded as reports of "Jubilees." In a Jubilee, hypoxic water advects onshore, pinning fleeing fish, which are easily caught by local residents (May 1973). Monitoring continues currently in many of these locations (National

Oceanic and Atmospheric Administration 2004). The variability of duration and frequency of estuarine hypoxia in the Gulf of Mexico led us to predict that in Aransas Bay, TX; Terrebonne Bay, LA, and Weeks Bay, AL, the P_{O2crit} would be lower and metabolic rates would be higher at low oxygen tensions. Oxygen consumption was significantly different among populations at the lowest P_{O2} treatment (1.8 kPa, Figure 2.3). Individuals from both Aransas Bay and Terrebonne Bay have high $\dot{M}O_2$ at 1.8 kPa, however only Terrebonne Bay is significantly different. The differences in $\dot{M}O_2$ at 1.8 kPa occur after long-term acclimation to controlled conditions, suggesting that there could be a genetic basis for the differences, or that differences could reflect a developmental or irreversible acclimation effect. The calculated P_{O2crit} values encompass the values previously calculated for *F. grandis* (~4.5 kPa, (Virani and Rees 2000)) and for its sister taxa *F. heteroclitus* (~4.6 kPa, (Cochran and Burnett 1996)). However, no significant differences were found in P_{O2crit} among populations.

If we assume the hypoxic responses are genetically determined (either due to differences in genes affecting metabolism, acclimation response or the rate of physiological response), there are several reasons for this lack of difference among populations in P_{O2crit}, yet a difference in $\dot{M}O_2$ at 1.8 kPa. One is simply that P_{O2crit} is more difficult to evolve because it is potentially affected by many more loci or has greater constraints. However, among different species of sculpins, there are phylogenetically independent correlations of P_{O2crit} with environment and many other metabolic parameters (Mandic, Todgham, and Richards 2009). These data among suggest that P_{O2crit} is readily evolved among closely related species. Alternatively, the samples size (five individuals per populations) does not provide the appropriate power.

Although larger samples size are always desirable, with five individuals and the empirically defined variance, the power of the test is 0.83 (probability of rejecting null hypothesis, i.e., not make a type II error) with a p-value > 0.20 for P_{O2crit}. Given these values, it is unlikely we falsely accept the null hypothesis of no differences among populations. Finally, unlike the comparison among species, we examined the differences among populations within a species. Within a species, populations are affected by migration that can inhibit the evolution of local adaptation (Endler 1977; Endler 1986; Slatkin 1987). Divergence among populations is a function of both the variation in selective pressure among populations and the magnitude of migration. The significant difference in $\dot{M}O_2$ at 1.8 kPa, but lack of divergence P_{O2crit} among populations of *F. grandis*, could reflect the difference in selection pressure in response to hypoxia among populations relative to the rate of migration. Thus, if these differences are evolving by natural selection, then we would suggest there is greater selection on metabolic rates at low partial pressure versus the selection for P_{O2crit}.

There are other reasons for these patterns that are not related to hypoxia *per se.* One is the experimental design. All measures of $\dot{M}O_2$ under hypoxia were done in series, thus all fish were initially exposed to 13.8 kPa, then to 10.8 kPa, then to 7.8 etc., down to the lowest oxygen partial pressure, 1.8 kPa, with 30 minutes spent at each treatment. Thus, at the lowest oxygen concentration, fish had been previously exposed to below normoxic conditions for two and a half hours. This serial exposure could initiate physiological processes that allow fish to better cope with low oxygen concentration, and would be similar to what occurs naturally. More importantly, all individuals in all populations were treated the same, and thus we can compare each oxygen treatment

among populations. Alternatively, other environmental factors such as salinity or temperature might differ among populations. However, one would expect these to be minimized through common gardening. Additionally, salinity and temperature were kept constant, as the same respirometer and ambient aquarium were used for all measurements. If population based differences existed for salinity or local temperature, one would expect to see them at multiple treatments. However, differences between populations were only observed at the lowest oxygen treatment, suggesting that hypoxia is the most likely cause for these differences.

There is one difference in the metabolic rate we suggest is due to age and not hypoxia related factors. For normoxic oxygen uptake there was only a significant difference in metabolism in a single collection, the 2006 collection from Pass Aux Heron. This effect disappeared if this population were combined with the 2007 individuals from the same location. The individuals from the 2006 Pass Aux Heron collection were overall larger and had less variation in size between individuals. *F. grandis* grow throughout their lifespan (Weatherley, Casselman, and Gill 1987) so it is likely that these individuals were older than the smaller individuals in the other collections. Thus, we propose that the difference in metabolism in this population is more likely to be an age related effect such as senescence, rather than one relating to divergent evolution in response to hypoxia. Indeed, differences in metabolism relating to ontogenic state have been observed in teleosts (Sloman et al. 2008).

The Effect of Body Mass

The more important finding of this research is the change of the body mass: metabolic rate relationship at different partial pressures of oxygen. The significant

relationship between metabolism and body mass remains across all oxygen treatments, regardless of population. However, body mass had a stronger effect (steeper slope) at intermediate oxygen partial pressure than at normoxic or the lowest oxygen partial pressure (1.8 kPa, Table 2.2). Thus, at intermediate oxygen concentrations, the steeper slope for log body mass *versus* log metabolism means that larger fish have metabolic rates more similar to normoxia (Figure 2.3). The observation that there is a stronger effect of body mass (steeper slope) at intermediate partial pressure is not well documented in the literature. The exponent (slope) for metabolism for many teleosts is often generalized ranging from 0.71 to 0.88 under normoxic conditions and 15-20°C (Peters 1983; Glazier 2005; Nilsson and Ostlund-Nilsson 2008). However, the exponent for *F. grandis* was not in this range but more similar to 0.66 at intermediate P_{O2} and approximately 0.5 at normoxia. These values are similar to those found by Almeida-Val et al. (Almeida-Val, Gomes, and Lopes 2006) for *Astronotus ocellatus*, a hypoxia tolerant cichlid. While these values fall outside the traditional distribution, several recent studies and reviews have suggested that the value of 0.75 may not be universal, either within or between species and may be subject to both ecological and evolutionary constraints (Julian et al. 2003; Douglas 2005; Almeida-Val, Gomes, and Lopes 2006; Seibel and Drazen 2007). At the lowest oxygen partial pressure we hypothesize that the small effect of body mass (slope = 0.37) is due to either a reduction in overall metabolism or greater reliance on anaerobic metabolism. That is, our measures of metabolism are only for $\dot{M}O_2$, and at 1.8 kPa much of the total metabolism could rely on anaerobic catabolism and thus be less affected by the ability to take up and deliver oxygen (Goolish 1991). The relative low effect of body mass at normoxia and greater effect at intermediate

metabolism, suggest that body-size metabolism relationship is sensitive to the environment.

The observation that P_{O2crit} is affected by body mass is controversial. Studies have found affects of body mass on P_{O2crit} in some species (Kalinin, Rantin, and Glass 1993; Sloman et al. 2006a; Nilsson and Ostlund-Nilsson 2008) but not in others (Cerezo and Garcia 2004; Sloman et al. 2008). Nilsson and Östlund-Nilsson (2008) argue that P_{O2crit} is unlikely to be influenced by body mass, arguing that the similar scaling values of oxygen consumption (0.79-0.88) and gill surface area (0.76-0.90) indicate that P_{O2crit} values should be independent of size. Our data compares metabolism within a species of adult fish that range from 2.5 to 41 grams. Among these individuals with similar body shapes and ways of life, there is a significant negative regression of P_{O2crit} with body mass ($p < 0.04$) even with this relatively small range of body sizes. The lower P_{O2crit} in large fish occurs even though the allometry of body mass and gill surface is less than one (Peters 1983; Nilsson and Ostlund-Nilsson 2008). Thus, larger fish have a proportionally smaller gill surface, yet appear to be able maintain more normal metabolism to lower oxygen concentrations. This is consistent with the observation by Graham (2006) that values for the scaling exponent for gill surface area range from 0.5-1.0 and have no clear relationship to respiration. The observation that larger fish have lower P_{O2crit} suggests that body mass is beneficial in hypoxic environments. Combining the two mass specific effects on P_{O2crit} and $\dot{M}O_2$ at intermediate oxygen concentrations; these observations suggest that fish with larger bodies are better able to maintain metabolism similar to normoxic levels for a wider range of oxygen concentrations (Figure 2.2, Table 2.2).

If this interaction between oxygen concentration and the effect of body mass on metabolism is a general phenomenon it could explain the differential habitat preference under hypoxia in different size fish (Burleson, Wilhelm, and Smatresk 2001). That is, it is possible that smaller individuals avoid hypoxia behaviorally in the environment, by either performing aquatic surface respiration, or staying in more shallow water, with higher oxygen saturation, because they are more sensitive. For *F. grandis,* we lack any behavioral or experimental evidence and thus this is speculative.

Conclusion

The data provide here, suggest that there are few difference in metabolic rate among populations, except at the lowest oxygen concentrations. Instead the most important parameter for surviving hypoxia is body size: larger fish have lower P_{O2crit}, and at intermediate oxygen concentrations have metabolic rates more similar to normoxic values. Thus, these data suggest that increase in body size is an effective strategy to minimize the effect of hypoxia. Additionally, these data suggest that the effect of body mass on physiology varies with the environment and thus its importance is dependent on the conditions used to measure physiological processes.

Table 2.1. Sample size and average mass from each collection site.

Population, West to East	Number of individuals (n)	Average Mass (g) (±std. dev.)
Aransas Bay, TX (1)	5	18.8±10.2
Matagorda Bay, TX (2)	5	11.6±8.1
Calcasieu Lake, LA (3)	6	9.6±9.2
Terrebonne Bay, LA (4)	5	10.5±7.1
Mississippi Sound, MS (5)	5	14.6±5.2
Pass Aux Herons, AL (6)	9	20.2±13.8
Weeks Bay, AL (7)	5	14.7±5.5

Table 2.2. Regression values from regression of $\log_{10}$ $\dot{M}O_2$ and $\log_{10}$ body mass.

Treatment	R^2	p-value	Slope
Normoxia	0.77	$6.7*10^{-18}$	0.51
13.8 kPa	0.81	$3.15*10^{-15}$	0.65
10.8 kPa	0.87	$1.5*10^{-18}$	0.62
7.8 kPa	0.88	$4.8*10^{-19}$	0.59
4.8 kPa	0.90	$6.0*10^{-21}$	0.57
2.8 kPa	0.87	$1.7*10^{-18}$	0.43
1.8 kPa	0.68	$7.5*10^{-11}$	0.37

Table 2.3. Analysis of covariance table for differences in $\dot{M}O_2$ between treatments

Source	Degrees of Freedom	Mean Square	F-value	Probability>F
Treatment	6	1.6104	296.86	<0.0001
Body mass	1	6.5168	1201.3	<0.0001
Treatment*Body mass	6	0.03374	6.2194	3.98*10-06
Error	266	0.0054249		

Table 2.4. Analysis of Variance between populations at 1.8 kPa treatment.

Source	Degrees of Freedom	Mean Square	F-value	Probability>F
Populations	6	0.0127	2.9606	0.020053
Error	33	0.0042895		
Total	39			

Table 2.5. Analysis of covariance table for comparison of P_{O2crit} values between populations.

Source	Degrees of Freedom	Mean Square	F-value	Probability>F
Population	6	0.027868	1.5631	0.19774
Body Mass	1	0.045732	2.565	0.12133
Population*Body mass	6	0.016143	0.90541	0.50622
Error	26	0.017829		

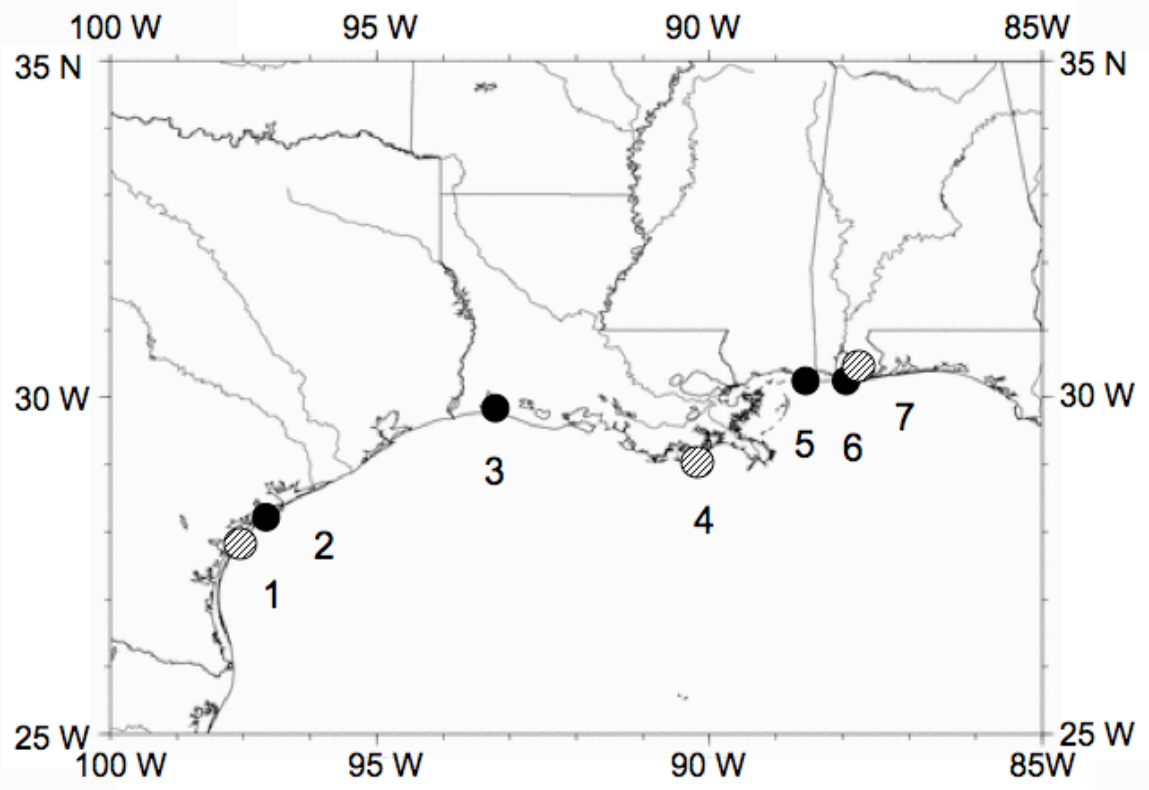

Figure 2.1. Map of Gulf of Mexico collection sites. Collection sites from west to east are: 1) Aransas Bay, TX (PA), 2) Matagorda Bay, TX (PO), 3) Calcasieu Lake, LA (LC), 4) Terrebonne Bay, LA (LV), 5) Kiln Co, MS (MS), 6) Pass Aux Heron, AL (DA), 7) Weeks Bay, AL (WB).

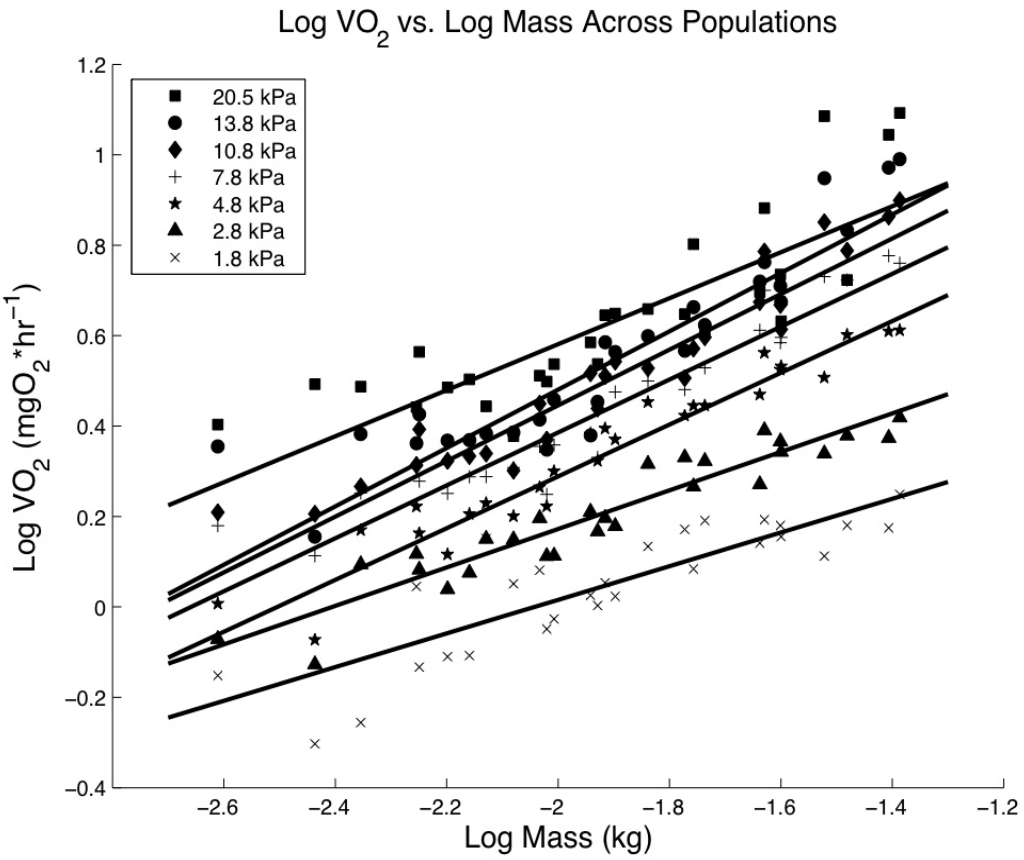

Figure 2.2. Metabolic rate versus body mass for seven oxygen concentrations. The relationship between log body mass and log metabolism was determined using all individuals from all populations. Oxygen consumption ($\dot{M}O_2$) was measured 3 times per individual, and the mean of these replicate measures at each oxygen treatment are plotted. R^2 range from 0.67 to 0.90. The seven oxygen concentrations were: 20.5, 13.8, 10.8, 7.8, 4.8, 2.8, & 1.8 kPa. There is a significant difference between slopes (ANCOVA $p<3.9*10^{-6}$, degrees of freedom 6, 266).

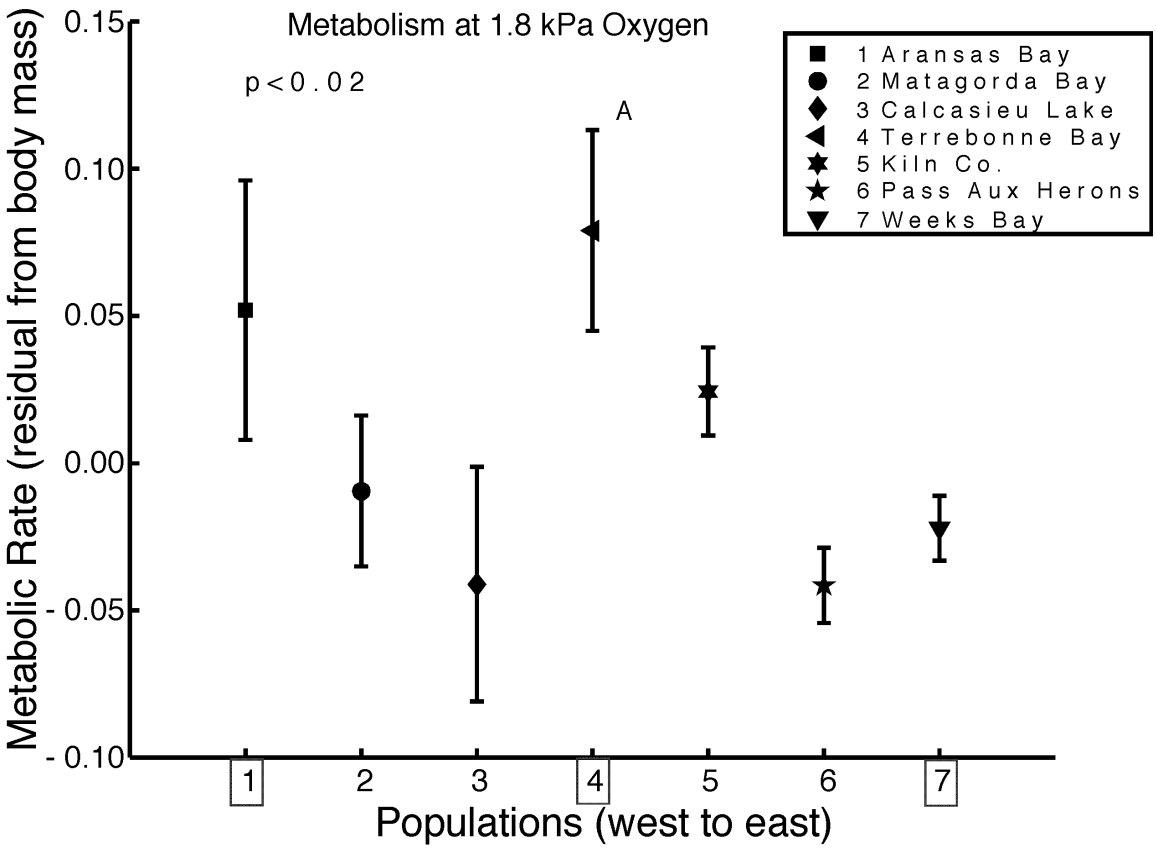

Figure 2.3. Population specific metabolic rates at lowest oxygen treatment. Average $\dot{M}O_2$ at 1.8 kPa were corrected for body mass by using the residual from log-log regression of body mass with metabolism. Populations are listed west to east, the numbers correspond to those listed in Figure 2.1, boxes around 1, 4, and 7, correspond to locations designated as degraded (Engle, Summers, and Gaston 1994; Engle, Summers, and Macauley 1999). Error bars are one standard error. Analysis of variance resulted in a significant difference between populations (ANOVA $p < 0.02$, degrees of freedom 6, 33). **A.** Tukey-Kramer post-hoc test determined population (#4) Terrebonne, to be significantly different from the Pass Aux Heron (#6) population.

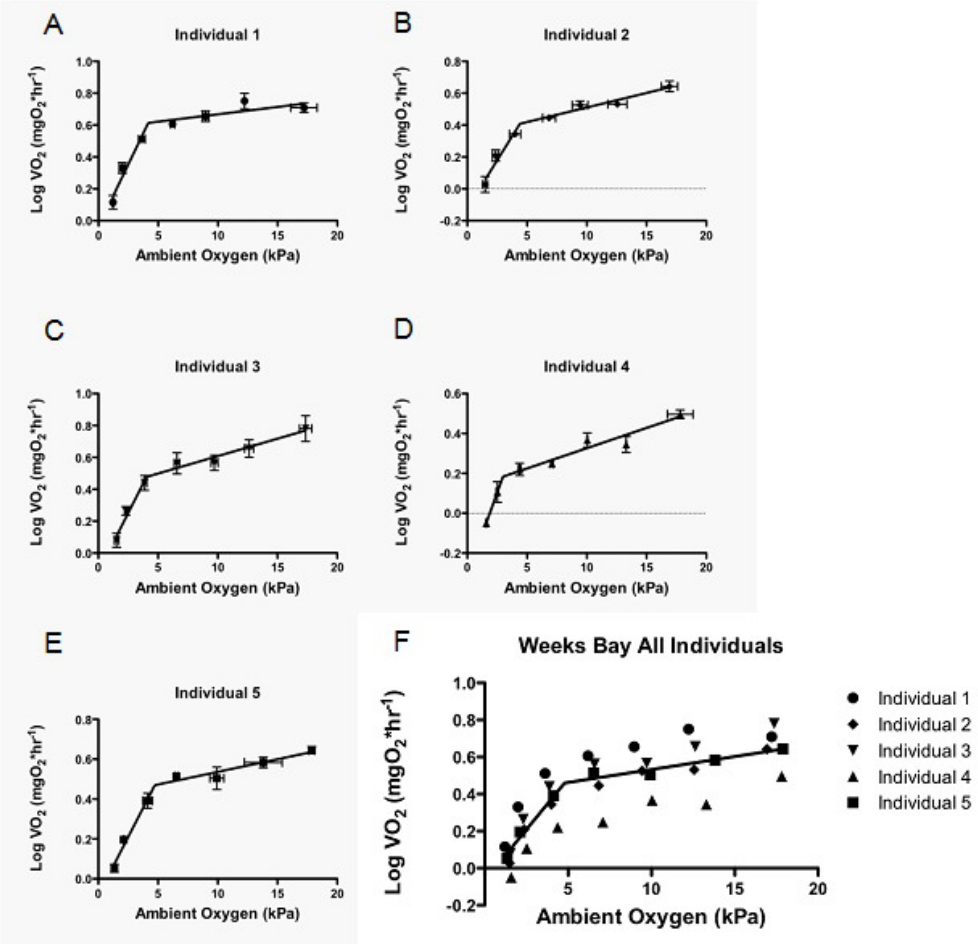

Figure 2.4. Critical oxygen tension. Metabolic rates versus oxygen partial pressure for (A-E) five individuals from the Weeks Bay population (#7) and (F) for the entire population using individual averages. Plotted are segmented linear regressions that are used to determine critical oxygen tensions (P_{O2crit}).

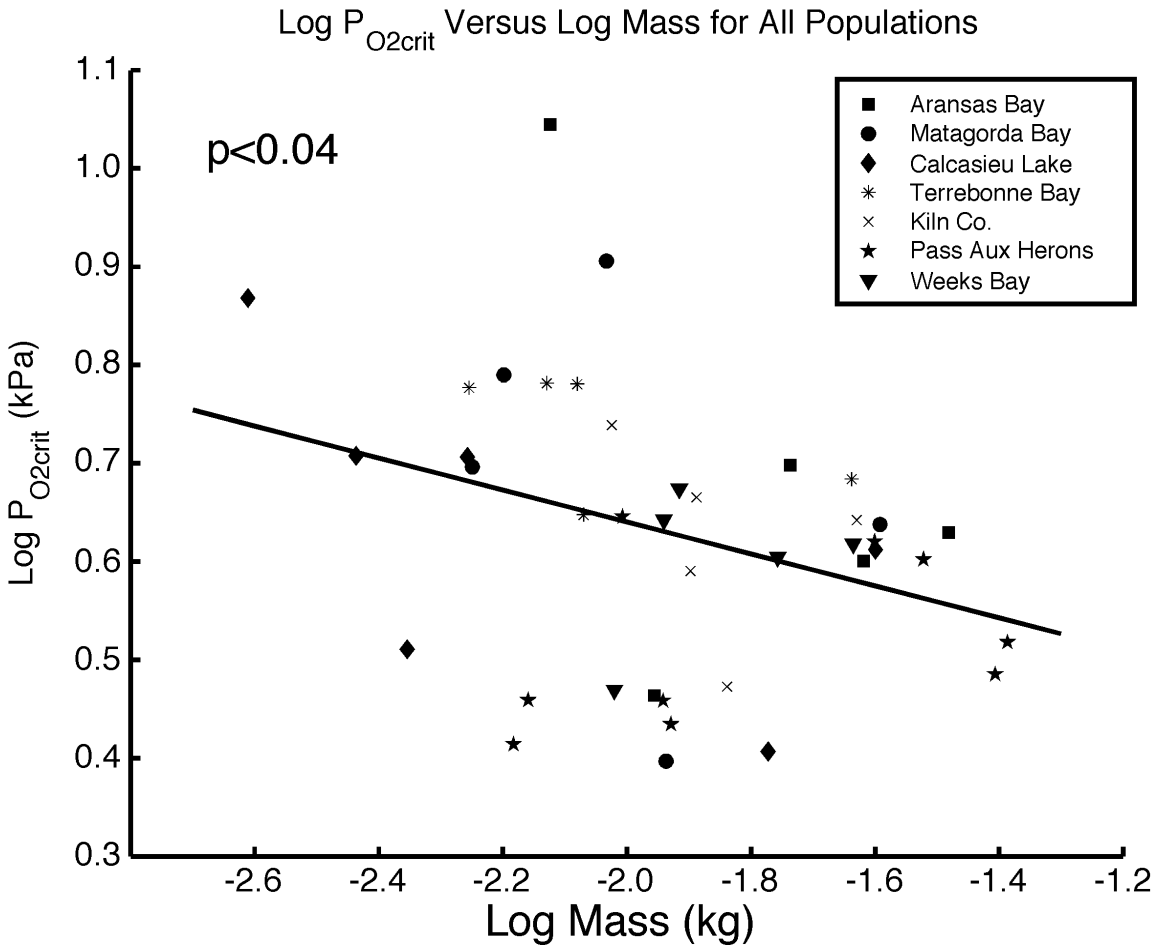

Figure 2.5. Body mass affects critical O_2 tension. Critical oxygen tension (log P_{O2crit}) is plotted versus log body mass for all 40 individuals. Symbols represent different populations. There is a significant regression with body mass (R^2=0.11, p<0.04).

Chapter 3 Time Course for Hypoxic Metabolic Gene Expression

3.1 Summary

Hypoxic conditions are becoming more common in estuaries along the Gulf of Mexico. To better understand the time course for physiological response to hypoxia, we exposed individuals for 0, 4, 8, 12, 24, 48 and 96 hours to a partial oxygen pressure of 2.8 kPa (~13.5% air saturation) and quantified the change in mRNA expression for 314 metabolic genes. During hypoxia, the maximum expression of mRNAs for different genes occurs at different time points. For the distribution of structural genes, the expression of mRNA is significantly over represented at zero exposure (Fisher-Exact test $p < 0.05$). For genes with peak expression at zero oxidative phosphorylation genes are under-represented, but oxidative phosphorylation genes have a significant over-representation after 96 hours (Fisher-Exact test, $p < 0.05$). Although most genes have a peak in expression after exposure to hypoxia, there were only 14 genes with significant differences ($p < 0.01$) in mRNA expression and the changes in expressions were small (less than 2-fold). Most of these significant differences occur with 48 or 96 hours of exposure. These significant differences appear to reflect a convergence among individuals. That is, after 4-24 hours of exposure, many of these 14 genes had changes in expression, but it varied significantly among individuals. However, these differences among individuals were smaller with greater exposure time. Finally, these patterns of expression among genes are correlated, suggesting that one or a few transcription factors affect the changes in mRNA expression.

3.2 Introductory material

The teleost fish *Fundulus grandis* lives in estuaries along the Gulf of Mexico that experience periodic hypoxia. Hypoxia is on the rise in the Gulf of Mexico affecting both the near shore and offshore environments. The increase frequency of hypoxia is the Gulf of Mexico is associated with the large subsurface hypoxic zone covering a geographic area approaching 20,000 km^2, first reported in the 1970's (Rabalais and Turner 2001) and recorded annually since (Rabalais and Turner 2001). This zone is located offshore, but has been documented to depths as shallow as 4 to 5 m. Importantly, estuaries in the Gulf of Mexico also experience periodic hypoxia to varying degrees: between Anclote Key, FL and Rio Grande, TX; 5.9% to 29.3% of estuaries surveyed were affected by hypoxia (Engle, Summers, and Macauley 1999).

Hypoxic environments can affect organisms on a variety of biological timeframes, from rapid biochemical changes to long-term evolutionary adaptation (Hochachka et al. 1996; Gracey, Troll, and Somero 2001; Hochachka and Somero 2002; Wu 2002; Webster 2003; Mandic, Todgham, and Richards 2009). Immediate responses include behavioral changes to avoid hypoxia zones (Keister, Houde, and Breitburg 2000; Wannamaker and Rice 2000; Burnett and Stickle 2001; Breitburg 2002; Bell, Eggleston, and Wolcott 2003b). When it is not possible to avoid hypoxic water, organisms can become quiescent or, in shallow systems the fish may perform aquatic surface respiration (ASR), gulping the oxygenated layer of water right at the surface (Virani and Rees 2000; Wu 2002; Watters and Cech 2003). Physiologically, the first response to hypoxia is often an increase in ventilation rate, increasing oxygen delivery to the blood, to compensate for lowered environmental oxygen. In oxygen regulators, organisms maintain their blood

oxygen despite decreasing environmental oxygen concentrations. The increased ventilation will maintain the rate of metabolism until a critical environmental oxygen concentration, the PO_{2crit}, is reached. At oxygen concentrations less than the PO_{2crit} increased ventilation can no longer maintain metabolism. On a cellular and molecular level, gene expression often changes.

Hypoxia induced gene expression changes and halted development in zebrafish (*Danio rerio*) embryos during exposure for 24 hours. Normal development and gene expression was resumed when the embryos were returned to normoxic conditions (Ton 2003). Similarly, a comparison of tissue specific responses to hypoxia in *Gillichthys mirabilis* over 144 hours (Gracey, Troll, and Somero 2001) identified 126 hypoxia regulated genes, corresponding to metabolism, locomotion, protein synthesis, antigrowth and proliferation, and amino acid metabolism. Most changes in gene expression occurred in the liver or skeletal muscles in the first 24-72 hours. There were few changes in cardiac or brain patterns of gene expression. Long-term (three week) exposure in *D. rerio* emphasized the importance of tissue specific differences. In gill 367 (2.5%) genes were differentially expressed, and the primary response was repression: two-thirds (250) of differentially expressed genes being repressed, and one-third (117) induced (van der Meer et al. 2005). In a similar study examining cardiac gene expression, nearly the same number of genes were differentially expressed (376), but the response was primarily the induction of mRNA expression: approximately two-thirds (260) of the genes had induced expression and a minority were repressed (Marques et al. 2008), almost exactly opposite of the gill study.

Determination of pooled yet tissue specific gene expression responses to hypoxia provide insight into the physiology and the biochemistry of tissues. However, none of these studies examined the variation among individuals, instead they relied on pooling several individual to get "an average" response precluding statistical evaluation (Whitehead and Crawford 2005). Without information about the variation among individuals, the change in any molecular trait could be a common response to the stress, or represent individual variation in hypoxic response. For example, if one or a few individuals had large fold changes, the average response could exceed the common 2-fold change often used as a criterion of differential expression. Yet with the appropriate sampling of individuals, this change may not be statistically significant. Additionally, understanding the variation among individuals within a population provides insight into the variation that could contribute to evolutionary adaptation (Crawford and Oleksiak 2007).

For *Fundulus grandis*, short term (3 hours) exposures to decreasing oxygen concentrations (20.5 kPa to 1.8 kPa) cause a gradual decline in metabolism above 7 kPa (Everett In Press-a), below this, MO_2 could no longer be maintained. The specific inflection point depended on body mass, with larger fish having lower PO_{2crit} (Everett In Press-a). These changes in metabolism were rapid. To better understand the effect of hypoxia, we examine the cellular response to hypoxia over four-day exposure (0, 4, 8, 12, 24, 48, and 96 hours). Specifically, we quantified changes in cardiac mRNA expression for metabolic genes (Table 3.1) in four individuals at each time point. The results presented here suggest that in cardiac tissue most responses take 48 to 96 hours and are relatively small.

3.3 Materials and methods

Animals and Hypoxic Exposure

Fundulus grandis were collected from Pass Aux Herons, AL in May 2007. 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 six weeks of pseudo-winter, temperatures were slowly increased to 24° C, and the lighting changed 14:10 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 prior to beginning hypoxic exposure and allowed to settle overnight. Exposure to hypoxia was carried out in a 45-gallon glass aquarium. Individuals subjected to hypoxia were exposed to 2.8 kPa O_2.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 ambient aquarium were continuously monitored and controlled using a 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 probes were calibrated via manufactures instructions. The galvanic probe was calibrated to 100% air saturation, made by vigorously bubbling air through 100 ml water for 20 minutes. The probe was calibrated at the start of the experiment, and checked for drift daily after that. The surface of the water was covered completely in bubble wrap to prevent aquatic surface respiration (ASR) and reoxygenation. Prior to the initiation of hypoxia, four individuals were sampled to be normoxic controls. Subsequently, oxygen in the system was dropped to 2.8±0.3 kPa over the course of 30 minutes. This concentration was held for 96 hours, and four individuals were sampled at each time point: 4, 8, 12, 24, 48, and 96 hours. Fish were sacrificed via cervical dislocation and heart tissues were collected and stored in RNAlater (Ambion) according to manufacturer's instructions.

RNA Preparation

RNA was extracted from four 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). 40 ml of 2 M Sodium Acetate was added to each sample followed by 400 ml 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 16,000g 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 16,000g for 30 min. The remaining RNA pellet was rinsed with 400 µl of 70% ethanol and further purified using RNAClean (Agencourt) 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 1996), using an Amino Allyl MessageAmp II-96 kit (Ambion). Briefly, this method amplifies the signal by using T7 RNA polymerase 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) 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 3.1; (Paschall et al. 2004a)). Average lengths of cDNAs were 1.5Kb with a majority including the N-terminal methionine. Amplified cDNA were spotted onto epoxide slides (Corning) using an inkjet printer (Aj100, ArrayJet, Scotland).

3.3.4 Hybridization

Twenty pmol of Cy3 and Cy5 labeled aliquots were vacuum dried together and resuspended in 10 µl of hybridization buffer (5X SSPE, 1% SDS, 50% formamide, 1 mg/ml polyA, 1 mg/ml sheared herring sperm carrier DNA, and 1mg/ml BSA) for a final concentration 2 pmol/µl for each sample. Immediately prior to use, slides were blocked in a solution consisting of 5% ethanolamine, 100 mM Tris (pH 7.8), and 0.1%SDS for 30 minutes. The slides were then washed in 50 °C 4X SSC, 0.1% SDS solution for one hour and rinsed with autoclaved water. Finally, slides were boiled for two minutes, given a final rinse in autoclaved water, and spun dry (800 rpm for 3 minutes). Labeled RNAs (2pmol/µl each of Cy3 and Cy5) in hybridization buffer (see above) were heated to 92°C for two minutes, quick cooled to 42 °C, 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 4X SSC to prevent the hybridizations from drying out. Samples were hybridized for approximately 48 hours at 42 °C. Following hybridization, slides were washed in decreasing concentrations of SSC and SDS (4X + 0.1% SDS-0.1X SSC no SDS) and then spun dry (800 rpm for 3 minutes). Slides were scanned using the Packard Bioscience ScanArray Express microarray scanner (PerkinElmer Life Sciences), with laser wavelengths set to 633 and 543 nm. Resulting .tiff images were imported into spot grids built in ImaGene (Biodiscovery) for each array, and spot signals were collected as fluorescence intensities for each dye channel.

Hybridization Design

Individuals from different time points were hybridized together in a loop design (Kerr, Martin, and Churchill 2000; Oleksiak, Churchill, and Crawford 2002). A “loop

design" does not rely on a reference, rather two different experimental samples label with Cy3 or Cy5 fluorescent dyes hybridized together, and each individual is measured on two slides, once with Cy3 and once with Cy5. This creates a loop of Cy3⇒Cy5 with 28 arrays (time with individual # as subscript):

$$0_2 \Rightarrow 4_6 \Rightarrow 8_{11} \Rightarrow 12_{16} \Rightarrow 24_{21} \Rightarrow 48_{26} \Rightarrow 96_{33} \Rightarrow 4_7 \Rightarrow 12_{17} \Rightarrow 48_{27} \Rightarrow 0_3 \Rightarrow 8_{12} \Rightarrow 24_{22} \Rightarrow$$

$$96_{34} \Rightarrow 8_{13} \Rightarrow 48_{28} \Rightarrow 4_8 \Rightarrow 24_{23} \Rightarrow 0_5 \Rightarrow 12_{19} \Rightarrow 96_{35} \Rightarrow 48_{29} \Rightarrow 12_{20} \Rightarrow 8_{14} \Rightarrow 0_1 \Rightarrow 24_{25} \Rightarrow$$

$$4_9 \Rightarrow 96_{32} \Rightarrow \text{first sample.}$$

Each slide had 6 arrays, and each array had two individuals hybridized, for 12 individuals per slide.

Data Processing and Statistical Analysis

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 plus two standard deviations of the negative control were removed from all individuals, and were not analyzed. Because the photo multiplying tube (PMT) in the ScanArray Express has a saturation fluorescence value of 65535, genes whose values were saturated across most individuals and treatment in the loop were also removed. The hypoxic treatment may 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, this spot was replaced with a missing data value in both Cy3 and Cy5 individuals, as these were presumed to be from an auto-fluorescing spot.

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:

$$y_{ijk} = m + A_i + D_j + I_k + e_{ijk}$$

where, m is the overall average signal, A_i is the effect of the *ith* array (one of eight arrays within a time point), D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), I_k is the fixed effect of the Individual (one of 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 (REML). The variance of the standardized means from this mixed model for the four individuals in each time point was calculated for each gene and averaged across all genes for each time point. The standardized means for each gene across all 7 time points (28 individuals) has a mean of zero and a variance of one. To examine the variance among individuals across all genes, the mixed model described above was used for all 28 individuals across all time points.

A similar procedure was used to calculate ANOVA on each gene across all treatments, with the following ANOVA model:

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

where, m is the overall average signal, A_i is the effect of the *ith* array (one of twenty-eight arrays), D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), T_m is the fixed effect of time for the mth time point (one of seven time points), $I_k(T_m)$ is the effect

of individuals within time point (one of 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 was performed using Macintosh's version (de Hoon et al. 2004) of Eisen's Cluster and Treeview (Eisen et al. 1998). In JMP 7 a Fisher's exact test was used to determine the statistical significance of gene pathway distribution between treatments. Dunnett's post-hoc test was used to determine which time points differ from the zero time point.

3.4 Results

Out of 373 possible genes, 48 genes had fluorescent signals below negative controls, and 10 genes had fluorescent signals saturated across all treatments, and were also removed. Thus 314 genes were used in analysis of the time course of hypoxia exposure.

Differences Among Individuals

For the four individuals in each time point, with each individual having three replicates per array and two arrays (one with Cy3 and the other with Cy5), there are 3 and 16 degree of freedom for the main effect (difference among individuals). Among the seven time points, the four hour time point had the fewest number of genes with a significant difference ($p < 0.01$) in mRNA expression and 96 hours had the most (Table 3.2). The average variance among individuals across all genes is greatest at the zero time point. This average variance is the mean variance across all genes for each time point, and was calculated by using the variance of the standard means among individuals within each time point. Thus, this variance represents the proportional variation among individuals and not the technical variation. Additionally, because these are standard

means (average of zero for each gene among all 28 individuals); genes with larger expression do not necessarily have larger variance.

Differences Among Exposure Times

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. Among all seven time points, 14 genes had significant changes ($p < 0.01$) in mRNA expression (Table 3.3, Figure 3.1). Most significant differences have a small fold change of less than 1.5 fold (Figure 3.1 A & B). Comparison among means for each time point relative to the zero hour control used the Dunnett's test. The probabilities from the Dunnett's test were used in the volcano plots (Figure 3.1B). Among the 14 genes with significant differences in mRNA expression, eight have a significant difference ($p < 0.01$) with the zero time point (Table 3.3). Of these eight, seven are different after 96 hours of exposure. The 48-hour exposure had four significant differences and three of these are also different at 96 hours. The 4-hour time exposure is the only other time point with a significant difference from zero, and this gene is also different at 48 and 96 hours. Thus, the expression of seven of the eight genes (out of fourteen significantly different, which are significant with Dunnett's test) are different at 96 hours. The hierarchical cluster groups 4, 24, 48 and 96 hour exposures (Figure 3.1A), and most of these change become larger and more significant with time (Figure 3.1B). Notice however, this trend does not include 8 and 12 hours.

Pair-wise correlation of the mean expression of the 14 significant genes reveals two patterns (Figure. 3.2): genes with positive or negative correlations. In the lower right are a cluster of the eight genes that have significantly greater expression at 48 and

96 hours (see Figure 3.1). All these genes are positively correlated with each other and negatively correlated to five of the other six genes. These six genes tend to be expressed earlier and three of these have significant positive correlations among each other.

Maximum Gene Expression

To examine patterns of expression beyond the 14 significant genes, we sorted the maximum expression levels at each time point using the standardized least square means for all 314 genes ((Figure 3.3); where the standardized least square mean of mRNA expression for each gene has a mean of zero and a variance of 1 across all 7 time points). Even though there are only 14 significant genes, and most of this significant difference in expression occurs at 48 and 96 hours, there are waves of maximum expression (Figure 3.3). This pattern mirrors the number of significantly expressed genes (Figure 3.1), with the highest number of genes having maximum, though not always significant, expression after 96 hours of hypoxia exposure.

There are distinct patters of gene expression within the distribution of maximized gene expression over time. To determine if the maximum peaks of expression are disproportionally associate with a specific time point we applied 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 maximum expression of specific pathways have significant distribution within some time points. Fatty acid metabolism, structural genes, and translational genes have more maximally expressed genes in the control ($p<0.05$, $p<0.01$, $p<0.05$). Oxidative phosphorylation genes are underrepresented in the control ($p<0.05$). Alcohol metabolism is significantly represented at 48 hours ($p<0.05$). Oxidative phosphorylation

and ubiquinol have significantly more maximally expressed genes at 96 hours than at any other time points ($p<0.01$, $p<0.05$).

3.5 Discussion

In cardiac tissue in the teleost fish *Fundulus grandis,* 14 genes (4.5%) exhibited significant differences ($p < 0.01$) in gene expression across all seven time treatments (0-96 hours; Table 3.3). This number is proportionally similar to the findings in previous studies (Gracey, Troll, and Somero 2001; Ton et al. 2002; Ton 2003; Marques et al. 2008). Interestingly more genes were up regulated over the course of 96 hours of hypoxia exposure than down regulated. This is similar to cardiac response in *D. rerio*, where 2.5% of genes had differential expression, with two-thirds of these being up regulated (Marques et al. 2008). In *F. grandis*, the eight genes up regulated in later exposures included genes for glycolysis, NADH, cytochrome c oxidase and fatty acid metabolism, whereas, both citrate cycle genes and P-450 genes are repressed in these later stages. Different sets of genes hit their peak of gene expression at each time point (Figure 3.3). These gene sets span a wide variety of metabolic pathways or functions, and functional groups overlap between time points. However, despite the overlap, there is significance in the distribution of several gene families. Fatty acid metabolism, structural genes, and translational genes are significantly over represented under normoxic conditions (Fisher-Exact test $p < 0.05$), indicating suppression under hypoxic conditions. Oxidative phosphorylation genes are underrepresented at zero, but have a significant over representation at 96 hours (Fisher-Exact test, $p < 0.05$). Together, these data set suggest a wave of changes in gene expression that produces significant

differences at 48-96 hours with greater expression for metabolic genes in the oxidative phosphorylation pathway. Similar to other research on hypoxia (Gracey, Troll, and Somero 2001), the enrichment of translation genes with a maximum expression at zero hours, suggest that hypoxia my repress protein synthesis.

All of the significant changes are relatively small (i.e. less than 2-fold or less than $\log_2$ 1.0, Figure 3.1). Recall that the ANOVA is testing whether the variation among time points is greater than within time-point (i.e., variance among individuals). Thus, these relatively small fold changes in expression will only be significant if the variation among individuals is only approximately half as large (F value of 2.06 yields a p-value of 0.01 at 3, 16 degree of freedom). Notice, that among the four individuals within each time point, on average, 12% of genes (38 out of 314) have significant differences among individuals within a time point. Additionally, the variation among individuals tends to be smaller at 48 and 96 hours (Table 3.2). Thus, we suggest that a partial explanation for the small number of significant genes is the large variation in inter-individual gene expression. To provide support for this concept, we compare the expression of the 14 significant genes among all 28 individuals (Figure 3.4). Only the 8 individuals from the 96 and 48-hour exposure time points form a cohesive 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. An example is the four-hour exposure, although it has the fewest number of significant genes that differ among individuals within time point (Table 3.2), three of the genes with significant difference in expression among individuals are among the 14 significant genes that differ between time points. At four hours of exposure the one gene that is significantly different between time points

(Dunnett's test $p < 0.01$) is SLC37A4 (Glucose 6-phosphate translocase), but this significant increase relative to the zero time point is due to two individuals (#7 and #8, Figure 3.4). Thus for the 14 genes with significant differences in expression, variation among individuals tends to get smaller with greater time of hypoxia exposure (Table 3.2). Yet, 96 hours has the greatest number of genes that differ among individuals (Table 3.2). If these patterns are meaningful, it suggests that all individuals are more similar with 2-3 days of hypoxia but only for the genes that respond to hypoxia.

Genes that are frequently expected to be induced such as LDH-B and HIF-1α did not show significant change over time (Virani and Rees 2000; Gracey, Troll, and Somero 2001; Ton 2003; Martinez et al. 2006; Hoogewijs et al. 2007). For HIF-1α, there is no expected change in mRNA because HIF-1α is constitutively expressed, and its activity is controlled through post-translational modification (Hoogewijs et al. 2007; Li and Brouwer 2007). The lack of induction of HIF-1α, yet a significant response to hypoxia in *F. grandis* supports this hypothesis. Similarly, there was little change in LDH-B in the study by (Gracey, Troll, and Somero 2001) in cardiac tissue. This is also reflected in enzyme activity studies performed by Martinez et al. (2006), where there was no significant change in enzyme activity between hypoxic and normoxic treatments in *F. grandis* cardiac tissue. It should be emphasized however, that these results are specific to cardiac studies. Several studies have shown differential profiles of gene expression response to hypoxia over time in various tissues, reflecting the role of each tissue in hypoxic response (Gracey, Troll, and Somero 2001; Ton 2002; Ton 2003; van der Meer et al. 2005; Ju et al. 2007; Boswell et al. 2009).

Correlations in mRNA Expression

The most well defined control of gene expression during hypoxia is the HIF transcription factor family. During normoxia, the α subunit of the HIF transcription factor is hydroxylated by prolyl hydrolases, and subsequently rapidly degraded. However, during hypoxia the protein stabilizes, 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). HIF is the most well known of the transcription factors associated with the hypoxic response and is by far the most dominant (Rocha 2007; Kenneth and Rocha 2008). However, there are a number of other controls. The NF-κB, AP-1, p53 and Myc families of transcription factors are also known to affect hypoxic gene expression. Several of these factors effect 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).

Pair wise correlation for the mean mRNA expression for each time of exposure across the 14 significant genes revealed two patterns: positive and negative correlations (Figure 3.2). While the view across all genes is quite complicated, looking at the fourteen significant genes, there are clear delineations. These genes cluster with similar patterns of expression, one group is up regulated, the other down regulated. This pattern is repeated within each pathway, such that the decreases in the SOD and citric acid cycle genes are positively correlated and negatively correlated with NADH, glycolysis, and fatty acid metabolism. These significant correlations occur even though there is much

variation among individuals (Figure 3.4). Taken together these data suggest that, although the timing of induction varies among individuals, the coordination of gene expression does not. Hypoxic gene expression is regulated by a few transcription factors and signaling proteins described above, and while several families are involved, the overall response is dominated by HIF (Hoogewijs et al. 2007; Rocha 2007; Kenneth and Rocha 2008). If similar mechanisms regulate hypoxic gene expression in *F. grandis*, these data are consistent with the pattern that one or a few transcription factors are affecting the patterns of gene expression across treatments.

Summary

Although there are many genes with peak expression during the exposure to hypoxia, only 14 genes have significant change in mRNA expression, and the magnitude of these changes are small (less than 2 fold). Surprisingly, in the early hours of exposure there is much variation among individuals. This inter-individual variation for hypoxia responsive genes is less apparent with greater exposure time (48-96 hours). Even with this complex pattern of response, the large number of correlations among genes suggests that a few transcription factors may be responsible.

Table 3.1. 384 microarray pathways

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
TOTAL METABOLIC GENES	329

Table 3.2: Differences among individual for each time point

Time	# significant genes among individuals	Average Variance Among Individuals	Average Variance Among Individuals for 14 Hypoxia Affected Genes	#Significant Time Dunnett's
0	40	1.353	0.955	n/a
4	20	0.958	0.756	1
8	34	1.011	0.649	0
12	41	0.706	0.376	0
24	36	0.839	0.544	0
48	47	0.891	0.474	4
96	49	1.021	0.503	7

Table 3.3. Genes with significant difference in mRNA expression among time points. Dunnett's test is pos-hoc test with specific comparison to zero time point. EC is the enzyme commission number. P-value is from the ANOVA for the effect of time (E is raised to the 10^{th} power). Dunnett's_X, where X represent one of 6 time points > 0 (i.e., 4, 8, 12, 24, 48 & 96).

Cluster*	Description	EC	pathway	Gene Abbreviation	Pval_ Time	Dunnette's _4_pval	Dunnette's _8_pval	Dunnette's _12_pval	Dunnette's _24_pval	Dunnette's _48_pval	Dunnette's _96_pval
1	Cytochrome P450 3A56		Cytochrome	cyp3a56	6.8E-03	0.046	0.719	0.528	0.998	0.994	0.012
2	NA_3			NA_3	8.0E-03	0.891	0.347	0.035	0.032	0.042	1.000
3	Cytochrome P450 1A1	1.14.14.1	Cytochrome	CYP1A1	3.0E-04	0.998	1.000	0.177	1.000	**0.001**	0.066
4	Superoxide dismutase [Cu-Zn]	1.15.1.1	Xenobiotic_	SOD1	7.4E-03	1.000	1.000	0.993	0.974	0.315	0.018
5	Dihydrolipoyl dehydrogenase,	1.8.1.4	Citrate cycle_	DLD	7.5E-03	0.173	0.991	0.584	0.372	0.056	**0.009**
6	Dihydrolipoyllysine-residue succinyltransferase	2.3.1.61	TCA	DLST	1.6E-03	0.543	0.570	0.884	0.513	0.338	0.061
7	Acyl-CoA-binding protein		Fatty acid_	DBI	1.2E-04	0.374	0.985	0.349	0.461	0.036	**0.003**
8	Cytochrome c oxidase VIc-2	1.9.3.1	Cytochrome c oxidase	MT-CO1	2.1E-03	0.070	0.845	0.547	0.574	0.036	**0.001**
9	Glucose 6-phosphate translocase		Glycolysis	SLC37A4	8.7E-04	**0.005**	0.431	0.021	0.161	**0.004**	**0.001**
10	NADH-ubiquinone oxidoreductase PDSW	1.6.5.3	NADH	NDUFB10	8.4E-03	0.028	0.373	0.723	0.377	**0.009**	**0.003**
11	ATP-binding cassette, G, .8		Membrane Transport	ABCG8	5.0E-03	0.087	0.944	0.018	0.476	0.019	**0.007**
12	NA_6			NA_6	8.6E-03	0.764	0.821	0.377	0.533	0.057	0.225
13	Ubiquinol-cytochrome C reductase	1.10.2.2	Ubiquinol	UQCRQ	8.1E-04	0.114	0.136	0.044	0.019	**0.001**	**0.000**
14	NADH-ubiquinone oxidoreductase 1	1.6.5.3	NADH	MT-ND1	3.3E-03	1.000	0.916	0.997	0.767	0.877	0.041

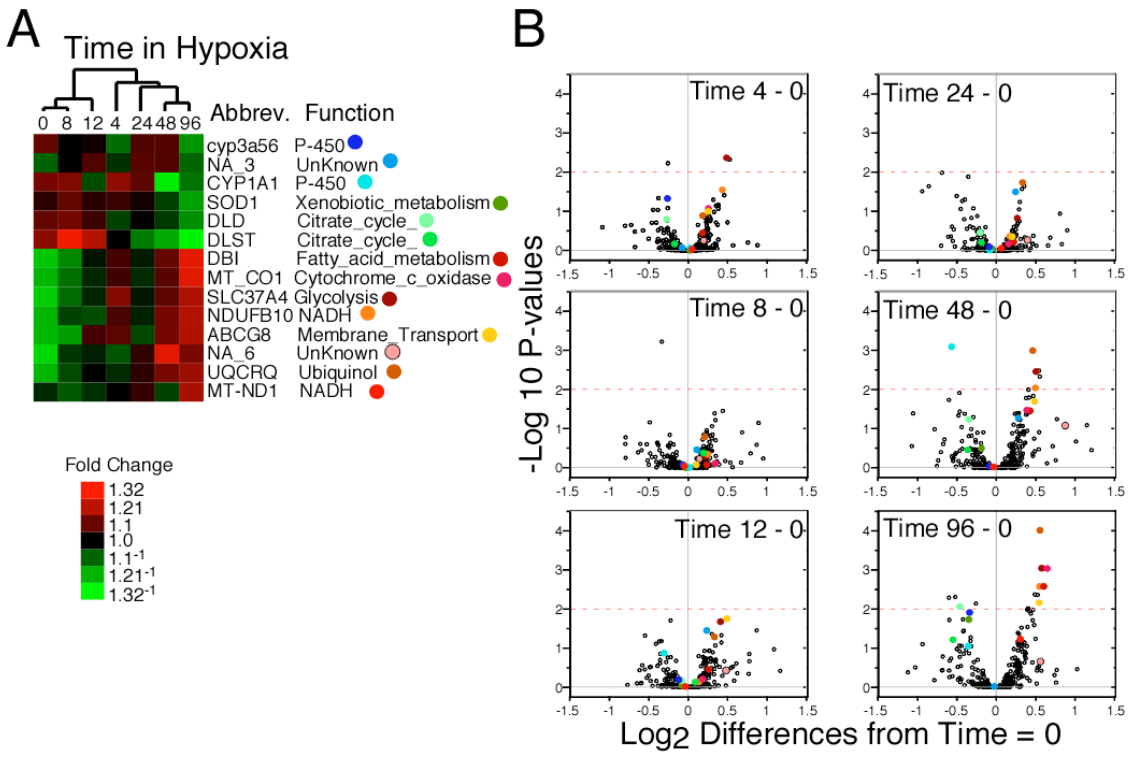

Figure 3.1. Patterns of hypoxia gene expression. A: Genes with significant difference in mRNA expression among exposure time to hypoxia (1.8 kPa) for 0,4,8,12,24, 48 and 96 hours. B. Volcano plots of $\log_2$ difference from zero time point and negative $\log_{10}$ p-values from Dunnett's Post-Hoc test. Detailed annotations are supplied in Table 3.3.

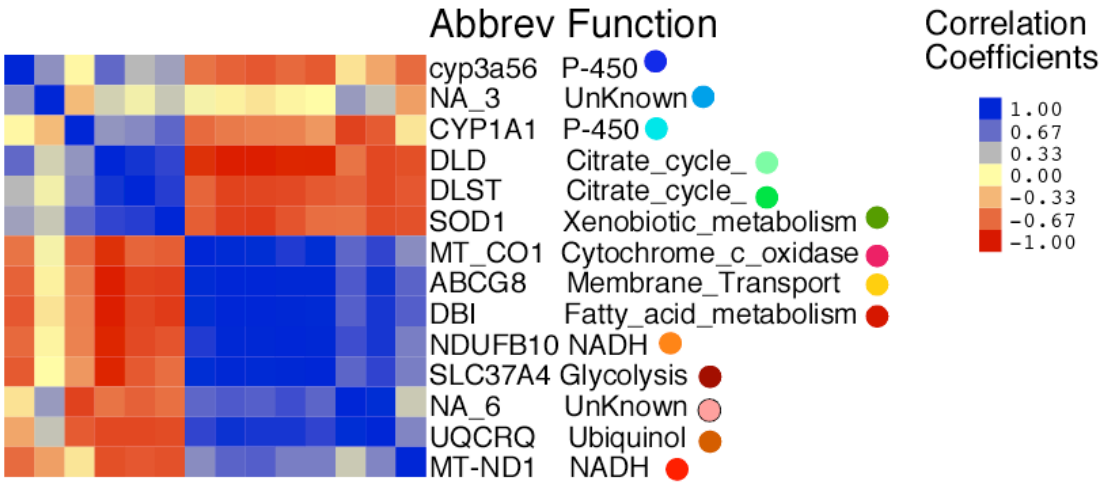

Figure 3.2. Correlation among genes. Pairwise correlations among 14 significant genes using the least square means for each time point (n=7). Correlation coefficients > 0.87 are significant at p-value of 0.01.

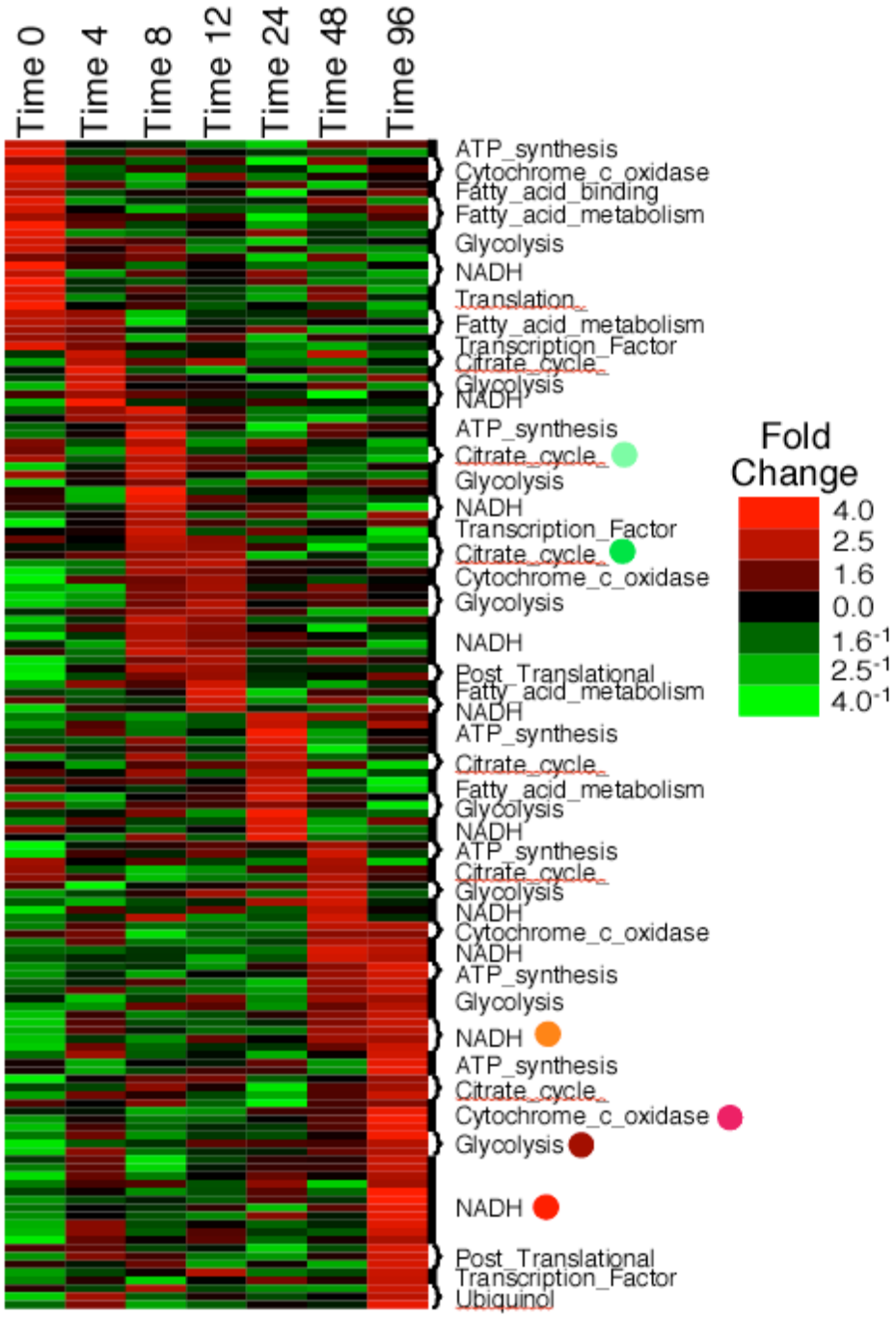

Figure 3.3. Maximum gene expression during exposure to hypoxia. Standardized least square means from for individuals exposed to hypoxia (1.8 kPa) for 4, 8, 12, 24, 48, and 96 hours. Pathways for groups of two or more genes are listed. Color dots symbolize significant genes in Figure 3.1.

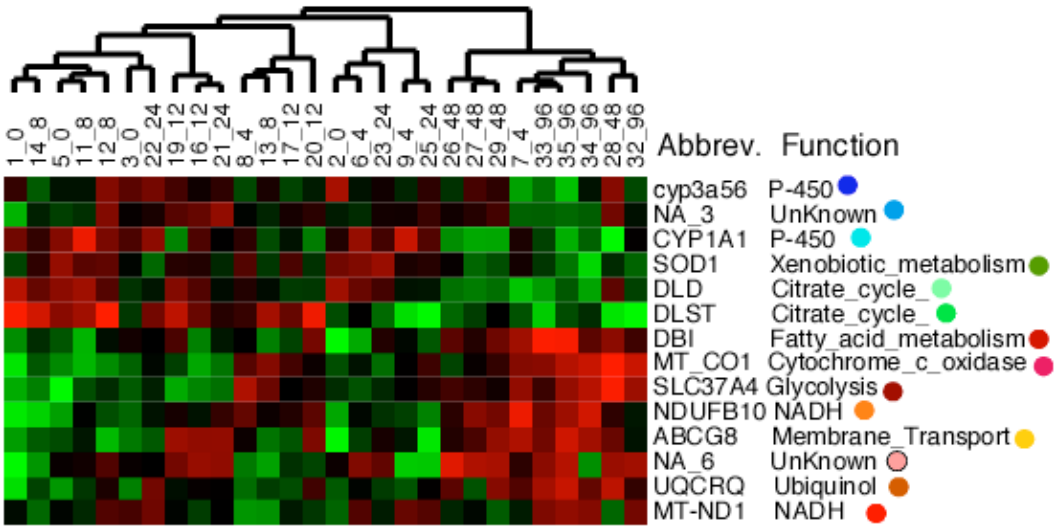

Figure 3.4. Hierarchical cluster of individuals. Cluster of individuals for genes with significant differences in expression. First number is the individual and the second number after the underscore is the exposure time. Difference between the grand means and the least square mean from the ANOVA with only individuals as fixed differences. Colored dots correspond to significant genes in Figure 3.1.

Chapter 4 Oxygen Concentration, Time, and Individual Variation Are Keys to Hypoxic Survival in *Fundulus grandis*

4.1 Summary

While the conventional definition of hypoxia is concentrations below 2 ml•L^{-1} (~7 kPa), it has become apparent that many organisms begin to exhibit symptoms of hypoxic stress at higher oxygen concentrations. To better understand the effects of variable oxgen concentrations and the interaction between oxygen concentration and time, individuals of *Fundulus grandis* were exposed to normoxia, moderate, or severe hypoxic conditions (7.8 or 1.8 kPa O_2) for 4 or 48 hours. Hypoxic effects were quantified by measuring the change in mRNA expression for 324 genes. There were significant changes in gene expression for both oxygen concentration and time. A total of 69 (21%) genes had significant changes ($p < 0.01$) in gene expression for at least one of the factors. To further elucidate the interaction between concentration and time, the response within each time point was analyzed. This demonstrated a progressive change; more genes had significant changes in expression after 48 hours than 4 hours (28 genes vs. 15 genes). Additionally, most changes occurred for the 1.8 kPa treatment (9 out of 15 genes after four hours, 18 out of 28 after 48 hours). Interestingly the differences in gene expression were not simply between the normoxic and hypoxic treatments. Instead both 7.8 and 1.8 kPa had a unique pattern of gene expression and there were more differences between the two hypoxia conditions (24 genes with significant difference in expression) than either has to normoxia (6 or 18 for 7.8 or 1.8 kPa). These results demonstrate that not just

hypoxia, but time spent at a specific concentration, determine an individual's response to hypoxic events.

4.2 Introductory material

Hypoxia is generally defined as oxygen concentrations less than or equal to 2 $ml \bullet L^{-1}$ (~7.1 kPa). Oxygen levels considered normoxic range from 2 $ml \bullet L^{-1}$ to 8 $ml \bullet L^{-1}$ (approximately 7.1 kPa-20.5 kPa) (Diaz and Rosenberg 1995; Rabalais and Turner 2001; Breitburg 2002). However, Vaquer-Sunyer and Duarte (2008) have recently pointed out that this definition may be in question. While 2 $ml \bullet L^{-1}$ is the classic definition, the authors point out the oxygen concentrations at which many organisms experience stress or even death are much more variable, thus the extent of hypoxic impact may be greatly underestimated. For *Fundulus grandis,* short term (3 hours) exposures to decreasing oxygen concentration (20.5 kPa to 1.8 kPa) cause a gradual decline in metabolism above 7 kPa (Everett In Press-a). Below 7 kPa metabolism, measured by oxygen consumption, could no longer be maintained. The specific inflection point depended on body mass, with larger fish having lower PO_{2crit} (Everett In Press-a). These changes in metabolism were rapid.

One approach for a more sensitive measure of an organism's response to hypoxia is to have quantifiable molecular or cellular indicators of hypoxic stress (Brouwer et al. 2005; Denslow, Garcia-Reyero, and Barber 2007). For instance a number of authors have been utilizing molecular genetics techniques, as well as quantifying physiological parameters such as reproductive success and enzyme activity, to develop new molecular biomarkers for hypoxic stress. Other groups have been using molecular techniques to

quantify thresholds of hypoxic stress in specific groups of organisms (Wiklund and Sundelin 2004; Brown-Peterson et al. 2005; Brouwer et al. 2007a; Brouwer et al. 2007b; Cheung et al. 2007; Ha and Choi 2009).

On a cellular and molecular level, gene expression often changes in response to stress. Hypoxia induced gene expression changes, and halted development in zebrafish (*Danio rerio*) embryos. Normal development and gene expression was resumed when the embryos were return to normoxic conditions (Ton 2003). A comparison of tissue specific responses to hypoxia in *Gillichthys mirabilis* over 144 hours (Gracey, Troll, and Somero 2001) identified 126 hypoxia regulated genes, corresponding to metabolism, locomotion, protein synthesis, antigrowth and proliferation, and amino acid metabolism. Long-term (three week) hypoxia exposure in *D. rerio* emphasized the importance of tissue specific differences. In gill 367 (2.5%) genes were differentially expressed, and the primary response was repression (van der Meer et al. 2005). In a similar study examining cardiac gene expression, nearly the same numbers of genes were differentially expressed (376), but the response was primarily the induction of mRNA expression (Marques et al. 2008). The effect of differential oxygen concentration was not studied in these examples.

Cooper et al (2002) demonstrated dose dependent shifts in antioxidant and metabolic enzyme activities in the estuarine fish *Leiostomus xanthurus*. Dalla Via et al. (1994) demonstrated changes in the concentration of components of metabolism including lactate and ATP in the sole, *Solea solea*; however changes were only observed in their two lowest oxygen treatments. Daphnia has been shown to have three different hemoglobin isoforms in circulation under normoxia, moderate, and severe hypoxia

(Lamkemeyer et al. 2005). Thus while many studies have compared the physiological effects of graded levels of hypoxia, few have compared the effects of different oxygen concentrations on gene expression.

In one such study zebrafish, *D. rerio*, Roesner et al. (2006) compared the expression of a number of globin genes, as well as two controls, LDH-B and PGK-1, under conditions of normoxia, mild, and severe hypoxia. They found only minor changes at mild hypoxia, and much more pronounced changes under severe hypoxia. This included a 65-75% reduction in hemoglobin expression after 48 hours, a 2.5 fold increase in myoglobin, and a transient increase in neuroglobin, which returned to normal by 48 hours. Brouwer et al (2007a; 2007b) examined changes in gene expression in response to moderate or severe hypoxia in grass shrimp, *Palaemonetes pugio*, for nuclear encoded genes (Brouwer et al. 2007b), and for mitochondrial genes (Brouwer et al. 2007a). In both studies little change in gene expression was observed under mild hypoxia, while many genes had significant changes in gene expression under severe hypoxia.

The goal of this study was to characterize the changes in gene expression over different oxygen concentrations that are near and below the range of critical oxygen tension for *F. grandis* and examine this effect over time. The differences in the response between the 1.8 and 7.8 kPa treatments suggest that 1.8 kPa is not just a more intense response than at 7.8 (two genes have shared response). Instead, both have the greatest response at 48 hours, but the cellular response between the treatments is different.

4.3 Materials and methods

Animals

Fundulus grandis were collected from Weeks Bay (WB) and Pass Aux Herons (DA), 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 six weeks of pseudo-winter, temperatures were slowly increased to 24° C, and the lighting changed 14:10 light/dark cycle and the fish were allowed to spawn. Fish were fed OSI Marine Flake *ad libitum* once daily in the evening.

Hypoxia Exposure

To minimize handling stress, fish were moved to experimental aquaria the evening prior to 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. Control tanks consisted of two 10 gallon glass aquaria maintained under the same temperature and salinity conditions as the test tank, each circulating via a single Aquarium Systems Millennium 2000 Power Filter. Ammonia

levels in all tanks were measured twice daily. Any rise in ammonia was controlled with Amquel.

Oxygen concentrations in the test tank were controlled via a system designed by LoligoSystems ApS (Hobro, Denmark). Oxygen levels in the ambient aquarium were continuously monitored and controlled using a 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 oxygen partial pressure was reached. The oxygen probe was calibrated via manufactures instructions. The galvanic probe was calibrated to 100% air saturation, made by vigorously bubbling air through 100 ml water for 20 minutes. The probe was calibrated at the start of the experiment, and checked for drift daily after that. The surface of the water in the hypoxic tank was covered completely in bubble wrap to prevent aquatic surface respiration (ASR) and reoxygenation.

Two oxygen partial pressures were used for the experiment: 1.8±0.3 kPa (severe hypoxia), and 7.8±0.3 kPa (moderate hypoxia), as well a normoxic control (~20.5 kPa). Each concentration (1.8 or 7.8 kPa) was tested individually, with control individuals being maintained and sampled simultaneously for each experiment. At the start of each experiment, oxygen in the system was dropped to the specified concentration over the course of 30 minutes. This concentration was held for 48 hours. A minimum of 6-7 individuals were sampled at each time point: 4 and 48 hours from both the experimental and control tanks (Table 4.1). Fish were sacrificed via cervical dislocation and heart

tissues were collected and stored in RNAlater (Ambion) according to manufacturer's instructions.

RNA Preparation

RNA was extracted from all 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). 40 ml of 2 M Sodium Acetate was added to each sample followed by 400 ml 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 16,000g 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 16,000g for 30 min. The remaining RNA pellet was rinsed with 400 µl of 70% ethanol and further purified using RNAClean (Agencourt) 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 1996), using an Amino Allyl MessageAmp II-96 kit (Ambion). Briefly, this method amplifies the signal by using T7 RNA polymerase 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) 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 (Paschall et al. 2004a). Average lengths of cDNAs were 1.5Kb with a majority including the N-terminal methionine. Amplified cDNA were spotted onto epoxide slides (Corning) using an inkjet printer (Aj100, ArrayJet, Scotland).

Hybridization

Twenty pmol of Cy3 and Cy5 labeled aliquots were vacuum dried together and resuspended in 10 µl of hybridization buffer (5X SSPE, 1% SDS, 50% formamide, 1 mg/ml polyA, 1 mg/ml sheared herring sperm carrier DNA, and 1mg/ml BSA) for a final concentration 2 pmol/µl for each sample. Immediately prior to use, slides were blocked in a solution consisting of 5% ethanolamine, 100 mM Tris (pH 7.8), and 0.1%SDS for 30 minutes. The slides were then washed in 50 °C 4X SSC, 0.1% SDS solution for one hour and rinsed with autoclaved water. Finally, slides were boiled for two minutes, given a final rinse in autoclaved water, and spun dry (800 rpm for 3 minutes). Label RNAs (2pmol/µl each of Cy3 and Cy5) in hybridization buffer (5X SSPE, 1% SDS, 50% formamide, 1mg/ml polyA, 1mg/ml sheared herring sperm carrier DNA, and 1mg/ml BSA) were heated to 92°C for two minutes, quick 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 4X

SSC to prevent hybridization from drying out. Samples were hybridized for approximately 48 hours at 42 °C. Following hybridization, slides were washed in decreasing concentrations of SSC and SDS (4X + 0.1% SDS-0.1X SSC no SDS) and then spun dry (800 rpm for 3 minutes). Slides were scanned using the Packard Bioscience ScanArray Express microarray scanner (PerkinElmer Life Sciences), with laser wavelengths set to 633 and 543 nm. Resulting .tiff images were imported into spot grids built in ImaGene (Biodiscovery) for each array, and spot signals were collected as fluorescence intensities for each dye channel.

Hybridization Design

Individuals from different time points were hybridized together in seven separate loop designs (Kerr, Martin, and Churchill 2000; Oleksiak, Churchill, and Crawford 2002). A "loop design" does not rely on a reference, rather two different experimental samples label with Cy3 or Cy5 fluorescent dyes hybridized together, and each individual is measured on two slides, once with Cy3 and once with Cy5. This creates a loop of Cy3⇒Cy5 with approximately 12 arrays per loop for instance (treatment with population # as subscript):

Loop 1: Treatment 1_1⇒Treatment 2_1⇒Treatment 3_1⇒Treatment 1_2⇒Treatment 2_2⇒Treatment 3_2⇒Treatment 4_1⇒Treatment 5_1⇒Treatment 6_1⇒Treatment 4_2⇒Treatment 5_2⇒Treatment 6_2⇒first sample

Treatments 1-6 represent each time-oxygen combination included in the experiment. For this experiment there were a total of 7 loops. The first six loops each had 12 individuals (12 Cy3⇒Cy5 arrays). The seventh loop contained only 7 individuals. Individuals from each time-dose treatment and population were included in each loop to evenly distribute

possible variance from these factors (see above example). Within this framework, individuals were randomly assigned to each loop (individuals were randomly selected from the 6-7 sampled per population and treatment (Table 4.1)). Each slide had 6 arrays, and each array had two individuals hybridized, for 12 individuals per slide. Individuals from each treatment were distributed across separate arrays to minimize loop affects.

Data Processing and Statistical Analysis

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. No genes showed consisted saturation fluorescence, however, genes with average fluorescent values less than the mean plus two standard deviations of the negative control were removes from all individuals and were not analyzed.

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 calculate ANOVA on each gene across all treatments initially the following ANOVA models was used:

$$y_{ijkm} = m + A_i + D_j + T_m + H_d + P_p + G_f + H_d*T_m + H_d*P_p + H_d*G_f +$$
$$T_m*P_p + T_m*G_f + P_p*G_f + T_m*H_d*P_p + T_m*P_p*G_f$$
$$+P_p*G_f*H_d + G_f*H_d*T_m + H_d*T_m*P_p*G_f + L_L + I_k(T_m*H_d) + e_{ijkm}$$

where, m is the overall average signal, A_i is the effect of the *ith* array, D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), T_m is the fixed effect of time for the *mth*

time point, H_d is the fixed effect of dose for the *dth* dose, P_p is the fixed effect of population effect for the *pth* population, G_f is the fixed effect of the *fth* gender, L_l is the effect of the *lth* loop, and $I_k(T_m)$ is the effect of individuals within time point and e_{ijk} is the error term. A_i, D_j, L_l, and $I_k(T_m)$ are random terms. In this case variance of the random terms is estimated by restricted maximum likelihood (REML). The results of this model demonstrated that gender and population had little effect on the sample variance, thus they were removed from the model and the following model was used:

$$y_{ijkm} = m + A_i + D_j + T_m + H_d + T_m * H_d + L_L + I_k(T_m * H_d) + e_{ijkm}$$

where, m is the overall average signal, A_i is the effect of the *ith* array, D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), T_m is the fixed effect of time for the mth time point, H_d is the fixed effect of dose for the dth dose, $T_m * H_d$ is the interaction term for dose and time, L_l is the effect of the lth loop, and $I_k(T_m)$ is the effect of individuals within time point and e_{ijk} is the error term. A_i, D_j, L_l, and $I_k(T_m)$ are random terms.

A similar procedure was used to examine the differences among individuals within each of the time treatments, using the following model:

$$y_{ijk} = m + A_i + D_j + I_k + e_{ijk}$$

where, m is the overall average signal, A_i is the effect of the *ith* array (one of eight arrays within a time point), D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), I_k is the fixed effect of the Individual (one of four individuals within each time point) and e_{ijk} is the error term. A_i, and D_j, are random terms. To examine the variance among individuals across all genes, the mixed model described above was used for all 79 individuals across all time points.

Hierarchical clustering of genes and individuals was conducted using Eisen's Cluster and Treeview for Windows. Post-Hoc testing including Tukey-Kramer and Student's t tests was performed in JMP 7. Matlab was used to produce figures.

4.4 Results

Out of 373 possible genes 52 had values below the negative controls and were removed. No spots were saturated across all treatments, so no genes were exclude due to saturation in the analysis. Thus, 324 genes remained in the analysis for hypoxic concentration response. In initial analysis with the full model, gender had 5 and population had 6 genes with significant differences in mRNA expression. As this is near the expected false positive rate with a p-value of 0.01, gender and population were ignored in all other analyses.

Differences among treatments

Among treatments there were 2 and 67 degrees of freedom for hypoxic dose and 1 and 67 degrees of freedom for time. A total of 69 (21%) genes were found to have significant differences in expression across all treatments ($p<0.01$). Of these genes, a total of 51 genes (16%) were significant between the oxygen treatments (normoxic control and two hypoxia doses) ($p<0.01$). There were 26 genes (8%) with significant differences in expression between the 4 Hour and 48 Hour time treatments. Additionally 15 genes (5%) were significant for the Dose x Time interaction. Of the 69 significant genes, 19 overlapped between factors (Dose, Time, Dose x Time) and 50 were unique to a single factor. Tukey-Kramer post-hoc testing for effect of dose across both time treatments demonstrated that the majority of differences (27 out of out of 51 genes

significant for dose) were between the 7.8 kPa and 1.8 kPa treatments (Table 4.2). There were fewer differences between the control and 1.8 kPa treatments (10), and only one difference between 7.8 kPa and the control. There were also 20 genes with significant p-values, for which the post-hoc test did not identify a specific significant comparison.

To better understand the effect of differential oxygen concentration, the three oxygen levels were compared within each time point (Figure 4.1, Figure 4.2). Within each of the time points, hierarchical cluster analysis groups the 7.8 kPa and normoxia treatments, with the 1.8 kPa treatment as its own branch (Figure 4.1). The differences between the 4 hour and 48 hour heat maps demonstrate patterns of changing gene expression over time. The greatest difference in gene expression is between the 1.8 kPa treatment and the other two treatments after 48 hours (Figure 4.2).

After four hours, 15 genes had significant changes in gene expression between treatments (ANOVA $p < 0.01$). However, by 48 hours, this had increased to 28 genes with changes in expression (ANOVA $p < 0.01$). No genes with significant changes in mRNA expression were shared between the four hour and 48 hour time points (Figure 4.1, Table 4.3). A complex pattern emerges with post hoc testing. There are only two genes with changes in gene expression by four hours between the control and 1.8 kPa treatments, and only one gene between the control and 7.8 kPa treatments (Tukey post-hoc test, $p < 0.01$; Figure 4.1, Figure 4.2). However, there are nine genes with significant differences in gene expression between the 1.8 and 7.8 kPa treatments (Tukey post-hoc test, $p < 0.01$; Figure 4.1, Figure 4.2). At four hours, among the 15 genes with significant differences in mRNA expression, only two genes share a difference from 1.8 kPa and both normoxia and 7.8 kPa (Figure 4.1). There were no genes where both hypoxia levels

(1.8 & 7.8 kPa) were different from normoxia. By 48 hours 16 genes have significant differences in expression between 1.8 kPa and normoxia, 5 between 7.8 kPa and normoxia, and 15 genes with significant differences in expression between 1.8 and 7.8 kPa (Tukey post-hoc test, $p < 0.01$; Figure 4.1, Figure 4.2). After 48 hours, 13 genes were different for more than one post-hoc comparison. Of these 13 comparisons, 9 are significant for 1.8 kPa and both normoxia and 7.8 kPa. Three comparisons are significant for 7.8 kPa versus normoxia and 1.8 kPa. One gene has significant changes in expression between both the 1.8 and 7.8 kPa and normoxia treatments. This indicates a complex response to changing oxygen concentration over time.

Correlation of the genes with significant differences across all treatments, within each time point reveals two different patterns between time points (Figure 4.3). Within each time point there are patterns of positive and negative correlation. In the four hour correlation most genes appear to have positive correlations, with a few negative correlations along the "edge." However, in the 48 hour correlation there are multiple complex modules of positive and negative correlation.

Differences among individuals

For the 6-7 individuals per time-dose treatment there were 71 and 319 degrees of freedom. Significant differences in gene expression existed between individuals for each of the six treatments ($p<0.01$) (Table 4.4). The 1.8 kPa-48hr treatment had the fewest number of differentially expressed genes between individuals (120) and the 48 hour normoxia had the most (169) (Table 4.2). This is the reverse of the four hour treatment. These differences among individuals within a treatment reflect the variance among individuals (Table 4.4).

4.5 Discussion

In this study most significant differences in mRNA expression occurred at 1.8 kPa versus either normoxia or 7.8 kPa. Similar to Everett et al, (Everett In Press-b) the greatest differences occurred after 48 hours of hypoxia exposure. Surprisingly, for the two doses (1.8 & 7.8 kPa) there were unique patterns of expression at 48 hours. That is, at 4 hours there were no genes with significant changes in expression for both 1.8 and 7.8 kPa, and at 48 hours only 13 (7.7%) are different for both 1.8 and 7.8 kPa.

While the physiological effects of environmental hypoxia on a number of organisms from both benthic and near shore habitats have been well characterized, little has been characterized about the underlying genetic factors which determine an organism's hypoxia tolerance. To better understand the effect of hypoxia, both time and dose need to be considered to fully understand an organism's ecological response, as the interaction between these factors may not be linear (Denslow, Garcia-Reyero, and Barber 2007). Measuring changes in gene expression has the potential to help resolve the complex interaction of time and dose by identifying specific biochemical pathways that are altered due to exposure in a quantifiable manner.

Previous studies have clearly demonstrated changes in gene expression in response to hypoxia (Gracey, Troll, and Somero 2001; Ton 2003; van der Meer et al. 2005; Brouwer et al. 2007a; Brouwer et al. 2007b; Ju et al. 2007; Marques et al. 2008; Boswell et al. 2009). Furthermore, many of these changes in gene expression have been shown to be variable over time (Gracey, Troll, and Somero 2001; Ton 2003; van der Meer et al. 2005; Brouwer et al. 2007a; Marques et al. 2008; Everett In Press-b). What is lacking in our characterization of the stress response to hypoxia is the effect of variable

oxygen concentration, and its interaction with time. That is, at what dose and after how long do organisms actually begin to experience detrimental hypoxic stress? The data presented here will help to answer these questions.

This study exposed individuals of *F. grandis* to two levels of hypoxia (moderate and severe hypoxia) over a 48 hour period to characterize changes in gene expression in relationship to hypoxic dose, and how these changes develop over time. The moderate hypoxia treatment (7.8 kPa) is above the average critical oxygen treatment for this species (around 4.6 kPa) (Virani and Rees 2000; Everett In Press-a), but at the upper range of the variation in critical oxygen tension among individuals (ranged from 2.4-7.8kPa) (Everett and Crawford, in press). Above the critical oxygen tension, individuals appear able to maintain near normal levels of aerobic metabolism (Everett and Crawford, in press), at least initially, only one gene exhibited significant changes in gene expression between 7.8 kPa and normoxia after four hours. Over time, however, it appears that the moderately lower levels of oxygen are exerting an effect, resulting in changes in gene expression. However, at 7.8 kPa, this effect is more limited than the 1.8 kPa treatment (5 versus 15 genes with significant changes in gene expression). The 1.8 kPa treatment is well below the calculated critical oxygen tension for this species, and reduction of metabolism (calculated as oxygen uptake) has been observed at this concentration (Virani and Rees 2000; Martinez et al. 2006; Everett In Press-a), therefore an underlying remodeling of gene expression is expected and has been previously documented (Everett, Antal, and Crawford, unpublished).

Genes with differential expression between different hypoxic doses included genes for glycolosis, oxidative phosphorylation, ATP synthesis, fatty acid metabolism,

ubiquinone biosynthesis, citrate cycle, and a handful of other pathways. Differential expression from normoxia in many of these genes in response to hypoxia is consistent with results from previous studies. Genes with differential expression over time included genes for the same pathways, many of which have been previously shown to have differential expression over time (Gracey, Troll, and Somero 2001; Ton 2003; van der Meer et al. 2005); (Everett, Antal, and Crawford, unpublished). Significant changes in gene expression were not observed in genes including LDH-B and HIF-1α, which are frequently associated with exposure to hypoxia (Virani and Rees 2000; Gracey, Troll, and Somero 2001; Ton 2003; Martinez et al. 2006; Hoogewijs et al. 2007). However, previous studies have demonstrated that the effect of hypoxia on gene expression is tissue specific, and lack of induction of LDH-B has been previously observed for cardiac tissue. Additionally, HIF-1α has been shown to be constitutively expressed, and function of this transcription factor is controlled at the protein level (Gracey, Troll, and Somero 2001; Ton 2002; Ton 2003; van der Meer et al. 2005; Hoogewijs et al. 2007; Ju et al. 2007; Boswell et al. 2009).

One difference between the current study and previous studies, however, was the inclusion of multiple hypoxic doses. Studies by Gracey et al (2001), Ton et al (2003); van der Meer et al. (2005), and Martinez et al. (2006), compared difference in gene expression between normoxia and a single hypoxic treatment. By including multiple hypoxic treatments we can capture more of the process of individuals reacting to hypoxic stress.

Vaquer-Sunyer and Duarte (2008) and Pollock et al. (2007), have pointed out the variability in species hypoxia tolerance is a major challenge to the current definition of

hypoxia. If species experience detrimental hypoxia stress at values much higher than $2mg \bullet L^{-1}$ using this value as the threshold for hypoxia becomes meaningless to conservation efforts etc. Previous studies that have examined the effects of hypoxic dose on organisms have been primarily physiological. These studies revealed species specific changes in behavior, metabolism, reproduction, and ventilation. The results here are consistent with these results, demonstrating changes in gene expression, which underlie changes in metabolism and can thus result in changes in behavior etc (Hoogewijs et al. 2007). While some species showed responses even at moderate levels of hypoxia, others did not experience change until severe hypoxia had been reached. This is also supported by the current data. At 1.8 kPa there were many more differences from normoxia than 7.8 (Figure 4.1). This is in line with the study (Brouwer et al. 2007a; Brouwer et al. 2007b) in grass shrimp that showed few changes in gene expression from normoxia in a moderate hypoxic treatment versus a severe hypoxic treatment.

Another factor in this study was the inclusion of two time points. Previous studies have demonstrated changes in gene expression over time with exposure to hypoxia. The results here are consistent with those findings. There are fewer differences in gene expression between treatments after 4 hours than after 48 hours (Figure 4.1, Figure 4.2). This is consistent between all pairs of treatments, including between the two hypoxia treatments.

The data above suggest a transition from intermediate to severe hypoxia. However, the differences in gene expression between the moderate (7.8 kPa) and severe (1.8 kPa) treatments suggest different responses at these two dose. That is, if mRNA expression affects a physiological response to maintain homeostasis, then there are

different response and physiological mechanism at work at these two doses. After four hours of hypoxic exposure, nine genes, out of 15 with significant changes in gene expression, were significantly different between the moderate and severe treatments. By 48 hours this had increased to15 genes out of 28, almost equal to the total number with significant change between the severe and control treatments (16). The expectation from previous studies is that gene expression at intermediate hypoxia would be a subset of the genes that respond at more severe hypoxia. Yet, this is not what occurs: most significant changes in gene expression are specific for one or the other hypoxic treatments. The response shared at 48 hours between the 7.8 and 1.8 kPa versus normoxia are genes with repressed expression (Figure 4.1) and only one of these is significant with the Tukey post-hoc test (CALM, Figure 4.1, ‡ and*).

The expectation of similar response with different oxygen concentration is based on the molecular mechanisms regulating the hypoxic response. The expression of a number of genes in model systems ranging from yeast to mice to bovine stem cells have been shown to be oxygen-dose dependent and related to the active concentration of the transcription factory HIF-1α (Kwast, Burke, and Poyton 1998; Vasconcelles et al. 2001). Moreover, activity of the transcription factor HIF-1α has been shown to vary exponentially over different oxygen concentrations (Jiang et al. 1996). The half life of HIF-1α is <5 minutes in normoxic conditions (Hoogewijs et al. 2007). This is because under normoxic conditions a family of prolyl-hydrolases (PHD1-3) catalyze the Fe (II) and oxygen dependent hydroxylation of both HIF-1α and HIF-2α in an oxygen dependent manner. Once hydroxylated the protein is rapidly ubiquitinated and degraded in the proteasome. At low oxygen concentration, these transcription factors are not

hydroxylated, not targeted for protein degradation, and thus have a much longer half-life. While HIF-1α is the better characterized of the α subunits, recently HIF-2α has also been implicated as a point of control of oxygen sensing and response to hypoxia (Hoogewijs et al. 2007; Aragones et al. 2008). Thus HIF activity and subsequent changes in gene expression are linked to cellular oxygen concentration.

Summary

Across hypoxic treatments 69 genes had significant differences in gene expression. The largest changes occurred only after 48 hours of hypoxia exposures, and the biggest effects were seen at the severe hypoxia treatment. Interestingly, there were also large numbers of genes with significant differences in expression between the moderate and severe hypoxia treatment. While some of these were also different than the normoxic control, many were unique to either moderate or severe hypoxia. This indicates that not just dose or duration, but a combination may be critically important to determining population survival in the event of an environmental hypoxia episode.

Table 4.1. Sampling of individuals (n) for each treatment. PO_2 for severe hypoxia is 1.8 kPa, and moderate hypoxia is 7.8 kPa.

	Four Hours-Normoxia	Four Hours-Moderate Hypoxia	Four Hours-Severe Hypoxia	48 Hours-Normoxia	48 Hours-Moderate Hypoxia	48-Hours-Severe Hypoxia
Pass Aux Heron, AL	6	7	7	6	7	7
Weeks Bay, AL	6	7	6	6	7	6

Table 4.2. Summary of ANOVA results. Results include ANOVA across both time and dose treatments, as well as across doses within each time point. Doses are 1.8 kPa, 7.8 kPa and Normoxia (N). Tukey-Kramer post hoc testing was performed both across all factors, as well as across doses within each time point. Cells marked with (*) contain genes for which changes in mRNA expression are shared between treatments. Genes with no specific significant comparison were not included in this table

	ANOVA			Tukey Across Time			Tukey within 4 hours			Tukey Within 48 hours		
	Dose	Time	DxT	1.8 vs. N	7.8 vs. N	1.8 vs. 7.8	1.8 vs. N	7.8 vs. N	1.8 vs. 7.8	1.8 vs. N	7.8 vs. N	1.8 vs. 7.8
# Significant t	51	26	15	10	1	27	2*	1	9*	16*	5*	15*

Table 4.3. Genes with significant changes in mRNA after 4 or 48 hours. Gene ID's are the same as listed in the hierarchical cluster analysis (Figure 4.1). Tukey-Kramer comparisons are listed. Cells marked with an (X) had a significant post-hoc comparison. Blank cells had a significant p-value, but no significant post hoc test. Gene's marked with a (*) were significant duplicates not included in the heat map.

Time	Cluster	GenBank #	Description	Gene ID	EC	KEGG pathway	p-value	7.8 kPa vs. N	1.8 kPa vs. N	1.8 vs. 7.8 kPa
H4	1	CN985186	Succinyl CoA 3 ketoacid coenzyme A transferase mitochondrial precursor	OXCT1	2.8.3.5	Synthesis and degradation of ketone bodies	0.004795			
H4	2	CN985282	Electron transfer flavoprotein beta subunit	ETFB			0.000994		X	X
H4	3	CN985302	NA 7	NA7			0.007203			X
H4	4	CN985144	Cytochrome P450 1A1	CYP1A1	1.14.14.1	Fatty acid metabolism	0.005715			X
H4	5	CN985166	N acetylserotonin O methyltransferase like protein	ASMTL			0.008924	X		
H4	6	CN985274	NADH ubiquinone oxidoreductase B22 subunit	NDUFB9	1.6.5.3	Ubiquinone biosynthesis	0.002927			X
H4	7	CV819930	ATP synthase lipid binding protein	ATP5G3	3.6.3.14	ATP synthesis	0.004574			
H4	8	CN985107	NADH ubiquinone oxidoreductase AGGG subunit mitochondrial precursor	NDUFB2	1.6.5.3	Ubiquinone biosynthesis	0.001114		X	X
H4	9	CN985188	NA 16	NA16			0.009471			
H4	10	CN985201	ATP synthase f chain mitochondrial	ATP5J2	3.6.3.14	ATP synthesis	0.00212			X
H4	11	CN985108	Pyruvate dehydrogenase E1 component beta subunit mitochondrial precursor	PDHB	1.2.4.1	Glycolysis / Gluconeogenesis	0.005522			

H4	12	CN980916	Cytochrome c oxidase polypeptide VIIb	COX7B	3.1.3.11	Glycolysis / Gluconeogenesis	0.003198			X
H4	13	CN985092	Cytochrome c oxidase polypeptide VIIa liver heart mitochondrial precursor	COX7A2	1.9.3.1	Oxidative phosphorylation	0.001546			X
H4	14	CN985113	60S ribosomal protein L28	RPL28			0.001135			X
H4*	15	CN985171	Cytochrome P450 1A1	CYP1A1	1.14.14.1	Fatty acid metabolism	0.001037			
H48	1	CN985184	Hexokinase D	GCK	2.7.1.1	Glycolysis / Gluconeogenesis	0.002829			
H48	2	CN984992	NA 29	NA29			0.006098	X		X
H48	3	CN983868	NADH ubiquinone oxidoreductase chain 3	MTND3	1.2.1.12	Oxidative phosphorylation	0.002398	X		X
H48	4	CN984990	Fatty acid binding protein heart	FABP7			0.00013	X		X
H48	5	CN985159	ATP binding cassette sub family G member 8	ABCG8			0.009106			X
H48	6	CN985165	NA 20	NA20			0.007573		X	X
H48	7	CN979833	Thioredoxin	TXN		Post-Translational	0.000508		X	
H48	8	CN985106	NA 11	NA11			0.007864		X	
H48	9	CN985047	Transketolase	TKT	2.2.1.1	Pentose phosphate pathway	0.007673			
H48	10	CN985202	Calmodulin	CALM			0.00545	X	X	
H48	11	CN985071	Fatty acid binding protein heart	FABP3			0.007603		X	
H48	12	CN985195	Phospholipase A2 precursor	PLA2G1B	3.1.1.4	Glycerolipid metabolism	0.003392	X		
H48	13	CN985015	Cytochrome c oxidase polypeptide VIII heart mitochondrial precursor	COX8H	1.9.3.1	Oxidative phosphorylation	8.5E-06		X	X

H48	14	CN985182	Betaine aldehyde dehydrogenase	ALDH9A1	1.2.1.8	Glycine, serine and threonine metabolism	0.001636		X	X
H48	15	CN985143	NA 3	NA3			0.000938		X	
H48	16	CN985137	Glycogen phosphorylase muscle form	PYGM	2.4.1.1	Starch and sucrose metabolism	0.004202		X	
H48	17	CN985112	Alcohol dehydrogenase class III	ADH5	1.1.1.1	Glycolysis / Gluconeogenesis	0.000157		X	X
H48	18	CN985196	Cytochrome P450 3A56	CYP3A56			0.009615			
H48	19	CO436101	Forkhead box protein P2	FOXP2			0.004768		X	
H48	20	CN985023	Alpha enolase	ENO1	4.2.1.11	Glycolysis / Gluconeogenesis	0.004435		X	X
H48	21	CN985197	Alanine aminotransferase	GPT	2.6.1.2	Glutamate metabolism	9.97E-05		X	X
H48	22	CN985111	Phosphoglycerate mutase 1	PGAM1	3.1.3.13	Glycolysis / Gluconeogenesis	3.13E-08		X	X
H48	23	CN985101	Cytochrome P450 1B1	CYP1B1	1.14.14.1	Fatty acid metabolism	0.000166		X	X
H48	24	CV821411	ATP synthase gamma chain mitochondrial	ATP5C1	3.6.3.14	ATP synthesis	0.007379			
H48	25	DR109378	5 aminolevulinic acid synthase	ALAS1	2.3.1.37	Glycine, serine and threonine metabolism	0.007461			
H48	26	CN985119	NADH ubiquinone oxidoreductase chain 3	MTND3	1.6.5.3	Ubiquinone biosynthesis	0.007988			X
H48	27	CN985217	NADH ubiquinone oxidoreductase chain 3	MTND3	1.6.5.3	Ubiquinone biosynthesis	0.008495			X
H48	28	CN985192	Phosphoglycerate mutase 1	PGAM1	3.1.3.13	Glycolysis / Gluconeogenesis	1.07E-06		X	X

Table 4.4. Differences among individuals at each treatment. Treatment abbreviations are as follows: H4 is four hours, H48 is 48 hours, 1.8 is severe hypoxia (1.8 kPa), and 7.8 is moderate hypoxia (7.8 kPa).

Treatment	Number if significant genes among individuals	Average variance among individuals across all genes	Average variance across individuals in hypoxia affected genes (69)
H4-1.8	151	0.984435902	1.061986584
H4-7.8	140	0.963078259	0.78025847
H4-Control	137	0.96235407	0.80288917
H48-1.8	120	0.932358438	1.086740837
H48-7.8	165	1.082386772	1.101895809
H48-Control	169	0.991727254	1.056813486

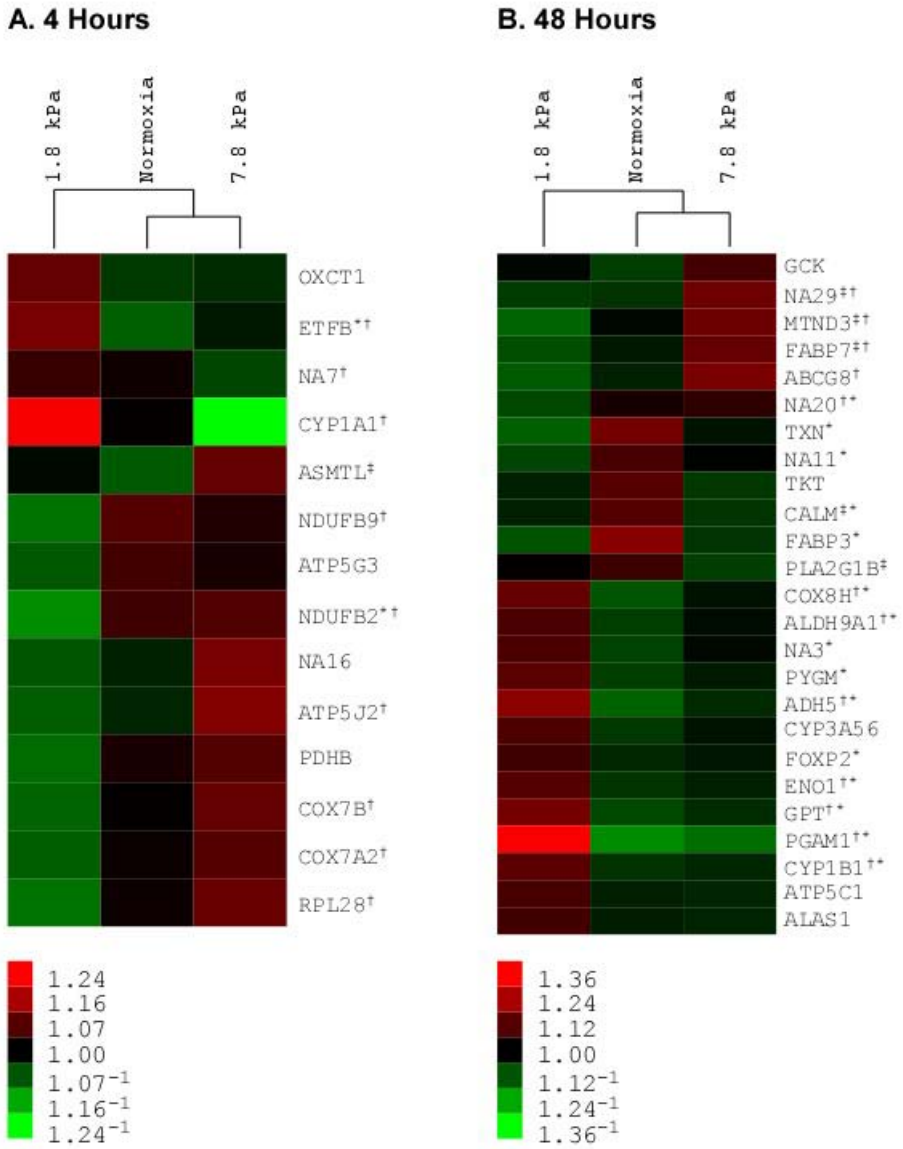

Figure 4.1. Patterns of Hypoxia Gene Expression. A: Genes with significant difference in mRNA expression among hypoxic doses 1.8 kPa and 7.8 kPa after four hours B. Genes with significant difference in mRNA expression among hypoxic doses 1.8 kPa and 7.8 kPa after 48 hours Note that for both factors small (less than 2 fold) changes in gene expression result in significant differences between factors. Significant comparisons from the Tukey-Kramer post-hoc test are shown as follows: (‡) is significant between 7.8 kPa and normoxia, (*) is significant between 1.8 kPa and normoxia, and (†) is significant between 1.8 kPa and 7.8 kPa.

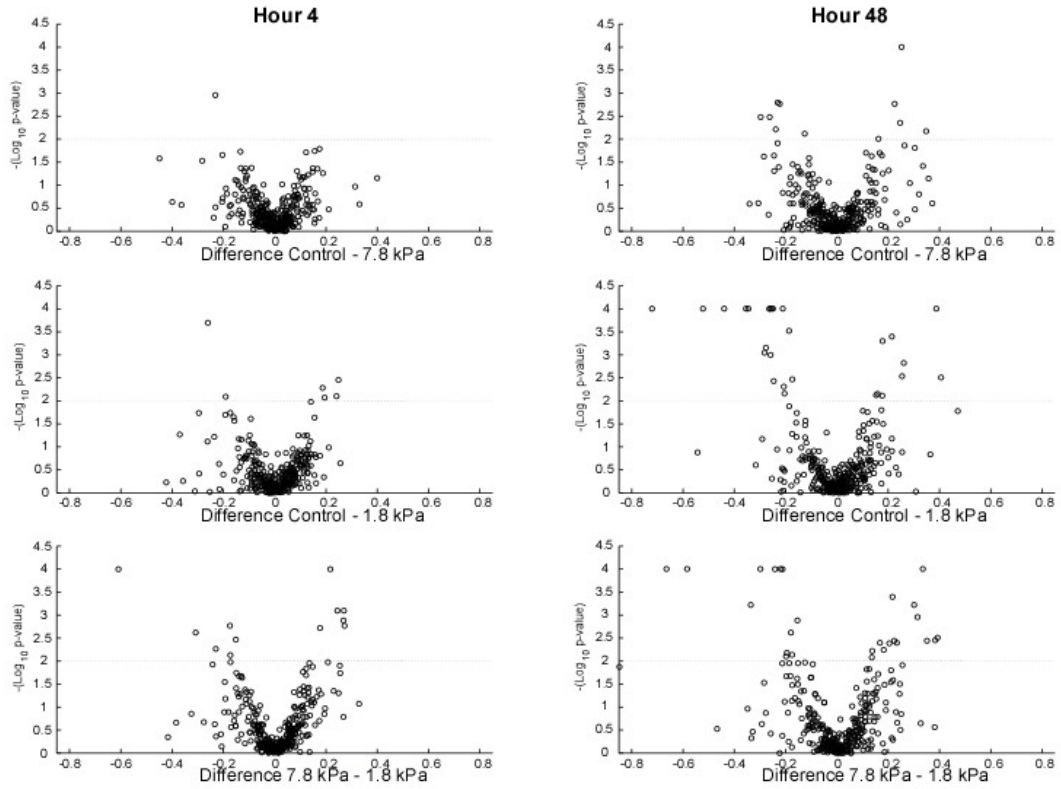

Figure 4.2. Patterns of Hypoxia Gene Expression. Volcano plots of difference between treatments and negative $\log_{10}$ p-values from Students t post hoc test. The differences in number of significant genes displayed here versus mentioned in the text are due to differences in post hoc testing.

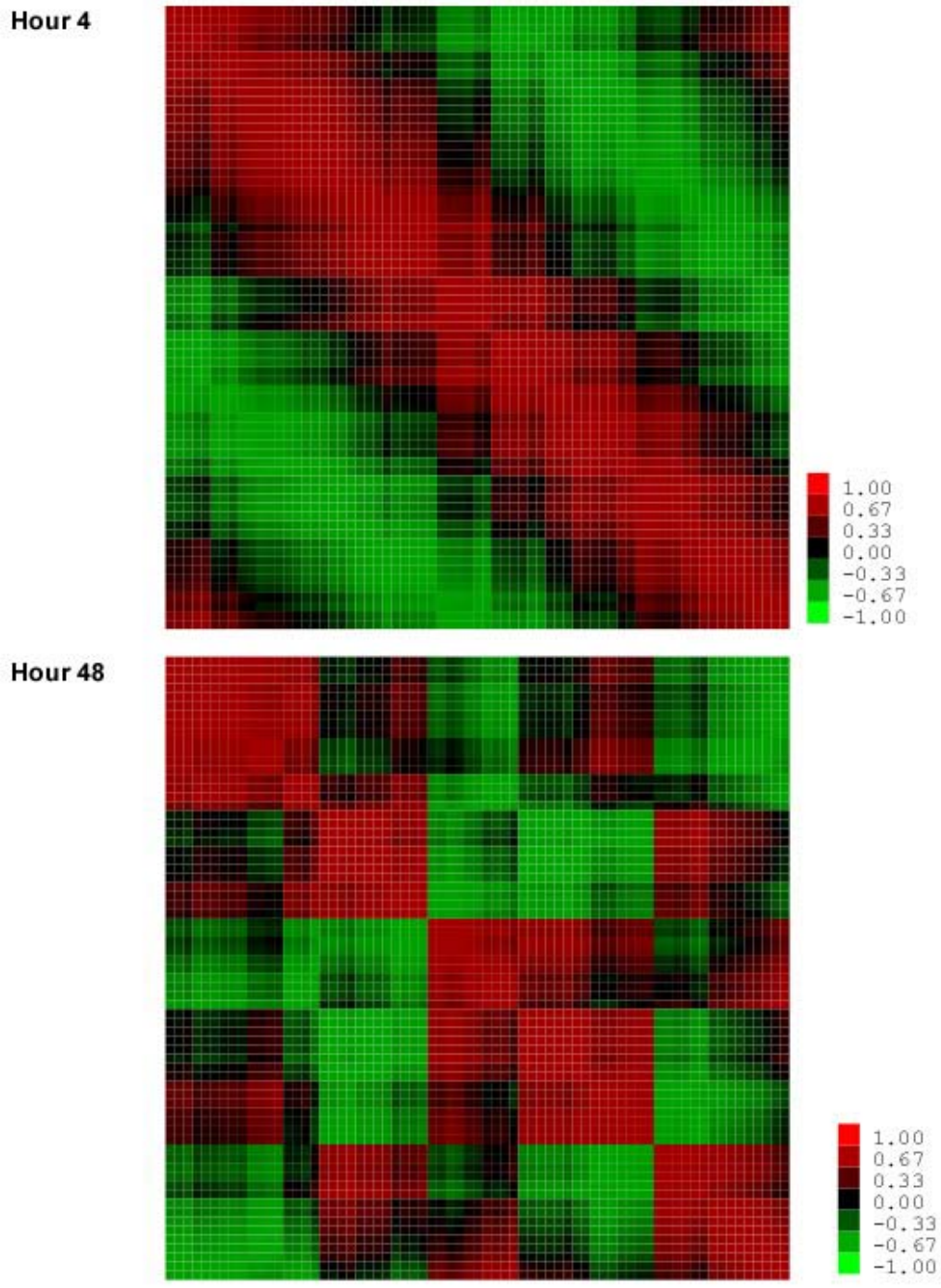

Figure 4.3. Correlation among genes. Pair wise correlations among 69 significant genes at each time point using the least square means for each time point

Chapter 5 Hypoxic Responses Among Six *Fundulus grandis* Populations

5.1 Summary

Are there differences in hypoxia response among populations with different exposure histories to hypoxia? To ascertain the difference among populations, individuals from six genetically distinct populations of *Fundulus grandis* were exposed to hypoxia (1.8 kPa) for 4 or 96 hours. Time in hypoxia had a significant effect on the expression of 609 genes (11%), while population affected the expression of 355 genes (7%). Genes with significant differences in expression among populations reflect geographic separation; there are more differences between populations separated by greater geographic distance. For the 59 genes with significant difference in expression for both hypoxia response and population, shared hypoxic histories appears to be more important than the neutral patterns expected by geographic distance. That is individuals from clean sites have a more similar response with one another, than with individuals from hypoxic sites. The majority of significant changes for the 609 hypoxia responsive genes take place after 96 hours of hypoxia exposure. It is at this 96-hours exposure time point that populations which have a high frequency of hypoxia events are most different from populations from more pristine environments. Despite the diversity of hypoxia response, correlation analysis reflects shared mechanisms of genetic control across all populations and time points.

5.2 Introductory material

Environmental factors can have profound effects on the evolution of organisms. Environmentally induced divergence affects can be observed in non-coding genetic markers, in gene expression, and in differences in physiological response (Pierce and Crawford 1997b; Pierce and Crawford 1997a; Oleksiak, Churchill, and Crawford 2002; Williams, Brown, and Crawford 2008; Everett In Press-a). For example, Whitehead and Crawford (2006) demonstrated adaptive differences in gene expression in *F. heteroclitus* with a temperature gradient along the east coast of the United States. A similar adaptive divergence in enzyme expression in response to the thermal gradient was shown to occur within and among seven *Fundulus* species (Pierce and Crawford 1997a). In addition to temperature, populations of F. *heteroclitus* have adaptive patterns of gene expression among polluted and non-polluted estuaries on the East coast (Fisher and Oleksiak 2007; Oleksiak 2008; Williams and Oleksiak 2008).

Evolutionary changes in gene expression have been demonstrated in other organisms as well. In blue mussel, *Mytilus edulus*, the Lap gene locus has been shown to differ between populations in response to variable salinity (Koehn et al. 1980). Division of Lepidopteron butterflies into high-altitude and low-altitude populations has been demonstrated to be dependent on expression of specific isoforms of PGI (Karl, Schmitt, and Fischer 2009). Thermal adaptation in the PGI locus in *Colias* butterflies has also been demonstrated (Watt 1977). These data suggest that populations subjected to different environments adapt to these environment by altering their proteins or patterns of gene expression. Importantly, data showing evolved adaptations can indicate which genes are important in environmental stress response (Crawford and Oleksiak 2007).

Hypoxia represents an increasing stress factor in the marine environment (Diaz and Rosenberg 1995; Rabalais and Turner 2001). Hypoxic environments can affect organisms on a variety of biological levels, from rapid changes in physiological state to long-term evolutionary adaptation (Hochachka et al. 1996; Gracey, Troll, and Somero 2001; Hochachka and Somero 2002; Wu 2002; Webster 2003; Mandic, Todgham, and Richards 2009). As a result of local adaptations within a population, hypoxia can drive divergence between populations. In Africa, hypoxic conditions can be variable between lake systems. Crispo and Chapman (2008) looked for divergence in microsatellite markers in populations of cichlids from lakes with different hypoxic histories; however no divergence was found in this species. Timmerman and Chapman (2004) found changes in the P_{O2crit}, the oxygen partial pressure at which individuals can no longer maintain metabolism, between populations of *Poecilia latipinna* with differing exposures histories of hypoxia. Differences in P_{O2crit} have also been demonstrated between populations of sculpins with variable exposure histories to hypoxia (Mandic, Todgham, and Richards 2009). Everett and Crawford (In Press-a) showed differences in oxygen consumption at 1.8 kPa between populations of *F. grandis*.

Hypoxia is widespread through estuaries of the Gulf of Mexico; however, individual estuaries are variable in their historical occurrence of hypoxia. Between Anclote Key, FL and Rio Grande, TX 5.9% to 29.3% of estuaries surveyed were affected by hypoxia (Engle, Summers, and Macauley 1999). This study, carried out as part of an EPA estuarine survey, recorded numerous estuaries with at least one oxygen minimum of less than or equal to 2 mg L^{-1} during the course of a four-year survey. However, of nine estuaries surveyed all four years, only three experienced hypoxia multiple years (Engle,

Summers, and Macauley 1999). Mobile Bay, AL has one of the longest recorded exposure histories of hypoxia, with records of hypoxic events dating to 1821 (May 1973). Table 5.1 summarizes known hypoxic and undegraded estuaries in the Gulf of Mexico used for this study. Thus, one might expect that populations from Port Aransas, TX, Leeville, LA, and Weeks Bay, AL, having a historically greater exposure to hypoxia, may evolve patterns of gene expression to adapt to these environments.

The goal of this study is to profile the changes in gene expression in *F. grandis* exposed to hypoxia over time, and to compare the response between populations. While previous studies have compared the physiological response to hypoxia between populations, or the gene expression response to hypoxia amongst pooled individuals, few studies compare gene expression response to hypoxia between populations. Understanding the variation among individuals within and between populations provides insight the variation that could contribute to evolutionary adaptation to hypoxic stress.

5.3 Materials and methods

Animals and Hypoxic Exposure

Fundulus grandis were collected from six locations along the Gulf of Mexico with variable histories of hypoxic conditions in February 2009. From west to east the sites include: 1) Aransas Bay, TX (PA); 2) Matagorda Bay, TX (PO); 3) Calcasieu Lake, LA (LC); 4) Terrebonne Bay, LA (LV); 5) Dauphin Island, AL (DA), and 6) Weeks Bay, AL (WB) (Figure 5.1, Table 5.1). Fish were either caught using minnow traps or purchased from local bait shops if the site of collection by the shop could be confirmed. Fish were transported back to the lab and maintained in closed re-circulating 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. Fish were collected during the winter, in January, so no pseudo-winter cycle was required upon return to the lab. Rather they were acclimated slowly to 24° C, and a 14:10 light/dark cycle and allowed to spawn. Fish were acclimated for a minimum of six weeks before experiments began. Fish were fed OSI Marine Flake *ad libitum* once daily in the evening. Fish were moved to the test tank the evening prior to 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 circulated back through the shared water supply, maintaining a temperature of at 24 ° C and a salinity of 15 ppt (See below for details). Within the tank water was circulated using two Eheim 1046 centrifugal water pumps. Ammonia levels in the test tank were measured twice daily.

Oxygen concentration in the test tank was controlled via a system designed by LoligoSystems ApS (Hobro, Denmark). Water from the shared recirculating system was pumped at a controlled rate into the bottom of a large degassing column made of a 4 foot length of 6 inch PVC pipe. After degassing (see below), deoxygenated water flowed out through an overflow port, and was gravity fed into the test tank. After circulating through the test tank, water flowed out through an overflow port, back into the central sump, where it was reoxygenated and underwent biological filtration. This system allowed maintenance of hypoxic oxygen levels in the test tank, while allowing biological filtration through the shared water supply. Oxygen levels in the test aquarium were

continuously monitored and controlled using a 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 bottom of the degassing chamber. Circulation was continuous, and N_2 gas was bubbled until the desired partial pressure was reached in the test chamber. The oxygen probes were calibrated via manufactures instructions. The galvanic probe was calibrated to 100% air saturation, made by vigorously bubbling air through 100 ml water for 20 minutes. The probe was calibrated at the start of the experiment, and checked for drift daily after that. The surface of the water was covered completely in bubble wrap to prevent aquatic surface respiration (ASR) and reoxygenation. The test chamber was divided into two parts, allowing hypoxia exposure of two populations simultaneously. Order of individuals to be tested representing the different populations was random. Prior to the initiation of hypoxia, ten individuals from each population were sampled as normoxic controls. Subsequently, oxygen in the system was dropped to 1.8±0.3 kPa over the course of 30 minutes. This concentration was held for 96 hours, and ten individuals from each population were sampled after 4 and 96 hours. Fish were sacrificed via cervical dislocation and heart tissues were collected and stored in RNAlater (Ambion) according to manufacturer's instructions.

RNA Preparation

RNA was extracted from all 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). 40 ml of 2 M Sodium Acetate was added to each sample followed by 400 ml 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 16,000g 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 16,000g for 30 min. The remaining RNA pellet was rinsed with 400 µl of 70% ethanol and further purified using RNAClean (Agencourt) 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 1996) using an Amino Allyl MessageAmp II-96 kit (Ambion). 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 UTP are incorporated during transcription. Cy3 and Cy5 dyes (GE Lifesciences) were then coupled to the amino-allyl labeled RNA.

Microarray

The amount of gene specific mRNA expression was measured using microarrays. Amplified cDNA sequences for 6,912 genes (7K-array) from *F. heteroclitus* cDNA libraries were spotted onto epoxide slides (Corning) using an inkjet printer (Aj100,

ArrayJet, Scotland). Approximately 200ng (0.5 ul of 400ng/ul) was printed on each spot. Each glass slide contained four, spatially separated 7K-arrays. Each array had herring sperm DNA spotted 12 times as negative controls, as well as 3 blank wells. The corners of each array had dye labeled corners (17 total) to aid in aligning spot-quantification software. Thus, 6,880 Fundulus genes and 32 controls were spotted on each array.

DNA spotted on arrays was amplified from isolated cDNA clones (approximate size ranged from 0.5 to 3.5 Kb with an average size of 1.5 Kb). Libraries used for the isolation of cDNAs were made from all 40 stages of Fundulus development, immediately post-hatch whole larvae, and adult tissues. Adult tissues were isolated from several individuals from each of nine populations from brain, liver, heart, muscle, spleen, eye, G.I., fat, gill, bone, gall bladder and epithelia. To enhance the discovery of stress genes, tissues were isolated from a subset of individuals expose for 12 hours to hypoxia, cold, heat, low and high salinity or hydrocarbons. All cDNAs were sequenced (ABI 3730) and annotated by similarity (BLASTX) to human, mouse, rat or dog RefSeq, or to Swiss-Prot. Annotation scheme, quality controls and pipeline are described in (Paschall et al. 2004b).

Hybridization

Thirty pmol of Cy3 and Cy5 labeled aliquots were vacuum dried together and resuspended in 10 µl of hybridization buffer (5X SSPE, 1% SDS, 50% formamide, 1 mg/ml polyA, 1 mg/ml sheared herring sperm carrier DNA, and 1mg/ml BSA) for a final concentration 2 pmol/µl for each sample. Immediately prior to use, slides were blocked in a solution consisting of 5% ethanolamine, 100 mM Tris (pH 7.8), and 0.1%SDS for 30 minutes. The slides were then washed in 50 °C 4X SSC, 0.1% SDS solution for one hour and rinsed with autoclaved water. Finally, slides were boiled for two minutes, given a

final rinse in autoclaved water, and spun dry (800 rpm for 3 minutes). Labeled RNAs (2pmol/μl each of Cy3 and Cy5) in hybridization buffer were heated to 92°C for two minutes, quick cooled to 42 °C, and applied to the slide, and a cover slip was gently placed over each zone. Each hybridization zone is 308 mm^2. Slides were placed in an air-tight chamber humidified with a paper soaked in 4X SSC to prevent the hybridization from drying out. Samples were hybridized for approximately 48 hours at 42 °C. Following hybridization, slides were washed in decreasing concentrations of SSC and SDS (4X + 0.1% SDS-0.1X SSC no SDS) and then spun dry (800 rpm for 3 minutes). Slides were scanned using the Packard Bioscience ScanArray Express microarray scanner (PerkinElmer Life Sciences), with laser wavelengths set to 633 and 543 nm. Resulting .tiff images were imported into spot grids built in ImaGene (Biodiscovery) for each array, and spot signals were collected as fluorescence intensities for each dye channel.

Hybridization Design

Individuals from different time points were hybridized together in seven separate loop designs (Kerr, Martin, and Churchill 2000; Oleksiak, Churchill, and Crawford 2002). A "loop design" does not rely on a reference, rather two different experimental samples label with Cy3 or Cy5 fluorescent dyes hybridized together, and each individual is measured on two slides, once with Cy3 and once with Cy5. There were a total of 5 loops with 32-36 arrays per loop and thus 64-72 samples (two samples one each labeled with either Cy3 or Cy5 per array). For example:

PA3_0 → PO4_0 → LC9_0 → LV10_0 → DA10_0 → WB5_0 → PA20_4 → PO17_4 → LC16_4 → LV13_4 → DA12_4 → WB16_4 → PA25_96 → PO25_96 → LC25_96 → LV21_96 → DA39_96 → WB25_96 → PO10_0 → LV7_0 → WB6_0 → PO14_4 →

LV15_4 → WB20_4 → PO42_96 → LV22_96 → WB26_96 → PA8_0 → LC4_0 → DA9_0 → PA16_4 → LC12_4 → DA16_4 → PA29_96 → LC23_96 → first sample

where each sample designation (i.e. PA3_0) is the population abbreviation, individual number and treatment (0, 4, or 96 hours). Individuals from each time treatment and population were included in each loop to evenly distribute possible variance from these factors. Individuals were randomly assigned to each loop (individuals were randomly selected from the 10 sampled per population and treatment). Each slide had 4 arrays, and each array had two individuals hybridized, for 8 individuals per slide. Individuals from each loop were distributed across separate array slides to minimize loop affects.

Data Processing and Statistical Analysis

All data processing was carried out in Microsoft Excel and SAS JMP Genomics 3.0. The microarray is printed with control spots of sheared Herring-sperm DNA, which do not bind to *Fundulus* sequence. Genes with average fluorescent values less than the mean of the four lowest negative controls were removed from all samples and were not analyzed. No genes were removed due to saturation fluorescence.

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 calculate ANOVA on each gene across all treatments initially the following ANOVA models was used:

$$y_{ijkm} = m + A_i + D_j + T_m + P_p + G_f + T_m*P_p + T_m*G_f + P_p*G_f + T_m*P_p*G_f + L_L + I_k(T_m*P_p) + e_{ijkm}$$

where, m is the overall average signal, A_i is the effect of the *ith* array, D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), T_m is the fixed effect of time for the *mth* time point, P_p is the fixed effect of population effect for the *pth* population, G_f is the fixed effect of the *fth* gender, L_l is the effect of the *lth* loop, and $I_k(T_m*P_p)$ is the effect of individuals within time point and population and e_{ijk} is the error term. A_i, D_j, L_l, and $I_k(T_m*P_p)$ are random terms. In this case variance of the random terms is estimated by restricted maximum likelihood (REML).

A similar procedure was used to examine the differences among individuals within each of the time treatments, using the following model:

$$y_{ijk} = m + A_i + D_j + I_k + e_{ijk}$$

where, m is the overall average signal, A_i is the effect of the *ith* array (one of eight arrays within a time point), D_j is the effect of the *jth* dye (with one of two dyes: Cy3 or Cy5), I_k is the fixed effect of the individual (one of ten individuals within each population/time point combination) and e_{ijk} is the error term. A_i, and D_j, are random terms. To examine the variance among individuals across all genes, the mixed model described above was used for all 170 individuals across all time points. For all ANOVA models a p-value cutoff of 0.01 was used.

Hierarchical clustering of genes and individuals was conducted using Eisen's Cluster and Treeview for Windows. Post-Hoc testing including Tukey-Kramer and Student's t tests was performed in JMP 7. Matlab was used to produce figures.

5.4 Results

Out of 6,915 possible genes, 1568 genes had fluorescent signals below negative controls, and were removed. Thus 5,347 genes were used in analysis of the time course and population effects of hypoxia exposure.

Differences among treatments

Among treatments there were 2 and 131 degrees of freedom for time in hypoxia (0 (normoxia), 4 or 96 hours), 5 and 131 degrees of freedom for populations, and 1 and 131 degrees of freedom for gender. A total of 1,265 (24%) genes had significant differences in mRNA expression across all treatments ($p<0.01$). Of these 1,265, 609 (11%) genes had significant differences in mRNA expression due to hypoxia exposure (time at hypoxia) alone, 355(7%) genes had significantly different expression due to population alone and 189 (3%) genes were affected by gender. Within the genes with a significant response to hypoxia (see below) only 24 (3%) genes had significant differences in gene expression for gender (ANOVA, $p<0.01$). As genders were sampled evenly among populations and treatments, and gender affects are not the focus of this study, these affects are not considered further. There were a total of 126 genes with a significant interaction term for time (Time vs. Population). Of these genes, 33 were also significant for time and 28 were also significant for population.

Tukey-Kramer post hoc testing for the 355 genes significant for population revealed that 143 genes were significant (Table 5.2) between at least one pair of populations, while 212 of the 355 genes had no specific significant comparison between populations despite significant p-values. The breakdown of significant comparisons for the 143 genes can be observed in Table 5.2. Overall, Port Aransas tended to have the

greatest number of pair-wise differences among all populations. Otherwise, the significant comparisons follow roughly geographic or genetic distance distributions, with the fewest number of differences between geographically close populations. The only large deviation from this trend is the comparison between two of the pristine populations, Lake Charles (LC) and Port O'Conner (PO), and between Port O'Conner and Port Aransas (PA), a site that experiences relatively frequent hypoxic events (Montagna and Ritter 2005). There are only 7 differences between the two pristine populations, while there are 19 differences between Port O'Conner and Port Aransas (PA), despite the fact that Port Aransas (PA) and Port O'Conner (PO) are geographically closer.

All populations were exposed to the same hypoxia regimen, exposure to a normoxic control at time 0, four hours of hypoxia and 96 hours of hypoxia. The 609 genes significant for hypoxia alone are the "Hypoxia responsive genes" for all further analysis. Dunnett's post hoc testing on the hypoxia responsive genes across all populations revealed a majority of genes (355, 58% of 609) had significant changes in gene expression after 96 hours of hypoxia exposure (Figure 5.2). After four hours 78 genes (13% of 609) had changes in expression due hypoxia exposure. Hierarchical cluster analysis for hypoxia exposure clustered the four hour and normoxic treatments (Figure 5.2).

To better understand the population specific effects of hypoxia, the relationships between the hypoxia responsive genes and genes affected by population, as well as the genes with significant interactions (Time vs. Population) were examined. Among the 355 genes significant for population, 59 also have a significant hypoxia response, but no significant interaction term. Out of these 59 genes, 20 had a significant difference in

expression when comparing pairs of populations using the Tukey-Kramer test. Among these 20 genes, Port Aransas again had the most pair-wise differences. Interestingly there are no differences between the populations from pristine sites (Port O'Conner, Lake Charles, and Dauphin Island), regardless of geographic or genetic distance. All other significant comparisons fall out on roughly geographic lines. This can also be seen in Figure 5.3. Hierarchical clustering groups the 96 hour samples together, however, within this branch, the three "Hypoxic" sites cluster together.

Populations were compared within each time point for the hypoxia responsive genes, and the response to hypoxia over time was profiled within each population individually. Within each population between 55 and 93 genes were significant overall for hypoxia response across time (Table 5.4). Additionally, Dunnett's test within each population revealed a pattern consistent with the pattern across populations, with greater changes in gene expression after 96 hours (Table 5.4, Figure 5.4). While the number of genes with significant changes in gene expression after four or 96 hours was similar between populations, few significant genes were shared among populations. After four hours of hypoxia no significant genes were shared among all six populations. A single gene was shared amongst five populations, two genes were shared between three populations, and four between two. All other genes were significant for a single population. After 96 hours of hypoxia exposure four genes with significant changes in gene expression were shared among all six populations. Four genes are shared between five populations, seven are shared in four populations, 26 are shared between three populations, and 31 are shared between two populations. The majority (135 out of 207

significant in Dunnett's test at 96 hours) of genes with significant changes in mRNA expression after 96 hours are unique to a single population (Figure 5.4).

To further examine the response among mRNA expression patterns, correlation in mRNA expression among all 609 hypoxia responsive genes was carried out. These correlations used the least square means for each individual, calculated in the mixed model ANOVA for individuals. Thus the 609 genes were correlated for all 170 individuals across populations and treatments. Correlation reveals two patterns: both positive and negative correlation (Figure 5.5). Genes which are positively correlated (upper right) are up regulated from the normoxic control by 96 hours, while those that are negatively correlated tend to be down regulated.

5.5 Discussion

Cardiac tissues from 170 individuals spanning 6 populations exhibited significant changes in gene expression in response to hypoxia in 609 (48% of all significant) genes. After 96 hours of hypoxia, more genes were up regulated than down regulated (52%), which is consistent with previous findings (Marques et al. 2008; Everett In Press-b), though the relative percentage is lower. Genes with changes in expression after four hours included transcription factors, genes for ATP synthesis, fatty acid metabolism and cytochrome P450 stress proteins. Genes with significant changes in mRNA expression after 96 hours across all populations included genes for ubiquinone biosynthesis, glycolysis, oxidative phosphorylation, ATP synthesis, post-translational modification, and other metabolic pathways. These results are comparable to previous studies. One difference between the changes in gene expression observed in this study and previous

chapters (Chapter 3, Time course for Hypoxia exposure) is the apparent lack of significant distributions (overrepresentation or underrepresentation) of members of specific biochemical pathways after exposure to hypoxia over time. Chapter 3 demonstrated significant changes in the distribution of members of some biochemical pathways between treatments, specifically with genes for oxidative phosphorylation underrepresented under normoxic conditions, but overrepresented after 96 hours of hypoxia (Fisher exact test). While significant overrepresentation or underrepresentation of members of a specific pathway were not repeated here, members of the oxidative phosphorylation pathway do have significant changes in gene expression after 96 hours. This observed difference between the two studies may be an artifact of sampling and annotation, as the previous study included only 379 well annotated metabolic genes, and Fisher exact tests are best for small sample sizes.

The differences described above were observed for the full model, across all populations and times. One goal of the current study was to examine differences among populations with variable exposure history to hypoxia. Differences in gene expression between populations with variable exposure history to an environmental stressor has been previously described (Pierce and Crawford 1997a; Oleksiak, Churchill, and Crawford 2002; Whitehead and Crawford 2006; Fisher and Oleksiak 2007). *F. grandis* has been shown to follow an isolation by distance model (Williams, Brown, and Crawford 2008). Geographically distinct populations are expected to have changes in gene expression following the same model (Whitehead and Crawford 2006); genes under natural selection for an environmental stressor exceed this model. Studies have demonstrated population specific differences in hypoxia response (Timmerman and Chapman 2004; Everett In

Press-a). In the full model, a total of 355 genes had significant changes in gene expression between populations. Tukey-Kramer post-hoc testing on these genes revealed that many pair-wise changes between populations roughly followed the expectations of genetic distance; there were greater numbers of differences between populations separated by greater geographic distances (Table 5.2). Additionally, individuals from Port Aransas exhibited the greatest number of differences compared to other populations, even compared to the geographic nearest neighbor, Port O'Conner. There were 19 genes with significant differences in gene expression between the Port O'Conner and Port Aransas populations, the most for any geographically close pair. It is interesting that although most of the variation among populations appears to be best explained by neutral demographic properties, the large unexpected difference between Port O'Conner and Port Aransas suggest adaptive divergence.

There were 59 genes significant for both hypoxia response and population effects. The post-hoc comparison of these genes for populations reveals a complex pattern (Table 5.3). Port Aransas still had the highest number of significant differences from all other populations, but these differences did not follow geographic comparisons, for example, Weeks Bay, the furthest sampling location geographically, only had two genes with significant changes in mRNA expression from Port Aransas. For these 59 genes, there were no significant pair-wise comparisons between all three of pristine populations: Port O'Conner, Lake Charles, and Dauphin Island. A similar grouping pattern is observed in Figure 5.3 among individuals from the sites with higher frequencies of hypoxia. While all of the populations form one a cluster after 96 hours, within this cluster, the three hypoxic sites, Weeks Bay, Port Aransas, and Leeville are clustered together, indicating a

similarity between these sites in this analysis. This suggests that over time, individuals from populations with similar exposure histories to hypoxia are more similar in their response to hypoxia, at least within the 59 genes significant for both hypoxia exposure and population.

Comparing the gene expression profiles for hypoxia exposure (time at hypoxia) within each population, a complex pattern emerges. There are similar numbers of genes with significant changes in gene expression over time within each population. Additionally, the results of Dunnett's test within each population were numerically similar across populations (Table 5.4). However, while the overall number of genes significant at each time point remains similar, the distribution of significant genes is more complex. After 96 hours, despite the numerical similarity between populations, only four genes with significant changes in gene expression were shared among all six populations. These included enolase, a transmembrane protein, a gene annotated as a human esophageal protein, and a hypothetical protein. As these genes are significant across all populations, they apparently have a role in the hypoxia response in *F.grandis*. Four genes are shared between five populations, seven are shared in four populations, 26 are shared between three populations, and 31 are shared between two populations. The populations which share genes do not exhibit any specific patterns associated with their hypoxic history or geography. Thus while there are certainly both differences and similarities between populations in their hypoxic response, the patterns of changes in gene expression between populations are complex and appear to be both a function geographic distances and potentially hypoxia exposure histories.

Correlations of gene expression revealed two patterns of gene expression: positive and negative correlations. Even across all 609 hypoxia responsive genes there are clear delineations. These genes cluster with similar patterns of expression, one group is up regulated, the other down regulated. These significant correlations occur even though there is much variation among populations (Figure 5.5, Table 5.2, and Table 5.3). Taken together these data suggest that although the induction of gene expression varies among individuals and populations, the coordination of gene expression does not. Hypoxic gene expression is regulated by a few transcription factors and signaling proteins, and while several families are involved, the overall response is dominated by HIF (Hoogewijs et al. 2007; Rocha 2007; Kenneth and Rocha 2008).

The most well defined control of gene expression during hypoxia is the HIF transcription factor family. During normoxia, the α 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). HIF is the most well known of the transcription factors associated with the hypoxic response and is by far the most dominant (Rocha 2007; Kenneth and Rocha 2008). However there are a number of other controls. The NF-κB, AP-1, p53 and Myc families of transcription factors are also known to affect hypoxic gene expression. Several of these factors effect 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 similar mechanisms regulate hypoxic gene expression in *F. grandis*, these data are consistent with the pattern that one or a few transcription factors are affecting the patterns of gene expression across treatments. Despite clear genetic diversity between populations and diversity of gene expression response (only two genes shared across all populations) the correlation makes it clear that there is a shared mechanism of control across populations.

Summary

There were 609 genes with significant differences in gene expression for exposure to hypoxia over time. Dunnett's post hoc testing reveals that the majority of changes in gene expression take place after 96 hours of hypoxia exposure. While this pattern is maintained within all six populations exposed to hypoxia, only four genes have significant changes in gene expression across all populations. There were 355 genes with significant differences in gene expression between populations. Post-hoc testing demonstrated that most of the pair-wise differences reflect geography; there are more differences between populations separated by greater geographic distance. There were 59 genes significant for both hypoxia response and population. Within these genes, populations with shared hypoxic histories appear similar despite geographic separation. Despite the diversity of hypoxia response, correlation analysis likely reflects shared mechanisms of genetic control across all populations and time points.

Table 5.1. Study Sites and their record of hypoxia from the literature

Site	Abbreviation	Record of Hypoxia	Reported by
Port Aransas/Aransas Bay, TX	PA	First in 1988, recurring 1988-1996	Ritter and Montagna (1999); Engle et al. (1999)
Port O'Conner/Matagorda Bay, TX, mouth of the bay	PO	No record, reported as an undegraded reference	Engle et al. (1994)
Lake Charles/Lake Calcasieu, LA	LC	No record, reported as an undegraded reference	Engle et al. (1994)
Terrebonne-Bartaria/Leeville, LA	LV	Offshore zone to mouth of bay, One year measurement out of four year study	Rabalais and Turner (2001); Engle et al. (1999)
Weeks Bay, AL/Mobile Bay interior	WB	Long record of hypoxia, dating to 1820's	May (1973); US EPA, Engle et al. (1994); Engle et al. (1999).
Mobile Bay-Dauphin Island AL/Pass Aux Heron	DA	No record found	

Table 5.2. Number of significant pair-wise comparisons between populations in the Tukey-Kramer post-hoc test across all factors for the 355 genes significant for populations

Population	PA	PO	LC	LV	DA	WB
PA	-	19	44	37	33	43
PO	19	-	7	22	18	20
LC	44	7	-	5	12	14
LV	37	22	5	-	4	5
DA	33	18	12	4	-	3
WB	43	20	14	5	3	-

Table 5.3. Significant pair-wise differences between populations (Tukey-Kramer) for the 59 genes significant for both population and hypoxia response.

Population	PA	PO	LC	LV	DA	WB
PA	-	1	6	4	4	2
PO	1	-	0	5	0	3
LC	6	0	-	2	0	0
LV	4	5	2	-	1	0
DA	4	0	0	1	-	1
WB	2	3	0	0	1	-

Table 5.4. Significant changes in gene expression for the hypoxia responsive genes within each population. H4 and H96 are the four and 96 hour hypoxia treatments.

Population	Total # of Significant Genes	Significant at H4 (Dunnett's test, $p<0.01$)	Significant at H96(Dunnett's test, $p<0.01$)
PA	76	9	62
PO	93	10	68
LC	72	6	57
LV	71	4	56
DA	55	7	42
WB	72	12	62

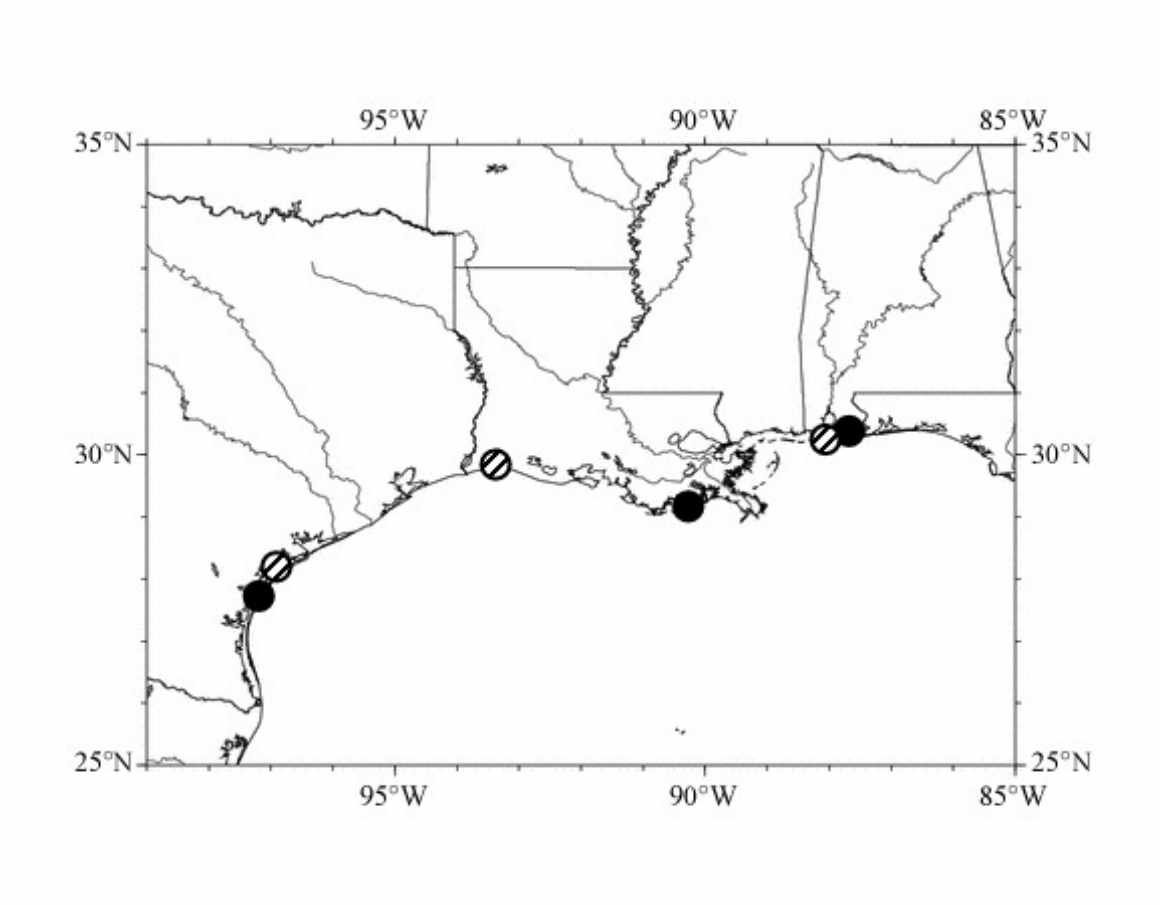

Figure 5.1. Map of Gulf of Mexico collection sites. Collection sites from west to east are: 1) Port Aransas, TX (PA) (Aransas Bay), 2) Port O'Conner (PO) (Matagorda Bay), 3) Lake Charles, LA (LC) (Lake Calcasieu), 4) Leeville, LA (LV) (Terrebonne Bay), 5) Dauphin Island, AL (DA) (Pass Aux Herons), 6) Weeks Bay, AL (WB). Sites are named with the closest city; the two letter abbreviation corresponds to the abbreviation in the text. Hashed sites reference sites, while solid sites are hypoxia sites as defined by Engle et al. (1999).

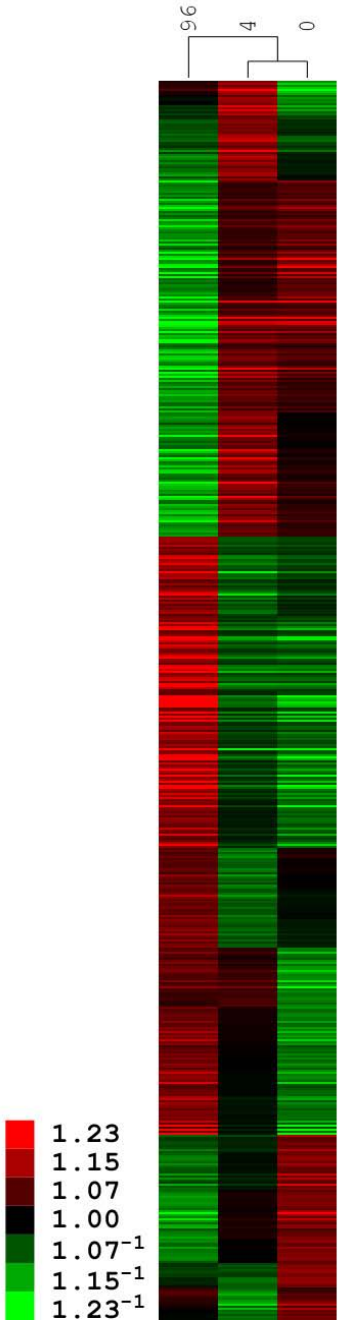

Figure 5.2. Hierarchical clustering of the 609 hypoxia responsive genes. Cluster was based on the ratio of least squared means for each gene compared to the grand mean for each gene. Colors represent fold change from the grand mean.

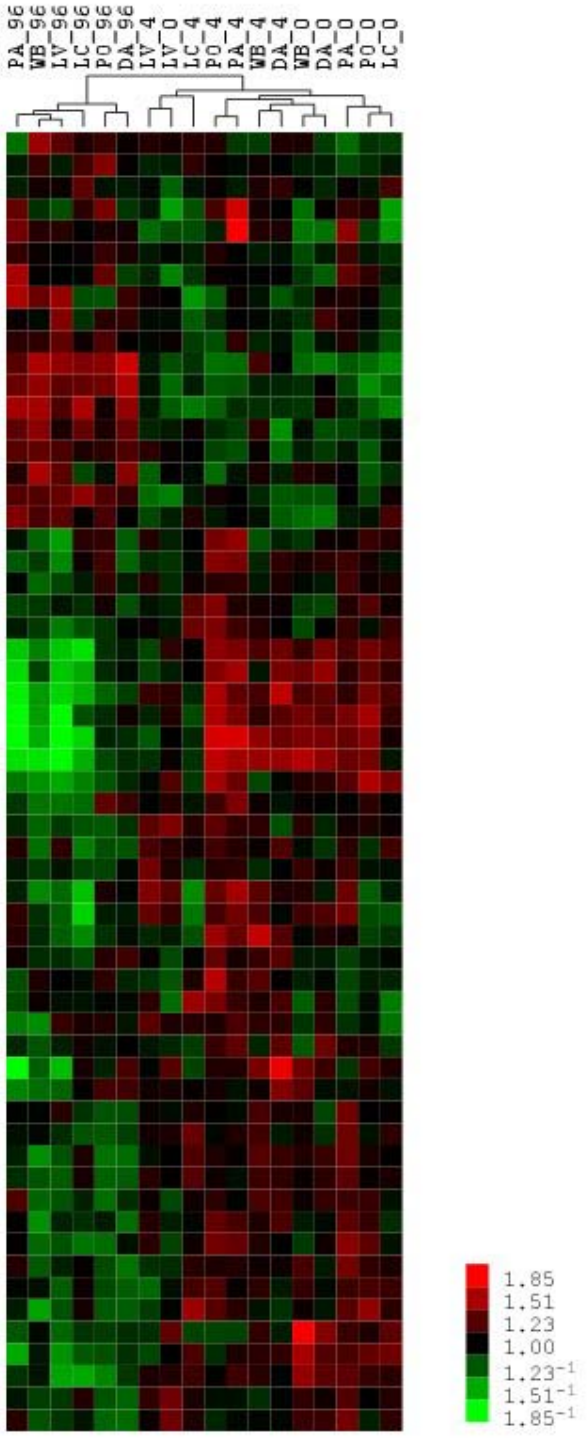

Figure 5.3. Hierarchical cluster analysis of the 59 genes significant for both population and time. Colors represent fold change from the Grand Mean. Each column is the average of all individuals in the Population/Time treatment. Labels are Population_time. Note that the 96 hour samples cluster together. Within the 96 hour branch, Port Aransas, Leeville, and Weeks Bay, the "hypoxia affected" populations cluster together.

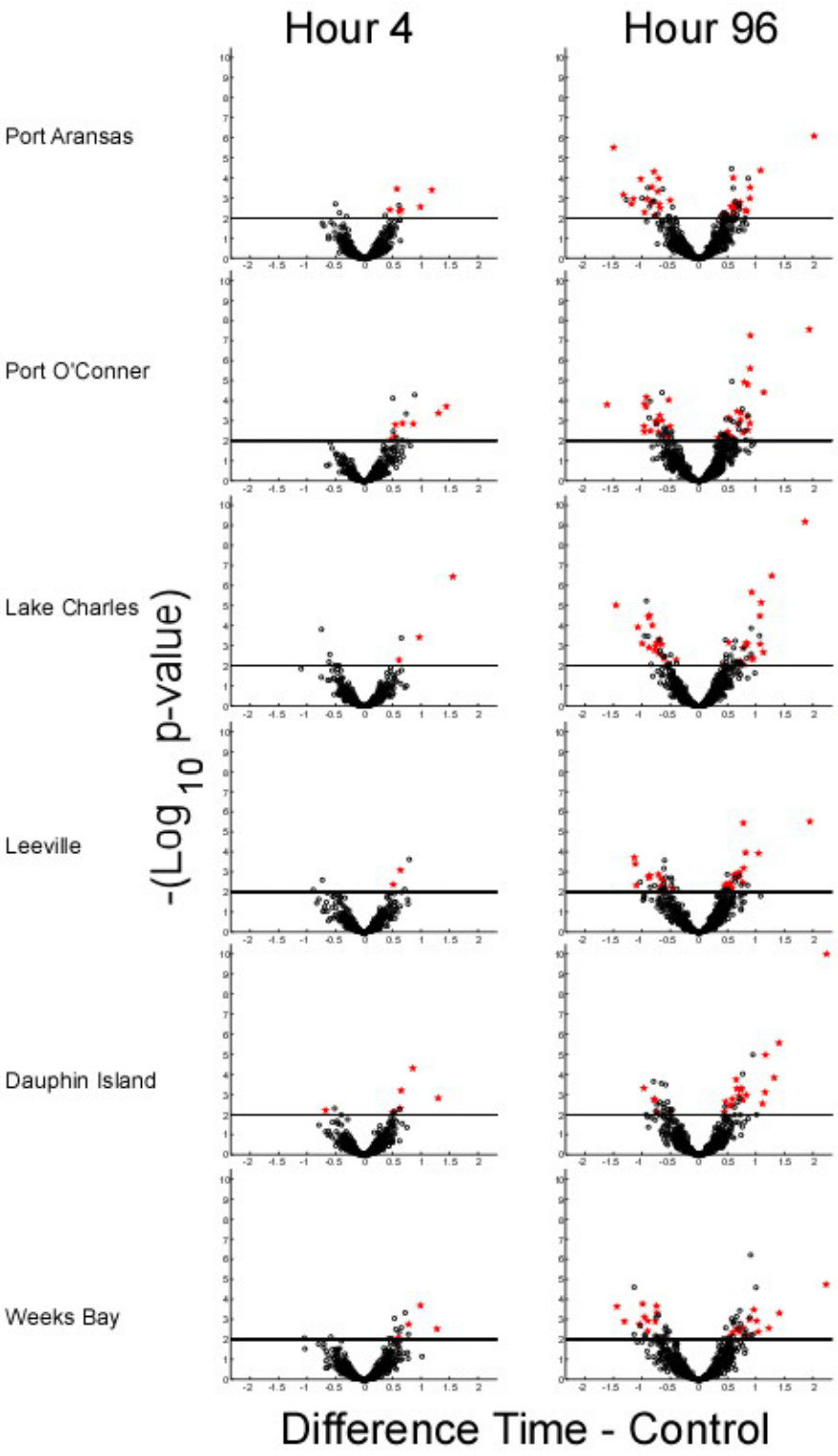

Figure 5.4. Volcano plots. Changes in gene expression over time for each population. Values were calculated using Dunnett's test and the difference in least squared mean between normoxic and hypoxic treatments. Highlighted genes are shared between at least two populations.

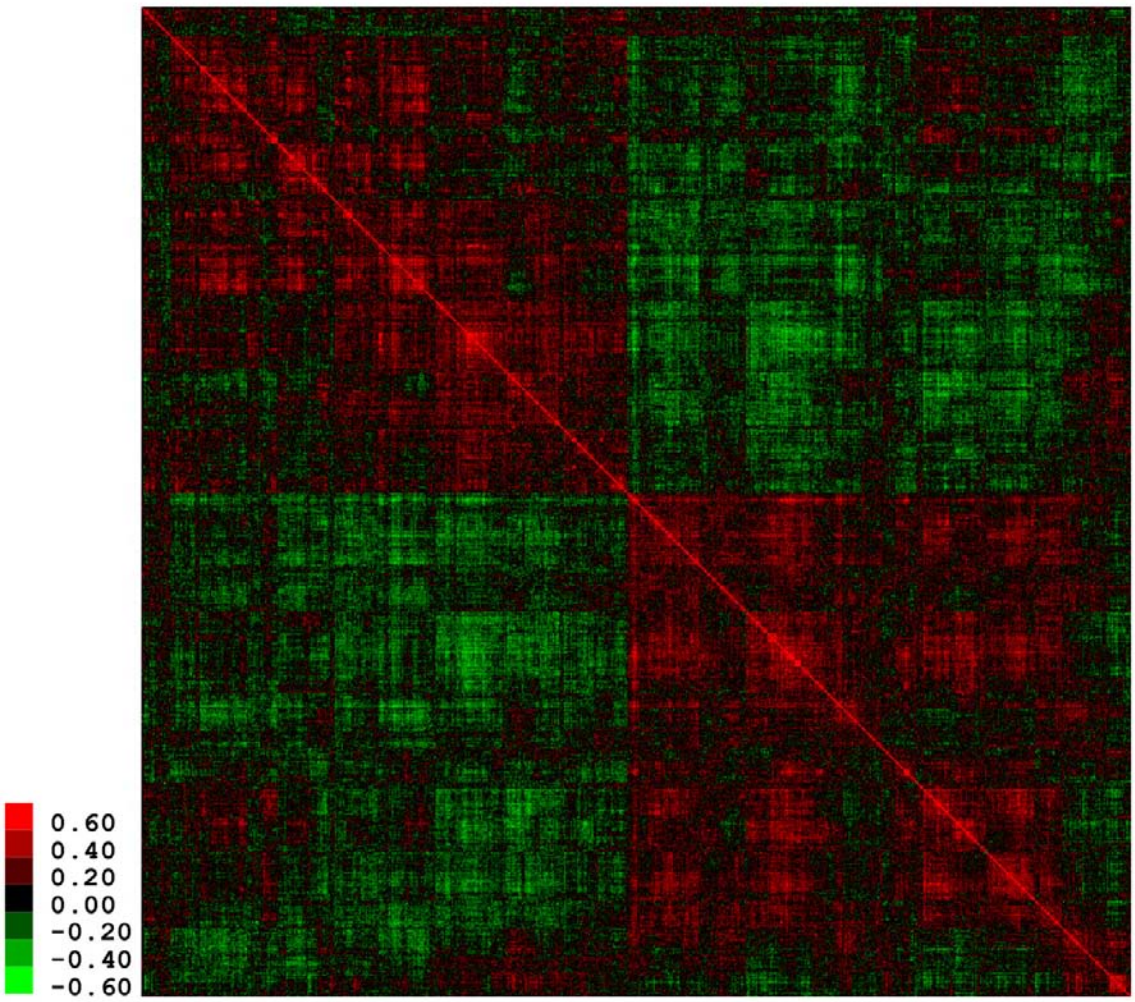

Figure 5.5. Correlations in gene expression for the hypoxia responsive genes. Correlations were calculated on the least squared means for each individual spanning all populations and time-points.

Chapter 6 Conclusions

The stimulus for this dissertation research was to better understand the effect of hypoxia and how it varies among populations. As outlined in the introduction, hypoxia is important ecologically, environmentally and for human health. Thus, understanding the genes that respond to and affect hypoxic physiology provides insights into environmental policy and human disease.

The strategy to undertake this research was first to define whole animal response to decreasing oxygen concentration. Specifically, by measuring total metabolism at seven different oxygen concentrations in seven different populations, I defined the critical concentration of oxygen where metabolism can no longer be maintained and how this varied among populations. This information provided a foundation to investigate the patterns of gene expression that responded to hypoxia. Gene expression was used because it is an early and sensitive measure of the effect of hypoxia. Using the information from the whole-body metabolic study, I designed a series of experiments to define the important parameters affecting hypoxic gene expression: time and dose. Thus, chapter 3 examined the temporal variation in gene expression with hypoxia, and chapter 4 examines the concentration dependent effect of hypoxia on gene expression. Using the results from these experiments, I examine the variation in gene expression among six populations.

In chapter 2 of this dissertation, I profiled the response in whole body metabolism, measured as oxygen uptake, across seven decreasing oxygen concentrations, and compared metabolism and P_{O2crit} , the critical oxygen tension, which is the oxygen

concentration at which individuals can no longer maintain metabolism, between populations from across the Gulf of Mexico. While measurements of oxygen uptake and PO_{2crit} in fish species are not uncommon, few studies compare populations. In one study, Timmerman and Chapman (2004) found that populations of *Poecilia latipinna* with different exposure histories to hypoxia had different P_{O2crit} values. An additional study in sculpins suggest similar results (Mandic, Todgham, and Richards 2009). A study comparing populations of trout found differences in cardiac hypoxia tolerance (Faust, Gamperl, and Rodnick 2004). In chapter 2, P_{O2crit} was successfully calculated for seven populations, and ranged between 2.4-7.8 kPa, across individuals. No significant difference in P_{O2crit} was observed between populations. However, there was a significant difference in metabolism ($\dot{M}O_2$) between populations at the lowest oxygen concentration. Individuals from both Aransas Bay and Terrebonne Bay have high $\dot{M}O_2$ at 1.8 kPa, however only Terrebonne Bay is significantly different. The differences in $\dot{M}O_2$ at 1.8 kPa occur after long-term acclimation to controlled conditions, suggesting that there could be a genetic basis for the differences or, that differences could reflect a developmental or irreversible acclimation effect.

The more important finding of this chapter is the change of body mass: metabolic rate relationship at different partial pressures of oxygen. The observation that there is a stronger effect of body mass (steeper slope) at intermediate partial pressure is not well documented in the literature. The exponent (slope) for metabolism for many teleosts is often generalized ranging from 0.71 to 0.88 under normoxic conditions and 15-20°C (Peters 1983; Glazier 2005; Nilsson and Ostlund-Nilsson 2008). However, the exponent for *F. grandis* was not in this range but more similar to 0.66 at intermediate P_{O2} and

approximately 0.5 at normoxia. These values are similar to those found by Almeida-Val et al. (Almeida-Val, Gomes, and Lopes 2006) for *Astronotus ocellatus*, a hypoxia tolerant cichlid. While these values fall outside the traditional distribution, several recent studies and reviews have suggested that the value of 0.75 may not be universal, either within or between species and may be subject to both ecological and evolutionary constraints (Julian et al. 2003; Douglas 2005; Almeida-Val, Gomes, and Lopes 2006; Seibel and Drazen 2007). The significant relationship between metabolism and body mass remains across all oxygen treatments, regardless of population. However, body mass had a stronger effect (steeper slope) at intermediate oxygen partial pressure than at normoxic or the lowest oxygen partial pressure (1.8 kPa). Thus, at intermediate oxygen concentrations, larger fish have metabolic rates more similar to normoxia.

There was also a significant relationship between P_{O2crit} and body mass. The observation that P_{O2crit} is affected by body mass is controversial. Studies have found affects of body mass on P_{O2crit} in some species (Kalinin, Rantin, and Glass 1993; Sloman et al. 2006a; Nilsson and Ostlund-Nilsson 2008) but not in others (Cerezo and Garcia 2004; Sloman et al. 2008). Nilsson and Östlund-Nilsson (2008) argue that P_{O2crit} is unlikely to be influenced by body mass, arguing that the similar scaling values of oxygen consumption (0.79-0.88) and gill surface area (0.76-0.90) indicate that P_{O2crit} values should be independent of size. However, Graham (2006) points out that values for the scaling exponent for gill surface area range from 0.5-1.0 and have no clear relationship to respiration. .My results demonstrated a significant relationship between body mass and P_{O2crit}, with larger individuals possessing lower P_{O2crit} values. These findings suggest that an increase in body size confers greater tolerance to hypoxia in this species.

In chapters 3 and 4 I profile the gene expression response to hypoxia in *F. grandis*. Both of these studies focused on changes in gene expression in metabolic genes, both using the same 384-gene microarray. Chapter 3 profiles the changes in gene expression over time to hypoxia. In this chapter, I exposed individuals to 2.8 kPa of hypoxia, below the average P_{O2crit} for *F. grandis*, for 0, 4,8,12, 24, 48, and 96 hours of hypoxia, and changes in gene expression between these time points were quantified. During hypoxia, the maximum expressions of mRNAs for different genes occur at different time points. For structural genes, the expression of mRNA is significantly over represented at zero exposure (Fisher-Exact test $p < 0.05$). While the expression of oxidative phosphorylation genes are under-represented at zero but have a significant over-representation after 96 hours (Fisher-Exact test, $p < 0.05$). This change in the distribution of peak expression among biochemical pathways is previously unreported in the literature.

Only 14 genes had significant changes in gene expression over time, and most of these occurred after 48 or 96 hours of hypoxia exposure. These significant differences appear to reflect a convergence among individuals. That is, after 4-24 hours of exposure, many of these 14 genes had changes in expression, but it varied significantly among individuals. However, these differences among individuals were smaller with greater exposure time. Finally, these patterns of expression are positively or negatively correlated, suggesting that one or a few transcription factors affect the changes in mRNA expression. This is consistent with the observation that hypoxic gene expression is regulated by a few transcription factors and signaling proteins, and while several families are involved, the overall response is dominated by HIF. While significant changes in

HIF-1α expression were not observed in these data, this is not unusual. HIF-1α is ubiquitously expressed, and its activity is regulated at the protein level. Decreasing oxygen concentration prevents oxygen dependent hydroxylation and subsequent degredation of the protein, allowing the subunit to stabilize and combine with its other subunits to affect transcription (Hoogewijs et al. 2007; Rocha 2007; Kenneth and Rocha 2008).

Chapter 4 profiles the changes in gene expression across different hypoxic concentrations. In this study individuals from two populations of *F. grandis* were exposed to three oxygen treatments, 20.5 kPa (normoxia), 7.8 kPa, and 1.8 kPa. The 7.8 kPa treatment is above the average P_{O2crit} for *F. grandis*, while 1.8 kPa is below. The lowest treatment, 1.8 kPa, is also the concentration where differences in metabolism were observed between populations. Individuals were exposed for either 4, or 48 hours. There were significant changes in gene expression both for dose and time. A total of 69 (21%) genes had significant changes ($p < 0.01$) in gene expression for at least one of the factors. To examine the relationship between these factors, the effects of dose within each time point were examined. This demonstrated a progressive change; more genes had significant changes in expression after 48 hours than 4 hours (28 genes vs. 15 genes). Additionally, most changes occurred for the 1.8 kPa treatment (9 out of 15 genes after four hours, 18 out of 28 after 48 hours). There was an interesting pattern of gene expression observed between the three oxygen concentrations. Both 7.8 and 1.8 kPa had a unique pattern of gene expression and there were more differences between the two hypoxia conditions (24 genes with significant difference in expression) than either has to normoxia (6 or 18 for 7.8 or 1.8 kPa).

A number of studies have examined the effects of different oxygen concentrations on physiological parameters. A study in the estuarine fish *Leiostomus xanthurus* by Cooper et al (2002) demonstrated dose dependent shifts in antioxidant and metabolic enzyme activities. Dalla Via et al. (1994) demonstrated changes in the concentration of components of metabolism including lactate and ATP in the sole, *Solea solea*; however changes were only observed in their two lowest oxygen treatments. Daphnia has been shown to have three different hemoglobin isoforms in circulation under normoxia, moderate, and severe hypoxia (Lamkemeyer et al. 2005). Few studies have compared the effect of different hypoxic treatments on gene expression. One study in grass shrimp compared the gene expression response in nuclear (Brouwer et al. 2007b) and mitochondrial (Brouwer et al. 2007a) gene expression. For nuclear genes, no significant changes in gene expression were observed under moderate hypoxic conditions, whereas, numerous genes exhibited changes in gene expression after 3 days of exposure to severely hypoxic conditions. I observed changes in gene expression under both moderate and severe hypoxic conditions, and significant difference between the two conditions. The difference in gene expression between hypoxic treatments has not been previously reported in the literature. It appears that not just hypoxic exposure, but the amount of time spent at a specific concentration which determines an organism's hypoxia response.

The question arises, how comparable are these results to previous studies? Are the times and hypoxic concentraitions relevant? The hypoxic oxygen concentrations used in the literature can be quite variable, and are reported in a variety of units, making direct comparisons between studies difficult difficult. In many cases the hypoxic concentrationss used in previous studies were either set simply to be below the 2 $ml \bullet L^{-1}$

threshold, or a value specifically set to compare to another study (Faust, Gamperl, and Rodnick 2004; van der Meer et al. 2005; Brouwer et al. 2007a; Brouwer et al. 2007b; Marques et al. 2008). The hypoxia treatments here were based on physiological data, defining the P_{O2crit} for the species, and using values above, (7.8 kPa) or below (2.8, 1.8 kPa) this value. This was the approach also used by Gracey et al. (2001), and also the physiological studies performed by Martinez et al. (2006). Other studies have used techniques such as hypoxic gradients, exposing individuals to a multitude of oxygen concentrations to determine a behavioral or physiological response (Cerezo and Garcia 2004). The summary of the data presented here, along with previous studies indicates it is difficult to make a direct comparison of hypoxia response and tolerance across species. While there are certainly some shared responses, overall survival and tolerance are based on a variety of factors including species, size, and population history.

Many studies have profiled the chronic response to hypoxia, exposing individuals for greater than 3 weeks. While important, profiling a more acute response to hypoxia has merit as well, many hypoxic zones exist for only hours to days depending on local conditions, and yet still may have detrimental effects on local ecosystems (Engle, Summers, and Gaston 1994; Engle, Summers, and Macauley 1999; Wu 2002). A closer examination of the long term studies reveals similar patterns of change to what was found in the results presented here. For instance, Brouwer et al. (2007) observed significant changes in gene expression in grass shrimp in response to severe hypoxia after 3 days of exposure. Many of the genes were upregulated, including several biochemical families upregulated in the current results. In the study by Gracey et al. (2001), close examination of the supplemental materials reveals similar patterns of cardiac gene expression as

observed in these results. However, since the focus of each of these studies was the long term effects of hypoxia, those were the primary results presented.

The two initial studies of gene expression response to hypoxia served a secondary purpose, determining the times and oxygen concentrations to maximize the changes in gene expression. Having determined the dose (1.8 kPa) and time (96 hours) that maximize these changes, in chapter 5 I set out to quantify changes in gene expression in response to hypoxia across thousands of genes, and between six populations of *F. grandis*. These six populations come from areas with differing exposure histories to hypoxia. Three are from relatively pristine sites, Port O'Conner, Lake Charles, and Dauphin Island, while the other three are from sites experiencing frequent hypoxic events, Port Aransas, Leeville, and Weeks Bay. Additionally, these populations have been previously determined to be genetically distinct (Williams, Brown, and Crawford 2008). Individuals from these six populations were exposed to normoxia (0 hours) or 4 or 96 hours of hypoxia, at an oxygen partial pressure of 1.8 kPa. Ten individuals from each population were sampled at each treatment. Hypoxia had a significant effect on the expression of 609 genes, while population affected the expression of 355 genes. Genes with significant difference in expression among populations reflect geographic separation; there are more differences between populations separated by greater geographic distance. A total of 59 genes had significant changes in mRNA expression for both hypoxia exposure and population effects. Within this group of genes, shared hypoxic histories appears to be more important than simply the neutral patterns expected by geographic distance. This was observed in Tukey-Kramer post hoc testing on these genes, there were no differences between the populations from pristine sites, despite

geographic separation. Hierarchical clustering of these genes also grouped individuals from the three hypoxic populations together after 96 hours of exposure, indicating similarity in their hypoxia response. Despite the diversity of hypoxia response, correlation analysis still reflects share mechanisms of genetic control across all populations and time points.

Previous studies in teleosts and other organisms have demonstrated adaptation in gene expression in response to a variety of environmental stressors (Watt 1977; Oleksiak, Churchill, and Crawford 2002; Whitehead and Crawford 2006; Oleksiak 2008). In teleosts, adaptation to hypoxia between populations has been demonstrated in physiological parameters, including P_{O2crit} and cardiac metabolism (Faust, Gamperl, and Rodnick 2004; Timmerman and Chapman 2004). However, little has been done to examine differences in gene expression response to hypoxia between populations. The observed changes in both metabolic and global gene expression demonstrate that in *F. grandis* there expression changes which underly observed physiological changes (Chapter 1, (Martinez et al. 2006). Additionally, there appears to be local adaptation in the hypoxic response between populations of *F. grandis* with differing exposure histories to hypoxia. While, some of these changes may be explained by local acclimation, all fish were common gardened for more than 6 weeks, which will reduce local differences. Additionally, similarities between individuals with similar exposure histories were observed across large geographic distances. Taken together, the most parsimonious explanation appears to be local adaptation to hypoxic conditions.

Summary

Hypoxia represents an environmental stressor to *F. grandis* individual from the Gulf of Mexico. This research has demonstrated that *F. grandis* cope with hypoxia through changes in metabolism and gene expression. Overall, the response to hypoxia is dependent on an individual's size (body mass), the oxygen concentration, and the duration of hypoxia exposure. Additionally, there appear to be some differences between populations with differing exposure history to hypoxia in the Gulf of Mexico. After 96 hours at severe hypoxia, individuals from pristine or hypoxic sites more closely resemble individuals from other pristine or hypoxic sites respectively, regardless of geographic separation. This indicates that hypoxia may be driving local adaptations in *F. grandis*. Future studies should focus on changes between populations with longer exposure times to hypoxia. Additionally, identifying specific changes in genes, such as changes in promoter regions, between populations which result in the observed changes in gene expression could help explain the mechanisms of hypoxia tolerance in this species.

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