MECHANISMS AND EVOLUTION OF HYPOXIA TOLERANCE IN FAMILY COTTIDAE

by

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ABSTRACT

A comparative phylogenetically independent contrast (PIC) analysis was employed to investigate the adaptive role of traits involved in hypoxia tolerance in sculpins, a group of closely related fish species that live in the nearshore marine environment. I demonstrated that there was a tight correlation between critical oxygen (O_2) tension (P_{crit}) and the distribution of species across an environmental gradient. Species of sculpins with the lowest P_{crit} inhabit the O_2 variable intertidal zone, while species with higher P_{crit} inhabit the O_2 stable subtidal zone. Low P_{crit} values in sculpins were associated with enhanced O_2 extraction capacity, with three principal traits accounting for 83% of the variation in P_{crit}: low routine O_2 consumption rate ($\dot{M}_{O_2}$), high mass specific gill surface area and high whole cell hemoglobin-oxygen (Hb-O_2) binding affinity. Variation in whole cell Hb-O_2 binding affinity was strongly correlated with the intrinsic affinity of Hb for O_2 and not to differences in the concentration of the allosteric Hb modulators ATP and GTP.

When environmental O_2 dropped below a species' P_{crit}, some species of sculpins behaviorally responded to the severe hypoxia by performing aquatic surface respiration (ASR) and aerial emergence. Although intertidal sculpins consistently performed these behaviors, the clustering of these species into a single phylogenetic clade did not allow us to draw conclusions regarding the relationship between ASR, aerial emergence and P_{crit} using PIC analysis. Three species of sculpins, which were chosen because of their low, medium and high P_{crit} values, exhibited dramatically varied mortality rates when exposed to severe hypoxia equivalent to 40% of their respective P_{crit}. Although ATP turnover rates were similar between the three species in the initial two hours of hypoxia exposure, the differences in the ability of the three species to survive severe hypoxia appeared to be associated with the concentration of on-board liver glycogen and the degree of liver glycogen depletion. However, when liver glycogen was

assessed in twelve species of sculpins at normoxia and compared with P_{crit}, there was no significant PIC correlation between P_{crit} and liver glycogen.

Overall, I have shown that there is a clear relationship between P_{crit} and the distribution of sculpins along the nearshore environment and that this is primarily related to differences in O_2 extraction capacity. When O_2 tensions are well below their P_{crit}, there are dramatic differences in behavioral, physiological and biochemical responses among these species of sculpins.

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ANOVA	analysis of variance
ASR	aquatic surface respiration
ATP	adenosine triphosphate
BMSC	Bamfield Marine Sciences Centre
°C	degrees Celsius
CO_2	carbon dioxide
CrP	creatine phosphate
cyt b	Cytochrome B
GTP	guanosine triphosphate
GTR	general time reversal
H^+	proton
Hb	hemoglobin
Hb P_{50}	partial pressure at 50% saturation of hemoglobin by oxygen
HPLC	high performance liquid chromatography
MCHC	mean cellular hemoglobin content
Mg^{2+}	magnesium
$\dot{M}_{O_2}$	oxygen consumption rate
N	Hill's coefficient
N_2	nitrogen
O_2	oxygen
PCR	polymerase chain reaction
P_{crit}	critical oxygen tension
pHi	intracellular pH
PIC	phylogenetically independent contrast
PO_2	partial pressure of oxygen
Ppt	parts per thousand
RBC	red blood cell
SE	Standard error

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CO-AUTHORSHIP STATEMENT

Chapter 2 and 3 of this thesis are co-authored. The research questions and the design of the experiments were conducted by Milica Mandic under the supervision of Jeffrey G. Richards. The vast majority of research and data analysis was accomplished by Milica Mandic. Anne E. Todgham provided expert advice regarding Family Cottidae in Chapter 2. Katherine A. Sloman provided expert advice and assisted with the behavioral experiment in Chapter 3. Following the conclusion of experiments, the manuscripts were written solely by Milica Mandic in consultation with Jeffrey G. Richards.

CHAPTER ONE: OVERALL INTRODUCTION

ENVIRONMENTAL HYPOXIA

Periods of low environmental oxygen (O_2; hypoxia) are common in the aquatic ecosystem, occurring in both freshwater and marine habitats. The aquatic environment is more susceptible to periodic hypoxia than terrestrial habitats due to relatively low capacitance of water for O_2. In ice covered lakes and ponds, severe hypoxia can ensue for a period of 4 to 6 months due to a lack of O_2 exchange with the atmosphere in combination with rotting dead plant material (van den Thillart et al., 1989). Seasonal fluctuations in environmental O_2 also occur in the highly vegetated small bodies of water that become isolated from the Amazon river when the water levels are low (Val, 1999). In addition to the long-term changes in O_2 levels in the waters of the Amazon basin, there are dramatic diurnal fluctuations due to photosynthesis and respiration of organisms (Val, 1999). This has also been recorded in marine environments such as estuaries and tidepools isolated from the tide (Burggren and Roberts, 1991; Landry et al., 2007). In tidepools emerged at night, O_2 levels can drop to nearly zero due to respiring biomass, while photosynthesis can cause O_2 to reach supersaturating levels of 400 to 600 torr of O_2 in tidepools emerged during the day (Truchot and Duhamel-Jouve, 1980). As hypoxia is common in the aquatic environment and as the frequency of hypoxia increases in water systems world-wide as a product of wide spread eutrophication (Diaz, 2001), there is a pressing need to understand how animals respond and adapt to low environmental O_2.

WHY IS HYPOXIA BAD?

The primary threat an animal exposed to environmental hypoxia faces is the reduced capacity for ATP production to maintain normal cellular functioning because of the reduction in mitochondrial oxidative phosphorylation. As ATP becomes limiting, the general cellular response is a failure in ion motive ATPases such as the Na^+/K^+ ATPase which leads to an influx

of Na^+ and efflux of K^+ (Krnjevic, 1993). This causes membrane depolarization and a large influx of Ca^{2+} through voltage gated Ca^{2+} channels (Boutilier and St-Pierre, 2000). Activation of Ca^{2+} dependent phospholipases and proteases due to increased Ca^{2+} concentrations in the cell leads to further membrane depolarization and cellular swelling (Choi, 1995). As membranes rupture, necrotic cell death occurs causing the animal to die (Boutilier and St-Pierre, 2000).

This pathway to necrotic cell death, elicited by a lack of O_2, occurs within minutes of hypoxia exposure in hypoxia sensitive animals. Animals deemed hypoxia tolerant can employ a number of defense mechanisms to prolong survival in hypoxia. However, if the severity and duration of hypoxia exposure exceeds an animal's ability to defend itself, the hypoxia-initiated cascade of events leading to cellular death will also occur in hypoxia tolerant animals (Boutilier and St-Pierre, 2000). Since the pathway to necrotic cell death caused by limited cellular O_2 is the same between a hypoxia tolerant and a hypoxia sensitive animal, this leads to the question of how an animal's tolerance to hypoxia is quantified and what mechanisms are utilized to prolong survival during hypoxia?

Determining Hypoxia Tolerance

An animal's tolerance to hypoxia can be predicted using several methods. One common technique is to determine an animal's critical O_2 tension (Chapman et al., 2002; Saint-Paul, 1984; Ultsch et al., 1978). The critical O_2 tension (P_{crit}) is the threshold environmental O_2 tension where an animal can no longer maintain O_2 consumption rate ($\dot{M}_{O_2}$) independent of the environmental O_2 tension. At P_{crit}, $\dot{M}_{O_2}$ begins to decrease in correspondence with a reduction in ambient O_2 levels (Pörtner and Grieshaber, 1993). The second common method of quantifying hypoxia tolerance is to determine the half maximal survival time (LT_{50}; Bickler and Buck, 2007) when animals are exposed to a steady level of severe hypoxia. Although both techniques are valid predictors of hypoxia tolerance, one method over the other may preferentially be utilized

depending on which hypoxia defense mechanisms are under investigation. When the defensive responses are geared towards increasing the efficiency of O_2 extraction from the environment to maintain routine metabolic rate, then P_{crit}, which quantifies the shift from routine $\dot{M}_{O_2}$ to depressed $\dot{M}_{O_2}$, should be used as a measure of an animal's ability to defend against changes in metabolic rate. When environmental O_2 levels drop too low for an adequate supply of O_2 to the tissues, however, other defensive mechanisms must be employed to maintain a balance between energy supply and demand. When investigating defensive responses that occur once routine $\dot{M}_{O_2}$ can no longer be maintained, LT_{50} measure may be a more appropriate indicator of an animal's ability to survive periods of cellular O_2 deficit.

DEFENSES AGAINST HYPOXIA

BEHAVIORAL AND MORPHOLOGICAL

Behavioral responses to low environmental O_2, such as aquatic surface respiration and aerial emergence, have been observed in a number of fish species. Aquatic surface respiration (ASR), whereby a fish moves up the water column to ventilate at the water-air interface is an effective strategy of maximizing O_2 extraction when the bulk water is hypoxic (Martin, 1995; Watters and Cech, 2003, Yoshiyama et al., 1995). Aquatic surface respiration is enhanced in some Amazon fish species, such as the *Colossoma macropomum*, through a morphological change of protruding the lower lip to more efficiently direct the surface layer of the water across the gills (Val, 1999). Aerial respiration is also an effective behavioral mechanism in maintaining O_2 extraction since fish that actively emerge out of water are able to maintain an aerial metabolic rate that is close to their aquatic metabolic rate during normoxia (Martin, 1996; Sloman et al., 2008; Wright and Raymond, 1978; Yoshiyama and Cech, 1994). However, if pressure from aerial predation becomes too great (Kramer et al., 1983; Sloman et al., 2006; Yoshiyama et al., 1995), or if fish do not have the capacity to employ these behavioral strategies, a suite of

physiological and biochemical defenses are available to help cope with low environmental O_2.

PHYSIOLOGICAL

Modifications to the respiratory cascade have long been thought to be important in species that frequently encounter low environmental O_2. A few studies have proposed that fish frequently exposed to bouts of hypoxia possess a large gill surface area for a greater capacity to extract O_2 at lower tensions compared to fish living in well-oxygenated habitats that typically possess smaller gill surface areas (Chapman et al., 2002; Saint-Paul, 1984; Timmerman and Chapman, 2004). For example, the hypoxia tolerant *Hoplias malabaricus* possesses a greater respiratory surface area than the hypoxia sensitive *Hoplias lacerdae* due to greater filament length and larger total number of secondary lamellae (Fernandes et al., 1993). In addition, although *Carassius carassius* and *Carassius auratus*, two species well known for their ability to withstand prolonged, severe hypoxia, have a small respiratory surface area in well-aerated water, during an exposure to hypoxia an increase in the respiratory surface by a ~7.5 fold occurs due to a remodeling of the gills. This is achieved through a decrease in intralamellar cell mass due to apoptosis and reduced cell proliferation, allowing protrusion of the secondary lamellae (Nilsson, 2007; Sollid et al., 2003).

Fish species possessing hemoglobins (Hbs) with a high affinity for binding O_2 can maintain O_2 uptake during hypoxia to a greater degree than fish species with a lower Hb-O_2 binding affinity. Therefore, it has been suggested that hypoxia tolerant species have a higher Hb-O_2 binding affinity than fish with a lower tolerance to environmental hypoxia (Hochachka and Somero, 2002; Jensen et al., 1998). The quintessential example of this is the hypoxia tolerant carp, *Cyprinus carpio*, which possesses a high Hb-O_2 binding affinity (P_{50} ~ 7 torr; Weber and Lykkeboe, 1978). Most likely the high Hb-O_2 binding affinity in *Cyprinus carpio* is due to specific amino acid sequence of the Hb protein, which has been shown to be the reason for the very high Hb-O_2 binding affinity in the bar-headed goose (Perutz, 1983). Since in many fish

species there are multiple Hb isoforms that exhibit functional heterogeneity, it has been proposed that isoform patterns play a large role in establishing the different Hb-O_2 binding affinities among fish species. Brix et al. (1999) demonstrated that triplefin fishes inhabiting the O_2 variable intertidal possess a greater number of Hb isoforms that consist of a greater proportion of the higher Hb-O_2 binding affinity isoforms than the triplefin fish species located in the deeper dwelling O_2 stable environments. Different Hb isoform patterns are also seen intraspecifically between individuals of a species reared under different O_2 tensions. Higher Hb-O_2 binding affinity isoforms are expressed in individuals of *Haplochromis ishmaeli* raised in a hypoxic environment than those reared under normoxic conditions (Rutjes et al., 2007).

Many fish species also have the ability to enhance Hb-O_2 binding affinity during hypoxia exposure through adjustments in concentrations of Hb modulators. The primary Hb modulators in fish are the organic phosphates ATP and GTP (Val, 2000), which bind to the cavity between two β chains of the Hb increasing the likelihood of the Hb remaining in the tense, deoxygenated state (Jensen et al., 1998). As environmental O_2 decreases, there is a decline in red blood cell (RBC) organic phosphate concentration leading to a decrease in the interaction of the modulators with Hb (Tetens and Lykkeboe, 1981; Weber and Lykkeboe, 1978; Wood and Johansen, 1972). This causes an increase in Hb-O_2 binding affinity and therefore an increase in O_2 extraction at the respiratory surface during hypoxia.

BIOCHEMICAL

When environmental hypoxia becomes too severe for a fish to maintain adequate O_2 extraction to support aerobic metabolism, a metabolic reorganization occurs in hypoxia tolerant species in order to maintain a balance between energy supply and demand (Hochachka et al., 1996). With a decrease in aerobic metabolism due to a lack of O_2, there is an up-regulation in the O_2 independent pathways of energy production, such as glycolysis and creatine phosphate (CrP) hydrolysis. However these pathways produce significantly less energy than oxidative

phosphorylation per unit glucose and a decrease in energy demand is necessary to match the decrease in energy supply. The reduction in energy utilization is primarily achieved through a regulated depression of metabolic rate by decreasing major energy consuming processes such as protein synthesis and ion pumping (Buck et al., 1993a; Buck et al., 1993b). This ability to depress metabolic rate has been considered one of the key hallmark defense mechanisms of a hypoxia tolerant animal (Boutilier and St-Pierre, 2000).

The Comparative Method and Assigning Adaptive Value

Because any of the previously mentioned behavioral, morphological, physiological and biochemical modifications can be clearly beneficial in allowing animals to exploit more O_2 variable environments, many studies have referred to these traits as adaptations to hypoxia tolerance. However, in these studies the term adaptation was loosely applied to any traits that aided in the survival of an animal without careful consideration of phylogeny. Therefore, it is difficult to discern traits that are true adaptations to hypoxia compared to traits that are valuable defenses against hypoxia but are highly conserved across tolerant and intolerant species. If similar responses occur between species which consistently experience periods of low environmental O_2 and those which are never exposed to hypoxic conditions, then most likely the responses are not adaptations to environmental hypoxia per se. The broad categorization of any trait which aids in the survival of an animal during an environmental perturbation as adaptive is not exclusive to hypoxia literature and has been prevalent in diving physiology prior to the study conducted by Mottishaw et al. (1999). Diving physiology literature provides a great example of the necessity of thoroughly testing the potential adaptive value of traits.

Historically, the ability of pinnipeds to dive for a prolonged period of time has been attributed to two key 'adaptations', bradycardia and peripheral vasoconstriction (Scholander, 1963). Both responses have been observed in pinnipeds during forced laboratory dives and voluntary sea dives, leading to the general conclusion that bradycardia and peripheral

vasoconstriction are integral adaptations to diving. However, despite the importance of bradycardia and peripheral vasoconstriction to the dive response, Mottishaw et al. (1999) demonstrated that the two characteristics did not vary significantly among pinnipeds despite a large variation in species-specific dive duration. With the application of an analytical evolutionary analysis, phylogenetically independent contrasts (PIC), Mottishaw et al. (1999) were able to conclude that bradycardia and peripheral vasoconstriction were not adaptations to the dive response as has long been the belief of diving physiologists. However, spleen mass, blood volume and Hb pool size were shown to correlate with dive duration in a phylogenetically independent manner, suggesting an involvement of these traits in the adaptation of pinnipeds to diving. Clearly a similar approach of utilizing more stringent statistical methods such as PIC is vital in determining if the characteristics involved in prolonging survival during hypoxia could be the result of natural selection. The additional strength of using methods involving an understanding of the phylogenetic history is the ability to elucidate if characteristics under study have evolved independently numerous times only in the species that experience the relevant environmental perturbation.

Part of the process involved in inferring evolutionary relationships among traits is employing a comparative method, ideally between closely related species that exhibit variation in the traits of interest (Garland et al., 2005). More closely related species are preferentially chosen to avoid the possibility that very distantly related species have undergone additional evolutionary change which may have confounding effects on the traits under investigation (Felsenstein, 1985; Garland et al., 2005). However, unless it is determined that all the species under study are equally distantly related from each other and form a 'star phylogeny', a serious statistical drawback of the comparative method is that the differential relatedness of the species creates phylogenetic non-independence (Felsenstein, 1985). Species cannot be treated as independent of each other since closely related species share more similar phenotypic and

genotypic characteristics than the more distantly related species. If the non-independence caused by a phylogenetic hierarchy is not corrected for, an increase in Type I error occurs in the data (Felsenstein, 1985; Garland et al., 1992; Garland et al., 2005). Application of the PIC method (Felsenstein, 1985) which uses phylogenetic information to transform species data into standardized independent contrasts effectively eliminates any non-independence from the data set.

SCULPINS: THE MODEL SYSTEM

A PIC corrected comparison of a group of closely related fish species, sculpins from the family Cottidae, was utilized in this thesis in an attempt to determine the adaptive value of traits long thought of as adaptations to hypoxia. Sculpins are an ideal comparative model for studying the evolutionary relationships among traits associated with hypoxia because of their differential distribution along the nearshore environment (Eschmeyer and Herald, 1983; Froese and Pauly, 2007). The nearshore displays a steep environmental gradient over a narrow geographical range. The intertidal zone shows severe diurnal fluctuations in O_2, and is in close proximity with the subtidal zone that shows little fluctuation in O_2 (Burggren and Roberts, 1991; Truchot and Duhamel-Jouve, 1980). Since species of sculpins exhibit a pattern of vertical zonation along the nearshore environment, they most likely experience drastically different selection pressures on the ability to tolerate fluctuating levels of environmental O_2. Thus they are an ideal group of species for studying adaptations to hypoxia.

THESIS OBJECTIVES

Despite an extensive mechanistic understanding of how hypoxia tolerant animals defend against hypoxia, there has been very little work conducted on the evolution of hypoxia tolerance in teleost fish. Focusing predominantly on characteristics involved in O_2 extraction efficiency, the purpose of my thesis was to begin to elucidate the adaptation of sculpins to hypoxia using PIC. To accomplish this, the three main objectives of my thesis research were to: 1) determine if

species of sculpins exhibited variation in hypoxia tolerance and if this variation is correlated to their distribution along the nearshore environment, 2) correlate traits believed to be involved in hypoxia tolerance with the observed hypoxia tolerance using PIC, and 3) quantify the responses of sculpins exposed to severe hypoxia.

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CHAPTER TWO: RESPIRATORY ADAPTATIONS TO HYPOXIA IN FAMILY COTTIDAE

INTRODUCTION

The ability of an animal to acquire O_2 from its environment has long been considered a major determinant of hypoxia tolerance (Hughes, 1973). Animals that possess a greater O_2 extraction capacity are able to maintain a routine metabolic rate at lower O_2 tensions and exploit more O_2 variable environments (Hochachka and Somero, 2002; Hughes, 1973). Potential modification to any of the multiple sites along the respiratory cascade, from O_2 uptake at the gills to O_2 use by the final electron acceptor in the mitochondrial electron transport chain, can, in theory, lead to enhanced O_2 extraction capacity from the environment and thereby increase hypoxia tolerance. Any mechanism that increases hypoxia tolerance may be a potential target of natural selection in organisms living in O_2 variable environments.

The Hb-O_2 binding system has long been considered a critical adaptation to low environmental O_2. Generally, Hbs of active fish living in stable, well-oxygenated water possess low Hb-O_2 binding affinity, while fish that inhabit variable O_2 environments, which routinely become hypoxic have high Hb-O_2 binding affinities (Hochachka and Somero, 2002; Powers, 1980; Wells, 1999). Interspecific variation in Hb-O_2 binding affinity can be attributed to differences in Hb multiplicity and heterogeneous Hb isoform expression (Brix et al., 1999; Wells et al., 1989; Wells, 1999) as well as mutations in amino acid sequences (Perutz, 1983). Apart from these intrinsic properties of the Hb, allosteric modulators, such as ATP and GTP, can also impact Hb-O_2 binding affinity. As effective modifiers of Hb-O_2 binding affinity, allosteric modulators may play an important role in the ability of Hb to efficiently function in O_2 transport over a range of environmental O_2 tensions (Wells et al., 1997).

A version of this chapter will be submitted for publication. Mandic, M., Todgham, A.E. and Richards, J.G. Respiratory Adaptations to Hypoxia in Family Cottidae.

Although there is a long standing contention that a high Hb-O_2 binding affinity is adaptive to surviving hypoxia, to date, most studies have compared distantly related species and therefore these studies have only been able to suggest that Hb plays a role in the evolution of tolerance to low O_2 levels. Assigning adaptive value to a trait requires two important parameters: 1) the careful selection of closely related species that differ in the frequency and magnitude of their exposure to the environmental perturbation under study and 2) the ability to demonstrate that the trait of interest is exhibited only by the species that typically experience the environmental perturbation. However, to eliminate inflated Type 1 errors that occur due to the phylogenetic non-independence that exists between closely related species, phylogenetically independent contrasts (PIC) are applied to the analysis (Felsenstein, 1985; Garland et al., 1992). This maintains the advantages of comparing closely related species, which is a critical component in the determination of adaptation of a trait within a PIC study (Garland et al., 2005). For studying the evolution of hypoxia tolerance, fish inhabiting the nearshore marine environment present an ideal model system.

The nearshore marine environment is a narrow geographical range encompassing two zones, the intertidal and the subtidal, that differ dramatically in the degree of variation in physical parameters such as O_2, temperature, salinity and pH (Burggren and Roberts, 1991; Truchot and Duhamel-Jouve, 1980). The intertidal zone is under heavy influence of the daily ebb and flow of the tides, and rocky pools (tidepools) within this zone experience severe diurnal fluctuations in abiotic factors. Of particular importance to the present study, tidepools emerged at night experience drops in O_2 to nearly zero within a few hours of emergence, while O_2 in tidepools emerged during the day can reach supersaturating levels of 400 to 600 torr (Truchot and Duhamel-Jouve, 1980; personal observation). The degree of variation in environmental O_2 is primarily dictated by the duration of emergence, therefore tidepools located high in the intertidal often experience more severe fluctuations in their environment than pools located lower in the

intertidal zone. The adjacent subtidal zone, however, shows little fluctuation in these physical parameters.

The steep environmental gradient along the nearshore environment has a strong effect on the vertical distribution patterns of species (Brix et al., 1999; Doty, 1946; Stillman and Somero, 1996). A group of closely related fish species, sculpins from the family Cottidae, exhibit a pattern of vertical zonation with different species inhabiting different portions of the nearshore environment (Eschmeyer and Herald, 1983; Froese and Pauly, 2007). Given the distribution pattern along the nearshore environment, different species of sculpins experience dramatically different magnitudes and frequencies of variation in their physical environment. It is likely, therefore, that species found in the intertidal zone, which most commonly experience hypoxia in the environment, have evolved mechanisms to tolerate hypoxia, while species inhabiting the relatively O_2 stable subtidal zone most likely lack these adaptations.

The objectives of the study were to determine the characteristics that are related to hypoxia tolerance in 12 species of sculpins that are found in different parts of the nearshore marine environment. Components of the respiratory cascade, such as gill surface area and whole blood Hb-O_2 binding affinity were quantified in normoxia-acclimated sculpins and the application of PIC aided in assigning adaptive value to these characters. Intrinsic Hb-O_2 binding affinity and concentrations of RBC allosteric modulators (eg. ATP and GTP) were also investigated to determine the underlying cause of variation in whole blood Hb-O_2 binding affinity.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

Marine sculpins were caught using handheld nets or seines during the lowest tidal cycles of June and August 2005, 2006 and 2007 at Ross Islets (48°52.4′N; 125°9.7′W), and Wizard's Rock (48°51.5′N; 125°9.4′W), near the Bamfield Marine Sciences Centre (BMSC), Bamfield,

British Columbia, Canada. Marine sculpins including tidepool (*Oligocottus maculosus*), fluffy (*Oligocottus snyderi*), and mosshead (*Clinocottus globiceps*) were caught in tidepools. The remaining marine sculpins were caught in the subtidal zone and include buffalo (*Enophrys bison*), padded (*Artedius fenestralis*), smoothhead (*Artedius lateralis*), cabezon (*Scorpaenichthys marmoratus*), Pacific staghorn (*Leptocottus armatus*), silverspotted (*Blepsias cirrhosus*) and scalyhead (*Artedius harringtoni*). The freshwater prickly sculpin (*Cottus asper*) was caught using baited minnow traps in Pachena Lake (48°50'11" N; 125°01'44" W) near BMSC during the same time period. Sculpins were transported to the University of British Columbia and the marine sculpins were held in re-circulating 12°C seawater (30 ppt salinity), which was obtained every two months from the Vancouver Aquarium. In 2006, shorthorn sculpins (*Myoxocephalus scorpius*) were brought in from the Atlantic Coast (Memorial University, Newfoundland) and held in the re-circulating saltwater system. Freshwater prickly sculpins were housed in an identical re-circulating system in 12°C freshwater. All sculpins were allowed to recover from transportation for at least 3 weeks before experimentation. Throughout the study period, fish were fed daily with bloodworms and frozen fish fillets, except 24 hours prior to experimental trials.

Whole animal respirometry (P_{crit} and routine $\dot{M}_{O_2}$) was performed on sculpins captured in 2005 and sculpins captured in 2006 were terminally sampled for analysis of gill surface area, concentrations of Hb modulators, affinity of Hb for O_2 in the presence and absence of Hb modulators, and Hb isoform profiles. To complete the Hb characterization, additional specimens were caught and terminally sampled in 2007 for measurements of RBC intracellular pH (pHi). The mean weights of fish used in 2006 are given in Table 2.1 and the weights of fish captured in 2005 and 2007 were generally not statistically different than those from 2006. Although there are statistical differences in some species, the variation in weight has no impact on

measurements of P_{crit} (unpublished data). We also had some initial concern that fish might differ from year to year in their physiological responses to hypoxia; however, no appreciable difference in measurements of P_{crit} from animals caught in separate years was observed (c.f. present study with Henriksson et al., In Press). All experimental procedures involving animals were done according to UBC protocol A05-0142.

EXPERIMENTAL PROTOCOLS

Whole Animal Respirometry (P_{crit} and routine $\dot{M}_{O_2}$)

Most fish maintain a stable $\dot{M}_{O_2}$ over a range of water O_2 levels, termed the O_2 independent pattern of $\dot{M}_{O_2}$, but as O_2 drops below a threshold, $\dot{M}_{O_2}$ decreases as water O_2 levels decline. This is often referred to as the O_2 dependent pattern of $\dot{M}_{O_2}$ (Pörtner and Grieshaber, 1993). The point at which $\dot{M}_{O_2}$ transitions from being independent of environmental O_2 to being dependent on environmental O_2 is referred to as the critical O_2 tension (P_{crit}) and is considered one potential indicator of an animal's tolerance to hypoxia (Chapman et al. 2002; Pörtner and Grieshaber, 1993). P_{crit} was determined for each sculpin species by measuring $\dot{M}_{O_2}$ in a sealed respirometer using a fiber optic O_2 probe (FOXY-R, Ocean Optics Ltd., Florida, USA). Briefly, fish were placed into a size-matched respirometer (20 mL/g) and held overnight under flow-through conditions (seawater for the marine sculpins and freshwater for the freshwater sculpins). Throughout the recovery and respirometry periods the respirometers were held in a temperature regulated water bath at 12°C. After the recovery period, the O_2 probe was secured into the respirometer and the respirometer sealed to prevent O_2 exchange between the inside of the respirometer and the outside water chamber. The O_2 probe was connected to a data acquisition system that recorded the steady decline of O_2 in the water as the fish consumed O_2 in the respirometer. The experiment was terminated either when the fish lost equilibrium or when

there was no further O_2 decline recorded by the probe. Mass specific $\dot{M}_{O_2}$ was calculated over 10 minute sequential periods and P_{crit} was determined as the first derivative of $\dot{M}_{O_2}$ vs. PO_2 using the visual basic program described by Yeager and Ultsch (1989). In our hands, we saw no differences in P_{crit} determined using closed or open respirometry, therefore the potential accumulation of CO_2 and ammonia that could occur during a typical trial does not affect $\dot{M}_{O_2}$. Routine $\dot{M}_{O_2}$ was calculated over the O_2 independent zone, which is well above the critical O_2 threshold.

Blood and Tissue Sampling

To obtain resting, normoxic tissue samples, individual fish were housed overnight in sampling baskets, which were submerged in well-aerated 580 liter tanks containing appropriate water (seawater for marine sculpins and freshwater for freshwater sculpins). The sample baskets were 5 liter plastic chambers with mesh sides and a 1 liter basin at the bottom. To sample a fish, the chamber was carefully removed, confining the fish to the 1liter basin and an overdose of benzocaine (250 mg/L, Sigma-Aldrich) was introduced into the chamber and the fish lost equilibrium within approximately one minute. The fish was removed, patted dry, weighed, and a blood sample was taken following caudal severance using a heparinized hematocrit (Hct) tube. Hematocrit tubes were placed on ice until processing could occur (<5 minutes). Liver, muscle, brain, heart and the left gill basket were dissected from each fish and immediately frozen in liquid N_2 and stored at -80°C until analysis. The right gill basket was placed into Karnovsky's fixative (Karnovsky, 1965) for later determination of gill surface area (see below).

From the sampled blood, 5 μL was added to 1 mL of Drabkin's solution (Sigma-Aldrich, USA) for determination of whole blood [Hb]. Approximately 2 μL of whole blood was set aside on ice for the analysis of whole blood Hb-O_2 affinity (see below), while the remainder of the blood was centrifuged (AUTOCRIT Ultra 3, Becton Dickinson and Company, New Jersey) at

13,700 g for 3 minutes. Hematocrit was obtained and RBCs were separated from plasma, and both were frozen in liquid N_2 and stored at -80°C.

On fish captured in 2007, blood samples were taken as described above, except whole blood was washed in heparinized Cortland saline (Wolf, 1963) and centrifuged at 5,000 g for 2 minutes at 5°C. The supernatant was drawn off and the RBC pellet was immediately frozen in liquid N_2 for later determination of pHi.

Preparation of RBC Hemolysates

Frozen RBC pellets were thawed on ice and 20 mM Tris buffer (pH 7.4) was added at 12 times the estimated RBC volume. Samples were vortexed and left on ice for 5 minutes before centrifugation at 15,000 g for 10 minutes at 4°C. From the resulting supernatant, aliquots (10-20 μL) were taken and frozen for determination of [ATP], [GTP], [Mg^{2+}] and [Hb]. Hemolysate [Hb] was used to standardize [ATP], [GTP] and [Mg^{2+}]. The remaining cell hemolysate was immediately stripped of Hb modulators (ATP and GTP) by loading the hemolysate onto Micro Bio-spin P30 Tris chromatography columns (Bio-Rad Laboratories) followed by centrifugation at 1000 g for 4 minutes at 4°C. Aliquots of the stripped Hb were set aside for the determination of stripped blood Hb-O_2 binding affinity (see below), met-Hb analysis, reconstituted Hb-O_2 binding affinity (see below) and Hb isoform analysis. All four aliquots were frozen and stored at -80°C.

In order to determine if ATP and GTP were the primary RBC Hb modulators in sculpins, measured RBC [ATP] and [GTP] were added back to the stripped Hb hemolysates in an attempt to reconstitute whole RBC Hb-O_2 binding affinity. Red blood cell hemolysates were thawed on ice and [Hb] quantified immediately. Samples of stripped Hb were reconstituted to the same [ATP]/[Hb] and [GTP]/[Hb] ratio measure as whole cell lysates (see below) and immediately analyzed for Hb-O_2 binding affinity as described below. To verify [ATP] and [GTP] in the reconstituted samples, concentrations of total NTP were determined spectrophotometrically

using the enzyme-coupled assays (glyceraldehydes-3-phosphate dehydrogenase and phosphoglycerate phosphokinase) as described by Bergmeyer (1983). There was no significant difference between the nominal sum of [ATP] and [GTP] and measured total [NTP] (data not shown).

ANALYTICAL PROCEDURES

Gill Morphometrics

Total gill surface area was determined according to the protocol described by Hughes (1984). The total number of filaments along one side of the 2nd gill was counted under a stereomicroscope and the length of every 5th filament was measured. Under a compound microscope the average lamellar spacing was determined by measuring the distance occupied by 10 lamellae. Between 7 and 8 lamellae were dissected free of the filament and the total area of each lamella was determined using AutoMontage software (Syncroscopy, Maryland, USA). Total gill surface area was calculated from $A = Lnbl$, where L is the total filament length (mm) on all gill arches from both gill baskets, n is the number of lamellae/mm on both sides of the filament, and bl is the average bilateral surface area of the lamellae (mm^2; Hughes, 1984).

Blood Hb-O_2 Affinity (P_{50})

The principles of measuring blood Hb-O_2 affinity (P_{50}) in small volumes of blood (<2 μL) are outlined by Reeves (1980). Briefly, approximately 1 μL of blood was sandwiched between two gas permeable membranes and loaded into a prototype PWee50 generously loaned by Dr. P. Frappell (LaTrobe U.; Australia). The analysis chamber was regulated at 12°C, and CO_2, O_2, and N_2 gases were mixed and delivered to the chamber by a Corning 192 Precision Gas Mixer. CO_2 was kept constant at 0.5%, but O_2 levels were varied throughout the experiment in order to determine the percent saturation of Hb at different O_2 levels. Percent saturation of Hb was measured using absorption at wavelengths of 393 nm and 435 nm. Blood samples were exposed to approximately 7 to 9 different O_2 tensions and measurements were recorded to

construct a linear section of the Hill plot for the determination of Hb P_{50} (Frappell et al., 2002). Hill's coefficient (n) was determined as the slope around half saturation in a plot of log(Y/(1-Y)) versus log PO_2.

Blood [ATP], [GTP] and Hb Isoforms

High performance liquid chromatography (HPLC) using Gilson 322 was used to determine blood [ATP] and [GTP] and Hb isoform profiles, according to the protocols outlined by Feuerlein and Weber (1994). Separate HPLC runs were performed for nucleotide triphosphates and Hb isoform determinations. Briefly, for the analysis of ATP and GTP, aliquots of frozen RBC hemolysates were thawed and immediately deproteinized with 3% perchloric acid and then neutralized with 3M Tris-Base. The samples were centrifuged at 20,000 g for 5 minutes at 4°C to remove any precipitates and 20 μL of the neutralized extract was injected onto an anion exchange Mono-Q 5/50 GL column (GE Health Care, USA). Following injection, buffer A (A=20 mM Tris, pH 8.0) was kept at 100% for 5 minutes. Between 5 and 22 minutes there was a linear decrease in buffer A to 70% buffer B (B=15 mM Tris, 0.5 mM NaCl, pH 8.0). Buffer B was then increased to 100% by 23.5 minutes and kept constant until 28.5 minutes when buffer A was increased to 100% by 30 minutes and maintained at that concentration for a subsequent 5 minutes (Feuerlein and Weber, 1994). Nucleotide triphosphates were detected at 254 nm and ATP and GTP peaks were identified by comparison to retention times of known standards and quantified by comparisons to a standard curve prepared daily. [ATP] and [GTP] were standardized to [Hb] determined on the RBC hemolysate.

For Hb isoform analysis, prepared stripped hemolysates were thawed on ice and buffer A was added to dilute Hb sample 7.2 times. The solution was filtered through a 0.2 μm low protein binding syringe filter (Acrodisc, Pall Corporation, USA) and 20 μL of the filtrate was injected onto the same anion exchange column used above. The HPLC separation protocol was identical to that used above and Hb isoform samples were monitored at 415nm. Hb isoforms were

identified according to the protocol described by Feuerlein and Weber (1994), who determined that the most cathodic isoforms will elute first and that the most anodic isoforms will elute last in an anion exchange column. To confirm that there was consistency between the current study and that of Feuerlein and Weber (1994), trout blood was run through the column, resulting in similar pattern of elution of isoforms between the two studies.

Magnesium, Met-Hb and RBC pHi

Red blood cell [Mg^{2+}] was determined in the frozen hemolysates using flame atomic absorption spectrometry (SpectrAA 240FS, Varian, Australia). Met-Hb was determined spectorphotometrically using the protocol established by Benesch et al. (1973) and samples always contained <15% met-Hb. To measure RBC pHi, approximately 100 μL of metabolic inhibitor cocktail (Pörtner et al., 1991) was added to frozen RBC pellets and vigorously mixed to facilitate cell lyses and left on ice for at least 10 minutes. Red blood cell pHi was measured using BMS 3 Mk2 (Radiometer, Copenhagen) capillary microelectrode at a regulated temperature of 12°C.

Phylogenetic Analyses

Genomic DNA was extracted from liver of 3 individuals of each species of sculpins using DNeasy Tissue Kit (Qiagen, Canada). Cytochrome b (*cyt b*) gene sequence was amplified from each genomic DNA sample by the polymerase chain reaction (PCR) using primers L14724 and H15915 from Schmidt and Gold (1993). The PCR product was gel purified and extracted using GenElute Gel Extraction Kit (Sigma-Aldrich, USA) and sequenced directly using BigDye Terminator v3.1 chemistry and high throughput sequence analysis (Applied Biosystems 3730S 48-capillary sequencer, NAPS, UBC). For each sample, the resulting PCR product was sequenced in both directions and a consensus sequence generated. Sequences were aligned using ClustalW and formatted as a nexus file in Mega 3.1 (Kumar, Tamura and Nei, 2004).

Sequences were imported into PAUP* (version 4, Sinauer Associates, Inc. Publishers, USA) to construct both maximum likelihood and maximum parsimony gene trees. Although both analyses gave similar results, maximum likelihood gene tree was chosen and Modeltest (Posada and Crandall, 1998) was used to determine the likelihood ratio test that best fit the sequence data. The tree was constructed using the general time reversal (GTR) DNA substitution model with a gamma distribution of 1.075 and proportion of invariant sites set to 0.545. Nucleotide frequency was relatively evenly distributed (A=0.237, C=0.388, G=0.126, and T=0.249). A heuristics search was used to create the tree with bootstrap analysis of 100 pseudoreplicates and the starting tree option of stepwise addition. Bayesian trees were also created using MrBayes 3.0 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

Phylogenetically Independent Contrasts

The maximum likelihood tree and branch lengths were imported into Mesquite (Maddison and Maddison, 2004) and the PDAP module (Midford et al., 2003) was used to analyze PIC. Additionally we imported 10,000 trees created through Bayesian analysis into Mesquite and performed the standardized contrasts on the simulated trees in order to determine the robustness of the PIC analysis in terms of the uncertainty in topology and branch lengths (Martins and Housworth, 2002). The PIC analysis using either maximum likelihood tree or Bayesian trees yielded similar results (Bayesian analysis not shown). The results of PIC analysis also did not change appreciably when branch lengths of the maximum likelihood tree were set to one (data not shown).

Since analysis can only be performed on complete data sets, we pruned the phylogenetic tree in Mesquite to include only the species for which there were available character data. Therefore, we excluded the outgroup, *Satyrichthys amiscus* whose *cyt b* sequence (Accession No. AP004441) was used to root the tree. The *cyt b* sequence from *Cottus bairdii* (Accession No. AY833333) was included in order to resolve a polytomy between *L. armatus*, *C. asper* and the

remainder of the sculpin family (cf. sculpin phylogeny in Kinziger and Wood, 2003). *Cottus bairdii* was subsequently pruned from the tree for PIC analysis. An additional tree was constructed (results not shown) containing only the species of sculpins with available character data and there was no significant effect on the results generated by PIC analysis.

It is critical to verify if independent contrasts have been adequately standardized by plotting the absolute value of the standardized independent contrast versus its standard deviation (Garland et al., 1992). There was no significant linear or nonlinear trend in the independent contrasts of each character, therefore the contrasts were adequately standardized according to methods described by Garland et al. (1992). The standardized independent contrasts of different characters were subsequently plotted and analyzed by regression analysis that were forced through the origin.

Statistical Analyses

Data are presented as means ± standard error. Phylogenetically independent contrast correlations were analyzed for significance in Mesquite, while conventional (non-PIC) correlations were analyzed for significance using SigmaStat 3.0. Multiple linear regression models were developed on phylogenetically standardized contrasts and analyzed for significance using SPSS 11.0. Statistical significance was assumed at $P<0.05$.

Results

Phylogeny and Species Distribution

The present study utilized 12 species of sculpins for which a well-resolved phylogeny was developed using the *cyt b* gene (Fig. 2.1). Among the marine sculpins available, representatives were captured from various areas of the intertidal and subtidal environment with a good distribution of maximum depth along the nearshore environment (Fig. 2.1; maximum depth from Froese and Pauly, 2007).

CRITICAL OXYGEN TENSION

Critical O_2 tension (P_{crit}) varied between the 12 species of sculpins (Fig. 2.1) and was significantly related to their individual maximum depth distribution. There was a significant PIC correlation between published species maximum depth location (Froese and Pauly, 2007) in the nearshore environment and P_{crit} (r^2=0.57, *P*=0.01, correlation not shown). Sculpins with high P_{crit} values are only found in the subtidal and deeper water environments while sculpins with the low P_{crit} values inhabit the intertidal zone (Fig. 2.1).

Routine $\dot{M}_{O_2}$ ranged from 2.2 to 4.5 μmol/g/hr among the 12 species of sculpins (Fig. 2.1). Using conventional correlation analysis there was no significant relationship between P_{crit} and routine $\dot{M}_{O_2}$ (Fig. 2.2A, *P*=0.15); however, when corrected for the phylogenetic relationship between species using PIC, the positive relationship was significant (Fig. 2.2B, *P*=0.04, PIC). As routine $\dot{M}_{O_2}$ increased there was a corresponding increase in P_{crit}.

There was an inverse relationship between P_{crit} and mass specific gill surface area (Fig. 2.2C, Table 2.1), such that as mass specific gill surface area decreased there was an increase in P_{crit}. The relationship was significant when tested with conventional correlation analysis (*P*=0.04, Fig. 2.2C) and improved when corrected for phylogeny (*P*=0.03, Fig. 2.2D). The large differences in total surface area between species was primarily due to large differences in filament number, filament length, lamellar area and body weight (Table 2.1). No significant relationship existed between species weight and mass specific gill surface area (r^2<0.01, *P*=0.94; data not shown), indicating that the variation in fish weight among species (see Table 2.1) had only a minor impact on mass specific gill surface area.

Whole blood Hb-O_2 P_{50} ranged from 23 torr in the intertidal *O. maculosus* to 58 torr in the subtidal *B. cirrhosus* (Table 2.4) and there was a significant positive correlation between whole blood Hb-O_2 P_{50} and P_{crit} with both conventional correlation analysis (*P*=0.01, Fig. 2.2E)

and with the application of PIC (P=0.03, Fig. 2.2F, PIC). The freshwater *C. asper* was removed from the correlation between P_{crit} and whole blood Hb-O_2 P_{50} because it was found in a previous study that freshwater adaptation and exposure caused a thickening of the gill membrane leading to an increase in respiratory diffusion distance (Henriksson et al., In press). The thickening of the gills caused a mismatch between P_{crit} and whole blood Hb-O_2 P_{50} that decreased with acclimation to seawater.

Variation in blood Hct (11 to 35%), [Hb] (0.1 to 1.3 mM), and a mean cellular Hb content (MCHC; 1.3 to 3.7) was observed among the species examined (Table 2.2); however, there was no relationship between these variables and P_{crit} using either conventional or PIC correlations (Table 2.5).

Phylogenetically corrected multiple linear regression analysis revealed that whole blood Hb-O_2 P_{50}, routine $\dot{M}_{O_2}$ and mass specific gill surface area combined explain 83% of the variation in P_{crit} among the species of sculpins (P=0.01). To maintain the number of species consistent, r^2 used for the multiple regression analysis was calculated after the removal of two species. *Artedius harringtoni* was removed due to lack of available data on whole blood Hb-O_2 P_{50}, and *C. asper* was removed due to confounding effects of freshwater adaptation on whole blood Hb-O_2 P_{50} (see above).

STRIPPED HB-O_2 BINDING AFFINITY AND RBC MODULATORS

Stripped Hb showed a similar pattern of Hb-O_2 P_{50} as whole blood Hb-O_2 P_{50}, but at much reduced O_2 tensions (Table 2.4). Both conventional (P=0.03, Fig. 2.3A) and phylogenetically independent contrasts (P=0.03, Fig. 2.3B) showed a significant positive correlation between whole blood Hb-O_2 P_{50} and stripped Hb-O_2 P_{50}.

Red blood cell [ATP]/[Hb] ranged between 0.69 and 2.43 in all species of sculpins except *E. bison* and *S. marmoratus* where [ATP]/[Hb] ratios were at nearly undetectable levels. There

was no significant relationship (conventional or PIC correlations) between [ATP]/[Hb] and P_{crit} (Table 2.5), or between [ATP]/[Hb] and whole blood Hb-O_2 P_{50} (Table 2.6). Red blood cell [GTP]/[Hb] ranged between 0.10 and 0.51 among sculpins except in *A. fenestralis* which exhibited very high levels (2.33) of GTP and *S. marmoratus* that had barely detectable levels of GTP (0.02; Table 2.2). Red blood cell [Mg^{2+}]/[Hb] did not vary appreciably between sculpins (Table 2.2). There was no correlation between [GTP]/[Hb] or [Mg^{2+}]/[Hb] and P_{crit} (Table 2.5, conventional and PIC) or between [GTP]/[Hb] or [Mg^{2+}]/[Hb] and whole blood Hb-O_2 P_{50} (Table 2.6, conventional and PIC).

Red blood cell pHi did not vary appreciably among the sculpin species (ranging between 7.18 and 7.42; Table 2.2) and there was no significant correlation (conventional or PIC corrected) between pHi and whole blood Hb-O_2 P_{50} (Table 2.6).

The estimated number of total Hb isoforms determined using anion-exchange chromatography varied between species but was generally between 3 and 10 isoforms (Table 2.3). The number of anodic Hb isoforms ranged between 2 and 9 among the sculpins examined, while cathodic Hb isoforms ranged between 0 in some species and up to 3 isoforms in other sculpin species (Table 2.3). There was no relationship (conventional or PIC) between the number of total, anodic, or cathodic isoforms and P_{crit} (Table 2.5) or whole blood Hb-O_2 P_{50} (Table 2.6).

The addition of measured RBC [ATP] and [GTP] to stripped Hb lysate almost fully reconstituted the whole blood Hb-O_2 P_{50}, and there was a significant correlation between reconstituted and whole blood Hb-O_2 P_{50} (conventional P=0.03, Fig 2.3C; PIC P=0.02, Fig. 2.3D). The correlations were significant despite two notable exceptions, *E. bison* and *S. marmoratus*, whose stripped Hb lysate we were unable to reconstitute back to whole blood Hb-O_2 P_{50}.

Although Hill's coefficient (n) did not vary to any great degree between different species of sculpins (Table 2.4), n did change depending on the amount of organic phosphates present in

the blood (Fig. 2.4). In whole blood, *n* was approximately 1.6 in all species examined (Table 2.4), and the removal of ATP and GTP increased *n* to about 2.6 (Fig. 2.4, Table 2.4) and this increase was seen across the species. When ATP and GTP were added back to stripped Hb lysates, *n* decreased back to approximately 1.7 (Table 2.4). There was no significant correlation between *n* in whole, stripped or reconstituted blood and whole blood Hb-O_2 P_{50} (Table 2.6, conventional and PIC).

DISCUSSION

Variation between sculpins in their ability to effectively extract O_2 from the environment may play a crucial role in dictating species distribution along the marine nearshore environment. I demonstrated a phylogenetically independent relationship between P_{crit}, habitat range, and the physiological parameters involved in environmental O_2 extraction. Intertidal sculpins experiencing diurnal fluctuations in O_2 possess a low P_{crit}, indicating an ability to maintain a routine $\dot{M}_{O_2}$ at lower environmental O_2 than subtidal or deeper water species that experience minimal fluctuations in O_2 and possess higher P_{crit} values. Strong effects of hypoxia on species distribution have also been demonstrated in other intertidal organisms such as the triplefin fishes (Brix et al., 1999), coral reef fishes (Nilsson et al., 2007) and on the distribution of populations of *Mytilus edulis* (Altieri, 2006), with the more hypoxia tolerant species located in the O_2 variable environments.

Critical O_2 tension is a composite measure of an animal's ability to extract O_2 from the environment. Whole animal O_2 extraction involves many components of the respiratory chain that can be differentially modified to maintain O_2 uptake during environmental hypoxia (Hughes, 1973). In our hands, 83% of the variability in P_{crit} among sculpins can be attributed to variation in routine $\dot{M}_{O_2}$, mass specific gill surface area, and whole blood Hb-O_2 binding affinity with species possessing a low P_{crit} having a low routine $\dot{M}_{O_2}$, large gill surface areas, and high whole

blood Hb-O_2 binding affinities. Although it has long been assumed that adjustments to the respiratory chain which enhance O_2 uptake would be adaptive to hypoxia survival (eg. Jensen, 1991 and Saint-Paul, 1984), this is the first study to demonstrate through phylogenetically independent contrasts convergent evolution of traits that aid in enhanced O_2 extraction capacity. This higher O_2 extraction capacity occurs in species of sculpins that are frequently exposed to O_2 variable environments.

Routine $\dot{M}_{O_2}$ is an important factor shaping the respiratory cascade because it is an index of the ultimate demand for O_2. In sculpins, although a simple correlation does not show a significant relationship, a phylogenetically independent correlation does indicate that there is a significant positive relationship between P_{crit} and routine $\dot{M}_{O_2}$ (Fig. 2.2B). A lower routine $\dot{M}_{O_2}$ could be beneficial to species that are frequently exposed to O_2 variable environments, such as the intertidal sculpins, as a lower $\dot{M}_{O_2}$ can be maintained over a greater range of PO_2 values than a higher $\dot{M}_{O_2}$.

Gill surface area is an important factor influencing an animal's ability to extract O_2 from the water. Within the sculpin family, there is a phylogenetically corrected significant correlation between P_{crit} and mass specific gill surface area (Fig. 2.2D). This indicates that a larger gill surface area in sculpins with a low P_{crit} is an adaptive modification to the respiratory chain as it provides an increased extraction efficiency of O_2 from the environment. A large gill surface area has previously been demonstrated in fish species frequently exposed to bouts of hypoxia such as the Amazonian *Colossoma macropomum* (Saint-Paul, 1984), salt marsh dwelling *Poecilia latipinna* (Timmerman and Chapman, 2004b), *Hoplias malabaricus* (Fernandes et al., 1993), and populations of *Pseudocrenilabrus multicolor*, *Gnathonemus victoriae* and *Petrocephalus catostoma* inhabiting dense swamp regions (Chapman et al., 2002).

Conflicting views exist on whether or not evolutionary adaptation occurs in whole blood Hb-O_2 binding affinity. Jensen (1991) has proposed that since a high whole blood Hb-O_2 binding affinity is often associated with hypoxia tolerant animals there must be a strong positive selection on Hb-O_2 binding affinities. However, a recent study by Milo et al. (2007) found that whole blood Hb-O_2 binding affinity does not vary among different mammals, while cooperativity between Hb subunits (Hill's coefficient) varies drastically. Under changing physiological conditions, however, the opposite trend is noted and Hill's coefficient remains constant, while the greatest change occurs with whole blood Hb-O_2 binding affinity. Milo et al. (2007), therefore, conclude that evolutionary adaptations act on Hill's coefficient, while 'physiological adaptations' act primarily on whole blood Hb-O_2 binding affinity.

Within sculpins, there is little variation in Hill's coefficient (Fig. 2.4), while a clear phylogenetically independent correlation exists between P_{crit} and whole blood Hb-O_2 binding affinity (Fig. 2.2F), suggesting that evolutionary adaptation within sculpins predominantly acts upon whole blood Hb-O_2 binding affinity. Sculpins that possess a higher P_{crit} have a lower Hb-O_2 binding affinity and are found lower in the nearshore environment. Meanwhile sculpins possessing higher Hb-O_2 binding affinity have an increased capacity to extract O_2 from the environment and thereby can tolerate more hypoxic conditions such as the ones that are routinely encountered in isolated tidepools at night.

Previous studies have suggested that an increase in O_2 carrying capacity can be accomplished through an increase in blood [Hb] and Hct (Chapman et al. 2002; Hochachka and Somero, 2002; Timmerman and Chapman, 2004a). Although sculpins inhabiting the tidepools do show higher Hct and [Hb] than the subtidal and freshwater species there is no significant correlation between hypoxia tolerance and these blood parameters under resting normoxic conditions. This is unlike the study conducted by Chapman et al. (2002), where they found that fish species dwelling in hypoxic swamps showed greater O_2 carrying capacity through higher

Hct and [Hb] than normoxic lake-dwelling fish species. In the sculpin family, it appears that the modifications to the O_2 carrying capacity of blood is primarily achieved through changes in whole cell Hb-O_2 binding affinities.

Whole blood Hb-O_2 binding affinity can be set by both the intrinsic properties of the Hb and the allosteric interactions between Hb and its modulators (Hochachka and Somero, 2002; Weber and Lykkeboe, 1978). Variation in whole blood Hb-O_2 binding affinity among different species of sculpins is primarily dictated by the intrinsic properties of the Hb protein. When all the major modulators of Hb are removed from the blood, the intrinsic (stripped) Hb-O_2 binding affinity significantly correlates with whole blood Hb-O_2 binding affinity in a phylogenetically independent manner (Fig. 2.3B). These differences in intrinsic Hb-O_2 binding affinity can be achieved through amino acid substitutions as seen in the bar-headed goose (Jessen et al., 1991; Perutz, 1983) or through variation in number and functional heterogeneity of Hb isoforms (Brix et al., 1999; Rutjes et al., 2007). Brix et al. (1999) found that the triplefin fishes located in O_2 variable tidepools and shallow water possess a greater number of Hb isoforms that are predominantly the higher Hb-O_2 binding affinity cathodic isoforms. Meanwhile triplefin fishes inhabiting the mid-depth and deeper waters express the lower O_2 affinity anodal isoforms and a decreasing number of Hb isoforms. In the Lake Victoria cichlid, *Haplochromis ishmaeli*, there is a functional switching of Hb isoforms during hypoxia acclimation to higher O_2 affinity isoforms (Rutjes et al., 2007). In sculpins, although a variation is seen in the number of Hb isoforms and proportion of anodic to cathodic Hb isoforms, there is no relationship between the number of total, anodic, or cathodic Hb isoforms expressed by a species and hypoxia tolerance. This suggests that the variation in intrinsic Hb-O_2 binding affinity may be due to differences in amino acid substitutions, which will be the focus of future work.

Whole blood Hb-O_2 binding affinity can also be dictated by the concentration of allosteric modulators such as ATP and GTP, whose binding to the Hb causes a reduction in

whole blood Hb-O_2 binding affinity. During hypoxia exposure, Hb-O_2 binding affinity increases through reductions in the allosteric modulators to bring about short-term improvements in O_2 uptake from the environment (Weber and Lykkeboe, 1978). ATP and GTP are the two major Hb modulators in most sculpins examined in this study as indicated by our ability to fully reconstitute whole blood Hb-O_2 binding affinity by adding measured [ATP] and [GTP] back to stripped Hb (Fig. 2.3D). However we were unable to reconstitute whole blood Hb-O_2 binding affinity in two species, *E. bison* and *S. marmoratus* suggesting that these two species may possess different Hb modulators. Neither ATP or GTP showed a significant phylogenetically independent correlation to whole blood Hb-O_2 binding affinity, indicating that even though the modulators may contribute to the overall Hb-O_2 binding affinity, they probably do not determine the variation seen between the different species.

Rutjes et al. (2007) has shown that hypoxia tolerant Lake Victoria cichlids have higher [ATP] and [GTP] under normoxic conditions compared with the relatively hypoxia intolerant salmonids. In sculpins a similar, although non-significant, trend is observed in RBC [ATP], with hypoxia tolerant species inhabiting the tidepools having higher [ATP] than the more hypoxia intolerant subtidal and freshwater species (Table 2.2). Higher [ATP] coupled with high whole blood Hb-O_2 binding affinity in hypoxia tolerant sculpins instill a significant capacity to endure not only severe hypoxia, but also large fluctuations in environmental O_2. A decrease in Hb modulators during hypoxia exposure has been demonstrated in other fish species (Jensen and Weber, 1982; Lykkeboe and Johansen, 1975; Weber and Lykkeboe, 1978) and presumably a similar regulation of Hb modulators occurs in hypoxia tolerant sculpins providing a plasticity in Hb-O_2 binding affinities necessary for coping with fluctuating environmental O_2.

The removal of organic phosphates, ATP and GTP, also causes an increase in Hill's coefficient that is consistent in all species examined (Fig. 2.4). An increase in Hill's coefficient indicates an increase in the degree of cooperativity between Hb subunits, adding an increased

benefit to species frequently exposed to hypoxia. In other species of fish, such as *Cyprinus carpio*, an increase in Hill's coefficient due to a decrease in Hb modulators is only maintained at pH below 6.5, and reverses at pHs above 6.5 (Tan and Noble, 1973). However, when temperature decreases from 20°C to 10°C, the shift between higher Hill's coefficient in stripped Hb hemolysates to a higher Hill's coefficient in whole blood occurs at a pH of 7.0. Since cooperativity of Hb subunits clearly involves a complex interaction of many physiological parameters such as temperature and pH, it is not surprising that there are difference between the current study and that of Tan and Noble (1973). In sculpins, it is an advantage to have the ability to increase Hb subunit cooperativity due to a decrease in Hb modulators at a relevant physiological pH since presumably a decline in RBC ATP and GTP will occur during an exposure to hypoxia to increase whole blood Hb-O_2 binding affinity. Coupling an increase in Hb subunit cooperativity with an increase in whole blood Hb-O_2 binding affinity will enhance O_2 extraction, aiding in survival during a bout of hypoxia.

Low routine $\dot{M}_{O_2}$, high gill surface area, and high whole blood Hb-O_2 binding affinity are characteristics that hypoxia tolerant species possess prior to hypoxia exposure. These traits allow an animal to maintain a 'normoxic' level of O_2 consumption even at significantly reduced O_2 tensions. This prolongs the period of time an animal can remain in low O_2 environment prior to eliciting a hypoxia response, such as a down-regulation of Hb allosteric modulators or a decrease in metabolic rate. For animals living in the nearshore marine environment this is ideal, as it allows for the ability to cope with diurnal fluctuations in O_2 levels without impacting cellular functioning. The focus of future work will be to examine a number of biochemical properties, such as the glycolytic enzyme capacity, during normoxia that may also help play a role in dictating hypoxia tolerance in sculpins. Additionally, future research will focus on

characterizing behavioral, physiological and biochemical responses of sculpins exposed to severe hypoxia.

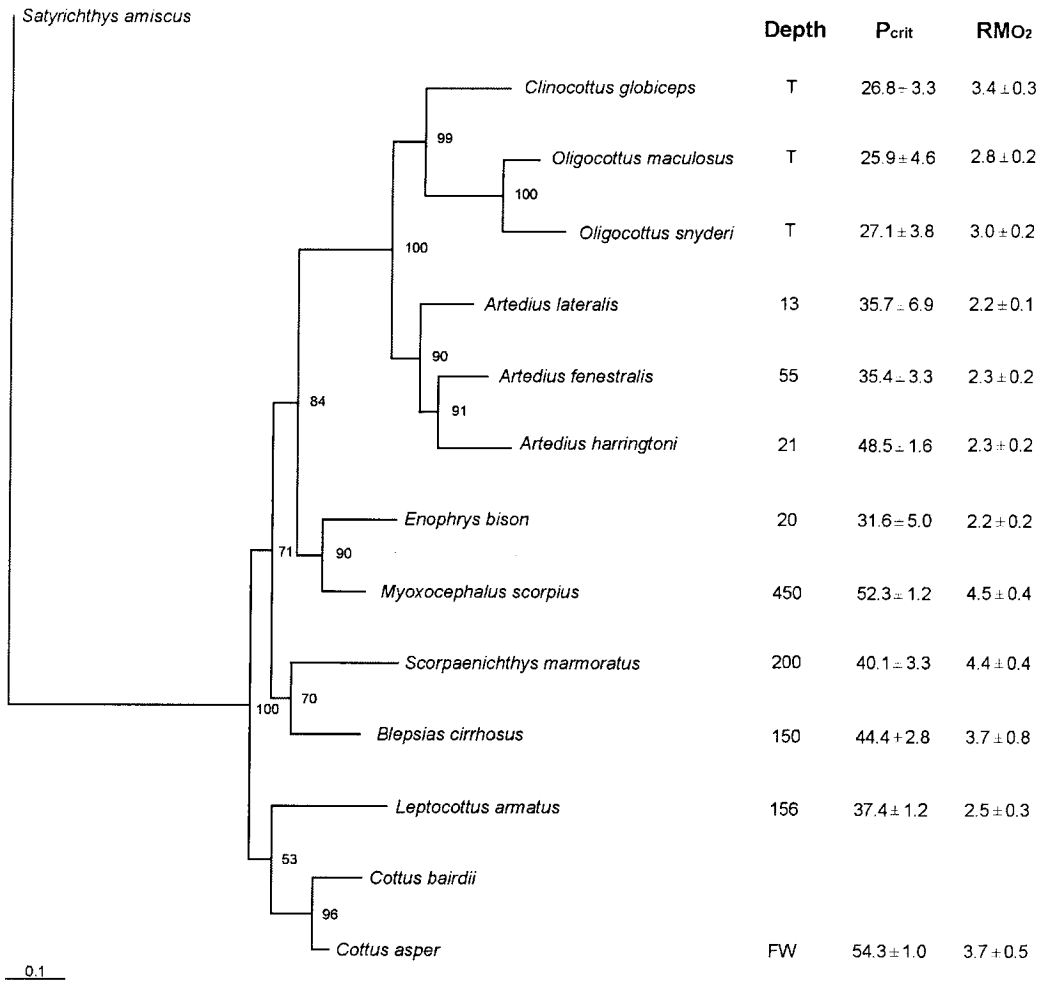

Figure 2-1. Phylogenetic relationship of 13 species of sculpins based on a maximum likelihood tree using *cyt b* sequences. Node bootstrap values are shown for groups with >50% support. *S. amiscus* is included as an outgroup species. Character data are presented for maximum depth of a species (meters), with T representing tidepool and FW representing freshwater. P_{crit} (torr) and routine $\dot{M}_{O_2}$ (μmol/g/hr) are also included with n = 6 to 9 except for *A. lateralis* and *A. harringtoni* where n = 4 and n = 3 respectively. Data are means ± SE.

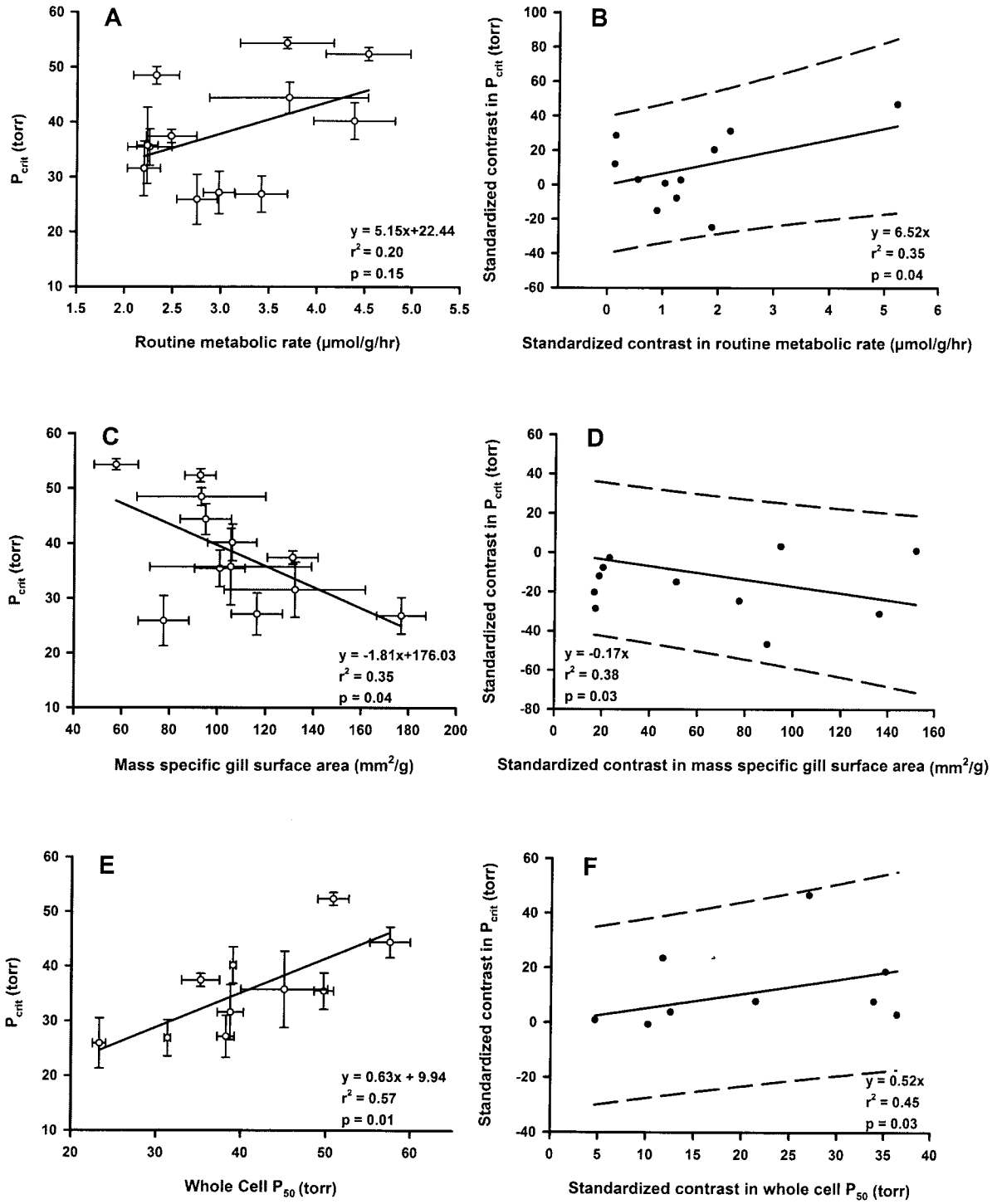

Figure 2-2. Relationship between P_{crit} and routine $\dot{M}_{O_2}$ (A,B), P_{crit} and mass specific gill surface area (C,D) and P_{crit} and whole blood Hb-O_2 P_{50} (E,F). (A,C,E) are conventional correlations and (B,D,F) are standardized independent contrasts. Error bars in (A,C,E) represent SE for x and y values. Dashed lines in (B,D,F) represent 95% prediction intervals.

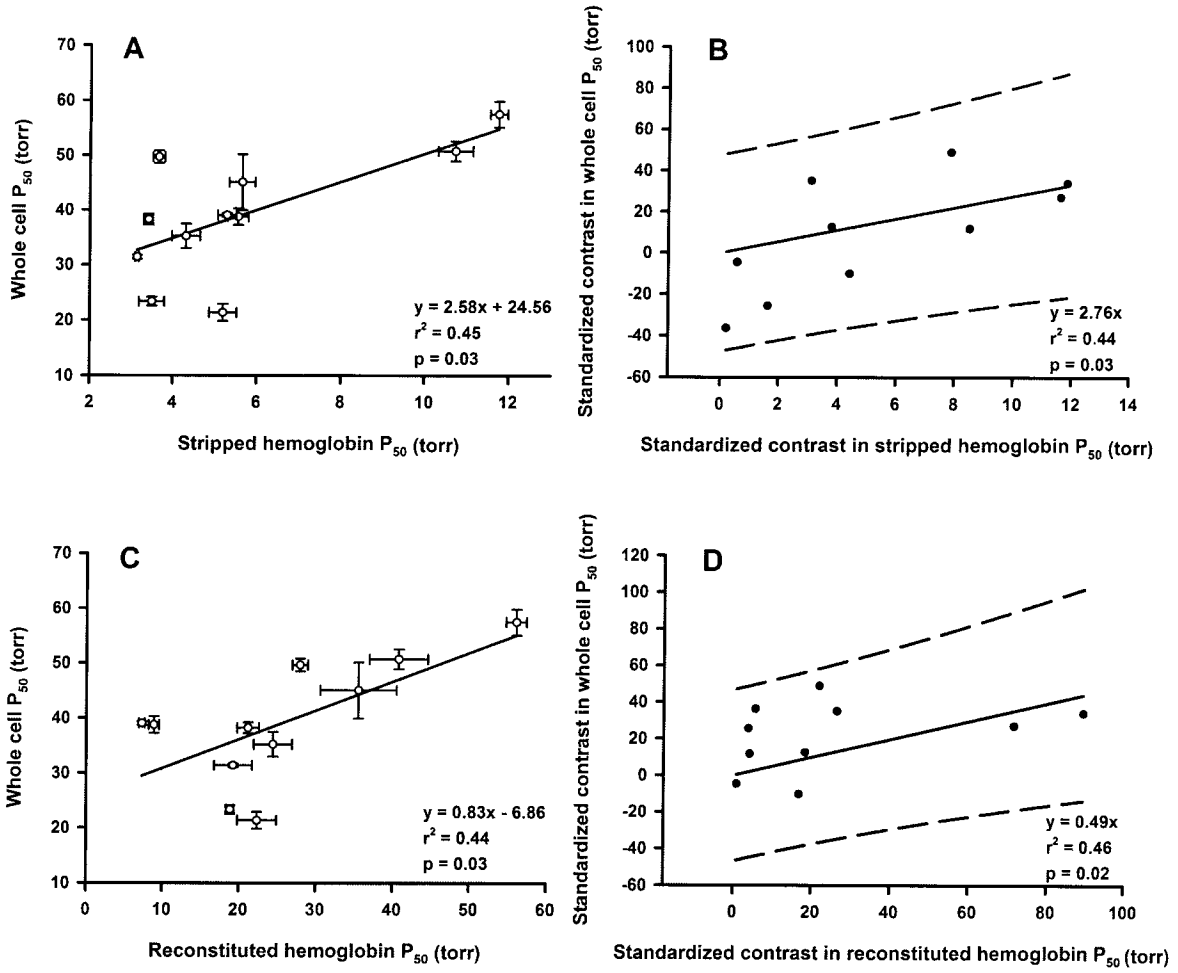

Figure 2-3. Relationship between whole blood Hb-O_2 P_{50} and stripped Hb-O_2 P_{50} (A,B) and whole blood Hb-O_2 P_{50} and reconstituted Hb-O_2 P_{50}. (A,C) are conventional correlations and (B,D) are standardized independent contrasts. Error bars in (A,C) represent SE for x and y values. Dashed lines in (B,D) represent 95% prediction intervals.

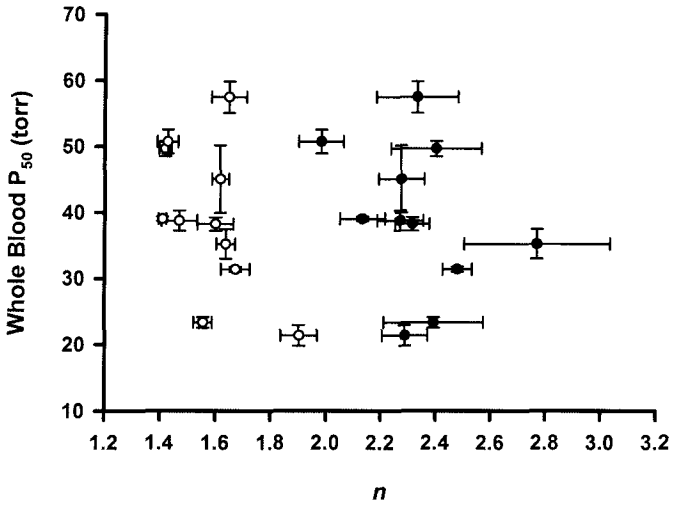

Figure 2-4. Relationship between whole blood Hb-O_2 P_{50} and Hill's coefficient (n) measured in whole blood (open circle) and stripped (filled circle). Error bars around the symbols represent SE for x and y values.

Table 2-1. Fish weight and gill morphometrics from 12 species of sculpins.

	Weight	Filament number	Filament length	Lamellar area	Total surface area	Mass specific surface area
O. maculosus	4.5±0.5	22±1	1.7±0.1	0.03±0.01	365±71	78±11
C. globiceps	1.6±0.2	25±1	1.3±0.1	0.03±0.01	285±45	177±10
O. snyderi	2.5±0.7	21±1	1.3±0.1	0.03±0.01	263±50	116±11
E. bison	10.9±5.3	38±5	2.0±0.3	0.03±0.01	935±350	132±30
A. fenestralis	16.9±2.1	35±1	2.8±0.1	0.05±0.01	1613±135	101±10
A. lateralis	15.2±1.4	38±1	2.5±0.1	0.07±0.03	1703±701	105±34
L. armatus	48.2±2.1	67±2	4.1±0.1	0.09±0.01	6320±588	131±11
S. marmoratus	20.8±2.9	59±3	2.9±0.2	0.04±0.01	2211±429	106±10
B. cirrhosus	2.6±0.4	28±1	1.3±0.1	0.02±0.01	264±51	95±11
A. harringtoni	4.1±1.1	27±1	1.5±0.2	0.02±0.01	286±32	93±27
M. scorpius	9.7±2.7	46±3	2.0±0.2	0.03±0.01	861±213	92±27
C. asper	19.3±1.8	33±1	2.3±0.1	0.06±0.01	1003±75	57±31

Data are means ± SE. Fish weight is presented in g, filament length in mm, lamellar area and total surface area in mm^2 and mass specific gill surface area is presented in mm^2/g. Sample size ranged from n = 8 to 9, except for *A. lateralis* where n = 4 and *A. harringtoni* where n = 3.

Table 2-2. Blood hematocrit, hemoglobin, mean cellular hemoglobin content, hemoglobin modulators, RBC intracellular pH from 11 species of sculpins.

	Hct	Hb	MCHC	ATP/Hb	GTP/Hb	Mg^{2+}/Hb	pHi
O. maculosus	35±2 (8)	1.3±0.1 (5)	3.7±0.2 (3)	1.82±0.18 (8)	0.25±0.06 (8)	29±2 (5)	7.35±0.03 (12)
C. globiceps	35±1 (8)	1.3±0.1 (6)	3.4±0.3 (2)	2.43±0.09 (8)	0.51±0.03 (8)	37±2 (4)	7.26±0.05 (2)
O. snyderi	37±3 (8)	1.3±0.1 (3)	3.2±0.7 (2)	2.18±0.06 (3)	0.10±0.01 (3)	N/A	7.26±0.02 (7)
E. bison	22±2 (8)	0.6±0.1 (7)	2.5±0.2 (7)	0.04±0.01 (6)	0.28±0.08 (6)	25±2 (3)	7.27
A. fenestralis	27±2 (8)	0.9±0.1 (8)	3.5±0.2 (8)	0.91±0.06 (8)	2.33±0.16 (8)	28±2 (8)	7.32±0.04 (6)
A. lateralis	24±2 (4)	0.9±0.1 (4)	3.5±0.4 (4)	1.44±0.09 (3)	0.40±0.07 (3)	24±1 (4)	7.30±0.06 (4)
L. armatus	11±1 (8)	0.1±0.1 (8)	1.3±0.2 (7)	1.47±0.13 (8)	0.33±0.05 (8)	35±2 (8)	7.29±0.02 (8)
S. marmoratus	23±1 (8)	0.8±0.1 (16)	2.8±0.1 (15)	0.01±0.01 (7)	0.02±0.01 (7)	22±1 (7)	7.18
B. cirrhosus	29±1 (8)	0.7±0.1 (6)	2.7±0.1 (14)	1.38±0.04 (3)	0.18±0.02 (3)	N/A	7.42±0.02 (12)
M. scorpius	25±2 (8)	0.7±0.1 (6)	2.7±0.2 (6)	0.69±0.13 (6)	0.22±0.03 (6)	28±1 (6)	N/A
C. asper	27±2 (8)	0.8±0.1 (15)	3.0±0.1 (15)	1.04±0.17 (8)	0.19±0.04 (8)	24±1 (7)	7.28±0.01 (8)

Data are means ± SE. Hematocrit (Hct) is presented in %, hemoglobin (Hb) in mM, mean cellular hemoglobin content (MCHC) in [Hb]/Hct, [ATP], [GTP] and [Mg^{2+}] are presented relative to [Hb]. pHi represents RBC intracellular pH. Numbers in brackets indicate sample size.

Table 2-3. Hemoglobin isoforms from 11 species of sculpins.

	Total Isoforms	Anodic Isoforms	Cathodic Isoforms
O. maculosus	4	4	0
C. globiceps	9	6	3
O. snyderi	4	3	1
E. bison	9	8	1
A. fenestralis	9	6	3
A. lateralis	10	7	3
L. armatus	5	5	0
S. marmoratus	4	4	0
B. cirrhosus	8	8	0
M. scorpius	3	3	0
C. asper	10	9	1

A representative from each species was used to determine the total number of hemoglobin isoforms, as well as the number of cathodic and anodic isoforms.

Table 2-4. Hb-O_2 P_{50} and Hill coefficient (*n*) in whole red blood cell, stripped blood, and reconstituted blood in 11 species of sculpins.

	Whole P_{50}	Stripped P_{50}	Reconstituted P_{50}	*n* (whole)	*n* (stripped)	*n* (reconstituted)
O. maculosus	23.3±0.8	3.5±0.3	18.9±0.5	1.56±0.03	2.39±0.18	1.66±0.06
C. globiceps	31.4±0.4	3.1±0.1	19.2±2.5	1.67±0.05	2.48±0.05	1.64±0.02
O. snyderi	38.7±1.0	3.4±0.1	21.2±1.4	1.60±0.06	2.31±0.06	1.71±0.06
E. bison	38.7±1.5	5.6±0.2	8.9±0.7	1.47±0.06	2.27±0.08	1.62±0.12
A. fenestralis	49.7±1.2	3.7±0.1	27.9±1.0	1.42±0.02	2.40±0.16	1.52±0.01
A. lateralis	45.1±5.1	5.7±0.3	35.5±5.0	1.62±0.03	2.27±0.08	1.48±0.02
L. armatus	35.2±2.2	4.3±0.3	24.4±2.5	1.64±0.03	2.77±0.27	1.67±0.07
S. marmoratus	39.0±0.4	5.3±0.2	7.3±0.4	1.41±0.01	2.13±0.08	2.10±0.15
B. cirrhosus	57.5±2.4	11.7±0.2	56.0±1.3	1.65±0.06	2.33±0.15	1.71±0.04
M. scorpius	50.7±1.8	10.7±0.4	40.7±3.8	1.43±0.04	1.98±0.08	1.59±0.04
C. asper	21.4±1.6	5.2±0.3	22.3±2.5	1.90±0.07	2.29±0.08	1.63±0.02

Data are means ± SE. All Hb-O_2 P_{50} values are represented in torr. For Hb-O_2 P_{50} and Hill coefficient (*n*) in whole red blood cell n = 6 to 9, and in stripped and reconstituted blood n = 3 to 4.

Table 2-5. Relationship between P_{crit} and hematological parameters, hemoglobin modulators, and hemoglobin isoforms of sculpins using conventional and phylogenetically independent contrast (PIC) correlations.

	Conventional			PIC		
Parameter	slope	r^2	*P*	slope	r^2	*P*
Hct	-0.53	0.16	0.23	0.31	0.04	0.57
Hb	-13.81	0.24	0.12	3.70	0.01	0.77
MCHC	-4.42	0.09	0.37	4.41	0.07	0.44
ATP/Hb	-5.79	0.21	0.15	-0.47	<0.01	0.92
GTP/Hb	-1.97	0.02	0.70	-0.50	<0.01	0.91
Mg^{2+}/Hb	-0.76	0.14	0.31	-0.46	0.05	0.55
Total Hb isoforms	0.17	<0.01	0.89	-0.45	0.02	0.66
Anodic Hb isoforms	1.24	0.07	0.43	-0.47	0.02	0.72
Cathodic Hb isoforms	-2.46	0.11	0.33	-1.32	0.02	0.64

Table 2-6. Relationship between whole RBC Hb-O_2 P_{50} and hemoglobin modulators, hemoglobin isoforms, RBC intracellular pH and Hill coefficients (*n*) of sculpins using conventional and phylogenetically independent contrast (PIC) correlations.

	Conventional			PIC		
Parameter	slope	r^2	*P*	slope	r^2	*P*
ATP/Hb	-3.58	0.06	0.46	1.43	<0.01	0.83
GTP/Hb	0.02	0.08	0.41	3.80	0.04	0.54
Mg^{2+}/Hb	-0.34	0.03	0.68	0.21	0.10	0.80
Total Hb isoforms	-0.01	<0.01	0.99	-0.83	0.04	0.54
Anodic Hb isoforms	-0.18	<0.01	0.92	-1.35	0.07	0.43
Cathodic Hb isoforms	0.43	<0.01	0.88	-0.02	<0.01	0.99
RBC pHi	62.14	0.12	0.32	48.05	0.09	0.40
n (whole)	-38.79	0.25	0.11	-0.01	0.17	0.21
n (stripped)	-16.89	0.09	0.37	-0.01	0.03	0.61
n (reconstituted)	-7.24	0.01	0.76	-0.01	0.02	0.67

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CHAPTER THREE: BEHAVIORAL, PHYSIOLOGICAL AND BIOCHEMICAL STRATEGIES IN RESPONSE TO HYPOXIA EXPOSURE IN FAMILY COTTIDAE

INTRODUCTION

Fish that experience hypoxia require a well-coordinated suite of responses to ensure survival. Upon exposure to hypoxia, fish may initially employ behavioral strategies of avoidance, followed by physiological and biochemical strategies to either enhance O_2 uptake from the environment or initiate responses to reduce tissue and cellular reliance on O_2. The common behavioral avoidance strategies utilized by fish include aquatic surface respiration (ASR) and aerial emergence (Martin, 1995; Watters and Cech, 2003; Yoshiyama et al., 1995), both of which allow an animal to access more O_2 rich environments. Aquatic surface respiration involves a fish selectively accessing the better oxygenated water-air interface, while voluntarily emergence involves moving out of the water to respire in air.

Although there is some discrepancy between studies, fish that actively emerge are generally able to maintain O_2 consumption rates in air that are similar, or reduced only by 25%, compared to those measured in water (Martin, 1996; Sloman et al., 2008; Wright and Raymond, 1978; Yoshiyama and Cech, 1994). In fact, tidepool sculpins forcibly emerged on moist substrate for 72 hours showed no measurable increase in whole body lactate levels suggesting that the fish are able to extract sufficient O_2 to maintain a completely aerobic metabolism under the relatively inactive conditions of aerial emergence (Sloman et al., 2008). Although there appears to be a limited physiological cost to aerial emergence, emergence behavior does involve an increased risk of aerial predation (Sloman et al., 2006; Yoshiyama et al., 1995). In response to perceived predation, fish will delay the performance of these behaviors (Sloman et al., 2008), and thus

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must invoke physiological or biochemical adjustments to survive periods of environmental hypoxia.

The ability to enhance O_2 uptake during confinement in hypoxia is primarily mediated by modifications to Hb-O_2 binding affinity. Reductions in RBC Hb modulators, such as ATP and GTP, occur in many fish species during hypoxia exposure, causing an increase in Hb-O_2 binding affinity leading to increased O_2 loading from the hypoxic environment (Jensen and Weber, 1982; Lykkeboe and Johansen, 1975; Weber and Lykkeboe, 1978). Tetens and Lykkeboe (1981) demonstrated a tight correlation between a stepwise increase in Hb-O_2 binding affinity and a decrease in RBC [ATP] in trout (*Oncorhynchus mykiss*). Decreases in RBC [ATP] and [GTP] directly affect Hb-O_2 binding affinity through a reduction in the allosteric binding of the modulators to the cavity between the β chains of the Hb molecule, and indirectly through an alteration of Donnan distribution of H^+ across the cell membrane resulting in an elevated RBC pH (Hochachka and Somero, 2002; Jensen et al., 1998).

If modulation of Hb-O_2 binding affinity is not sufficient to maintain adequate O_2 supply to tissues to meet metabolic requirements, an up-regulation of O_2 independent pathways for ATP production is one important component in prolonging survival during hypoxia. As O_2 becomes limiting, there is a switch in energy provision from one based on oxidative phosphorylation to one based primarily on substrate level phosphorylation (i.e. glycolysis and CrP hydrolysis; Hochachka et al., 1996). Another important defense mechanism against hypoxia is a down-regulation in cellular energy demand, primarily achieved through a significant decrease in major energy consuming processes such as protein synthesis and ion pumping (Buck et al., 1993a; Buck et al., 1993b, Lewis et al., 2007). Decreasing energy demand while attempting to maximize O_2 independent energy production are critical biochemical adjustments essential to hypoxia survival when processes involved in maximizing O_2 uptake fail.

Comparison among species of sculpins from the family Cottidae is an ideal way to understand the adaptive traits involved in hypoxia tolerance. The distribution of sculpins along the marine nearshore environment is associated with critical O_2 tension (P_{crit}), such that the species with lowest P_{crit} are found in the highly O_2 variable tidepools and the species with higher P_{crit} inhabit the O_2 stable subtidal zone (see Chapter 2). Previous work with sculpins has focused on elucidating the physiological characteristics of fish under normoxic conditions that might be of adaptive value for hypoxia survival, such as gill surface area and Hb-O_2 binding affinity (see Chapter 2).

The present study focused on the behavioral, physiological and biochemical defenses of different species of sculpins to hypoxia exposure. Specifically, I examined the relationship between P_{crit} and behavioral responses, such as ASR and aerial emergence, in 12 species of sculpins. Hepatic glycogen and CrP, were also assessed in different sculpins to determine if there is a relationship between capacity to sustain substrate-level phosphorylation and P_{crit}. Three species of sculpins, which were chosen because of their low, medium and high P_{crit} values, were exposed to severe hypoxia and the concentrations of Hb modulators and glycolytic metabolites were analyzed.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

Marine and freshwater sculpins were obtained near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada and housed at University of British Columbia as described in Chapter 2. Briefly, marine sculpins were caught using handheld nets or seines during the lowest tidal cycles of June and August 2006 at Wizard Islet (48°51.5′N; 125°9.4′W) and Ross Islets (48°52.4′N; 125°9.7′W), while freshwater sculpins, *Cottus asper*, were caught using baited minnow traps in Pachena Lake (48°50'11" N; 125°01'44" W). Marine sculpins obtained are representatives inhabiting various areas of the marine nearshore environment and

the specific maximum depth for each species is listed in Chapter 2 Figure 2.1. Briefly, *Oligocottus maculosus*, *Oligocottus snyderi*, and *Clinocottus globiceps* are found in the high to low tidepool areas, while the remainder of the species are found in the subtidal or deeper water, including *Enophrys bison*, *Artedius fenestralis*, *Artedius lateralis*, *Artedius harringtoni*, *Scorpaenichthys marmoratus*, *Leptocottus armatus*, and *Blepsias cirrhosus* (Eschmeyer and Herald, 1983; Froese and Pauly, 2007). An additional deep-water marine sculpin species, *Myoxocephalus scorpius*, was brought in from the Atlantic coast (Memorial University; Newfoundland).

Three separate experiments were conducted. In experimental series 1, ASR and aerial emergence behaviors of 12 species of sculpins were assessed in response to declining O_2. In experimental series 2, the concentration of available on-board fuels in the liver were determined for all 12 species of sculpins. In experimental series 3, Hb modulators and liver metabolites were measured in three species of sculpins that differ in their P_{crit}, *O. maculosus*, *A. lateralis* and *B. cirrhosus*, when subjected to severe hypoxia at O_2 tensions equivalent to 40% of respective P_{crit} levels for up to 6 hours. This relative level of hypoxia was expected to elicit a measurable hypoxic response that could potentially be sustained by each species for number of hours.

EXPERIMENTAL PROTOCOLS

Series 1. Behavior

The threshold O_2 tension at which individual sculpin displayed ASR and aerial emergence behavior was determined using published protocols (Sloman et al., 2006; Watters and Cech, 2003; Yoshiyama et al., 1995). Briefly, individual fish were weighed and placed into a 60 liter aquarium which was maintained at 12°C through partial submergence in a temperature regulated wet table. The aquarium was subdivided into 4 zones and a variation in water depth across the zones was achieved with a 30° angled ramp. Zone 1 included a strip of the entire water column with decreasing water depth in subsequent zones until zone 4 represented an area where

the ramp emerged from the water. Small stones covered the ramp to provide a more natural substrate for the fish and three sides of the aquarium were covered with black plastic to minimize disturbance during the behavior trials.

Fish were allowed to acclimate to the aquarium for one hour before experimentation. During the acclimation period, air was bubbled into the water through an air stone situated along the bottom of zone 1. At the start of the behavioral trial, air was switched to nitrogen (N_2) and water O_2 concentrations were decreased to near zero over a 1 hour period. Water O_2 concentrations were monitored using an Oxyguard Handy MKIII O_2 probe to ensure a consistent rate of O_2 decrease between trials. The O_2 probe was placed in different sections of the aquarium during a control trial and there was no noticeable difference in O_2 levels between the zones.

Every two minutes during a trial, behavior, O_2 concentration, and the zone in which the fish was located were recorded. The behavioral responses under observation were ASR and aerial emergence. The O_2 tension at which fish performed ASR was defined as when fish were seen ventilating at the surface of the water, and the O_2 tension at which aerial emergence occurred was defined as when the head and gill operculae were completely emerged and exposed to the air. The experiment was terminated and O_2 concentration was noted when fish either emerged or lost equilibrium.

Series 2. Metabolic Fuel

To determine the quantity of on-board metabolic fuel in 12 species of sculpins, I examined liver from the same fish that were terminally sampled for blood and various tissues in Chapter 2. Details regarding experimental set-up and results on various measurements on blood and gills have previously been reported in Chapter 2. In the present study, liver samples were analyzed for [ATP], [CrP], [glycogen] and [glucose].

Series 3. Relative Hypoxia Exposure

For this study, we chose three species of sculpins that varied in their P_{crit} and were found on separate clades of the sculpin phylogenetic tree (Chapter 2; Figure 2.1). *O. maculosus* had the lowest P_{crit} at 25.9 torr, *A. lateralis* possessed a P_{crit} at 35.7 torr and *B. cirrhosus* had the highest P_{crit} at 44.4 torr (Chapter 2). The three species of sculpins were selected to determine if the biochemical response caused by hypoxia at a level equal to 40% of a species' respective P_{crit} varied between the species.

Individual fish were placed in well-aerated 5 liter plastic chambers with mesh sides and a 1 liter basin in the bottom and were submerged in 69 liter glass aquaria. For each species, fish were equally divided in two separate aquaria and temperature was maintained at 12°C. Fish were allowed a 24 hour recovery period from handling during which time air was bubbled into all aquaria to maintain O_2 levels at approximately air-saturated water. At the onset of the experimental trial, air was switched to N_2 and within half an hour O_2 levels in all aquaria declined to levels corresponding to 40% of P_{crit} values for each species (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). O_2 levels in the aquaria were monitored with an Oxyguard Handy MKIII O_2 probe and maintained at this level for up to 6 hours.

For all three species of sculpins, fish were meant to be terminally sampled at normoxia, 2, and 6 hours of hypoxia. However, *B. cirrhosus* did not survive past 2 hours of hypoxia and a separate trial was conducted with this species to obtain samples from normoxia and 1 and 2 hours of hypoxia. There was no significant difference in samples from normoxia and 2 hours of hypoxia between the two trials for *B. cirrhosus* (t-test), therefore all samples have been combined. To sample a fish, individual chambers were removed from the aquaria and an overdose of benzocaine (250 mg/L, Sigma-Aldrich) was added to the water remaining in the basin. Fish lost equilibrium within a minute, at which point it was removed from the water, patted dry and weighed (*O. maculosus* = 5.3±0.3 g; *A. lateralis* = 8.4±0.5 g; *B. cirrhosus* =

8.0±0.6 g). Blood was sampled via caudal severance using a heparinized Hct tube and placed on ice. Samples of liver, muscle, heart and brain were dissected and immediately frozen in liquid N_2 and stored at -80°C until further analysis. Hematocrit tubes containing blood were centrifuged at 13,700 g for 3 minutes at which point Hct was calculated and packed RBC were separated from the plasma and both were frozen in liquid N_2 and stored at -80°C.

ANALYTICAL PROCEDURES

Liver Metabolites

Frozen liver samples from both Series 2 and Series 3 experiments were weighed and immediately sonicated on ice in 500 µL of 8% perchloric acid for 5 seconds using a Kontes Micro Ultrasonic Cell Disrupter (Kontes, Vineland, New Jersey). An aliquot of the homogenate was taken for later glycogen digestion and frozen at -80°C. The remaining homogenate was centrifuged at 20,000 g for 5 minutes at 4°C, and the supernatant neutralized with 3M K_2CO_3. Neutralized extracts were centrifuged to remove the precipitates and frozen at -80°C until later analysis. Liver samples from Series 2 were assayed for ATP, CrP, glycogen and glucose and samples from Series 3 were assayed for ATP, CrP, glycogen, glucose and lactate. Neutralized extracts were assayed spectrophotometrically for ATP, CrP, glucose and lactate according to protocols outlined in Bergmeyer (1983). The homogenate set aside for glycogen analysis was thawed, digested to glucose using amyloglucosidase and then assayed for glucose. Glycogen was expressed as µmol glycosyl units/g wet weight.

Red Blood Cell Hemoglobin Modulators

Red blood cell [ATP] and [GTP] were determined using high performance liquid chromatography (HPLC; Gilson 322) according to protocols outlined in Feuerlein and Weber (1994) with modifications presented in Chapter 2. Magnesium concentrations were determined on RBC hemolysates using flame atomic absorption spectrometry (SpectrAA 240FS, Varian, Australia).

STATISTICAL ANALYSIS

Phylogenetically independent contrast correlations were analyzed in the PDAP module (Midford et al., 2003) in Mesquite (Maddison and Maddison, 2004) using the maximum likelihood tree created in Chapter 2 (Figure 2.1). Conventional correlations (non-PIC) were analyzed in SigmaStat 3.0. Results from Series 3 were analyzed with a one-way ANOVA (SigmaStat 3.0) with time as the independent variable followed by the Holm-Sidak pos-hoc test. A two-way ANOVA could not be performed due to differing time points during hypoxia between the three species of sculpins. Alpha was set at 0.05 for all statistical tests.

RESULTS

SERIES 1. BEHAVIOR

Aquatic surface respiration was performed by 100% of the individuals of the three tidepool species, *O. maculosus*, *O. snyderi* and *C. globiceps* (Table 3.1). The remaining eight species of sculpins displayed ASR behavior at lower frequency, ranging from 0% of the individuals of *S. marmoratus* and *B. cirrhosus* to 88% of the individuals of *C. asper*. The O_2 tension at which ASR first occurred varied between species of sculpins, ranging from 23.3 torr in *C. globiceps* to 7.3 torr in *A. fenestralis*. Aerial emergence was consistently performed by the three tidepool species, *O. maculosus*, *O. snyderi* and *C. globiceps*, as well as *L. armatus* and *C. asper* and only a small percent of the individuals of *E. bison* and *M. scorpius* (Table 3.1). The remaining species of sculpins lost equilibrium without displaying aerial emergence (data not shown). Of the species which performed aerial emergence, the O_2 tension threshold for aerial emergence did not vary between the species and was around 10 torr. No correlation existed between percent of individuals of a species performing either ASR or aerial emergence and P_{crit} (Table 3.2; conventional and PIC correlations). In addition, there was no correlation (conventional or PIC) between the percent of individuals of a species performing these behaviors and the species' maximum depth (a proxy measure for habitat distribution; Table 3.3).

SERIES 2. METABOLIC FUEL

There was a high degree of variation in liver [glycogen], [CrP], [glucose] and [ATP] among the normoxia acclimated sculpins (Table 3.4), but there was no correlation between P_{crit} and liver metabolites (Table 3.5; conventional and PIC).

SERIES 3. RELATIVE HYPOXIA EXPOSURE

There was differential survival among the three species of sculpins upon exposure to 40% of their respective P_{crit}. *O. maculosus* survived the full 6 hour hypoxia exposure with no visible signs of distress, while 2 out of the 6 individuals of *A. lateralis* died between the 2 and 6 hour exposure. One hundred percent mortality was observed in *B. cirrhosus* shortly after the 2 hour exposure to hypoxia (data not shown).

Red blood cell [ATP] did not decrease significantly in *O. maculosus* during 6 hours of hypoxia (Fig. 3.1A). However, within 2 hour exposure to hypoxia, RBC [ATP] significantly decreased by 41% in *A. lateralis* and 66% in *B. cirrhosus* (Fig. 3.1A). Red blood cell GTP significantly declined within 2 hour exposure to hypoxia in *O. maculosus* and *A. lateralis* and within 1 hour hypoxia exposure in *B. cirrhosus* (Fig. 3.1B). The relative magnitude of decrease in RBC [GTP] was similar in all three species and ranged between 69% to 74%. There was no significant change in RBC Mg^{2+} during hypoxia exposure in *O. maculosus*, *A. lateralis* and *B. cirrhosus* (Table 3.6). Hematocrit levels did not significantly change during hypoxia exposure in *O. maculosus* and *B. cirrhosus* (Table 3.6), but in *A. lateralis*, Hct levels significantly increased within 6 hours of hypoxia exposure.

Upon hypoxia exposure, liver ATP decreased significantly within 2 hours in *O. maculosus* and *A. lateralis* (Fig. 3.2A). From 2 to 6 hours, ATP remained constant in *A. lateralis*, but significantly increased in *O. maculosus*. In *B. cirrhosus*, ATP significantly declined during the first hour of hypoxia but remained steady for the duration of the exposure (Fig. 3.2A). In all three species, there was a consistent 80 to 87% decrease in liver CrP during the first 2 hours of

hypoxia, but in *O. maculosus* and *A. lateralis* (Fig. 3.2B) the levels remained constant between 2 and 6 hr hypoxia exposure. Liver CrP decreased significantly within the first hour of hypoxia exposure in *B. cirrhosus* with no further decline in CrP (Fig. 3.2B).

In normoxic acclimated sculpins there was large variation in liver [glycogen] among the three species of sculpins, with *O. maculosus* possessing the highest and *B. cirrhosus* possessing the lowest amount of hepatic glycogen (Fig. 3.3). There was a significant decrease in liver [glycogen] in *O. maculosus* by 6 hours of hypoxia exposure. In *B. cirrhosus*, however, a significant drop in [glycogen] was observed within the first hour of hypoxia and by 2 hours there was a 75% decline in glycogen from normoxic levels. There was no significant change in liver [glycogen] in *A. lateralis*. Liver [glucose] remained constant throughout hypoxia in *O. maculosus* and *B. cirrhosus* (Fig. 3.4). In *A. lateralis*, there was no significant change in [glucose] between normoxic and hypoxic fish.

Liver [lactate] significantly increased during hypoxia exposure in *O. maculosus* and *B. cirrhosus* (Fig. 3.5). Within 2 hours of hypoxia exposure, there was a 32% increase in lactate in *O. maculosus* and a 126% increase in *B. cirrhosus*. There was an apparent increase in [lactate] in *A. lateralis* during hypoxia, although this trend was not significant.

DISCUSSION

Surviving periods of hypoxia involves employing a complex suite of responses to either extend an animal's ability to remain active or to defend against consequences of limited cellular O_2. Sustaining routine $\dot{M}_{O_2}$ in face of hypoxia can be achieved through behavioral responses such as ASR and aerial emergence or physiological adjustments such as enhancement in O_2 extraction. However, if O_2 levels drop below an animal's P_{crit}, a depression in metabolic rate and an increase in substrate level phosphorylation are utilized to prevent a catastrophic energy loss leading to necrotic cell death. Many authors (Jensen, 1991; Martin, 1995; Saint-Paul, 1984;

Wood, 1980) have argued adaptive value of behavioral, physiological, and biochemical traits for hypoxia survival, but have done so only in a qualitative fashion that lacks a thorough statistical analysis. The current study, as well as Chapter 2, are the first to employ a phylogenetically independent analysis of carefully selected species to ascertain the adaptive value of characteristics that have been long thought of as critical to an animal's ability to survive hypoxia.

A small number of studies have suggested that differences in ASR and aerial emergence behaviors in sculpins are adaptive and result from different selection pressures created by the differential distribution of the species of sculpins along the intertidal zone (Martin, 1996; Watters and Cech, 2003). The results of the current study support these assumptions to the extent that the tidepool species consistently exhibit these behaviors while the subtidal and deeper water species do not. However, PIC analysis showed no correlation between the presence of these behaviors and P_{crit}, nor between the presence of the behaviors and the distribution of the species of sculpins along the vertical tidal zone (Table 3.2 and 3.3). Therefore, there is no conclusive support for ASR or aerial emergence behaviors being adaptive to hypoxia survival. This may, in part, be due to the limited number of species that consistently display these behaviors thus affecting our ability to detect significance.

Despite the lack of a relationship between P_{crit} and the behavioral responses of sculpins to hypoxia, within the tidepool species examined, *O. maculosus*, *C. globiceps* and *O. snyderi*, there is interesting variation in the pattern of behavioral avoidance that is worthy of discussion. Although all the tidepool species possess similar P_{crit} values (*O. maculosus* = 25.9 torr; *C. globiceps* = 26.8 torr; *O. snyderi* = 27.1 torr; Chapter 2) there are differences in the O_2 threshold at which ASR and emergence behaviors are displayed. Despite low intraspecific variation, there is variation among the tidepool species in the O_2 tensions at which they display avoidance behaviors, suggesting that there are negative consequences associated with employing these avoidance responses. For example, *O. maculosus* are typically found high in the intertidal zone,

inhabiting barren tidepools that lack vegetation as protective covering. As a result, *O. maculosus* may be more susceptible to aerial predation, and therefore may elect to delay the onset of ASR and aerial emergence (Yoshiyama et al., 1995). Aerial predation has been thought to be a factor in delaying ASR and aerial emergence in other fish, such as *Mugil cephalus* and juvenile *Astronotus ocellatus* (Shingles et al., 2005; Sloman et al., 2006). In addition to aerial predation, the variation in O_2 thresholds for the avoidance behaviors may also be due to the differences in the efficiency of O_2 extraction in the three species of sculpins. In *O. snyderi* there is very little difference between ASR and emergence O_2 thresholds compared with the other two species, suggesting that in *O. snyderi* ASR may not be as efficient for maximizing O_2 uptake. This could be due to differences in head morphology and will be investigated as a potential explanation for the variation in the behavioral responses of the three tidepool species.

Sculpins inhabiting the O_2 stable subtidal and deeper water environments exhibited high variation in the number of individuals performing ASR and aerial emergence during exposure to hypoxia. Although a few subtidal species, *S. marmoratus* and *B. cirrhosus*, were never seen to ASR or aerial emerge, other species exhibited variation in this behavior, such that only a percentage of the population performed these behaviors in response to hypoxia (Table 3.1). Martin (1996) demonstrated high mortality rates in three species of subtidal and deeper water sculpins, *Icelinus borealis*, *Jordania zonope* and *Chitonotus pugentensis* when emerged for less than 2 hours, suggesting that subtidal and deeper water sculpins cannot aerially respire as efficiently as the tidepool species, which can survive out of water for at least 72 hours without any measurable deleterious effects (Martin, 1993; Martin, 1996; Sloman et al., 2008). It will be the focus of a future investigation to understand this differential capacity in aerial respiration by comparing species to determine what features, such as gill structure, may differ and therefore be essential for effective aerial emergence.

There were differences in hypoxia survival in three species of sculpins, *O. maculosus*, *A. lateralis*, and *B. cirrhosus*, exposed to the same relative levels of severe hypoxia. This suggests that despite experiencing hypoxia at levels equivalent to 40% of their respective P_{crit}s, the three species of sculpins differ in their physiological and biochemical defense mechanisms for coping with severe O_2 deficit. Among the three species examined there was a relationship between a species' P_{crit} and their ability to survive hypoxia for a prolonged period of time, such that no mortality occurred in the species with the lowest P_{crit}, *O. maculosus*, while 100% mortality occurred in the species with the highest P_{crit}, *B. cirrhosus*, shortly after the initial two hours of hypoxia exposure. Since the sculpins were subjected to hypoxia based on the same percent of P_{crit}, the differences in mortality rates reveal dramatically varied defensive capabilities to hypoxia when O_2 levels drop below P_{crit} levels.

One mechanism for combating hypoxia and maximizing O_2 uptake is the modulation of blood O_2 carrying capacity, through an increase in Hct (Tetens and Lykkeboe, 1981; Brauner and Wang, 1997) or an increase in Hb-O_2 binding affinity via decreases in Hb allosteric modulators (Jensen and Weber, 1982; Lykkeboe and Johansen, 1975; Weber and Lykkeboe, 1978). Despite previous work showing that an increase in Hct is beneficial in increasing blood O_2 carrying capacity (Brauner and Wang, 1997), an increase in Hct does not appear to be the primary means of modulation of blood O_2 carrying capacity in sculpins, since levels remained constant in *O. maculosus* and *B. cirrhosus*, and were only seen to significantly increase in *A. lateralis* by 6 hours of hypoxia exposure (Table 3.6). However, all three species of sculpins exhibited a decline in RBC [ATP] and [GTP] (Fig. 3.1), which should bring about an increase in Hb-O_2 binding affinity and enhance O_2 uptake at low environmental O_2. This is not surprising since a decrease in Hb modulators has been noted in many fish species, including Antarctic fish, such as *Pagothenia borchgrevinki*, which inhabit an O_2 stable environment, indicating that a hypoxia induced decrease in Hb modulators is a highly conserved trait amongst fishes (Wells et al.,

1989). However, there was interspecific variation in RBC [ATP] decline at 2 hours of hypoxia exposure, with a significant 41% and 66% decrease in *A. lateralis* and *B. cirrhosus* respectively and a non-significant decrease in *O. maculosus*. There appears to be a relationship between the degree of RBC [ATP] decrease and P_{crit} among the three species, such that the species with highest P_{crit}, *B. cirrhosus*, exhibited the highest decrease in RBC [ATP] and the species with lowest P_{crit}, *O. maculosus*, exhibited the lowest decrease in RBC [ATP].

An animal's ability to survive hypoxia is not only dependent upon maximizing O_2 uptake but also on the metabolic organization of the tissues (Hochachka and Somero, 2002). An important aspect of this metabolic organization is the ability to maintain metabolic ATP production during hypoxia exposure through an up-regulation of substrate level phosphorylation while limiting tissue energy demands via metabolic rate suppression (Boutilier, 2001; Hochachka et al., 1996; Hochachka and Somero, 2002). As a result, it has been postulated that there should be a relationship between substrate availability, in particular liver glycogen, and an animal's hypoxia tolerance. A survey of different fish species supports this contention with more hypoxia tolerant fish such as *Carassius carassius* and *Carassius auratus* possessing higher liver glycogen levels than more hypoxia intolerant fish such as *Oncorhynchus mykiss* and *Gadus morhua* (van den Thillart and van Raaij, 1995). Although there are large variations in liver glycogen, glucose and CrP and some variation in liver ATP between the 12 species of sculpins acclimated to normoxia in the present study (Table 3.4), there is no phylogenetically independent correlation between these liver substrates and P_{crit}. This suggests that the amount of on-board fuels may not play a dominant role in dictating P_{crit} in sculpins. However, P_{crit} may not be the relevant measure in this case as it is a surrogate measure of whole animal O_2 extraction and not necessarily reflective of the biochemical ability to alter energy use and provision at the tissue. Therefore, the focus of future work will be to employ another measure of hypoxia tolerance,

such as the LT_{50}, in order to determine the relationship between substrate availability and hypoxia tolerance in sculpins.

When liver ATP turnover, calculated from ATP utilization, CrP breakdown and lactate accumulation, is compared between *O. maculosus*, *A. lateralis* and *B. cirrhosus* exposed to two hours at 40% of their respective P_{crit}, there was little difference among species, with ATP turnover rates of 1.0 μmol/g/hr, 0.9 μmol/g/hr and 1.5 μmol/g/hr for *O. maculosus*, *A. lateralis* and *B. cirrhosus* respectively. Overall, independent of the differences in P_{crit}, the rate of ATP turnover was roughly constant between the three species exposed to hypoxia based on the same percent of P_{crit}. However, despite the lack of variation in liver ATP turnover, there was a considerable difference in mortality rates between the three species of sculpins. This suggests that differential defensive mechanisms do exist between the species, with the increased survivorship in sculpins that possess a low P_{crit} being a result of higher liver [glycogen] during hypoxia. Although there appeared to be a relationship between mortality rates and on-board levels of liver glycogen among these three species (Fig. 3.3), the relationship did not exist between liver [glycogen] and P_{crit} when examined broadly across 12 species of sculpins. This suggests that the link between liver [glycogen] and P_{crit} is not a generality but a phenomenon specific to the three species chosen for this experiment.

Liver glycogen mobilization appears to be an important defensive strategy against severe hypoxia in *O. maculosus* and *B. cirrhosus*. In both species, liver [glycogen] decreased dramatically over the duration of hypoxia exposure, although in *B. cirrhosus* the precipitous drop in glycogen occurred much sooner, at two hours of exposure to hypoxia, than in *O. maculosus* where glycogen did not significantly decrease until six hours of hypoxia exposure. In *A. lateralis*, a sculpin species with a mid-range P_{crit}, however, there was no significant change in liver [glycogen]. This may signify that *A. lateralis* relies primarily on the endogenous activation of glycogen breakdown in other tissues as the fuel source for ATP production during severe

hypoxia. On the other hand, it is difficult to draw concrete conclusions regarding liver glycogen in *A. lateralis* since the large variation may mask any potential mobilization of liver glycogen. This variability has been shown in other species, such as *Oncorhynchus mykiss* (Dunn and Hochachka, 1986), *Fundulus heteroclitus* (Fangue et al., In Press), *Rana temporaria* (Smith, 1950), and in both *A. lateralis* and *B. cirrhosus* sampled under normoxia on two separate occasions (cf. Fig. 3.3 and Table 3.4). Since glycogen content appears to be variable between individuals of the same species, it is not surprising that there is no definitive association between liver glycogen and P_{crit} in sculpins.

Liver lactate significantly accumulated in *O. maculosus* and *B. cirrhosus*, with an indication of a similar trend in *A. lateralis* in response to hypoxia (Fig. 3.5). However, the absolute increase of lactate was negligible in all three species over the entire hypoxia exposure. Despite the large liver glycogen depletions in *O. maculosus* and *B. cirrhosus*, [glucose] remained constant in the liver (Fig. 3.4) and [lactate] did not appreciably change, suggesting that the breakdown of liver glycogen into glucose was primarily destined for utilization by other tissues during hypoxia.

We quantified the behavioral response of 12 species of sculpins that range in their P_{crit} values and have determined that, contrary to the literature, no relationship exists between the location of a species along the marine nearshore environment or P_{crit} to the performance of ASR and aerial emergence. However, since only a limited number of species exhibited these behaviors, this had an impact on our ability to detect significance and is worthy of further investigation. Exposing *O. maculosus*, *A. lateralis*, and *B. cirrhosus*, to the same relative level of hypoxia revealed dramatically different mortality rates despite a similar ATP turnover rate in the initial two hours of hypoxia. Comparison of on-board liver [glycogen] among the three species would suggest that a species' ability to survive prolonged period of time in severe hypoxia is due to a higher liver [glycogen]. However, there is no correlation between liver [glycogen] at

normoxia and P_{crit} when examining broadly across 12 species of sculpins.

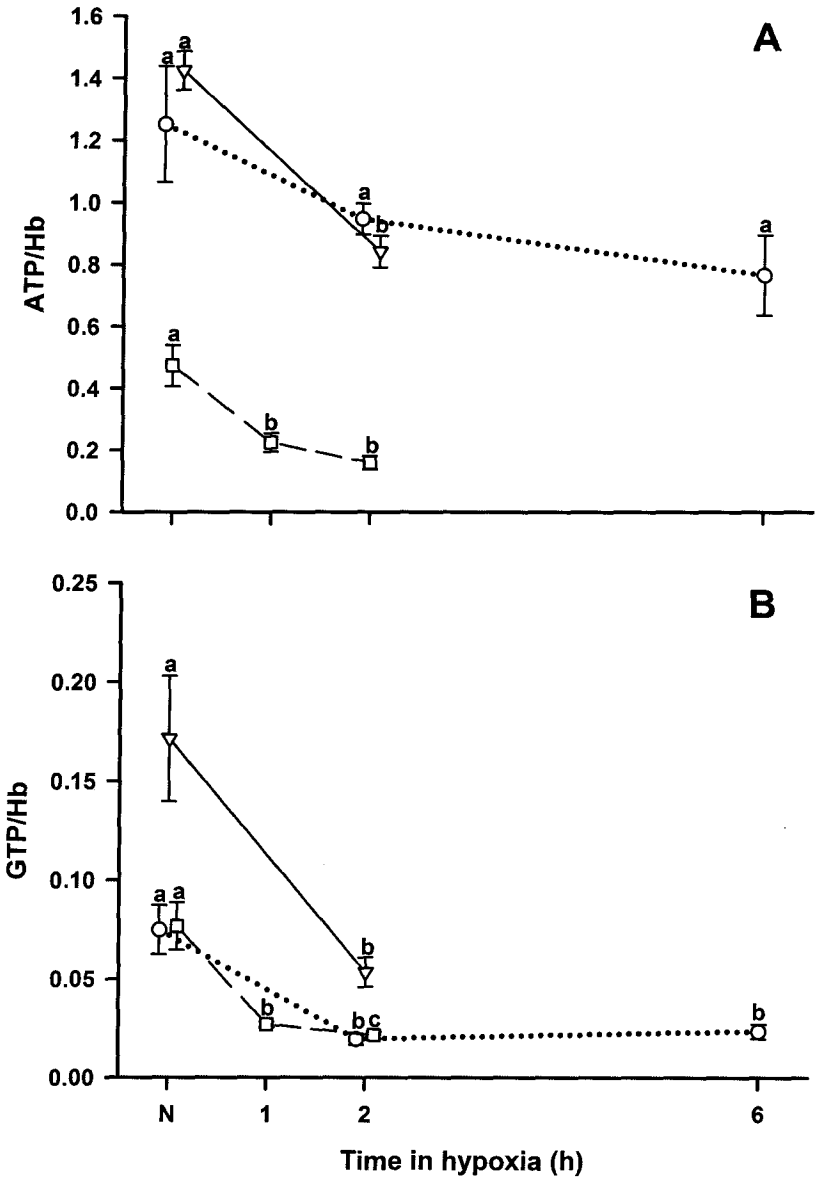

Figure 3-1. Red blood cell ATP (A) and GTP (B) in *O. maculosus* (circle, dotted line), *A. lateralis* (triangle, solid line) and *B. cirrhosus* (square, dashed line) exposed to normoxia and hypoxia equivalent to 40% of respective P_{crit} (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). For ATP and GTP n = 4 to 10, except for *O. maculosus* at 6h hypoxia where n = 2. Data was lost for *A. lateralis* at 6h hypoxia. Overlapping points are offset. Different letters indicate a significant effect of time within a species. Data are means ± SE.

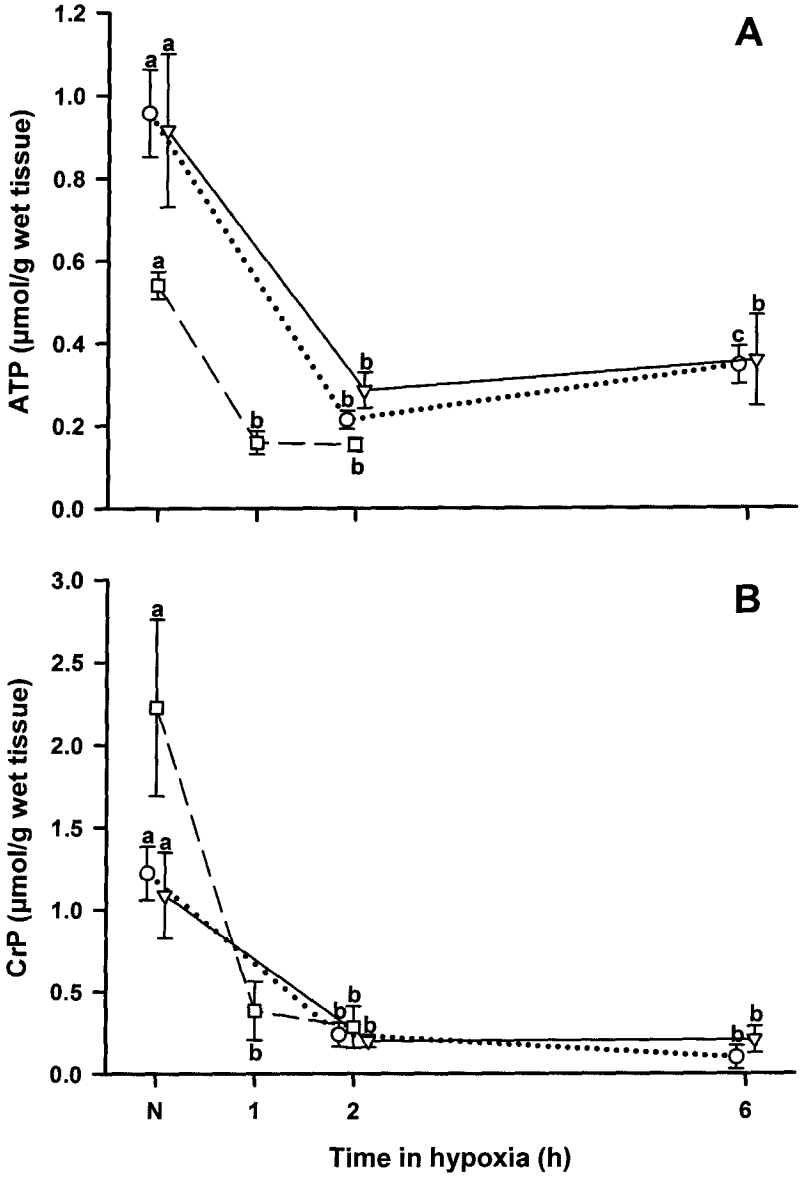

Figure 3-2. Liver ATP (A) and CrP (B) in *O. maculosus* (circle, dotted line), *A. lateralis* (triangle, solid line) and *B. cirrhosus* (square, dashed line) exposed to normoxia and hypoxia equivalent to 40% of respective P_{crit} (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). For ATP and CrP n = 6 to 10, except for *A. lateralis* at 6h hypoxia where n = 4. Overlapping points are offset. Different letters indicate a significant effect of time within a species. Data are means ± SE.

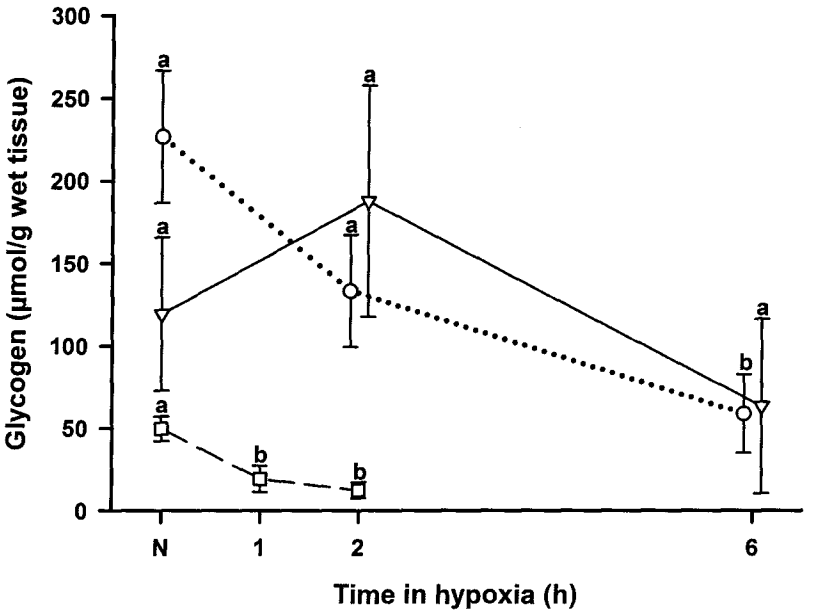

Figure 3-3. Liver glycogen in *O. maculosus* (circle, dotted line), *A. lateralis* (triangle, solid line) and *B. cirrhosus* (square, dashed line) exposed to normoxia and hypoxia equivalent to 40% of respective P_{crit} (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). For glycogen n = 6 to 10, except for *A. lateralis* at 6h hypoxia where n = 4. Overlapping points are offset. Different letters indicate a significant effect of time within a species. Data are means ± SE.

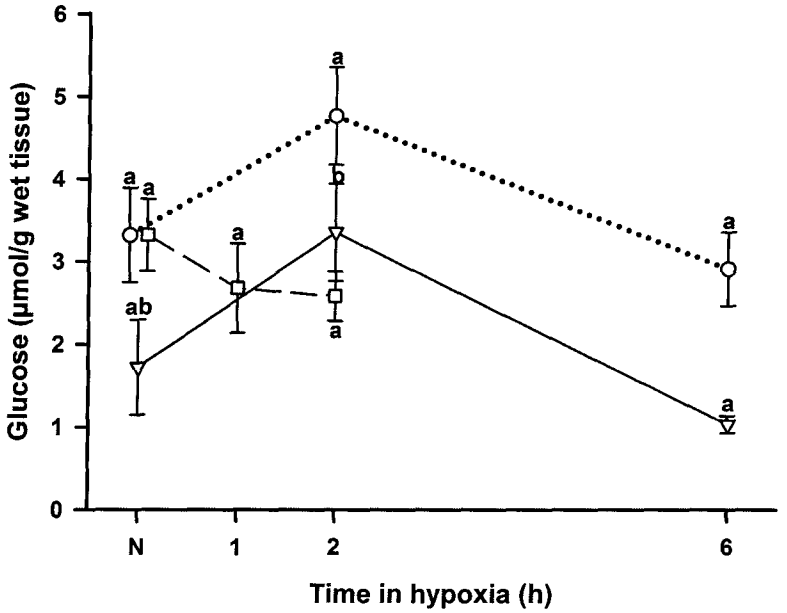

Figure 3-4. Liver glucose in *O. maculosus* (circle, dotted line), *A. lateralis* (triangle, solid line) and *B. cirrhosus* (square, dashed line) exposed to normoxia and hypoxia equivalent to 40% of respective P_{crit} (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). For glucose n = 6 to 10, except for *A. lateralis* at 6h hypoxia where n = 4. Overlapping points are offset. Different letters indicate a significant effect of time within a species. Data are means ± SE.

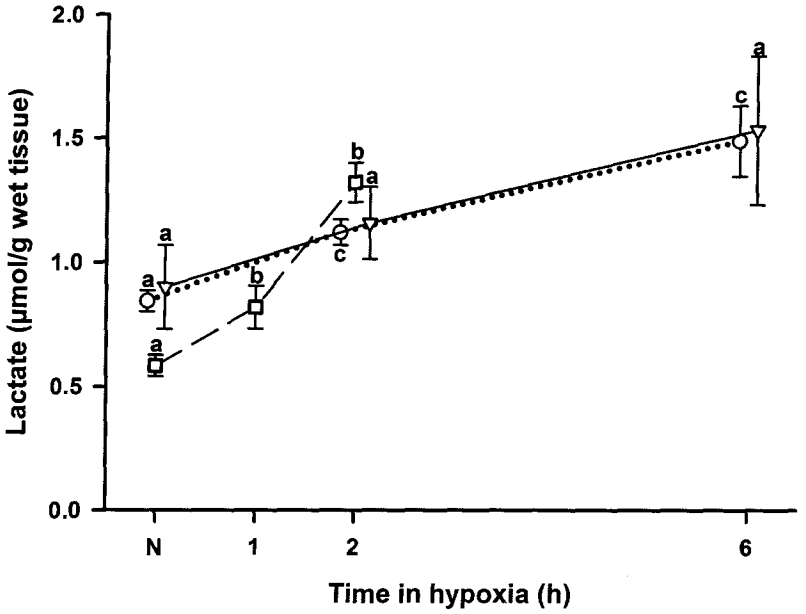

Figure 3-5. Liver lactate in *O. maculosus* (circle, dotted line), *A. lateralis* (triangle, solid line) and *B. cirrhosus* (square, dashed line) exposed to normoxia (~167 torr) and hypoxia equivalent to 40% of respective P_{crit} (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). For lactate n = 6 to 10, except for *A. lateralis* at 6h hypoxia where n = 4. Overlapping points are offset. Different letters indicate a significant effect of time within a species. Data are means ± SE.

Table 3-1. Fish weight and behavioral responses from 11 species of sculpins.

	Weight	ASR	OUT	% ASR	% OUT
O. maculosus	2.9±0.6	15.1±1.4	8.6±0.9	100	100
C. globiceps	1.5±0.2	23.3±1.0	10.0±0.4	100	100
O. snyderi	2.4±0.3	15.1±1.1	13.4±1.1	100	100
E. bison	2.4±0.2	11.7	12.3	17	17
A. fenestralis	16.2±0.6	7.3	-	17	0
A. lateralis	13.9±0.3	13.1±1.8	-	67	0
L. armatus	14.4±3.7	9.2±1.0	10.0±1.0	50	100
S. marmoratus	21.8±3.9	-	-	0	0
B. cirrhosus	2.1±0.2	-	-	0	0
M. scorpius	7.8±1.5	8.3±1.0	10.0±1.2	38	38
C. asper	10.4±1.5	11.3±1.3	9.3±0.8	88	100

Data are means ± SE. Weight is presented in grams, aquatic surface respiration (ASR) and aerial emergence (OUT) are presented in torr, and % ASR and % OUT represent the percent of individuals performing ASR or aerial emergence. 6 to 8 individuals were tested for each behavioral analysis.

Table 3-2. Relationship between P_{crit} and percent of individuals performing aquatic surface respiration (ASR) and aerial emergence (OUT) using conventional and phylogenetically independent contrast (PIC) correlations.

	Conventional			PIC		
Parameter	slope	r^2	*P*	Slope	r^2	*P*
% ASR	-0.09	0.13	0.28	0.04	0.02	0.68
% OUT	-0.05	0.05	0.51	0.01	<0.01	0.94

Table 3-3. Relationship between maximum habitat depth and percent of individuals of species performing aquatic surface respiration (ASR) and aerial emergence (OUT) using conventional and phylogenetically independent contrast (PIC) correlations.

	Conventional			PIC		
Parameter	slope	r^2	*P*	Slope	r^2	*P*
% ASR	-0.07	0.06	0.49	0.02	0.01	0.81
% OUT	-0.04	0.01	0.74	0.03	0.01	0.75

Table 3-4. Liver metabolites in 12 species of sculpins.

	ATP	CrP	Glycogen	Glucose
O. maculosus	1.5±0.2	3.5±0.6	250.4±38.2	2.0±0.3
C. globiceps	0.6±0.2	0.7±0.1	80.0±23.5	1.7±0.3
O. snyderi	0.9±0.2	0.9±0.3	229.8±52.5	2.0±0.4
E. bison	0.7±0.1	1.6±0.4	159.3±27.1	1.6±0.4
A. fenestralis	1.9±0.3	3.1±1.2	352.4±57.4	1.0±0.1
A. lateralis	1.4±0.3	2.2±0.6	328.7±64.4	1.0±0.2
L. armatus	1.0±0.1	0.6±0.1	98.2±17.0	1.4±0.2
S. marmoratus	1.3±0.1	0.4±0.1	394.2±34.6	1.4±0.2
B. cirrhosus	0.8±0.1	0.7±0.1	126.3±29.0	4.2±0.4
A. harringtoni	1.1±0.1	0.5±0.1	87.6±15.3	5.4±0.3
M. scorpius	1.1±0.1	0.5±0.1	296.2±37.3	0.3±0.1
C. asper	1.7±0.2	0.5±0.1	278.7±74.9	1.6±0.2

Data are means ± SE. Liver metabolites are presented in μmol/g of wet tissue. Sample size for liver metabolites ranged from n = 6 to 9, except for *A. harringtoni* where n = 2.

Table 3-5. Relationship between P_{crit} and liver metabolites using conventional and phylogenetically independent contrast (PIC) correlations.

	Conventional			PIC		
Parameter	slope	r^2	*P*	Slope	r^2	*P*
ATP	5.95	0.06	0.44	4.34	0.04	0.50
CrP	-4.76	0.28	0.08	-3.36	0.21	0.14
Glycogen	0.01	0.01	0.72	0.01	0.01	0.73
Glucose	1.18	0.03	0.59	1.12	0.04	0.53

Table 3-6. Hematocrit and red blood cell Mg^{2+} in *O. maculosus*, *A. lateralis* and *B. cirrhosus* exposed to normoxia and hypoxia.

	O. maculosus			*A. lateralis*			*B. cirrhosus*		
Parameter	N	2h	6h	N	2h	6h	N	1h	2h
Hct	48±2[a]	42±3[a]	45±3[a]	33±2[a]	33±2[a]	42±1[b]	35±3[a]	38±2[a]	39±2[a]
Mg^{2+}/Hb	26±3[a]	29±1[a]	29±4[a]	25±2[a]	23±1[a]	N/A	16±2[a]	19±3[a]	18±1[a]

Data are means ± SE. Hematocrit (Hct) is presented in % and $[Mg^{2+}]$ is presented relative to [Hb]. For Hct n = 6 to 10 except for *A. lateralis* at 6h hypoxia where n = 4 and for $[Mg^{2+}]$/Hb n = 4 to 10 except for *O. maculosus* at 6h hypoxia where n = 2. N represents normoxia and hours (h) represent time exposed to hypoxia equivalent to 40% of a species respective P_{crit} (*O. maculosus* = 10.4 torr; *A. lateralis* = 14.9 torr; *B. cirrhosus* = 17.6 torr). Different letters indicate a significant effect of time within a species.

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CHAPTER FOUR: GENERAL DISCUSSION

The goal of the research presented in this thesis was to begin to understand the adaptations responsible for hypoxia tolerance in sculpins. To accomplish this, a number of traits were examined using the comparative method in combination with PIC analysis in closely related species of sculpins. The application of this approach is essential in order to determine which traits are potentially adaptive for hypoxia tolerance, because it is provides insight into two important pieces of information: 1) if the traits have independently evolved numerous times, and 2) if the traits conferring tolerance have evolved only in the species that experience environmental hypoxia. In sculpins, a strong correlation exists between P_{crit} and the species distribution along the nearshore environment. Species of sculpins inhabiting the O_2 variable intertidal possess the lowest P_{crit}, while species of sculpins inhabiting the subtidal and deeper water possess higher P_{crit}. This ecological framework has allowed me to clearly demonstrate that variation in the ability of various sculpins to extract O_2 from their environmental is key to surviving low environmental O_2.

There are three principle components involved in the enhanced O_2 extraction capacity in sculpins possessing a low P_{crit}. According to phylogenetically independent multiple regression analysis, routine $\dot{M}_{O_2}$, mass specific gill surface area and whole blood Hb-O_2 binding affinity contribute to 83% of the variability in P_{crit} among species of sculpins. It appears that a low routine $\dot{M}_{O_2}$, high gill surface area and high Hb-O_2 binding affinity are critical traits involved in the ability of sculpins to survive variable O_2 environments. To date, this is the first study to demonstrate the adaptive value of these traits to hypoxia tolerance. This is in agreement with previous authors (Chapman et al., 2002; Hochachka and Somero, 2002; Saint-Paul, 1984; Wells, 1999) who have suggested that $\dot{M}_{O_2}$, gill surface area and especially Hb, play an important role

in the evolution of hypoxia tolerance in fish, although these authors were not able to directly demonstrate the adaptive value of the traits.

Hemoglobin has been considered for a long time one of the key proteins involved in the evolution of animals to environmental hypoxia. This is most likely true for sculpins, which show that the species with a lower P_{crit} possess a higher whole blood Hb-O_2 binding affinity while the species with a lower P_{crit} possess the lower whole blood Hb-O_2 binding affinity. The variation in whole blood Hb-O_2 binding affinity in sculpins is primarily determined by the intrinsic properties of the hemoglobin protein and not through influences of allosteric modulators. Recent studies have demonstrated the importance of the multiplicity and heterogeneity of hemoglobin isoforms in determining whole blood Hb-O_2 binding affinity (Brix et al., 1999; Wells et al., 1989; Wells, 1999). However, in sculpins there is no evidence that the number of total isoforms or the proportion of anodic to cathodic isoforms of hemoglobin play a significant role in whole blood Hb-O_2 binding affinity. This suggests that the variation in the Hb-O_2 binding affinity among species may be due to differences in the amino acid sequences. Although allosteric modulators do not contribute to setting the variation in whole blood Hb-O_2 binding affinity in sculpins, they are vital components in decreasing the high intrinsic O_2 binding affinity, allowing for unloading of O_2 at the tissues.

Since O_2 levels can drop to near anoxia in tidepools emerged at night (Truchot and Duhamel-Jouve, 1980) sculpins must be equipped to tolerate hypoxic conditions well below the point where O_2 can be efficiently extracted from the environment. Sculpins, specifically those species found in tidepools, employ behavioral responses to hypoxia such as ASR and aerial emergence. These avoidance behaviors are beneficial because they allow fish to maintain routine $\dot{M}_{O_2}$ despite O_2 tensions of the bulk water being at or below P_{crit} (Martin, 1996; Sloman et al., 2008; Wright and Raymond, 1978; Yoshiyama and Cech, 1994). Although Martin (1996) has

suggested that aerial emergence is an adaptation of sculpins to the fluctuating O_2 environment, a lack of a phylogenetically independent correlation between the avoidance behaviors and P_{crit}, suggests that ASR and aerial emergence may not play a large role in the evolution of hypoxia tolerance in sculpins. However, only a small number of species exhibited these behaviors, limiting our ability to detect significance, therefore allowing for the possibility that ASR and aerial emergence may still be important in the evolution of hypoxia tolerance in sculpins. However, for those sculpins that do not possess these behavioral responses to hypoxia, or for those that do but are restricted to hypoxic waters due to a risk of aerial predation, there must be physiological and biochemical defenses that allow sculpins to survive periods of environmental hypoxia.

The key biochemical defenses for surviving low environmental O_2 tensions is a large up-regulation of O_2 independent pathways for ATP production, such as substrate level phosphorylation, and a decrease in metabolic rate through a suppression of energy consuming pathways (Boutilier and St-Pierre, 2000; Hochachka and Somero, 2002). In order to understand how species of sculpins respond to hypoxia, *O. maculosus*, *A. lateralis* and *B. cirrhosus*, were chosen based on the difference in their P_{crit} and exposed to several hours of hypoxia. Despite experiencing O_2 tensions that were equivalent to 40% of respective P_{crit}, mortality rates varied dramatically between the three species of sculpins. There was no mortality in the species with the lowest P_{crit}, *O. maculosus*, while the species with highest P_{crit}, *B. cirrhosus* did not survive beyond a two hour hypoxia exposure. The large variation in mortality rates could be attributed to the dramatic differences in on-board liver [glycogen] between the three species of sculpins. However, liver [glycogen] was examined in twelve species of sculpins and PIC analysis showed that there is no relationship between normoxic levels of liver glycogen and P_{crit} in sculpins. The focus of future work will be to examine in greater detail biochemical defense strategies across a number of sculpin species using phylogenetically independent correlations.

Clearly, the P_{crit} of a sculpin is important as it is linked to the distribution of the species along the nearshore environment and O_2 extraction capacity. However, P_{crit} may be just one proxy measurement for hypoxia tolerance, since it is clear that when species of sculpins are exposed to O_2 tensions well below their P_{crit} threshold mortality rates differ dramatically despite the level of hypoxia being equivalent among the species. Therefore, a secondary measure for hypoxia tolerance such as the LT_{50} will be determined among the species of sculpins. It will be interesting to examine if the variation in P_{crit} is mirrored by a similar variation in LT_{50} and how these two measurements of hypoxia are linked to various aspects of metabolic change during hypoxia. With the application of PIC analysis, it will be possible to determine the role of these metabolic defenses in the evolution of hypoxia tolerance in sculpins.

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