Abstract

Variation in environmental conditions can sometimes impose severe limitations on organismal survival and reproductive success and organisms that live in variable environments have evolved complex traits to cope with or buffer against the environmental conditions. In my dissertation I examined how adaptive evolution and phenotypic plasticity play a role in the ability of nearshore species of sculpin to tolerate low levels of O₂ or hypoxia. I showed that constitutively expressed biochemical traits in the brain correlated with hypoxia tolerance among the different species independent of phylogenetic relationships, suggesting that these traits may have evolved via natural selection in response to hypoxia. A similar correlation was not seen in either the liver or the white muscle.

Next, I showed that transcriptionally mediated phenotypic plasticity is likely associated with the difference in hypoxia tolerance between two species of sculpin. The hypoxia-tolerant tidepool sculpin (Oligocottus maculosus) did not alter gene transcription during the ecologically relevant time-frames of hypoxia exposure (up to 8 hours), in contrast to the hypoxia-intolerant silverspotted sculpin (Blepsias cirrhosus). This suggests that the tolerant species may not rely on phenotypic plasticity during a typical environmental hypoxia exposure and instead may rely on constitutively expressed or fixed traits for survival. Only if hypoxia persists do the hypoxia tolerant tidepool sculpins alter gene transcription, for which a large set of genes showed transcriptional patterns that were divergent to the hypoxia intolerant silverspotted sculpin.

Lastly, I examined if similar transcriptional responses occur among three species of sculpin all with the same measured hypoxia tolerance. While a high proportion (65%) of clones showed similar transcription patterns among the species, a majority of genes associated with metabolism and protein production showed differences in both short and long exposures to hypoxia. As metabolism and
protein production both play a major role in hypoxic survival, transcriptional
differences in genes belonging to these biological processes suggests that the
species likely use different mechanism to achieve similar overall hypoxia tolerance
phenotype. Combined, this work demonstrates how phenotypic plasticity and
adaptive evolution play a role in the variation of hypoxia tolerance among species of
sculpin living in the nearshore environment.
Preface

A version of Chapter 2 has been published as “Mandic M., B. Speers-Roesch and J.G. Richards. 2013. Hypoxia tolerance is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiological and Biochemical Zoology 86: 92-105”. I designed and conducted all experiments, carried out all analyses and wrote the manuscript under the supervision of Dr. J.G. Richards. Dr. B. Speers-Roesch assisted in design and helped carry out the loss of equilibrium experiment. All authors provided editorial input to the manuscript.

A version of Chapter 3 has been published as “Mandic M., M.L. Ramon, A.Y. Gracey and J.G. Richards. 2014. Divergent transcriptional patterns are related to differences in hypoxia tolerance between the intertidal and the subtidal sculpins. Molecular Ecology 23: 6091-6103”. I designed all the experiments, carried out the analyses and wrote the manuscript under the supervision of Dr. J.G. Richards. Experiments were primarily conducted by me with assistance from Dr. M.L. Ramon in the preparation and hybridization of samples to microarrays. Dr. A.Y. Gracey provided technical expertise on microarrays. All authors provided editorial input to the manuscript.

Chapter 4 work was a collaboration with Drs. M.L. Ramon, A.C. Gerstein, A.Y. Gracey and J.G. Richards. I designed the experiments, primarily conducted all experiments with assistance from Dr. M.L. Ramon in preparation and hybridization of samples to microarrays and wrote the manuscript under the supervision of Dr. J.G. Richards. Dr. A.C. Gerstein provided expertise on the maximum-likelihood model analysis and Dr. A.Y. Gracey provided advice on microarray experiments.

All experiments in this dissertation were approved by the UBC animal care committee (A09-0611).
# Table of Contents

Abstract........................................................................................................................................ii
Preface..............................................................................................................................................iv
Table of Contents ..............................................................................................................................v
List of Tables .....................................................................................................................................ix
List of Figures ...................................................................................................................................x
Acknowledgements ..........................................................................................................................xii
Dedication .......................................................................................................................................xiv

Chapter 1  General Introduction ....................................................................................................1
  1.1 Environmental Stress - Hypoxia .........................................................................................3
  1.2 Time-Scale of the Hypoxia Response ..............................................................................4
  1.3 Levels of Biological Organization and the Hypoxic Response .......................................7
  1.4 Model Species: Sculpins (Superfamily Cottoidea) .......................................................8
  1.5 Research Goals ...............................................................................................................10

Chapter 2  Hypoxia Tolerance in Sculpins is Associated with High Anaerobic Enzyme Activity in Brain, but Not in Liver or Muscle .........................................................12
  2.1 Summary .........................................................................................................................12
  2.2 Introduction ....................................................................................................................13
  2.3 Materials and Methods..................................................................................................16
    2.3.1 Experimental animals .............................................................................................16
    2.3.2 Hypoxia tolerance experiments ..............................................................................17
    2.3.3 Analytical procedures ............................................................................................18
    2.3.4 Statistical analysis .................................................................................................21
2.4 Results ................................................................................................................................. 22
  2.4.1 Phylogeny ....................................................................................................................... 22
  2.4.2 Hypoxia tolerance .......................................................................................................... 23
  2.4.3 Scaling ........................................................................................................................... 23
  2.4.4 Brain ............................................................................................................................... 24
  2.4.5 Liver ............................................................................................................................... 24
  2.4.6 Muscle ........................................................................................................................... 25
2.5 Discussion .......................................................................................................................... 26
  2.5.1 Hypoxia tolerance .......................................................................................................... 26
  2.5.2 Brain enzymes ............................................................................................................... 27
  2.5.3 Standardization of enzyme activity ............................................................................... 28
  2.5.4 Muscle and liver enzymes ............................................................................................ 29
  2.5.5 Metabolites ................................................................................................................... 30
  2.5.6 Conclusions ................................................................................................................... 31

Chapter 3  Divergent Transcriptional Patterns are Related to Differences in Hypoxia Tolerance Between the Intertidal and the Subtidal Sculpins .......... 40
  3.1 Summary ........................................................................................................................... 40
  3.2 Introduction ........................................................................................................................ 41
  3.3 Materials and Methods ..................................................................................................... 44
    3.3.1 Experimental animals .................................................................................................. 44
    3.3.2 Hypoxia time-course .................................................................................................... 44
    3.3.3 Microarray hybridization ............................................................................................ 45
    3.3.4 Microarray probe sequences ....................................................................................... 47
    3.3.5 Statistical analyses ....................................................................................................... 48
  3.4 Results ............................................................................................................................... 48
3.4.1 Experiment 1: Relative hypoxia exposure time course ........................................ 49
3.4.2 Experiment 2: Absolute environmental hypoxia .............................................. 51
3.5 Discussion ............................................................................................................. 52
  3.5.1 Transcription of genes similarly induced or repressed by hypoxia in both species .................................................. 53
  3.5.2 Transcriptionally divergent response between the species .................... 54
  3.5.3 Relative vs. absolute levels of hypoxia ....................................................... 56
  3.5.4 Conclusions ............................................................................................ 57

Chapter 4 Variation Underlying Similar Phenotypes: Transcriptional Divergence in Hypoxia Responsive Genes Associated with Metabolism and Protein Synthesis in Three Species of Sculpin with the Same Hypoxia Tolerance .......................................................... 66
  4.1 Summary ........................................................................................................... 66
  4.2 Introduction ...................................................................................................... 67
  4.3 Materials and Methods .................................................................................. 70
    4.3.1 Experimental animals ............................................................................... 70
    4.3.2 Critical O₂ tension .................................................................................... 70
    4.3.3 Hypoxia exposure ...................................................................................... 71
    4.3.4 Microarray experiments .......................................................................... 71
    4.3.5 Statistical analysis .................................................................................... 71
  4.4 Results and Discussion .................................................................................... 73
    4.4.1 Energy metabolism .................................................................................... 74
    4.4.2 Protein production, protein localization and protein folding .................. 76
    4.4.3 Transcription patterns of hypoxia intermediate-tolerant species versus a hypoxia-tolerant species .................................................. 77
    4.4.4 Wild caught versus lab-reared species .................................................... 78
4.4.5 Short-term versus long-term responses................................................................. 78
4.4.6 Conclusion ............................................................................................................... 79

Chapter 5 General Discussion....................................................................................... 91

5.1 Major Findings and Implications .......................................................................... 91
  5.1.1 Measures of hypoxia tolerance ........................................................................... 91
  5.1.2 Multiple candidate traits underlie variation in hypoxia tolerance among sculpins
                                           .................................................................................................................. 92
  5.1.3 Short- versus long-term hypoxia exposure ....................................................... 95
  5.1.4 The role of constitutive differences in phenotype versus phenotypic plasticity in
                                           the intertidal sculpins ...................................................................................... 96
  5.1.5 Convergent evolution of hypoxia tolerance ..................................................... 98

5.2 Future Directions ...................................................................................................... 98

5.3 Concluding Thoughts .............................................................................................. 100

Bibliography .................................................................................................................. 103

Appendix A .................................................................................................................... 130

Appendix B .................................................................................................................... 131

Appendix C .................................................................................................................... 133
List of Tables

Table 2.1  Body weights of species of sculpin used in the LOE_{50} experiment and in analytical procedures

Table 2.2  Tissue enzymes in 12 species of sculpin

Table 2.3  Tissue metabolites in 12 species of sculpin

Table 3.1  Experimental design of O_{2} exposures and sampling times for each species

Table 3.2  Euclidean distance of the transcriptional response between the tidepool sculpin and the silverspotted sculpin measured for the 72 hour time course

Table 4.1  Summary of clones with similar and different transcription patterns among the three species of sculpin

Table 5.1  Summary of traits examined in 5 species
List of Figures

Figure 1.1 Time-scale of a stress response ................................................................. 11
Figure 2.1 Phylogenetic relationship among 15 species of sculpin based on a
maximum-likelihood tree using cytochrome b sequences........................................ 33
Figure 2.2 Relationship between $P_{\text{crit}}$ and $\text{LOE}_{50}$................................................................. 34
Figure 2.3 Relationships between $P_{\text{crit}}$ and maximal enzyme activities of lactate
dehydrogenase (LDH), pyruvate kinase (PK), creatine phosphokinase (CPK) and
citrate synthase (CS) in the brain of 12 species of sculpin........................................ 34
Figure 2.4 Relationship between $P_{\text{crit}}$ and soluble protein concentration in brain,
 liver, and muscle. ........................................................................................................ 35
Figure 3.1 Venn diagram depicting the number of clones whose transcription
significantly changed in response to hypoxia............................................................. 58
Figure 3.2 Trajectory of transcription in the tidepool sculpin and the silverspotted
sculpin exposed to relative environmental hypoxia over 72 hours and absolute
environmental hypoxia at 24 hours. .............................................................................. 59
Figure 3.3 Transcription levels of genes associated with metabolism (A-D),
regulation of cell number (E-H) and immunity (I) in the tidepool sculpin and
silverspotted sculpin. ..................................................................................................... 60
Figure 3.4 Transcription levels of genes associated with protein production (A-E),
protein localization (F) and protein folding (G-H) in the tidepool sculpin and the
silverspotted sculpin. ..................................................................................................... 62
Figure 4.1 A sculpin phylogenetic tree based on cytochrome b sequences............. 80
Figure 4.2 Schematic representation of genes associated with metabolism............ 81
Figure 4.3 Schematic representation of genes associated with protein production,
localization and folding ................................................................................................. 82
Figure 4.4 Transcript levels of genes associated with metabolism in the smoothhead
sculpin (black line, square symbol), sailfin sculpin (blue line, diamond symbol) and
Pacific staghorn sculpin (red line, inverted triangle symbol) exposed to 72 hours of
hypoxia .......................................................................................................................... 83
Figure 4.5 Genes for which all three species showed similar significant changes in transcription in response to 72 hours of hypoxia................................................................. 85
Figure 4.6 Similar transcription patterns in genes for which only a subset of species showed a significant effect of time in hypoxia................................................................. 86
Figure 4.7 Transcript levels of genes associated with protein production, localization and folding in the smoothhead sculpin (black line, square symbol), sailfin sculpin (blue line, diamond symbol) and Pacific staghorn sculpin (red line, inverted triangle symbol) exposed to 72 hours of hypoxia................................................................. 88
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Chapter 1

General Introduction

A fundamental goal of comparative physiology is to understand how environmental stresses have shaped the functional physiological responses of an organism and how these responses contribute to defining the geographical distribution of a species. Although “stress” is defined in different ways in different scientific fields, environmental stress can be defined as an environmental factor that results in a loss of fitness of an organism or a population, i.e. results in loss of reproductive output (Bijlsma and Loeschcke 2005, Schulte 2014). As organisms most often experience environmental conditions that are not optimal for maximum fitness, the definition of stress is more strictly applied to environmental conditions that have a significant impact on the fitness of individuals in a population (Bijlsma and Loeschcke 2005). Thus environmental stresses impose a strong influence on a species’ ability to survive in a habitat. By observing the environments where organisms exist, we can acquire insight on their evolved stress responses that compensate or buffer against the (potentially) deleterious effects of the environmental conditions.

There are different aspects of environmental stress that can influence the adaptive trajectory of an organism, mainly the frequency, intensity and the timescale of exposure to the environmental stress. Focusing on the temporal aspect, an environmental stress may be of short or long duration, or sustained within or across generations, with the differences in the length of time of exposure to stress influencing the mechanisms used to alleviate the effects of stress (see Fig. 1.1). For example, immediate or acute responses of an organism may be sufficient for initial survival to an environmental stress or if the length of the stress exposure is short.
Longer durations of stress within an individual’s lifetime may lead to improvement in the performance of the individual via reversible phenotypic plasticity or via irreversible developmental plasticity if the exposure to stress occurs during developmental stages (Stearns 1989, Gabriel 2005, Gabriel 2006, Ghalambor et al. 2007). Epigenetic changes may improve compensatory responses of individuals across a few generations, while across many generations evolutionary change may shift populations or species closer to adaptive optimum for the environmental stress (Burggren 2014, Schulte 2014). Thus, compensatory mechanisms that work at different time-scales can decrease the negative effect of environmental stress and improve survival and fitness of individuals within a population or species.

Phenotypic plasticity may impact adaptive evolution and in turn, adaptation may play a significant role in the evolution of phenotypic plasticity. One viewpoint regarding the impact of phenotypic plasticity on adaptation is that environmentally-induced phenotypic variation constrains adaptive evolution by decreasing the effect of natural selection on genotype (Stebbens 1977, Grant 1991). In contrast, others propose that phenotypic plasticity may facilitate adaptation through a process known as 'genetic assimilation', whereby the phenotypic change that allows survival and reproduction of individuals in response to a new environmental stress becomes constitutively expressed in the population even if the environmental stress is no longer present (Robinson and Dukas 1999, Ghalambor et al. 2007). Therefore phenotypic plasticity, whether through constraint or facilitation of adaptation, plays a role in the evolutionary trajectory of a population or a species in response to environmental stress.

Adaptation, in turn, can impact the evolution of phenotypic plasticity as natural selection may favor either environmentally-induced plastic traits or fixed, constitutively expressed traits (Berrigan and Scheiner 2004, Callahan et al. 2008). More specifically, several other strategies may be selected over phenotypic plasticity in heterogeneous environments ('heterogeneous' encompassing either changes from non-stress to stress conditions or from one type of environmental stress to a different one). These include the 1) specialist: single phenotype is produced that is optimal for one set of environmental conditions, 2) generalist:
single phenotype is produced that is moderately successful in most environments; also termed ‘conservative bet-hedging’ and 3) diversified bet-hedger: equal probability of producing alternate phenotypes (Philippi and Seger 1989, Wilson and Yoshimura 1994, Dewitt and Langerhans 2004, Botero et al. 2015). Theoretical modeling and empirical studies have examined the environmental conditions that may favor one strategy over another in a heterogeneous environment and these include predictability of environmental change, reliability of environmental cues, frequency and intensity of change, length of environmental stress and costs associated with phenotypic plasticity (Moran 1992, Padilla and Adolph 1996, Schlichting and Smith 2002, Gabriel 2005, Botero et al. 2015). If environmental change is predictable, environmental cues are reliable or there is low cost associated with altering phenotype, selection should favor phenotypic plasticity in response to an environmental stress. As the role of adaptation and phenotypic plasticity on the stress response of a population or species are interlinked, my doctoral research examined the interplay between these two processes in hopes of gaining a better understanding how organisms evolved to cope with an environmental stress, with a specific focus on low O₂ availability or hypoxia in the environment.

1.1 Environmental Stress - Hypoxia

Periodic hypoxia is a widespread abiotic stress, occurring across diverse aquatic environments such as ponds, lakes, rivers, marine coastal areas and regions of intermediate oceanic depth (400-1000 meters; Diaz and Breitburg 2009). Water is 1000 times denser than air and 50 times more viscous; O₂ diffuses 10,000 times slower through water than air and there is 20 to 40 times less O₂ contained in water than in the same volume of air, depending on temperature and salinity (Graham 1990). As a consequence, in part, of these physical properties of water, aquatic ecosystems are particularly susceptible to fluctuations in O₂ and hypoxia occurs when respiration exceeds the production and diffusion of O₂ in the environment. Occurrence of hypoxic events is also attributed to many other interacting components of the aquatic ecosystem, including density of vegetation, loads of
organic materials and the degree and duration to which bodies of water become isolated. Together, these elements create complex, heterogeneous aquatic environments that are marked with intermittent decreases in O$_2$ levels to varying degrees of magnitude, frequency and duration (Burggren and Roberts 1991, Val 1999). This has led to the evolution of a variety of behavioral, morphological, physiological, biochemical and molecular responses among fishes (Nikinmaa 2002).

Hypoxic events can last for hours, days or even months. Short-term hypoxia is prevalent and often severe in coastal waters such as salt marshes, estuaries and intertidal zones (Truchot and Duhamel-Jouve, 1980). Long-term hypoxia can occur in river systems such as the Amazon during the dry season when water levels decrease and there is low water mixing coupled with high water plant cover and high organic decomposition (Val, 1999), as well as in shallow temperate lakes and ponds that are prone to ice coverage in the winter (van den Thillart et al. 1989). While it is clear that hypoxia is a natural occurrence in aquatic habitats, the extensiveness, severity and duration of hypoxic zones has dramatically increased since around the 1950s as a result of anthropogenic eutrophication of both the freshwater and coastal ecosystems (Diaz and Breitburg, 2009). A number of these hypoxic areas have been classified as dead zones due to massive mortality of vertebrate and invertebrate organisms, leading to increasing global concern about the impact of hypoxia, especially anthropogenic-induced hypoxia, on the survival of productive ecosystems (Diaz 2008, Vaquer-Sunyer and Duarte 2008). Clearly, it is critical that we understand the mechanisms organisms use to cope with hypoxic stress in order to identify the effect of ever-increasing hypoxic challenges on biological diversity.

### 1.2 Time-Scale of the Hypoxia Response

Rapid changes in the environment necessitate immediate behavioural, physiological and metabolic responses in order to maintain function, and many acute modifications to hypoxia exposure have been noted across diverse fish taxa. Behavioural responses in fishes include the avoidance of low O$_2$ zones (Dalla Via et al. 1998, Lefrancois and Domenici 2006), reduction of spontaneous swimming
activity (Chapman and McKenzie 2009), aquatic surface respiration and air-breathing (Martin 1995, Yoshiyama et al. 1995, Graham 1997, Watters and Cech 2003). A common acute respiratory modification to low O₂ conditions is the hypoxic ventilatory response, which is the increase in ventilation volume as a result of changes in breathing frequency and/or amplitude in order to increase arterial PO₂ (reviewed in Perry et al. 2009). Bradycardia has also been shown to occur in a number of fish species within minutes of hypoxia exposure, the benefits of which include increased time for O₂ diffusion in the myocardium and a reduction in myocardial O₂ demand (Farrell 2007, Gamperl and Driedzic 2009). Hypoxia-induced acute responses may be sufficient for short-term survival, which in turn may allow phenotypic changes to occur that can enhance the capacity of the organism to endure hypoxia exposure (Richards 2009).

Phenotypic plasticity or the modification of phenotypic expression in response to changing environmental conditions is viewed as an important strategy in adaptation to variable, heterogeneous environments (e.g. Stearns 1989, Moran 1992, Via et al. 1995, Gabriel 2005, Ghalambor et al. 2007, Pfenning et al. 2010). Indeed, reversible phenotypic plasticity, irreversible developmental plasticity and epigenetics have all been noted to play a role in hypoxic stress response of fishes. For example, transcription of genes involved in glycolysis, oxidative phosphorylation, iron/heme metabolism cell proliferation and protein metabolism have been found to change in fish species in response to hypoxic exposure (Gracey et al. 2001, Ju et al. 2007, Everett et al. 2011, Leveelahti et al. 2011). These hypoxia-induced gene transcription patterns have been presumed to alter phenotype and therefore are considered an important part of the plastic response. Other reversible phenotypic changes as a result of long-term hypoxic events include gill remodeling to increase respiratory surface area (Sollid et al. 2003, Nilsson 2007), decreases in hemoglobin (Hb) modulators that enhance Hb-O₂ binding affinity (Weber and Lykkeboe 1978, Tetens and Lykkeboe, 1981) and changes in traits associated with both energy supply and energy demand (Hochachka 1986). Energy supply during hypoxic exposure becomes limited but changes in the concentration of enzymes associated with carbohydrate metabolism over time may increase capacity of
pathways of ATP production (Martinez et al. 2006, Martinez 2011). To conserve the lower energy supply, fishes such as the goldfish (*Carassius auratus*) turn down processes with high energy expenditure, profoundly reducing metabolic energy demand (van Waversveld et al. 1989). This can occur rapidly at the onset of hypoxia but is maintained throughout the hypoxic exposure and lowering metabolic demand to match hypoxia-induced reduction in energy supply is one of the classic strategies hypoxia-tolerant species use to combat the effects of long-term hypoxia (van Waversveld et al. 1989, Lutz and Nilsson 1997, Bickler and Buck 2007, Lewis et al. 2007, Hochachka 1986, Hochachka et al. 1996, Boutilier 2001). In addition to reversible phenotypic plasticity, alteration of phenotypic expression over generations via epigenetics has been noted in zebrafish (*Danio rerio*) where parental exposure to hypoxia increased offspring hypoxia tolerance as measured by time to loss of equilibrium (Ho and Burggren 2012), while changes in adult critical oxygen tension were also found in zebrafish as a result of developmental exposure to hypoxia (Robertson et al. 2014). The many noted examples of phenotypic changes in response to low O$_2$ across diverse fish lineages suggests that phenotypic plasticity is an important strategy for increasing survival and reducing the impact of hypoxic stress.

Intraspecific and interspecific variation in fixed physiological traits in different taxa have been thought to have evolved in response to O$_2$ availability in the environment. Specifically, higher gill surface area and greater total gill filament length, have been found in populations living in O$_2$-poor environments versus well-oxygenated environments in sailfin mollies (*Poecilia latipinna*; Timmerman and Chapman 2001) and African cyprinids, *Barbus neumayeri* (Chapman et al. 1999) respectively. It has been thought that a number of changes at the level of the hemoglobin have evolved in response to hypoxic selective pressure, including high concentration of Hb and hematocrit among fishes in the Lake Victoria region (Chapman et al. 2002), higher number of Hb isoforms with a greater proportion of the higher Hb-O$_2$ affinity between intertidal and deeper water triplefin fishes (Brix et al. 1999), and amino acid substitution leading to higher Hb-O$_2$ affinity in the high altitude bar-headed goose in comparison to low altitude geese species (Perutz 1983,
Jessen et al. 1991). These studies are informative examples of how populations or species that vary in O₂ regimes have potentially evolved differences in fixed physiological traits. However, many studies examining traits underlying hypoxia tolerance have not employed methods to test if the traits have evolved by natural selection in response to hypoxic pressure. The most direct method to ascertain if a trait is adaptive is to measure the fitness of alternate alleles in a population (Endler 1986, Dalziel et al. 2009), a task that may be difficult to accomplish in many taxa. Alternately, comparative phylogenetic analysis may be used to determine if candidate traits are potential adaptations (Pierce and Crawford 1997, Feder et al. 2000, Darveau et al. 2005a, Garland et al. 2005). These include, for example, a phylogenetically corrected examination of traits in closely related species with varying levels of hypoxia tolerance or a comparison of traits in populations or species with independently evolved similar level of hypoxia tolerance (instances of parallel or convergent evolution are thought to be a result of natural selection; Endler 1986). The use of these methods is critical in determining if candidate traits are potential adaptations to environments with periodic decreases in O₂.

### 1.3 Levels of Biological Organization and the Hypoxic Response

Coordination and integration of many traits contribute to complex phenotypes such as hypoxia tolerance. As such, many investigators in the field of comparative physiology have called on a systematic examination of levels of biological hierarchy as an integrative, mechanistic approach to understanding how species respond to environmental challenges (Dalziel et al. 2009, Gaston et al. 2009, Mykles et al. 2012, Somero 2012). This ‘vertical integration’ of traits (Mykles et al. 2012), from gene transcription, protein function or amount, biochemical and cellular pathways, physiological traits, morphological characteristics to behavioural responses, is important in order to understand how variation in environmental stress leads to variation in stress response among different populations or species. Technological advances and the advent of the ‘-omic’ era in particular have provided an array of tools, such as whole-genome sequencing data, transcriptomics, epigenomics, proteomics and metabolomics, to address the relationship between

### 1.4 Model Species: Sculpins (Superfamily Cottoidea)

August Krogh has famously stated “For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied” (Krogh 1929). This quote exemplifies the comparative physiology approach of carefully considering the most appropriate organisms to study given the questions under investigation. The comparative approach of examining populations and species that have adapted to different environmental conditions has been a staple of physiology as it allows us to infer the evolutionary processes that had shaped the physiological and ecological landscape (Garland et al. 2005). Included in this approach is the explicit incorporation of phylogenetic information and the application of phylogenetically independent contrasts (PIC) in order to take into account phylogenetic relationships and species relatedness when drawing inference from correlative data (Felsenstein 1985, Garland et al. 1992). To address the question of how hypoxia tolerance evolved several criteria need to be considered when selecting model systems: 1) organisms must show variation in hypoxia tolerance, 2) the level of hypoxia tolerance matches the likelihood of hypoxic exposure in the habitat of the chosen species and 3) phylogenetic
relationships need to be elucidated in order to take into account non-independence in the data as a result of species relatedness. As such, one ideal model study system to address important questions regarding evolution of hypoxia tolerance are sculpins, small benthic fish species living in the nearshore environment.

The marine intertidal zone is an aquatic environment susceptible to $O_2$ fluctuations as a result of the ebb and flow of tides, with hypoxic and even anoxic (no $O_2$) events occurring in isolated tidepools at night (Truchot and Duhamel-Jouve 1980, Richards 2011). In contrast, hypoxia is less frequent or severe in the subtidal zone and $O_2$ levels remain stable and high in the pelagic and deeper coastal waters. Marine sculpins, a group of fishes belonging to the superfamily Cottoidea, are distributed along the nearshore environment with species inhabiting the intertidal zone, subtidal zone and deeper waters. As predicted by the habitat of the species, sculpins living in the intertidal zone are more hypoxia-tolerant than subtidal species and sculpins from both environments are more hypoxia-tolerant than the deeper water species (Mandic et al. 2009b). A number of behavioural responses and fixed physiological traits have been found to underlie variation in hypoxia tolerance among the different sculpin species. Gill morphology and hemoglobin properties significantly correlated with critical $O_2$ tension ($P_{\text{crit}}$; a proxy measure of hypoxia tolerance) independent of phylogenetic relationships, where the more hypoxia-tolerant species were found to have larger gill surface area and higher Hb-\(O_2\) binding affinity compared to the more intolerant species of sculpin (Mandic et al. 2009b). These traits suggest that the variation in $O_2$ extraction and delivery may partially underlie the variation in hypoxia tolerance among sculpin species. In response to acute hypoxia exposure, the hypoxia-tolerant intertidal species consistently displayed behavioural modifications, mainly aquatic surface respiration followed by aerial respiration, while less tolerant species did not exhibit these behaviours or performed them at low frequencies (Mandic et al. 2009a). Overall, these previous studies have shown that the sculpins are a tractable model system as they have begun to address the question of what underlying traits may be important in adaptation to environmental hypoxia. In particular, the past studies and this thesis have begun to apply the approach of using multiple biological levels
(behavioural, physiological, biochemical and gene transcription) with temporal time-scales (acute responses, phenotypic plasticity, fixed putative adaptations) to examine how variation in a complex phenotype may have evolved in response to an environmental stress.

1.5 Research Goals

The overall objective of my doctoral research was to examine how adaptation and phenotypic plasticity have shaped the hypoxic response in sculpin species distributed along the nearshore environment. Specifically, I focused on how variation in fixed biochemical traits and transcriptionally-induced phenotypic plasticity correlated with hypoxia tolerance among the different sculpin species. My first objective was to determine if the previously assessed critical O$_2$ tension ($P_{crit}$) in species (Mandic et al. 2009b) is related to hypoxia tolerance in sculpins. I quantified loss of equilibrium of 50% of a population (LOE$_{50}$) in 11 species of sculpin and correlated this measure of hypoxia tolerance to the previously assessed $P_{crit}$ values (Chapter 2). My second objective was to determine whether the variation in biochemical traits associated with energy metabolism, explained, at least in part, the differences in the ability of the species to tolerate low levels of O$_2$. To address this objective, I quantified maximal activity of enzymes and metabolite concentrations associated with ATP production in brain, liver and white muscle of 12 species of sculpin under normoxic conditions (Chapter 2). Phylogenetically independent contrast analysis (Felsenstein 1985) was applied to all correlations to take into account phylogenetic relationships, allowing for more confidence that the significant correlations were less likely a result of relatedness and instead can be considered putative adaptive traits.

My subsequent objectives focused on defining the relationship between levels of hypoxia tolerance and phenotypic plasticity via changes in gene transcription. Specifically, my third objective was to determine how species with different levels of hypoxia tolerance transcriptionally respond to hypoxic stress over time. To address this objective, I assessed gene transcription patterns of the hypoxia-tolerant tidepool sculpin (*Oligocottus maculosus*) and the hypoxia-
intolerant silverspotted sculpin (*Blepsias cirrhosis*) during exposure to hypoxia (Chapter 3). Both species were exposed to two sets of hypoxic conditions; a relative hypoxia exposure where individuals were exposed to O$_2$ tensions scaled to the species’ hypoxia tolerance (65% $P_{crit}$) and an absolute hypoxia exposure where individuals were exposed to a single environmental O$_2$ tension. My fourth objective was to determine if species of sculpin with similar tolerances to environmental hypoxia also exhibit convergence at the level of gene transcription. Specifically, gene transcription patterns over short- and long-term hypoxia exposure were assessed in three similarly hypoxia-tolerant species of sculpin, in order to provide insight into whether the species evolved similar hypoxia tolerance via convergent or divergent mechanisms (Chapter 4).

**Figure 1.1 Time-scale of a stress response**

![Time-scale of a stress response](image)

- Acute Response
- Phenotypic plasticity
- Developmental plasticity
- Epigenetics
- Adaptation

- minutes to hours
- hours to days
- generation
- few generations
- many generations

Time
Chapter 2

Hypoxia Tolerance in Sculpins is Associated with High Anaerobic Enzyme Activity in Brain, but Not in Liver or Muscle

2.1 Summary

We assessed hypoxia tolerance in 11 species of fish from the superfamily Cottoidea (commonly called sculpins) that are known to differ in their critical O\textsubscript{2} tensions (P\textsubscript{crit}) and examined whether hypoxia tolerance correlated with larger substrate stores and higher maximal activity of enzymes associated with anaerobic ATP production (especially glycolysis). Among the sculpins studied, there was large variation in time to loss of equilibrium (LOE\textsubscript{50}) at 6.4±0.1 torr with values ranging between 25 and 538 minutes and the variation in LOE\textsubscript{50} was correlated with P\textsubscript{crit}. Our measures of time to LOE\textsubscript{50} and P\textsubscript{crit} were regressed against maximal enzyme activities of lactate dehydrogenase (LDH), pyruvate kinase (PK), creatine phosphokinase (CPK) and citrate synthase (CS) as well as the concentrations of glycogen, glucose and creatine phosphate in the brain, liver and white muscle. In the brain, there was a phylogenetically independent relationship between P\textsubscript{crit} and tissue LDH, PK, CPK and CS activities expressed relative to tissue mass. Hypoxia-tolerant sculpins (those with low P\textsubscript{crit} values) had higher levels of brain LDH, PK, CPK and CS activities expressed relative to tissue mass.

\footnote{A version of this chapter has been published: Mandic M., B. Speers-Roesch and J.G. Richards. 2013. Hypoxia tolerance is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiological and Biochemical Zoology 86: 92-105}
CPK, and CS than hypoxia-intolerant sculpins. Similarly, LOE50 regressed against brain LDH, PK and CPK activities expressed relative to tissue mass, with the more hypoxia-tolerant species (ie. higher LOE50) having higher enzyme activities. However, when the phylogenetic relationship among our sculpins was taken into account, only the relationship between hypoxia tolerance and LDH activity remained significant. When enzyme activities were expressed relative to total soluble protein in the tissue, the only relationships that remained were between brain LDH activity and $P_{\text{crit}}$ and LOE50. In liver and white muscle, there were no relationships between the measures of hypoxia tolerance and enzyme activity or metabolite content. Overall, our analysis suggests that hypoxia-tolerant sculpins maintain higher maximal activities of some of the enzymes involved in anaerobic metabolism in the brain and this may be an adaptation to hypoxia.

2.2 Introduction

In order to survive periods of low environmental O2 (hypoxia), fish must be able to acquire more O2 from the hypoxic environment or respond at the biochemical level by reducing energetic demands or enhancing O2-independent means of ATP production. Initial compensatory mechanisms during environmental hypoxia are geared towards enhancing O2 extraction and delivery to the tissues in order to maintain aerobic energy production. However, if the severity of hypoxia increases and tissue O2 levels drop, fish face limitations to their metabolic energy supply as a result of reduced capacity to fully oxidize carbohydrate, lipid and amino acid fuels. To maintain metabolic energy balance and prevent hypoxia-induced cellular death, hypoxia-tolerant animals are thought to down-regulate ATP consuming pathways and shift ATP generation toward substrate-level phosphorylation (i.e., anaerobic or O2-independent energy production) by increasing glycolytic flux as well as creatine phosphate (CrP) hydrolysis (e.g. in Astronotus ocellatus, Richards et al. 2007). Hypoxia-intolerant species are thought to be unable to reduce ATP demand during hypoxia and therefore quickly exhaust their limited substrates for O2-independent energy production (Boutilier and St-Pierre 2000; Boutilier 2001). As such, at low O2 tensions, hypoxia survival is
dictated by the capacity to reduce cellular ATP demands and up-regulate or sustain prolonged periods of ATP generation via substrate-level phosphorylation. It has been postulated, therefore, that hypoxia tolerance in fishes and other ectotherms is associated with a greater capacity to generate ATP via substrate-level phosphorylation (Richards 2009).

Higher substrate levels for anaerobic metabolism have long been associated with hypoxia tolerance (Van den Thillart and Van Raaij 1995; Chippari-Gomes et al. 2005). For example, the impressive hypoxia tolerance of goldfish (Carassius auratus) and Crucian carp (Carassius carassius) is, in part, attributed to large hepatic glycogen stores, which are accumulated prior to the onset of severe hypoxia during wintering under the ice (Walker and Johansen 1977; Hyvarinen et al. 1985). The winter glycogen levels in the goldfish and Crucian carp are 10 to 45-fold higher than in the hypoxia-intolerant Atlantic cod (Gadus morhua) and rainbow trout (Oncorhyncus mykiss; Van den Thillart and Van Raaij 1995), suggesting that high glycogen stores may be an important adaptive trait for hypoxia survival in fishes. Hypoxia tolerance is also associated with higher maximal activities of some glycolytic enzymes, particularly lactate dehydrogenase (LDH; Almeida-Val et al. 2000; Chippari-Gomes et al. 2005; Farwell et al. 2007). In centrarchids, the more hypoxia-tolerant pumpkinseed, Lepomis gibbosus, has higher levels of LDH in white muscle than the hypoxia-intolerant bluegill, Lepomis macrochirus (Farwell et al. 2007). While variation in maximal enzyme activity appears to be related to hypoxia tolerance, it is unclear what effect this variation has on overall metabolic flux since in vivo flux rates are regulated primarily by allosteric and covalent modification of regulatory enzymes (e.g. hexokinase, phosphofructokinase and pyruvate dehydrogenase; Newsholme and Crabtree 1986). Despite the lack of a clear mechanistic understanding of how maximal enzyme activity affects metabolic flux rates, variation in maximal enzyme activities seen among species and populations of fish has been attributed to natural selection (e.g. Crawford and Powers 1989; Pierce and Crawford 1997), thus suggesting that this variation is functionally important. In particular, Pierce and Crawford (1997) showed a significant correlation between habitat temperature and maximal enzyme activity of LDH, glyceraldehyde 3-
phosphate dehydrogenase and pyruvate kinase (PK) in the heart of 11 species of fish from the genus *Fundulus*, and the authors argued strongly that maximal enzyme activity was under selection.

In order to determine if environmental hypoxia acts as a selective pressure on substrate concentration and maximal activity of enzymes involved in anaerobic metabolism, we chose to examine these variables in sculpins, a group of small benthic fishes belonging to superfamily Cottoidea. We have previously shown that there is variation in critical O$_2$ tension, or P$_{crit}$, which is a whole animal measure of O$_2$ extraction capacity, among the species of sculpin studied and that P$_{crit}$ correlates with habitat. The more hypoxia-tolerant species (lower P$_{crit}$) inhabit the O$_2$-variable tidepools and the more hypoxia-intolerant species (higher P$_{crit}$) are located in the more stable subtidal and deeper waters (Mandic et al. 2009). A multi-species comparison is only effective, however, if the phylogenetic relationship is taken into account since species’ means cannot be treated as statistically independent due to the differences in the degree of relatedness among the species (Felsenstein 1985; Garland et al. 2005). Therefore, application of the phylogenetically independent contrast (PIC) analysis, which uses phylogenetic information to transform species data into standardized independent contrasts effectively eliminates non-independence from the data set (Felsenstein, 1985). Taking into account the phylogenetic relationship among sculpins, we hypothesize that the hypoxia-tolerant species have larger anaerobic substrate stores and higher maximal activity of enzymes associated with O$_2$-independent ATP generating pathways compared with the more hypoxia-intolerant species of sculpin.

In this study, we quantified the concentrations of the three main substrates of anaerobic ATP production (glycogen, glucose and CrP) and the maximal enzyme activities of LDH, PK, creatine phosphokinase (CPK), and citrate synthase (CS) in brain, liver and muscle of 12 species of sculpin acclimated to normoxia. We chose to examine LDH and PK because maximal activity of these enzymes has previously been shown to be under selection in fish exposed to environmental stress (Pierce and Crawford 1997). Creatine phosphokinase was examined because it is part of an enzyme system that plays an important role in anaerobic generation of ATP via CrP.
hydrolysis. An enzyme from aerobic metabolism, CS, was also selected for analysis in order to compare variation in anaerobic enzymes with an enzyme involved in aerobic metabolism. Brain, liver, and muscle were selected for analysis because it is likely that tissue-specific profiles have evolved in response to differences in O$_2$ supply, energetic demand, and metabolic role of the tissue (Martinez et al. 2006). Brain is well known as a hypoxia-sensitive tissue (Boutilier 2001) and the maintenance of stable cellular ATP is critical to survival. Liver and muscle were chosen because previous studies have shown that these two tissues respond differently to long-term hypoxia exposure (Martinez et al. 2006), with liver showing increases and muscle showing decreases in glycolytic enzyme activity. Analysis was performed in normoxia acclimated sculpins (precluding measurements of lactate) in order to assess whether standing variation in substrate levels and maximal enzyme activities were correlated with independent measures of hypoxia tolerance ($P_{crit}$ and time to loss of equilibrium analysis; LOE$_{50}$). Using PIC analysis, hypoxia tolerance was regressed against substrate concentrations and maximal enzyme activity to ascertain if environmental hypoxia has been a selective pressure in defining the ability to generate ATP under low O$_2$ conditions.

2.3 Materials and Methods

2.3.1 Experimental animals

The sculpins used in this study were collected between June and August of 2006, 2007 and 2008. Sculpins collected in 2006 and 2007 were terminally sampled (Mandic et al. 2009), and muscle, liver and brain tissue from those animals were used in this study for analysis of metabolites and maximal enzyme activities. Sculpins collected in 2008 were used for the determination of LOE$_{50}$ and additional $P_{crit}$ analysis on two species of sculpin, Myoxocephalus polyacanthocephalus and Hemilepidotus hemilepidotus. Marine sculpins, including Oligocottus maculosus, Oligocottus snyderi, Clinocottus globiceps, Enophrys bison, Artedius fenestralis, Artedius lateralis, Leptocottus armatus, Blepsias cirrhosus, Scorpaenichthys marmoratus, Myoxocephalus scorpius, M. polyacanthocephalus and H. hemilepidotus.
were caught using handheld nets or seines during the lowest tidal cycle 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 and held in flow-through seawater (12°C, 33 ppt salinity). The freshwater sculpin (Cottus asper) was caught using baited minnow traps in Pachena Lake (48°50’11” N; 125°01’44” W) during the same time periods and were housed in freshwater re-circulating system held at 12°C. All sculpins were allowed to recover from capture for at least one week before experimentation. In our experiments, variable sample sizes across the different species of sculpin exist as a consequence of year-to-year variation in our ability to collect sufficient numbers of individuals of species that are found at low abundance in the wild. Fish were fed daily with mussels, bloodworms and frozen fish bait, except 24 hours prior to experimental trials. All experiments were conducted according to guidelines set out by the Canadian Council for Animal Care and approved institutional protocols.

2.3.2 Hypoxia tolerance experiments

Hypoxia tolerance is a complex trait and warrants a comparison of multiple methods of measurement. We have employed two approaches to assess hypoxia tolerance in sculpins, including the analysis of the time for 50% of a group of sculpins to show loss of equilibrium (LOE50) and Pcrit (water PO2 at which an animal no longer sustains routine O2 consumption and the rate declines as PO2 decreases in the water). For the majority of the species used in this study, Pcrit was taken directly from Mandic et al. (2009). The present study included two new species, M. polyacanthocephalus and H. hemilepidotus and Pcrit was determined using the same methods as described in Mandic et al. (2009).

2.3.2.1 Time to loss of equilibrium (LOE50)

To determine LOE50, individuals from two randomly selected species were placed into separate 40 L aquaria and allowed to recover overnight under flow-through conditions. A small submersible pump was placed in each aquarium to ensure adequate mixing and an Oxyguard Handy MKIII O2 probe was suspended in
each aquarium to monitor water $PO_2$. The aquaria were partially submerged in a
wet table with flow-through water to keep the temperature at 12°C.

At the end of the recovery period, water flow was stopped and air bubbling
into the tanks was switched to $N_2$ to induce hypoxia. The rate of water $PO_2$ decrease
was constant among all the trials and within 30 min water $PO_2$ stabilized at 6.4±0.1
torr ($n = 24$). Water $PO_2$ was monitored every 10 minutes and adjusted by bubbling
either $N_2$ or air into the aquaria as required. Fish behavior was also monitored every
10 min and the time an individual showed loss of equilibrium and unresponsiveness
to prodding was recorded. Once a fish reached these endpoints, it was removed
from the tank and immediately euthanized in an overdose of benzocaine (125 mg
mL$^{-1}$, Sigma-Aldrich, USA).

For species in which individuals were collected in abundance, multiple trials
were conducted with eight individual fish per trial. Four trials were carried out for
$O. maculosus$ and $L. armatus$, three trials for $C. globiceps$, $B. cirrhosis$ and $C. asper$, and
two trials for $A. fenestralis$. For $A. lateralis$, $E. bison$, $H. hemilepidotus$, $M. polyacanthocephalus$ and $O. snyderi$, a single trial of six individuals was conducted as
a consequence of a much lower capture success from the wild.

2.3.3 Analytical procedures

2.3.3.1 Tissue collection and metabolite assays

Resting, normoxic tissue samples of brain, muscle and liver were obtained as
described in Mandic et al. (2009). Briefly, individual fish were isolated overnight in
sampling chambers which were submerged in well-aerated 580 L tanks. A sampling
chamber was removed from a tank at which time the fish was immediately exposed
to an overdose of benzocaine (125 mg mL$^{-1}$, Sigma-Aldrich, USA) and within one
minute the fish lost equilibrium and was quickly sampled. Liver, brain, and muscle
were rapidly dissected from the fish, frozen in liquid $N_2$, and stored at -80°C for up
to 8 months until analysis. Sampling of each tissue, from dissection to freezing, took
less than 10 sec, so all tissues were sampled within 30 sec of the loss of equilibrium.

Aliquots of frozen liver, brain, and muscle were weighed into 2 mL tubes and
immediately sonicated on ice in 6 to 10 volumes of 8% perchloric acid using three, 6
sec bursts of a Kontes Micro Ultrasonic Cell Disrupter (Kontes, Vineland, New Jersey) set on its highest setting. 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 K₂CO₃. Neutralized extracts were centrifuged to remove the precipitates and frozen at -80°C until later analysis.

Neutralized extracts were thawed on ice and assayed for ATP, CrP and glucose according to protocols outlined in Bergmeyer (1983). The homogenate set aside for glycogen analysis was thawed, partially neutralized with 3M K₂CO₃, buffered in 0.45M sodium acetate (pH 4.8) and digested to glucose using amyloglucosidase (Bergmeyer 1983). The homogenate was then neutralized with 3M K₂CO₃ and assayed for glucose according to the protocols outlined in Bergmeyer (1983). Total glycogen content was calculated by subtracting free glucose measured in neutralized extracts from glucose obtained in digested samples. Glycogen was expressed as µmol glycosyl units/g wet weight.

2.3.3.2 Enzyme activity assays

Aliquots of frozen liver, brain, and muscle were quickly weighed to prevent the tissues from thawing and placed in 10 to 20 volumes of ice-cold homogenizer buffer (50 mM HEPES, 5 mM dipotassium ethylene diaminetetraacetic acid, 0.1% Triton X-100, pH 7.4) and immediately sonicated on ice using three, 6 sec bursts set on the highest speed setting. The homogenate was centrifuged at 10,000 g for 2 minutes at 4°C and the resulting supernatant was divided in separate aliquots for analysis of lactate dehydrogenase (LDH), pyruvate kinase (PK), creatine phosphokinase (CPK) and citrate synthase (CS). The high-speed centrifugation may have resulted in some loss of activity due to enzymes bound to subcellular structures, but since all our samples were treated identically, this allows for a valid relative comparison of enzyme activity among the different species of sculpin.

Maximal enzyme activities were determined spectrophotometrically (VersaMax, Molecular Devices, Sunnyvale, California) by measuring the accumulation or disappearance of NADH at 340 nm (LDH, PK and CPK) and the
appearance of 5-thio-2-nitrobenzoic acid (TNB) as a result of the reaction of free CoA with 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm (CS) at 25°C over a 10 minute incubation period. The assay conditions were as follows (in mM): LDH (50 Tris, pH 7.4, 2.5 pyruvate, 0.6 NADH), PK (50 Tris, pH 7.4, 0.5 NADH, 5 ADP, 0.01 fructose-1,6-bisphosphate, 100 KCl, 10 MgCl₂, 5 phosphoenolpyruvate, 50 U/mL LDH), CPK (50 Tris, pH 7.4, 20 creatine phosphate, 1.5 ADP, 12 AMP, 1.5 NAD, 20 D-glucose, 6.5 dithiothreitol, 25 MgCl₂, 2 U/mL hexokinase and 1.5 U/mL glucose-6-phosphate dehydrogenase), and CS (50 Tris, pH 8.0, 0.5 oxaloacetate, 0.3 acetyl-CoA, 0.15 5,5-dithiobis-2-nitrobenzoic acid). Substrates such as pyruvate (LDH), phosphoenolpyruvate (PK), creatine phosphate (CPK) and oxaloacetate (CS) were at saturating levels. Kinase and phosphatase inhibitors were not used in this study to avoid inadvertent modification to two of the kinase enzymes under study, PK and CPK. Kinases and phosphatases are known to modify PK by increasing the Michaelis-Menten (K_m) of the enzyme with increasing phosphorylation and to avoid the complicating factors of phosphorylation, saturating levels of substrate PEP were used to measure maximal enzyme activity (Riou et al. 1976). There is also some evidence that the activity of CPK may be regulated by phosphorylation and dephosphorylation, but the extent of regulation appears small (Lin et al. 2009). Background reaction rates were determined by omitting the following substrates from the control reaction rates: pyruvate (LDH), phosphoenolpyruvate (PK), creatine phosphate (CPK) and oxaloacetate (CS). We used empirically determined extinction coefficients to quantify maximal activity for each enzyme. Total soluble protein was determined in each homogenate using the Bradford protein assay [Sigma-Aldrich (Bradford 1976)]. Maximal enzyme activities were normalized to tissue weight and total soluble protein.

Functional properties of an enzyme can be modified through binding with subcellular components such as membranes, mitochondria and F-actin (Brooks and Storey 1995), and hypoxia has been shown to alter the ratio of free vs. bound enzymes in muscle, liver and brain (Lushchak et al. 1998). To ascertain if percent of bound enzymes differs among species of sculpin we tested the activity of free and
bound LDH in muscle, liver and brain of 3 species of sculpin that differ in hypoxia tolerance. Tissue samples were homogenized as described above and the homogenate was spun in a centrifuge at 10,000 g for 2 minutes at 4°C. The supernatant was removed and set aside to test the maximal activity of free LDH, while the pellet was re-suspended in 1 mL of 50 mM Tris buffer and centrifuged. The supernatant was drawn off and the pellet was re-suspended in 200 μL of 50 mM Tris buffer, frozen at -80°C, thawed and assayed for maximal activity of bound LDH. In the liver there was no detectable bound LDH activity, while in the brain only 1.6% was bound for all 3 species (data not shown). In the muscle, there was 15.9±0.8%, 20.1±1.9% and 22.0±2.4% bound enzyme activity in *O. maculosus*, *M. scorpius* and *C. asper* respectively, although the differences among the species was not significant (ANOVA, *P*=0.095).

### 2.3.4 Statistical analysis

Data are presented as means ± SEM. The Kaplan-Meier survival curve analysis in SigmaStat 3.0 was used to determine the LOE₅₀ median value for each sculpin species. There was no significant difference in LOE₅₀ between trials within a species and consequently data from individual trials were combined for each species to establish the LOE₅₀. To compare maximal enzyme activity of free vs. bound enzymes among species we used a one-way ANOVA (SigmaStat 3.0). Regressions were determined using the least squares regression analysis and correlations were determined using Pearson’s correlation analysis (SigmaStat 3.0) as well as the phylogenetically independent contrast (PIC) analysis using PAUP (Midford et al. 2005) in Mesquite (Maddison and Maddison 2004) in order to remove the effect of phylogeny from the regressions and correlations.

For PIC analysis, phylogeny based on *cytochrome b* sequences originally published in Mandic et al. (2009) was modified to include *H. hemilepidotus* (accession no. EF521369) and *M. polyacanthocephalus* (accession no. AB114909; Fig. 2.1). Analysis used to create the trees follows the steps outlined in Mandic et al. 2009. Briefly, a maximum-likelihood tree was constructed with bootstrap analysis of 1000 pseudoreplicates in PAUP (v. 4, Sinauer Sunderland, United States) and the
maximum-likelihood tree was imported into Mesquite for PIC analysis. In addition, Mr. Bayes v. 3.0 was used to generate a consensus tree from 10 000 Bayesian trees (Huelsenbeck and Ronquist 2003). The maximum-likelihood and the Bayesian consensus tree show similar phylogenetic relationships among species, therefore maximum-likelihood tree is presented with both bootstrap and posterior probability values for each clade (Fig. 2.1). In order to determine if variation in tree topology and branch length has an effect on PIC analysis (Martins and Housworth 2002) we used both the maximum-likelihood tree and the Bayesian consensus tree for all analysis. Analysis of PIC using the Bayesian consensus tree are not discussed unless a difference in significance of a regression occurred as a result of the two types of trees.

All the comparisons conducted in this study were planned prior to statistical analysis, therefore accounting for multiple comparisons of the results using techniques such as sequential Bonferroni analysis and false discovery rate are not reported. The goal of this study was to explore the data maximally and to minimize the possibility of introducing a Type I statistical error. Consequently, there may be an inflated Type I error associated with our analysis and as such we caution the reader that further analysis and explicit testing of the significant results reported herein should be performed. Statistical significance was assumed at \( P<0.05 \).

2.4 Results

2.4.1 Phylogeny

In the present study, cytochrome \( b \) sequences from 15 species of sculpin were used to construct a well-resolved phylogeny (Fig. 2.1) and the relationships among the species are in general agreement with published molecular phylogenies (e.g. Ramon and Knope 2008, Mandic et al. 2009). Both the maximum-likelihood tree presented in Fig. 2.1 and Bayesian consensus tree are used in PIC analysis of the following correlations and regressions between hypoxia tolerance and enzyme and metabolites of energy producing pathways in sculpins.
2.4.2 Hypoxia tolerance

Time to loss of equilibrium at 6.4±0.1 torr ranged between 25 and 538 min in our study species. *Blepsias cirrhosus* was the most hypoxia-intolerant species with the lowest LOE$_{50}$ at 25 min and *O. maculosus* was the most hypoxia-tolerant species with the highest LOE$_{50}$ at 538 min. There was a negative relationship between LOE$_{50}$ and $P_{\text{crit}}$, where species with low $P_{\text{crit}}$ had correspondingly high LOE$_{50}$ values. This relationship was significant when analyzed with Pearson’s correlation analysis (dashed line on Fig. 2.2); however, when corrected for the phylogenetic relationship among species using PIC, the correlation was no longer significant (solid line on Fig. 2.2). The critical O$_2$ tensions used in this analysis were previously published (Mandic et al. 2009) with the exception of *M. polyacanthocephalus* ($P_{\text{crit}}$ 28.2±1.3 torr; n = 6) and *H. hemilepidotus* ($P_{\text{crit}}$ 38.5±1.5 torr; n = 4).

2.4.3 Scaling

Allometric scaling is well known to affect metabolic rate and other physiological and biochemical processes (e.g. Somero and Childress 1980; Darveau et al. 2005a; Darveau et al. 2005b). In our sculpins, there was significant variation in body weight (Table 2.1), which could influence the measures of hypoxia tolerance and maximal enzyme activities. In order to determine if the variation in body weight impacted LOE$_{50}$, we regressed LOE$_{50}$ against body weight across species and no significant relationship was observed. Furthermore, we previously demonstrated that variation in body weight was not related to $P_{\text{crit}}$ (Mandic et al. 2009). When tissue maximal enzymes were regressed against body weight there were no significant relationships, with the exception of muscle PK activity expressed as total soluble protein, which increased with increasing body weight (0.15x-0.58, $r^2$=0.42, $P$=0.022). However, when $P_{\text{crit}}$ or LOE$_{50}$ were regressed against residual PK activity the relationships were not significant ($P_{\text{crit}}$: y=17.31x+36.65, $r^2$=0.02, $P$=0.659; LOE$_{50}$: -523.43x+278.58, $r^2$=0.07, $P$=0.450). Overall, our analysis suggests that the variation in body weight seen among the sculpins used in the present study does not affect our determination of LOE$_{50}$, $P_{\text{crit}}$ or maximal enzyme activity.
2.4.4 Brain

Both $P_{\text{crit}}$ (Fig. 2.3A) and LOE$_{50}$ (4.31x-332.08, $r^2=0.66$, $P=0.004$, least squares regression; 3.21x, $r^2=0.49$, $P=0.025$, PIC regression) regressed to brain LDH activity when standardized to tissue mass and analyzed using least squares and PIC regressions. Hypoxia-tolerant sculpins (low $P_{\text{crit}}$, high LOE$_{50}$) had higher brain LDH activity compared with the hypoxia-intolerant species. The same trend was seen between $P_{\text{crit}}$ and activities of PK, CPK, and CS in the brain (expressed relative to tissue mass), with all three enzymes showing higher activities in species of sculpin with lower $P_{\text{crit}}$. The regressions were significant when analyzed with least squares and PIC regression analyses (Fig. 2.3B,D). Using least squares regression analysis, there was a positive regression between brain PK ($7.65x-393.35$, $r^2=0.41$, $P=0.046$, least squares regression) and CPK ($0.55x-217.48$, $r^2=0.45$, $P=0.035$, least squares regression) activities and LOE$_{50}$ but not when phylogeny was taken into account. There was no relationship between brain CS activity and LOE$_{50}$.

When enzyme activities were expressed relative to total soluble protein (Appendix A), only brain LDH activity showed a relationship to $P_{\text{crit}}$ ($-34.98x+74.69$, $r^2=0.36$, $P=0.041$) and LOE$_{50}$ ($730.78x-524.09$, $r^2=0.69$, $P=0.003$) using least squares regression analysis. When corrected for the phylogenetic relationships among species, LOE$_{50}$ ($620.00x$, $r^2=0.65$, $P=0.005$) regressed to brain LDH activity but not $P_{\text{crit}}$ although the $P$-value approached significance ($-35.20x$, $r^2=0.31$, $P=0.058$). Brain PK, CPK, and CS standardized to total soluble protein did not show a relationship with $P_{\text{crit}}$. There was a negative relationship between total soluble protein in the brain and $P_{\text{crit}}$ (Fig. 2.4A), but not LOE$_{50}$.

There was variation in brain ATP and CrP levels among the species of sculpin in normoxia (Table 2.3), but there was no relationship between these metabolite levels and $P_{\text{crit}}$ or LOE$_{50}$. Due to a limitation in tissue size, we were unable to measure glycogen levels in the brain.

2.4.5 Liver

Species of sculpin exhibited a range of LDH, PK, CPK and CS activities in the liver (Table 2.2; Appendix A); however, there was no relationship between $P_{\text{crit}}$ or
LOE<sub>50</sub> and the activity of any enzyme expressed relative to tissue mass or total soluble protein. There was no relationship between soluble liver protein and LOE<sub>50</sub> or P<sub>crit</sub> using least squares regression analysis. The phylogenetically corrected correlation between soluble liver protein and P<sub>crit</sub> was significant (Fig. 2.4B), suggesting that species of sculpin with higher P<sub>crit</sub> also exhibited higher levels of total soluble protein in their liver. However when the data were analyzed with PIC using the Bayesian consensus tree instead of the maximum likelihood tree, there was no relationship between soluble liver protein and P<sub>crit</sub>. This inconsistency in our PIC correlations is likely attributed to the differences in branch lengths between the two types of trees, indicating that the correlation may be an artifact of a specific tree topology.

Liver ATP, CrP and glycogen varied among the different species of sculpin (Table 2.3), but despite this variation, there was no relationship between the liver metabolites and P<sub>crit</sub> or LOE<sub>50</sub>.

### 2.4.6 Muscle

Activities of LDH, PK, CPK and CS varied among the sculpins examined (Table 2.2, Appendix A). Despite this variability, there was no relationship between our measures of hypoxia tolerance (P<sub>crit</sub> or LOE<sub>50</sub>) and muscle enzyme activities either when expressed relative to tissue mass or when expressed relative to total soluble protein. Soluble muscle protein did not correlate with P<sub>crit</sub> (Fig. 2.4C) or with LOE<sub>50</sub>.

Similar to liver and brain, there were differences in the levels of muscle ATP, CrP, and glycogen among species of sculpin (Table 2.3). There was a negative relationship between muscle CrP and P<sub>crit</sub>, where species of sculpin with low P<sub>crit</sub> exhibited high levels of muscle CrP (-1.19x+63.937, r<sup>2</sup>=0.33, P=0.050, least squares regression; -1.47x, r<sup>2</sup>=0.46, P=0.016, PIC regression). When LOE<sub>50</sub> was regressed against muscle CrP levels, there was no significant pattern among sculpins. Neither P<sub>crit</sub> nor LOE<sub>50</sub> regressed against muscle ATP and glycogen.
2.5 Discussion

2.5.1 Hypoxia tolerance

The comparative approach in tandem with phylogenetic analysis is a powerful tool in providing evidence of selection on phenotypic traits once it is determined that there is variation in tolerance among species as a consequence of an environmental pressure. Environmental hypoxia may be an important agent of selection among sculpins, as different species are exposed to varied frequency, duration and magnitude of low levels of O$_2$. Loss of equilibrium and unresponsiveness to stimuli pose severe impediments to survival in the wild, therefore an important indicator of tolerance to hypoxia is the length of time that is required for individuals of a species to reach these endpoints, which is expressed as LOE$_{50}$. Sculpins exhibited a range of LOE$_{50}$ values (Fig. 2.2), indicating that variation in hypoxia tolerance exists in this group of closely related species.

The ability of an animal to acquire O$_2$ from its environment is considered to be critical to hypoxia survival. Indeed, among the species of sculpin investigated herein, we observed a strong relationship between LOE$_{50}$ and $P_{\text{crit}}$, supporting the notion that $P_{\text{crit}}$ is an indirect measure of hypoxia tolerance as previously suggested in the literature (Hagerman 1998; Chapman et al. 2002; Mandic et al. 2009; Seibel 2011; Speers-Roesch et al. 2012). In sculpins, there was a correlation between LOE$_{50}$ and $P_{\text{crit}}$, where species with high LOE$_{50}$ values exhibited correspondingly low $P_{\text{crit}}$ values (Fig. 2.2). However, the $r^2$ value for the correlation indicated that only 46% of the variability in $P_{\text{crit}}$ was associated with the variability in LOE$_{50}$, and the correlation became non-significant with the application of PIC. This would suggest that beyond O$_2$ extraction, other characteristics, such as metabolic rate suppression and capacity to generate energy via substrate-level phosphorylation, may play important roles in contributing to the variation in hypoxia tolerance among sculpins.

As O$_2$ levels drop below the point where oxidative phosphorylation can generate adequate ATP to sustain metabolic functions (ie. below a species $P_{\text{crit}}$) there is an increased reliance on glycolysis and CrP hydrolysis for the production of
ATP (DiAngelo and Heath 1987; Boutilier et al. 1988; Van Waarde et al. 1990). This switch in energy production during low O₂ exposure occurs in both hypoxia-tolerant and hypoxia-intolerant species. Therefore the distinguishing characteristic between hypoxia-tolerant and -intolerant species is not the recruitment of anaerobic pathways, but instead it is thought to be the ability to generate sufficient flux through these pathways during exposure to hypoxia. As such, we predicted that the hypoxia-tolerant species would have high tissue levels of substrates to support these pathways or that the hypoxia-tolerant species would possibly exhibit higher maximal enzyme activities as suggested in previous studies (e.g. Martinez et al. 2011).

### 2.5.2 Brain enzymes

The brain is an energetically demanding organ and as such is highly susceptible to decreases in O₂ (Lutz 1992; Erecinska and Silver 2001; Nilsson 2001). To prevent rapid neural death during exposure to hypoxia, tolerant species increase their reliance on glycolysis and increase blood flow to the brain in order to maintain a constant supply of ATP (Storey 1987; Nilsson et al. 1994; Johansson et al. 1995). Our data are consistent with this idea as higher PK and LDH activities expressed relative to tissue mass suggest that maximal capacity for glycolysis is greater in the more hypoxia-tolerant sculpins, indicating that these species may be capable of producing more ATP during a sudden drop in environmental O₂. Although the brain primarily relies on glycogen and glucose for energy supply (reviewed in Bolanos et al. 2009), CrP hydrolysis also occurs to buffer ATP consumption (Lutz et al. 2003) and high CPK activity expressed relative to tissue mass, as seen in sculpins with low Pcrit, could confer a comparable benefit of higher ATP production during hypoxia. Similar to the trend observed among the enzymes involved in the anaerobic pathways, there was a significant regression between CS, an enzyme involved in the tricarboxylic acid cycle, and hypoxia tolerance as measured by Pcrit. Sculpins with a low Pcrit had greater CS activity expressed relative to tissue mass than the species with a high Pcrit and this could be advantageous as it is likely that glucose oxidation still occurs in the brain during hypoxia due to increased blood flow, as seen in the
crucian carp (Nilsson et al. 1994). Increased blood flow to the brain would allow the delivery of any $O_2$ that the animal is able to extract from the environment as well as glucose mobilized from sources such as the liver.

Up-regulating the amount of enzymes and hence enzymatic activities can take time, therefore it could be beneficial to maintain high levels of these enzymes even under normoxic conditions. This is particularly relevant in sculpins, where they can experience sudden bouts of short-term hypoxia in their natural environment, such as for sculpins isolated in tidepools at night. Indeed, in the present study we show in sculpins that hypoxia tolerance, quantified by measures of $P_{crit}$, regressed against LDH, PK, CPK and CS activities expressed relative to tissue mass (Fig. 2.3). The relationships remained significant when phylogenetic independent contrast analysis was conducted, indicating that the variation in enzyme activities among sculpin species may be the result of adaptation to hypoxia. However, when LOE$_{50}$ was used as an index of hypoxia tolerance in sculpins, only brain LDH exhibited a phylogenetically independent relationship to hypoxia tolerance. There was no relationship between brain CS and LOE$_{50}$, and while LOE$_{50}$ significantly regressed against brain PK and CPK, the relationships were not significant when phylogeny was taking into account, possibly attributing the variation in these enzymes to phylogenetic distance among species.

Our data suggests that $P_{crit}$ rather than LOE$_{50}$ regressed more strongly against brain maximal enzyme activities expressed relative to tissue mass in sculpins. However, the discrepancy between $P_{crit}$ and LOE$_{50}$ in the regressions with brain enzyme activities may be a result of decreased statistical power in the LOE$_{50}$ regressions. Maximal enzyme values for $M. scorpius$ and $S. marmoratus$ could not be included in the regressions with LOE$_{50}$ since LOE$_{50}$ values are not known for these two species, potentially leading to a loss in the ability to detect significant relationships between maximal enzyme activities and LOE$_{50}$.

### 2.5.3 Standardization of enzyme activity

Standardization of enzyme activity to a common denominator may be achieved in multiple ways. For example, enzyme activity can be expressed relative
to tissue mass, total soluble protein, or in case of mitochondrial enzymes, relative to cytochrome oxidase (COX) or many other denominators depending on the specific goal of the analysis (Leary et al. 2003). In the current study, enzyme activities discussed thus far have been expressed relative to tissue mass. When total soluble protein was used as a denominator, $P_{\text{crit}}$ and LOE$_{50}$ only regressed significantly against brain LDH activity. Interestingly, there was a phylogenetically independent regression between $P_{\text{crit}}$ and total soluble protein, where sculpins with low $P_{\text{crit}}$ displayed high soluble brain protein levels as opposed to the lower levels of soluble brain protein exhibited by species with high $P_{\text{crit}}$ (Fig. 2.4). Although the underlying reason behind this trend is unclear, one possible explanation is that the high soluble protein levels in the brain increase protein stability, which may be important in animals that are prone to environmental perturbations such as hypoxia. Proteins may have low intrinsic stabilities as a trade-off for increased degree of flexibility to undergo rapid catalytic conformational changes during periods of environmental stress and are therefore greatly dependent on high cellular protein concentration to maintain functional protein structure (Hochachka and Somero 2002). Alternatively, reversible brain swelling has been known to occur during hypoxia in some fishes such as the common carp (Van der Linden et al. 2001), and it is possible that high levels of soluble proteins in hypoxia-tolerant species of sculpin may be a mechanism to negate the negative effects of large intracellular volume increases on protein function. However, there was no relationship between brain soluble proteins and LOE$_{50}$ and further investigation is necessary to confirm if there are high levels of soluble proteins in the brains of tolerant species of sculpin and if so, to test whether these high levels of soluble proteins play a role in maintaining protein function during exposure to hypoxia.

### 2.5.4 Muscle and liver enzymes

There were no significant regressions among different enzyme activities and $P_{\text{crit}}$ or LOE$_{50}$ in either the muscle or the liver. During hypoxia exposure, energy demand in the muscle can decrease as a result of reduction in spontaneous locomotor activity (Nilsson et al. 1993) and a rapid decrease in protein synthesis.
rates (Smith et al. 1996). Therefore, unlike the brain, energy demand can be significantly reduced in the muscle negating the need to up-regulate activities of enzymes associated with anaerobic pathways. In support of this idea, muscle glycolytic enzyme activities in *Fundulus grandis* were seen to significantly decrease (Martinez et al. 2006) and muscle CPK mRNA levels were down-regulated in *Gullichthys mirabilis* (Gracey et al. 2001) in response to low O2 exposures. Lack of a relationship between hypoxia tolerance and enzyme activities in the muscle could also be attributed to other selection pressures acting on glycolysis and CrP hydrolysis such as burst swimming for predator avoidance or prey capture. In the liver, many glycolytic enzymes are also common to gluconeogenesis, a process where lactate transported from other areas of the body is re-synthesized into glucose (Knox et al. 1980). Many of the enzymes are readily reversible and therefore, a lack of regression to hypoxia tolerance may reflect the dual function of these enzymes.

### 2.5.5 Metabolites

Sustaining flux during hypoxia requires not only elevated enzyme capacities but sufficient substrate reserves as well. The remarkable hypoxia/anoxia tolerance of goldfish and crucian carp have been partly attributed to large reserves of liver glycogen (Van den Thillart et al. 1980; Hyvarinen et al. 1985) and in general, level of glycogen deposition is thought to be affected by hypoxia (Van den Thillart and Van Raaij 1995). Therefore it is plausible that higher glycogen or CrP levels may occur in the more hypoxia-tolerant species. The results for muscle CrP appear to support this prediction, as there was a phylogenetically independent regression between hypoxia tolerance and muscle CrP, although this was only the case when $P_{\text{crit}}$ was used as a measure of hypoxia tolerance and not LOE50. Although elevated levels of muscle CrP in hypoxia-tolerant sculpin species may be beneficial for maintaining ATP during exposure to hypoxia, without further measurements of creatine levels and pH buffering capacity it is difficult to ascertain the mechanism contributing to the variation of CrP levels among the sculpin species. Unlike the muscle, there was no relationship between CrP and hypoxia tolerance in the brain or the liver.
suggesting that this mode of energy production is not likely sustained long in these tissues during a bout of hypoxia. Hydrolysis of CrP may primarily be utilized in the initial stages of hypoxia until exogenous glucose can be delivered to the tissue or tissue glycogen mobilized.

There was high variation in liver glycogen among the differentially tolerant species of sculpin. However, neither $P_{\text{crit}}$ or $\text{LOE}_{50}$ regressed against liver glycogen. The lack of trend between hypoxia tolerance and liver and muscle glycogen in sculpins is contrary to the prediction that species more frequently exposed to environmental hypoxia store greater amounts of glycogen in their tissues than species that rarely experience low $O_2$. This may be attributed to the highly labile glycogen stores that can be influenced by many factors, including temperature, diet, and burst swimming activity (Van den Thillart and Raaij 1995). Although the amount of substrate available may be an important factor for hypoxic survival, there was no clear relationship in sculpins that the more hypoxia-tolerant species exhibit higher levels of glycogen or CrP than the more intolerant species.

2.5.6 Conclusions

Increased reliance on substrate-level phosphorylation for ATP production is a critical component of hypoxia survival. In the brain, sculpins with low $P_{\text{crit}}$ and high $\text{LOE}_{50}$ expressed high LDH activities regardless of whether the maximal enzyme activity was expressed relative to tissue mass or total soluble protein. As these relationships remained significant when phylogeny was taken into account, one possibility is that selection is acting on maximum capacity for flux through anaerobic pathways for ATP production during hypoxia in the brain. Although these significant correlations in the brain are intriguing, they must be viewed cautiously because of the inflated Type II error associated with the multiple comparisons. The results observed in the brain are in contrast with the results seen in liver and the white muscle, where there were no relationships between hypoxia tolerance and maximal enzyme activities. A significant, phylogenetically independent relationship was found between $P_{\text{crit}}$ and total soluble protein in the brain, although the functional significance of this is unknown. Further investigation is necessary to
ascertain if high total soluble protein in the brain of hypoxia-tolerant species of sculpin is an important factor to survival in hypoxia.
**Figure 2.1** Phylogenetic relationship among 15 species of sculpin based on a maximum-likelihood tree using cytochrome b sequences. Satyrichthys amiscus is included as an out-group. A similar tree has been previously published (Mandic et al. 2009) but did not include Hemilepidotus hemilepidotus and Myoxocephalus polyacanthocephalus. Numbers above lines show percent of 1000 maximum-likelihood bootstrap replicates with values shown for groups with more than 50% support. Italicized numbers below lines show posterior probability from Bayesian Markov Chain Monte Carlo analysis.
Figure 2.2 Relationship between $P_{\text{crit}}$ and LOE$_{50}$.
Pearson’s correlation (dashed line; -0.04x+46.57, $r^2=0.46$, $P=0.023$) and phylogenetically independent contrast (PIC) correlation (solid line; -0.03x, $r^2=0.31$, $P=0.073$) are plotted on species data that are presented as mean ± SE. Dotted lines represent 95% prediction lines for the PIC correlation. Numbers represent different species: 1) Oligocottus maculosus, (2) Clinocottus globiceps, (3) Oligocottus snyderi, (4) Myoxocephalus polyacanthocephalus, (5) Enophrys bison, (6) Artedius fenestralis, (7) Artedius lateralis, (8) Leptocottus armatus, (9) Blepsias cirrhosus, (10) Cottus asper, and (11) Hemilepidotus hemilepidotus.

Figure 2.3 Relationships between $P_{\text{crit}}$ and maximal enzyme activities of lactate dehydrogenase (LDH), pyruvate kinase (PK), creatine phosphokinase (CPK) and citrate synthase (CS) in the brain of 12 species of sculpin.
$P_{\text{crit}}$ and brain LDH (A; -0.29x+76.55, $r^2=0.75$, $P<0.001$, least squares regression; -0.30x, $r^2=0.80$, $P<0.001$, phylogenetically independent contrast (PIC) regression), $P_{\text{crit}}$ and brain PK (B; -0.44x+74.58, $r^2=0.34$, $P=0.045$, least squares regression; -0.54x, $r^2=0.45$, $P=0.016$, PIC regression), $P_{\text{crit}}$ and brain CPK (C; -0.04x+70.32, $r^2=0.57$, $P=0.004$, least squares regression; -0.04x, $r^2=0.60$, $P=0.003$, PIC) and $P_{\text{crit}}$ and brain CS (D; -8.45x+71.22, $r^2=0.36$, $P=0.039$, least squares regression; -8.67x,
Least squares regression (dashed lines) and PIC regressions (solid lines) are plotted on species data that are presented as mean ± SE. Dotted lines represent 95% prediction lines for the PIC regressions. Numbers represent different species: (1) *Oligocottus maculosus*, (2) *Clinocottus globiceps*, (3) *Oligocottus snyderi*, (4) *Myoxocephalus polyacanthocephalus*, (5) *Enophrys bison*, (6) *Artemius fenestralis*, (7) *Artemius lateralis*, (8) *Leptocottus armatus*, (9) *Blepsias cirrhosus*, (10) *Cottus asper*, (11) *Scorpaenichthys marmoratus*, and (12) *Myoxocephalus scorpius*.

**Figure 2.4** Relationship between $P_{\text{crit}}$ and soluble protein concentration in brain, liver, and muscle.

Brain (A; $-0.29x+75.84$, $r^2=0.52$, $P=0.008$, least squares regression; $-0.29x$, $r^2=0.71$, $P<0.001$ phylogenetically independent contrast (PIC) regression), liver (B; $0.12x+19.23$, $r^2=0.09$, $P=0.337$, least squares regression; $0.29x$, $r^2=0.36$, $P=0.040$, PIC regression), and muscle (C; $-0.23x+53.99$, $r^2=0.12$, $P=0.262$, least squares regression).
regression; -0.29x, \( r^2 = 0.18 \), \( P = 0.173 \), PIC regression). See figure 2.3 captions for more detail.
Table 2.1  Body weights of species of sculpin used in the LOE<sub>50</sub> experiment and in analytical procedures.

Body weights are expressed as grams. The first data column refers to body weights of fish used in the LOE<sub>50</sub> experiment and the second data column refers to body weights of fish used to assess maximal enzyme activities and metabolites. Data are means ± SE.

<table>
<thead>
<tr>
<th>Species</th>
<th>LOE&lt;sub&gt;50&lt;/sub&gt; experiment</th>
<th>Analytical experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. maculosus</em></td>
<td>2.92±0.22</td>
<td>4.50±0.50</td>
</tr>
<tr>
<td><em>C. globiceps</em></td>
<td>1.21±0.06</td>
<td>1.59±0.20</td>
</tr>
<tr>
<td><em>O. snyderi</em></td>
<td>3.85±1.30</td>
<td>2.54±0.71</td>
</tr>
<tr>
<td><em>M. polyacanthocephalus</em></td>
<td>14.42±2.32</td>
<td>10.50±1.30</td>
</tr>
<tr>
<td><em>E. bison</em></td>
<td>4.28±1.61</td>
<td>10.91±5.29</td>
</tr>
<tr>
<td><em>A. fenestralis</em></td>
<td>6.52±2.32</td>
<td>16.91±2.14</td>
</tr>
<tr>
<td><em>A. lateralis</em></td>
<td>14.48±3.58</td>
<td>15.16±1.38</td>
</tr>
<tr>
<td><em>L. armatus</em></td>
<td>32.26±1.58</td>
<td>48.22±2.07</td>
</tr>
<tr>
<td><em>S. marmoratus</em></td>
<td>N/A</td>
<td>20.84±2.88</td>
</tr>
<tr>
<td><em>B. cirrhosis</em></td>
<td>7.20±0.54</td>
<td>2.63±0.37</td>
</tr>
<tr>
<td><em>M. scorpius</em></td>
<td>N/A</td>
<td>9.73±2.65</td>
</tr>
<tr>
<td><em>C. asper</em></td>
<td>30.47±2.10</td>
<td>19.34±1.84</td>
</tr>
<tr>
<td><em>H. hemilepidotus</em></td>
<td>8.13±1.28</td>
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</tr>
</tbody>
</table>
Table 2.2  Tissue enzymes in 12 species of sculpin.

Tissue enzymes are presented in μmol/min/g wet tissue. Numbers in parentheses represent sample size. Data are means±SE.

<table>
<thead>
<tr>
<th>Species</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDH</td>
<td>PK</td>
</tr>
<tr>
<td><em>O. maculosus</em></td>
<td>3.06±0.81 (16)</td>
<td>5.62±0.64 (16)</td>
</tr>
<tr>
<td><em>C. globiceps</em></td>
<td>6.01±1.73 (10)</td>
<td>4.27±0.99 (10)</td>
</tr>
<tr>
<td><em>O. snyderi</em></td>
<td>4.76±1.02 (8)</td>
<td>3.14±0.62 (8)</td>
</tr>
<tr>
<td><em>M. polyacanthocephalus</em></td>
<td>1.59±0.11 (8)</td>
<td>4.06±0.71 (8)</td>
</tr>
<tr>
<td><em>E. bison</em></td>
<td>6.85±1.67 (11)</td>
<td>6.48±1.16 (11)</td>
</tr>
<tr>
<td><em>A. fenestralis</em></td>
<td>1.64±0.20 (8)</td>
<td>3.68±0.42 (8)</td>
</tr>
<tr>
<td><em>A. lateralis</em></td>
<td>2.01±0.48 (7)</td>
<td>3.49±0.51 (7)</td>
</tr>
<tr>
<td><em>L. armatus</em></td>
<td>3.98±0.50 (12)</td>
<td>4.12±0.37 (12)</td>
</tr>
<tr>
<td><em>S. marmoratus</em></td>
<td>3.38±0.49 (12)</td>
<td>6.06±0.54 (12)</td>
</tr>
<tr>
<td><em>B. cirrhosis</em></td>
<td>1.81±0.20 (12)</td>
<td>4.86±0.36 (12)</td>
</tr>
<tr>
<td><em>M. scorpius</em></td>
<td>3.09±0.47 (7)</td>
<td>4.94±0.37 (7)</td>
</tr>
<tr>
<td><em>C. asper</em></td>
<td>1.94±0.17 (8)</td>
<td>2.55±0.32 (8)</td>
</tr>
</tbody>
</table>
Table 2.3  Tissue metabolites in 12 species of sculpin.

Tissue metabolites are presented in μmol/g wet tissue. Numbers in parentheses represent sample size. Data are means ± SE.

<table>
<thead>
<tr>
<th></th>
<th>Brain</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>CrP</td>
<td>ATP</td>
</tr>
<tr>
<td>O. maculosus</td>
<td>0.7±0.1</td>
<td>1.7±0.4</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>(10)</td>
<td></td>
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<td>(8)</td>
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<tr>
<td>C. globiceps</td>
<td>0.4±0.1</td>
<td>0.6±0.2</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>(8)</td>
<td></td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>O. snyderi</td>
<td>0.8±0.3</td>
<td>1.8±0.4</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>M. polyacanthocephalus</td>
<td>0.8±0.1</td>
<td>2.0±0.2</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>E. bison</td>
<td>1.1±0.1</td>
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<td>(7)</td>
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<tr>
<td>S. marmoratus</td>
<td>0.9±0.1</td>
<td>2.3±0.4</td>
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<td>(8)</td>
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<tr>
<td>B. cirrhosis</td>
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<td>0.8±0.2</td>
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<td>(10)</td>
<td></td>
<td></td>
<td>(9)</td>
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<tr>
<td>M. scorpius</td>
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</tbody>
</table>
Chapter 3

Divergent Transcriptional Patterns are Related to Differences in Hypoxia Tolerance Between the Intertidal and the Subtidal Sculpins²

3.1 Summary

Transcriptionally mediated phenotypic plasticity as a mechanism of modifying traits in response to an environmental challenge remains an important area of study. We compared the transcriptional responses to low oxygen (hypoxia) of the hypoxia-tolerant intertidal fish, the tidepool sculpin (Oligocottus maculosus) with the closely related hypoxia-intolerant subtidal fish, the silverspotted sculpin (Blepsias cirrhosus) to determine if these species use different mechanisms to cope with hypoxia. Individuals from each species were exposed to environmental O₂ tensions chosen to yield a similar level of tissue hypoxia and gene transcription was assessed in the liver over time. There was an effect of time in hypoxia, where the greatest transcriptional change in the silverspotted sculpin occurred between 3 to 24 hours in contrast to the tidepool sculpin where the largest transcriptional change occurred between 24 and 72 hours of hypoxia. A number of genes showed similar hypoxia-induced transcription patterns in both species (e.g. genes associated with glycolysis and apoptosis) suggesting they are involved in a conserved hypoxia

² A version of this chapter has been published: Mandic M., M.L. Ramon, A.Y. Gracey and J.G. Richards. 2014. Divergent transcriptional patterns are related to differences in hypoxia tolerance between the intertidal and the subtidal sculpins. Molecular Ecology 23: 6091-6103
response. A large set of genes showed divergent transcriptional patterns in the two species, including fatty acid oxidation and oxidative phosphorylation, suggesting that these biological processes may contribute to explaining variation in hypoxia tolerance in these species. When both species were exposed to a single environmental $O_2$ tension, large transcriptional responses were seen in the hypoxia-intolerant silverspotted sculpin while almost no response was observed in the hypoxia-tolerant tidepool sculpin. Overall, divergent transcription patterns in response to both magnitude and duration of hypoxia provide insights into the processes that may determine an animal’s capacity to tolerate frequent bouts of hypoxia in the wild.

3.2 Introduction

The evolution of phenotypic plasticity is an important evolutionary mechanism for adaptation of animals to variable environments (see reviews West-Eberhard 1989, Ghalambor et al. 2007). In many aquatic ecosystems, periodic decreases in $O_2$ tension (hypoxia) are common and create spatial and temporal environmental heterogeneity. As a result, hypoxia is considered to be an important selective pressure in the evolution of aquatic organisms (Nikinmaa 2002). In particular, the marine rocky intertidal zone is susceptible to rapid and pronounced changes in $O_2$ tension due to the predictable influence of the tidal cycle. When tidepools are isolated from the ocean at night, $O_2$ levels drop to near anoxic levels as a consequence of flora and fauna respiration. While the severity and frequency of hypoxia is significantly reduced in the connecting subtidal environment, periodic hypoxia still occurs as a result of the lower mixing of water associated with the protected nearshore environment (Truchot and Duhamel-Jouve 1980, Richards 2011). In contrast, $O_2$ tensions are generally high and stable in the pelagic zone and up to mid-range depths of $\sim$400 m (reviewed in Diaz and Breitburg 2009). This spatial and temporal variation of hypoxic events, along with variation in other abiotic factors, heavily influences species ranges resulting in a strong vertical zonation pattern along the nearshore (Trussell and Etter 2001, Altieri 2006).
Sculpins (superfamily Cottoidea) are a group of predominantly benthic fish species that inhabit environments from the intertidal and nearshore to deeper, mid-range water depths and the greatest diversity of sculpin species occur in the Northeastern Pacific (Nelson 2006, Froese and Pauly 2013). Phylogenetic analysis indicates that the direction of evolution of sculpins is from the subtidal to the intertidal environment, suggesting that the ancestral species are found primarily in the O₂ stable subtidal and the more derived species are found in the O₂ variable intertidal (Ramon and Knope 2008). The distribution of sculpins along the nearshore environment is related to their hypoxia tolerance with the more hypoxia-tolerant species inhabiting the intertidal and the less hypoxia-tolerant species inhabiting deeper subtidal waters. Variation in hypoxia tolerance among sculpins is explained, at least in part, by variation in physiological traits involved in O₂ extraction and biochemical traits involved in energy production (Mandic et al. 2009, Chapter 2). However, the extent to which phenotypic plasticity, versus constitutive differences in phenotype, play a role in hypoxic survival in the heterogeneous nearshore environment is unknown among sculpins that differ in hypoxia tolerance.

Identifying the mechanisms that result in phenotypic plasticity remains an important question in ecology and evolution (Aubin-Horth and Renn 2009). Environmentally induced changes in phenotypic traits may be achieved through multiple mechanisms, including changes in allelic sensitivity and gene transcription (Via et al. 1995, Dewitt et al. 1998), as well translational and post-translation modification. Particular attention has been given to understanding the role of environmentally induced changes in gene transcript levels and the characterization of transcriptomic reaction norms (Schlichting and Pigliucci 1993, Schlichting and Smith 2002, Aubin-Horth and Renn 2009) because changes in gene transcription have been shown to result in phenotypic plasticity in response to environmental change (as reviewed by Schlichting and Pigliucci 1993) and because of the availability of high-throughput methods (e.g. microarrays and RNA-seq; Aubin-Horth and Renn 2009). Multiple studies have examined transcription patterns during exposure to short-term (Gracey et al. 2001, Ju et al. 2007, Everett et al. 2011, Leveelahti et al. 2011), long-term (van der Meer et al. 2005) and developmental
hypoxia (Ton et al. 2003) and these studies provide insight into the genes and pathways that are potentially involved in hypoxia-induced phenotypic plasticity. However, despite our general understanding of the effects of hypoxia on gene transcription in fish, no study has attempted to assess how natural variation in hypoxia tolerance among fishes affects the gene transcription patterns that form the basis of phenotypic plasticity.

The goal of this study was to compare the hypoxia-induced gene transcription patterns between the hypoxia-tolerant tidepool sculpin (*Oligocottus maculosus*) and the hypoxia-intolerant silverspotted sculpin (*Blepsias cirrhosus*) to determine if, and how, differences in transcriptional plasticity are associated with differences in hypoxia tolerance. We have conducted this work to gain insight into the biological processes that may change in response to hypoxia exposure and contribute to defining differences in whole animal hypoxia tolerance and species distribution patterns across the rocky intertidal zone. Since the ability to acquire O$_2$ from the environment differs between the two study species due to differences in gill surface area and hemoglobin-O$_2$ binding affinity (Mandic et al. 2009), we used an experimental treatment that scaled our hypoxia exposures to the species-specific hypoxia tolerance. Therefore, both species experienced the same level of hypoxemia and tissue hypoxia (see Speers-Roesch et al. 2012; this treatment is referred to as the “relative hypoxia exposure”) and we examined changes in transcript levels for up to 72 hours of hypoxia. While this approach may lead to fewer differences in gene transcription between the two species than may occur if both species were exposed to a single environmental O$_2$ tension, any differences in transcriptional patterns are more likely to be involved in defining hypoxia tolerance. In addition, we also exposed the two species of sculpin to a single environmental O$_2$ tension for 24 hours to examine the direct effect of environmental hypoxia, which is an important factor that determines species distribution (termed the “absolute environmental hypoxia”). In both hypoxia exposures, we examined the transcript profile in the liver, which is a tissue critical to energy metabolism and as a result is vital in an animal’s defense against hypoxia (van den Thillart et al. 1980). To narrow the focus of our analyses, we posed the following questions: 1) Is there temporal variation in
gene transcript levels and how does it vary between the two species of sculpin? 2) Are there similar transcriptional patterns in genes between the two species? 3) What are the genes or biological processes for which these species exhibit a divergent transcriptional pattern? 4) How does gene transcription differ between these species when exposed to absolute environmental hypoxia, especially in comparison to relative environmental hypoxia of the same duration?

3.3 Materials and Methods

3.3.1 Experimental animals

Sculpins were collected in July and August of 2010 near the Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada. Tidepool sculpins (*Oligocottus maculosus*), a fish species found along the intertidal zone and most densely populated in the upper mid tidepool region (Green 1971), were caught using handheld nets while beach seines were used to capture silverspotted sculpins (*Blepsias cirrhosus*), a fish species typically found in the kelp and eelgrass beds of the subtidal zone (Dean et al. 2000, Elfird and Konar 2013). Collection occurred during the lowest tidal cycle at Wizard’s Rock (48°51.5′N; 125°9.4′W). The sculpins were transported to The University of British Columbia and held in fully aerated recirculating seawater at 12°C and 30 ppt salinity, replaced monthly with natural, sand-filtered seawater from the Vancouver Aquarium. The fish were allowed to acclimate to constant laboratory conditions for two months prior to experimentation. During this time, fish were fed daily with a combination of bloodworms and krill, except 24 hours before an experiment.

3.3.2 Hypoxia time-course

Fish were transferred to a custom-built recirculating system, the Low Oxygen Control and Monitoring Aquatic Research System (Integrated Aqua Systems, Inc.), designed to closely regulate O$_2$ levels and temperature. Individuals from each species were split into four separate, 36 gallon insulated tanks capable of independent control of O$_2$ tensions, with a front-facing window. Fish were allowed to recover for 24 hours under full air saturation, following which access to the air-
water interface was restricted by a Plexiglas cover. We exposed the two species of sculpin to hypoxia at O\textsubscript{2} tensions that corresponded to 65\% of the species-specific $P_{\text{crit}}$, which is a measurement of O\textsubscript{2} extraction and a proxy for hypoxia tolerance ($P_{\text{crit}}$ of 25.9 Torr for tidepool sculpin and 44.4 Torr for silverspotted sculpin, Mandic et al. 2009). A previous study of gene transcription in *Gillichthys mirabilis* (Gracey et al. 2001) found that this level of hypoxia induced hypoxic stress while allowing fish to survive for up to 6 days of hypoxia. Specifically, two tanks of the hypoxia-tolerant tidepool sculpin, were exposed to O\textsubscript{2} tensions set at 17 Torr and two tanks of the hypoxia-intolerant silverspotted sculpin, were exposed to 29 Torr (“experiment 1: relative hypoxia exposure”). Additionally, O\textsubscript{2} tensions were set to 23 Torr in the third tank of each species (“experiment 2: absolute environmental hypoxia exposure”). Normoxic conditions (~158 Torr) were maintained in the fourth tank of individuals for both species (Table 3.1).

For experiment 1, liver, muscle, heart, brain and gill from individuals of both species were sampled (only liver was used in the microarray experiments and other tissues were archived) at 3, 8, 24, 48 and 72 hours of relative hypoxia exposure, with two normoxic fish sampled at each hypoxic time point. For experiment 2, the same tissues were sampled at 24 hours. As experiment 1 and 2 were conducted simultaneously we used the same normoxic control samples. Briefly, fish were removed from the tank and immediately euthanized by exposure to an overdose of benzocaine (125 mg mL\textsuperscript{-1}; Sigma-Aldrich). Tissues were dissected, flash frozen in liquid N\textsubscript{2}, and stored at -80\°C until analysis.

### 3.3.3 Microarray hybridization

For each species, total RNA was isolated from liver of five normoxic and four hypoxic samples per time point, for a total of 29 samples per species, using TRIzol (Invitrogen) according to the manufacturer’s protocol. The total RNA was purified further using glass-fiber filter columns (Qiagen) according to the manufacturer’s instructions. Double-stranded cDNA was prepared by reverse-transcribing 1\mu g of liver RNA in a 20 \mu l reaction containing 20 pmols of T7-dT\textsubscript{15}VN primer, 2 \mu l MMLV HP RT 10X Reaction Buffer, 2 \mu l 100 mM DTT, 2 \mu l 50x aminoallyl dNTP mix (25 mM
dATP, 25 mM dCTP, 25 mM dGTP, 15 mM dTTP, 10 mM aminoallyl-dUTP), 20 U of RNaseOut (Invitrogen) and 100 U of MMLV-reverse transcriptase (Epicentre). The RNA and primer mixture was heat-denatured at 65°C for 10 minutes, placed on ice for 10 minutes and the remaining components were added. The reaction was incubated at 40°C for 2 hours and stopped by heating to 65°C for 15 minutes. Next, the reaction was neutralized by the addition of 10 μl 1M HEPES, 3 μl NaOAc and purified using a Qiagen QIAquick PCR purification kit (Qiagen). Half the cDNA was labeled with Cy5 and the other half with Cy3. Fluorescently-labeled cDNA samples were resuspended in 50 μl of hybridization buffer (358.8 mM LiCl, 200 mM LiMes, 50 mM EDTA, 0.1% LDS, 0.1% BSA) with the addition of 1 μl of poly A and 1 μl of tRNA, they were then competitively hybridized for 18 hours at 65°C to a custom cDNA array from woolly sculpin (Clinocottus analis; see section below, “Microarray probe sequences”) using an interwoven loop design (Kerr and Churchill 2001, Vinciotti et al. 2005). Each array then underwent a SDS wash (0.5 x SSC, 0.01% SDS at 50°C) for 10 minutes followed by a 0.06 X SSC wash for 10 minutes to remove unhybridized cDNA. Competitive hybridization was performed only between time point samples within a species and no direct cross-species hybridizations were performed. We used a loop design where each RNA sample was hybridized to either 2 or 4 arrays with fluor-reversal (two loops, one for each species of sculpin, Appendix B). The additional two technical replicates for some of the biological replicates build internal spokes of a hybridization loop in order to connect the loop to create more reliable comparison among the non-adjacent samples (Kerr and Churchill 2001).

The heterologous, or cross-species, microarray platform used for this analysis has been validated by numerous studies (e.g. Renn et al. 2004, Boswell et al. 2009, Chelaifa et al. 2010), but great care must be taken when designing and interpreting heterologous arrays as certain experimental designs can confound estimates of gene transcript levels (Kassahn 2008, Machado et al. 2009). For example, in experiments that competitively hybridize two different species on an array (e.g. Cy3 label for species A and Cy5 label for species B onto microarray platform developed for species C), relative transcription levels between species...
could emerge from differences in transcript abundance or from a difference in sequence divergence that could unknowingly affect how well each species hybridizes to the array (Kassahn 2008, Machado et al. 2009). However, issues with sequence divergence can be avoided in heterologous experiments by competitively hybridizing samples from the same species, rather than between species (e.g. Cy3 label for species A and Cy5 label for species A onto microarray platform developed for species C). Under these analytical conditions, both samples will be similarly affected by sequence divergence and relative expression should solely reflect transcript abundance (Kassahn 2008). In this study, we only conducted competitive hybridization on samples from the same species that differed by hypoxia exposure time. This approach avoids false positives caused by the skewing of the hybridization ratios, which would occur as a result of large sequence divergence when two different species are hybridized together on an array. Further, the current study focused only on clones whose transcript levels were significantly altered in response to hypoxia exposure in both species and we avoid drawing any conclusions regarding the clones that were found to significantly change in only one of the species. This conservative approach of analysis eliminates the possibility of drawing conclusions from false negatives that may be due to poor hybridization.

3.3.4 Microarray probe sequences

The custom woolly sculpin array was an anonymous array consisting of approximately 9600 cDNA clones (array elements). A previous study (Ramon and Gracey, unpublished) generated approximately 800 EST (expression sequence tag) sequences from the cDNA clones on the array and an additional 900 were sequenced for this study. The 900 clones were chosen based on a preliminary data analysis. We selected a number of clones belonging to each of the broad clusters that were generated via hierarchical clustering. The clones were sequenced using the following protocols.

Briefly, clones were grown overnight in 96-well plates, their inserts amplified using flanking vector-specific primers, and the 5’ end sequence of the cDNA inserts were obtained by Sanger sequencing. The resulting 5’-end EST sequences were then
clustered using two consecutive rounds of CAP3 assembly (Huang and Madan 1999). The resulting non-redundant contigs were annotated as the genes for which they had the greatest identity using SwissProt when the EST sequences yielded a hit with an E-value of less than $1 \times 10^{-4}$. We determined after sequencing ten 96-well plates that we had approximately 1% redundancy within our normalized libraries.

### 3.3.5 Statistical analyses

Raw data were log$_2$ transformed and lowess normalized using the maanova package (Wu et al. 2013) in R (R Core Team 2013). Differential expression for each clone across time points was detected using linear mixed models (‘array’ as a random effect, and ‘dye’ and ‘time points’ as fixed effects) with 100 permutations and the F test statistic. Significance was estimated via false discovery rate (Storey 2002) using the qvalue package (Dabney and Storey 2013). Clones showing a main effect of time in hypoxia ($q<0.01$) were identified for each species and clones significant to both species (overlap dataset) were further analyzed (2408 clones, Fig. 3.1). We chose to use a high level of significance ($q<0.01$) rather than the more standard $q<0.05$ to account for minor effects on error estimates of variance due to uneven technical replicates across the biological samples. Genes (annotated ESTs) from the overlap dataset were assigned to gene ontology biological process categories using searches in EMBL-EBI QuickGO (www.ebi.ac.uk/QuickGO/) and Uniprot (http://www.uniprot.org) databases. A principal component analysis was performed in EMA (Easy Microarray data Analysis) package (Servant et al. 2013) in R on the overlap dataset for both species simultaneously. Venn diagrams were created using VENNY (Oliveros 2007).

### 3.4 Results

Transcript levels of 2408 clones significantly changed in response to hypoxia exposure in both the hypoxia-tolerant tidepool sculpin and the hypoxia-intolerant silverspotted sculpin (Fig. 3.1). A principal component analysis, performed on these hypoxia-responsive clones, showed a clear separation between three clusters of time points. One cluster included the late time points for the relative hypoxia
exposure (24hr, 48hr, 72hr) in the tidepool sculpin, while the second cluster included the early time-points for the relative hypoxia exposure (3hr, 8hr, 24hr) and the absolute hypoxia exposure time point (24hr) in the silverspotted sculpin. The third cluster included the 0hr time point for both species as well as the 3hr relative hypoxia and 24hr absolute hypoxia exposure in the tidepool sculpin and the late time-points for relative hypoxia exposure (48hr, 72hr) in the silverspotted sculpin (Fig. 3.2).

3.4.1 Experiment 1: Relative hypoxia exposure time course

A total of 235 genes were assigned a UniProt identification from clones whose transcription significantly changed over time in both species. The annotated genes were broadly categorized into a number of gene ontology biological processes. The biological processes roughly fell into general categories of metabolism, regulation of cell number, immunity, protein production, protein localization and protein folding (Figs. 3.3, 3.4).

In the tidepool sculpin, relative hypoxia exposure resulted in a general increase in transcript abundance for genes associated with glycolysis (Fig. 3.3A), positive regulation of apoptosis (Fig. 3.3E), negative regulation of cell proliferation (Fig. 3.3H), chromosome organization (Fig. 3.4A), regulation of translational initiation (Fig. 3.4C) and protein localization (Fig. 3.4F). In particular, there were generally small changes in transcript level during the initial stages of the time-course, followed by large increases in transcript levels between 24hr and 72hr of hypoxia exposure. The silverspotted sculpin also exhibited an increase in transcript levels of genes involved in glycolysis, positive regulation of apoptosis, regulation of translational initiation and protein localization, but the transcript levels changed during the first 24hr of hypoxia and subsequently generally returned to levels that were close to those seen in the normoxic samples. Unlike the tidepool sculpin, the silverspotted sculpin exhibited a variable transcriptional response in the genes belonging to negative regulation of cell proliferation (Fig. 3.3H) and an increase or no change followed by a decrease in transcript levels of genes associated with chromosome organization (Fig. 3.4A).
There was an overall decrease in the transcript levels of genes assigned to oxidative phosphorylation (Fig. 3.3B), fatty acid oxidation (Fig. 3.3C), response to reactive O$_2$ species (Fig. 3.3D), negative regulation of apoptosis (Fig. 3.3F) and positive regulation of cell proliferation (Fig. 3.3G) between 24hr and 72hr of hypoxia exposure in the tidepool sculpin, with the exception of a transient increase at 48hr in oxidative phosphorylation genes. In contrast, transcript levels of oxidative phosphorylation, fatty acid oxidation and negative regulation of apoptosis genes decreased in the silverspotted sculpin only in the initial stages of hypoxia, at which point there was a steady increase in transcript levels for the duration of hypoxia exposure (Fig. 3.3B,C). The transcriptional response in the silverspotted sculpin was more variable for genes associated with responses to reactive O$_2$ species and positive regulation of cell proliferation, where some of the genes exhibited an increase in transcript level while others a decrease (Fig. 3.3D,G).

Genes associated with RNA processing (Fig. 3.4B), translational elongation (Fig. 3.4D) and protein folding (Fig. 3.4G,H) showed a decrease in transcript levels during the first 24hr hypoxia exposure in the tidepool sculpin, followed by an increase for the rest of the hypoxia exposure. On the other hand, in the silverspotted sculpin there was a decrease in transcript abundance of genes belonging to RNA processing, heat shock proteins (HSPs, protein folding, Fig. 3.4H) and translational elongation (Fig. 3.4D) for the majority of the hypoxia exposure, with the exception of a transient increase at 48hr in translational elongation genes. The remainder of the protein folding genes in the silverspotted sculpin exhibited either a decrease or increase in transcript levels in response to hypoxia (Fig. 3.4G).

In the tidepool sculpin, there was variation in transcript abundance of hypoxia-induced immune response genes and protein maturation genes during the initial 24hr of hypoxia. This was followed by a larger divergence in the transcript levels of the genes, where the transcript levels for some genes decreased while others increased for the remainder of the hypoxia exposure (Fig. 3.3I, Fig. 3.4E). In the silverspotted sculpin, the hypoxia-induced immune response genes and protein maturation genes exhibited a similar pattern of transcription to the tidepool sculpin; however, in the silverspotted sculpin a change in transcript level of the genes
occurred in the first 24 hours with an increase in transcript levels for some genes and a decrease in others. By 48 hours of hypoxia exposure, gene transcript abundance returned close to the normoxic levels (Fig. 3.3I).

To identify the genes that showed the greatest difference in transcriptional response among species in response to hypoxia exposure, we calculated Euclidean distance of the transcriptional time-course curves between the tidepool and silverspotted sculpins. The two genes with the highest Euclidean distances were DNA damage-inducible transcription 4 protein and Diablo homolog, both genes which are associated with positive regulation of apoptosis. Five other genes with high Euclidean distance measures were also associated with regulation of cell number. Other biological processes represented by genes with high Euclidean distance scores were glycolysis, regulation of translation initiation, immune response, chromosome organization and protein folding (Table 3.2, complete list of genes in Appendix C).

3.4.2 Experiment 2: Absolute environmental hypoxia

There was little or no difference in gene transcript levels between relative environmental hypoxia exposure (29 Torr) and absolute environmental hypoxia exposure (23 Torr) at 24hr for the silverspotted sculpin. In the tidepool sculpin, however, gene transcript levels were different between 24hr relative (17 Torr) and absolute (23 Torr) environmental hypoxia exposures. Genes for most of the biological processes in the tidepool sculpin either increased or decreased during relative environmental hypoxia, but the same genes did not change relative to normoxic levels in the absolute environmental hypoxia exposure (e.g. glycolysis, response to reactive O\(_2\) species, regulation of translational initiation, protein maturation) or transcription occurred in the opposite direction than that determined for relative environmental hypoxia exposure (e.g. oxidative phosphorylation, translational elongation, protein folding). Principal component analysis also demonstrated that the 24hr relative and absolute hypoxia time-points clustered together in the silverspotted sculpin, while in the tidepool sculpin, the two time-points were grouped in separate clusters (Fig. 3.2).
3.5 Discussion

The intertidal environment has many of the characteristics that have been proposed to favor the evolution of phenotypic plasticity, including frequent environmental change, temporal variation and reliable environmental cues (Moran 1992, Schlichting and Smith 2002). To identify genes or pathways that may have evolved to mediate phenotypic plasticity in response to environmental change, it is common to use transcriptomic analysis (Schlichting and Pigliucci 1993). In the present study, both the hypoxia-tolerant tidepool sculpin and the hypoxia-intolerant silverspotted sculpin altered the transcription of a large number of genes in response to relative environmental hypoxia. However, there were temporal differences in the transcriptional response between the two species. In the silverspotted sculpin, the greatest transcriptional change occurred rapidly, within 3 to 24 hours of hypoxia exposure. In the tidepool sculpin, on the other hand, the greatest transcriptional response for the most part occurred later at 24 to 72 hours of hypoxia exposure, which is beyond the typical timeframe that tidepool sculpins experience hypoxia in the intertidal zone (approximately 6 to 8 hours; Richards 2011). Although protein translation is thought to occur quickly following transcription (e.g. in sea urchins; Ben-Tabou de-Leon and Davidson 2009), it must be noted that changes in gene transcription levels are not always matched by changes in protein abundance or activity, necessitating functional analysis. Despite this inconsistent link between gene transcription and function, it is thought that genes which show rapid and transient changes in transcription may be most associated with phenotypic plasticity (Aubin-Horth and Renn, 2009). The species-specific temporal patterns suggest that rapid changes in gene transcription, and therefore phenotypic plasticity mediated via gene regulation, are not likely an important component of survival in initial stages of hypoxia for the tidepool sculpin, which inhabits the most O₂ variable environment. However, it is possible that this species does display phenotypic plasticity as a result of translational or post-translational regulation.
It is also possible that the tidepool sculpin is a phenotypic generalist and does not alter phenotype in response to fluctuations in $\text{O}_2$ over environmentally realistic timeframes, in spite of living in an environment that appears to be conducive to the evolution of plasticity. If the lack of a transcriptional response within ecologically relevant timeframes is an indicator that the tidepool sculpin is a phenotypic generalist, several factors may contribute to favoring a fixed generalist phenotype strategy over phenotypic plasticity. As hypoxia exposure in the intertidal is a cyclic and often a daily event, the metabolic costs associated with continually altering phenotype may be greater than the benefit received via matching phenotype to the environmental condition. The lag time between the cue for environmental change and the production of a new phenotype could also cause a mismatch between the environment and the phenotype (Padilla and Adolph 1996) and it is possible that a time lag coupled with the short duration of a tidal cycle may constrain the benefits of phenotypic plasticity in the intertidal and instead favor the evolution of a ‘resilient’ generalist.

3.5.1 Transcription of genes similarly induced or repressed by hypoxia in both species

Both species of sculpin similarly increased or decreased transcription of genes belonging to a number of biological processes, including glycolysis, positive regulation of apoptosis, immune response and protein maturation, albeit the greatest change in transcription of these genes occurred during different stages of hypoxia in each species. The transcription patterns of the later time-course of the tidepool sculpin shared similarities to the transcription patterns of the silverspotted sculpin during the initial stages of hypoxia. This was also seen in genes associated with negative regulation of apoptosis, chromosome organization, regulation of translational initiation and protein localization, except in these biological processes the direction of transcriptional change switched in the later half of the hypoxia exposure in the silverspotted sculpin. Therefore, while the tidepool sculpin exhibited little transcription change during initial hypoxia exposure followed by large changes in the later stages of hypoxia, in the silverspotted sculpin
transcription was initially altered first in one direction then reversed for the remainder of the hypoxia exposure.

Similar induction or repression patterns of gene transcription between the two species may be a result of either shared phylogenetic history or simply be a widespread, common response to hypoxia exposure that is observed in all fishes regardless of hypoxia tolerance. For example, both species of sculpin induced transcription of genes associated with glycolysis. The increase in transcription of glycolytic enzymes has been shown in a number of other fish species (e.g. Gracey et al. 2001, Ton et al. 2003, van der Meer et al. 2005, Ju et al. 2007) and indeed increased reliance on glycolysis during hypoxia has been well documented in fishes, reptiles and mammals and appears to be independent of hypoxia tolerance (Kelly and Storey 1988, Churchill et al. 1994, van den Thillart and van Raaij 1995, Webster 2003). Far less is known about the responses of the other biological processes to hypoxia in fishes, including apoptosis and protein maturation and therefore it is difficult to ascertain whether similar transcriptional changes between the two sculpin species during low O\textsubscript{2} exposure is widespread among fishes or if it is a consequence of the sculpin-specific ancestry.

3.5.2 Transcriptionally divergent response between the species

Transcription of genes involved in oxidative phosphorylation, fatty acid oxidation, cell proliferation, translational elongation, protein folding and antioxidant defenses differed between the two species, such that, for the most part, gene transcription decreased in the tidepool sculpin during hypoxia while in the silverspotted sculpin the transcriptional response was more variable. In contrast to the tidepool sculpin, transcription of genes associated with oxidative phosphorylation and fatty acid oxidation primarily increased during hypoxia in the silverspotted sculpin, while a split response of both an increase and decrease was seen among the genes associated with antioxidant defense and protein folding. These fundamentally divergent responses, despite the fact that they occur over different time frames in the species, may still highlight important biological processes that contribute to explaining the variation in hypoxia tolerance among the
fish species. However, as noted previously, transcriptional change does not always relate to a functional change at higher levels of biological organization, thus further studies are need to elucidate the role of these biological processes in the evolution of hypoxia tolerance.

The ability to suppress metabolic energy demands during hypoxia exposure may be a potential functional consequence of the transcriptional differences between the two species of sculpin. Energy suppression, which has been linked to decreases in sodium pump activity, protein synthesis, urea synthesis and gluconeogenesis, has been postulated to be the hallmark of hypoxia tolerance (Boutilier and St-Pierre 2000, Boutilier 2001, Bickler and Buck 2007, Richards 2009). If an animal is able to decrease energy demand it may be able to maintain energy balance despite a decrease in energy supply, thus prolonging survival during hypoxia. In the tidepool sculpin, transcriptional data suggests hypoxia-induced decreases in energetically expensive biological processes such as cell proliferation and translational elongation or biological processes dependent on the presence of O₂, such as fatty acid oxidation, oxidative phosphorylation and ROS. Conversely, transcription of these genes did not decrease in the silverspotted sculpin, likely indicating the species may not down-regulate energy demand and therefore be unable to conserve their limited energy supply during hypoxia. As a result, the difference in hypoxia tolerance between the two species may be partially explained by the ability of the species to decrease energetically expensive biological processes, perhaps partially mediated by changes in gene transcription.

Energy producing pathways requiring O₂ are compromised during hypoxia and as a result there is an increased reliance on anaerobic glycolysis (as shown previously, e.g. in Carassius auratus, van den Thillart et al. 1980). In the tidepool sculpin, a decrease in the transcription of fatty acid oxidation genes and oxidative phosphorylation genes coupled with increased glycolytic transcription suggests a shift to O₂-independent production of energy during hypoxia. In the silverspotted sculpin, despite an increase in transcription of glycolytic genes, there was also an overall induction of genes involved in oxidative phosphorylation and fatty acid oxidation (after a brief, transient decrease at 3 hours of hypoxia). This
counterintuitive response in the silverspotted sculpin is likely detrimental if the changes in transcript level are associated with function changes as they could result in increased production of ROS and fatty acid metabolites. Furthermore, the metabolic costs of increasing transcript levels of genes from futile biological pathways could result in a more severe disruption of metabolic energy balance in the silverspotted sculpins than in tidepool sculpin and contribute to their lower hypoxia tolerance. Silverspotted sculpins have roughly half the hepatic glycogen stores than tidepool sculpins and they use these glycogen stores at a higher rate when exposed to hypoxia (Chapter 2, Speers-Roesch et al. 2013), further supporting the notion that silverspotted sculpins have high metabolic demands that are not suppressed during hypoxia exposure. Previous functional studies and the transcriptional data from the current study suggest that the lower capacity of $O_2$-independent production of energy in the silverspotted sculpin precludes the species from being able to down-regulate oxidative phosphorylation and fatty acid oxidation and thus conserve the limited energy supply during hypoxia.

### 3.5.3 Relative vs. absolute levels of hypoxia

When both species were exposed to the same environmental $O_2$ tension (23 Torr), there was a notable lack of a transcriptional response in the tidepool sculpin compared with large transcriptional changes in the silverspotted sculpin. The silverspotted sculpin showed similar transcriptional patterns at 24 hours between the relative (29 Torr) and absolute (23 Torr) hypoxia exposures. In contrast, substantial differences were seen in the tidepool sculpin at 24 hours between the relative (17 Torr) and absolute (23 Torr) hypoxia exposure, as demonstrated by the separation of clustering patterns of the time-course points for this species in the principal component analysis. For the tidepool sculpin, a more severe environmental hypoxia was required to induce changes in gene transcription than in the silverspotted sculpin, highlighting the greater ability of the tidepool sculpin to utilize other mechanisms of combating hypoxia, such as greater $O_2$ extraction capacity. Likely, the transcriptional differences between the two species of sculpin reflect the patterns of species distribution across the marine nearshore.
environment. Collectively, this study highlights not only large differences in the induction or repression of genes involved in certain biological processes between hypoxia-tolerant and -intolerant species, but the large variation in the initiation of a molecular response, both temporally and in response to different magnitudes of hypoxia.

3.5.4 Conclusions

There was significant temporal variation in the transcriptional response to relative hypoxia exposure between the two sculpins. Despite living in a highly variable intertidal environment, the tidepool sculpin does not alter phenotype via changes in transcription unless hypoxia persists for longer than a typical tidal cycle or O2 levels become too low. In contrast, the silverspotted sculpin very quickly altered gene transcription during exposure to hypoxia, suggesting that the phenotypic traits already expressed were unable to support survival in low O2 environments. During sustained hypoxia, genes associated with a number of biological processes exhibited large transcriptional divergences between the two species. In the tidepool sculpin, the predominant pattern was a decrease in transcription while in the silverspotted sculpin the transcriptional response was more variable. This would suggest that the tidepool sculpin is better able to conserve limited energy stores by lowering energy expenditure associated with metabolically expensive biological processes during periods of extended hypoxia exposure, although further functional studies will be required to corroborate this finding. Ultimately, these transcriptional differences may play a significant role in determining the capacities of the species to tolerate fluctuating O2 in the environment and therefore determine the species distribution along the nearshore environment.
**Figure 3.1** Venn diagram depicting the number of clones whose transcription significantly changed in response to hypoxia.

The number in the black circle represents the tidepool sculpin, the number in the light gray circle represents the silverspotted sculpin and the number in the dark gray area represents the common clones whose transcription significantly changed in response to hypoxia in both species. Significance set at $q<0.01$. 

![Venn diagram with numbers: Tidepool 3470, Silverspotted 2408, Common 904]
**Figure 3.2** Trajectory of transcription in the tidepool sculpin and the silverspotted sculpin exposed to relative environmental hypoxia over 72 hours and absolute environmental hypoxia at 24 hours. Relative time points are indicated by square symbols and absolute time points are indicated by diamond symbols and (Abs). The tidepool sculpin is represented with black and the silverspotted sculpin is represented with red. The numbers in the brackets indicate the percent of variation in transcription among the time points explained by each of the respective principal components. The 0hr time points for both species overlap, but were offset for clarity.
**Figure 3.3** Transcription levels of genes associated with metabolism (A-D), regulation of cell number (E-H) and immunity (I) in the tidepool sculpin and silverspotted sculpin.

Both species were exposed to relative environmental hypoxia for 72 hours (time-course, 17 Torr and 29 Torr, respectively). Additionally the tidepool sculpin and the silverspotted sculpin were exposed to absolute environmental hypoxia (23 Torr) for 24 hours and the resulting transcription of genes are represented in the light blue strips for each of the species. Genes represented in panel A are 6-phosphofructokinase liver type (red), 6-phosphofructokinase muscle type (black), alpha-enolase (blue), glucose-6-phosphate isomerase (green) and pyruvate kinase isozymes M1/M2 (purple); in panel B, ATP synthase subunit d (red), ATP synthase subunit e (black), ATP synthase subunit gamma (green), ATP synthase subunit O (purple), cytochrome b-c1 complex subunit 10 (blue); in panel C, 3-hydroxybutyrate dehydrogenase type 2 (blue), enoyl-CoA hydratase (black), hydroxyacyl-coenzyme A dehydrogenase (green), medium-chain specific acyl-CoA dehydrogenase (red); in panel D, mitochondrial uncoupling protein 2 (blue), peroxiredoxin-1 (black), phospholipid hydroperoxide glutathione peroxidase (purple), protein DJ-1 (green), tropomyosin alpha-1 chain (red); in panel E, catenin beta-1 (yellow), cell death activator CIDE-B (blue), death-associated protein-like 1 (coral), Diablo homolog (light blue), DNA damage-inducible transcript 4 protein (red), eukaryotic translation initiation factor 4 gamma 2 (tan), eukaryotic translation initiation factor 5A-1 (orange), poly(U)-binding-splicing factor PUF60 (black), kinase isozymes M1/M2 (purple), RAF proto-oncogene serine/threonine-protein kinase (magenta), serine/threonine-protein kinase Sgk1 (gray); in panel F, annexin A4 (red), annexin A5 (blue), Keratin, type I cytoskeletal 18 (black), macrophage migration inhibitory factor (green); in panel G, coronin 1A (red), eukaryotic translation initiation factor 5A-1 (blue), macrophage migration inhibitory factor (gray), peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (purple), peroxiredoxin-1 (red), peroxisome proliferator-activated receptor delta (black), ubiquitin-conjugating enzyme E2 A (green); in panel H, cystathionline gamma-lyase (black), fatty acid-binding protein, heart (red), RAF proto-oncogene serine/threonine-protein kinase (blue); in panel I,
beta-2-microglobulin (tan), C-C motif chemokine 25 (yellow), collectin-12 (blue), complement C1q subcomponent subunit C (black), complement C3 (red), complement factor H (magenta), coronin-1A (orange), cytochrome b-245 light chain (purple), glucose-6-phosphate isomerase (pink), macrophage migration inhibitory factor (gray), major histocompatibility complex class I-related gene protein (coral), mannan-binding lectin serine protease 1 (light blue), myeloid differentiation primary response protein MyD88 (green).
Figure 3.4 Transcription levels of genes associated with protein production (A-E), protein localization (F) and protein folding (G-H) in the tidepool sculpin and the silverspotted sculpin.

Genes represented in panel A are phospholipid hydroperoxide glutathione peroxidase (blue), protein SET (black), ubiquitin-conjugating enzyme E2 A (red); in panel B, 40S ribosomal protein S7 (blue), methyllosome protein 50 (green), poly(U)-binding-splicing factor PUF60 (black), ribosome biogenesis protein NSA2 homolog (red); in panel C, eukaryotic translation initiation factor 1b (black), eukaryotic translation initiation factor 4 gamma 2 (red), eukaryotic translation initiation factor 4E-binding protein 3 (blue); in panel D, 40S ribosomal protein S21 (green), 40S ribosomal protein S7 (purple), 60S ribosomal protein L10 (magenta), 60S ribosomal protein L13 (blue), 60S ribosomal protein L30 (red), 60S ribosomal protein L39 (gray), elongation factor 1-gamma (black), eukaryotic translation initiation factor 5A-1 (yellow); in panel E, complement C1q subcomponent subunit C (black), complement C3 (blue), complement factor H (green), mannan-binding lectin serine protease 1 (magenta), peptidyl-prolyl cis-trans isomerase FKBP1A (purple), signal peptidase complex catalytic subunit SEC11A (red); in panel F, ADP-ribosylation factor 5 (black), ADP-ribosylation factor 6 (red), catenin beta-1 (purple), centrosomal protein of 57 kDa (black), clathrin light chain A (green), eukaryotic translation initiation factor 5A-1 (light blue), importin-7 (gray), keratin type I cytoskeletal 18 (yellow), translocation protein SEC63 homolog (blue); in panel G, peptidyl-prolyl cis-trans isomerase F (blue), peptidyl-prolyl cis-trans isomerase FKBP1A (green), peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (magenta), T-complex protein 1 subunit eta (gray), T-complex protein 1 subunit theta (purple), T-complex protein 1 subunit zeta (black); in panel H, 10 kDa heat shock protein (red), heat shock protein 75 kDa (green), heat shock protein HSP 90-alpha (black), heat shock protein HSP 90-beta (blue). See figure 3.3 legend for more detail.
<table>
<thead>
<tr>
<th>Species</th>
<th>Normoxia</th>
<th>Relative Hypoxia (3, 8, 24, 48, 72 hours)</th>
<th>Absolute Hypoxia (24 hours)</th>
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<tr>
<td>Tidepool</td>
<td>158 Torr</td>
<td>17 Torr</td>
<td>23 Torr</td>
</tr>
<tr>
<td>Silverspotted</td>
<td>158 Torr</td>
<td>29 Torr</td>
<td>23 Torr</td>
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Table 3.1 Experimental design of O₂ exposures and sampling times for each species
Table 3.2 Euclidean distance of the transcriptional response between the tidepool sculpin and the silverspotted sculpin measured for the 72 hour time course.

Values are presented for the top twenty genes from biological processes presented in figures 3.3 and 3.4 and the remaining Euclidean distances can be found in the supplementary material.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene UniProt ID</th>
<th>Gene Ontology Name</th>
<th>Euclidean distance</th>
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</thead>
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<tr>
<td>DNA damage-inducible transcript 4 protein</td>
<td>DDT4</td>
<td>GO:0043065~positive regulation of apoptosis</td>
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<td>GO:0008285~negative regulation of cell proliferation</td>
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<td></td>
<td></td>
<td>GO:0006446~regulation of translational initiation</td>
<td></td>
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<tr>
<td>Ubiquitin-conjugating enzyme E2 A</td>
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Chapter 4

Variation Underlying Similar Phenotypes: Transcriptional Divergence in Hypoxia Responsive Genes Associated with Metabolism and Protein Synthesis in Three Species of Sculpin with the Same Hypoxia Tolerance

4.1 Summary

The evolution of phenotypic convergence among taxa can emerge from similar or differing genetic mechanisms, making it difficult to predict the evolutionary change underlying phenotypes. In this study, we exposed three species of closely related marine fish from the superfamily Cottoidea (smoothhead sculpin [Arteius lateralis], sailfin sculpin [Nautichthys oculofasciatus] and Pacific staghorn sculpin [Leptocottus armatus]) to 72 hours of hypoxia to assess if transcriptional patterns differed among species with similar hypoxia tolerances. Individuals of each species were exposed to 23 torr and sampled at 3 and 8 hours (termed ‘short-term exposure’) and 24, 48 and 72 hours (termed ‘long-term exposure’) and mRNA transcription was assessed with a custom designed microarray. A high proportion (65%) of clones on the microarray were shown not to differ in their hypoxia-induced transcriptional patterns in the three species. However, the majority of genes associated with metabolism and protein production differed in transcription patterns among the species in both the short- and long-term hypoxia exposures. These transcriptional data suggest that the three species of sculpin utilize metabolic pathways, specifically amino acid catabolism, glycolysis and fatty acid oxidation, differently to generate ATP in hypoxia. Transcription of genes involved in different steps of protein production, including mRNA processing, protein translation and
protein localization increased in the smoothhead sculpin, decreased in the sailfin sculpin and was variable in the Pacific staghorn sculpin. As changes in metabolism and protein production are considered integral to hypoxic survival, differences in the transcription patterns of genes associated with these processes suggests that the three sculpin species may have evolved similar hypoxia tolerance, in part, through different mechanisms.

**4.2 Introduction**

Natural selection has long been implicated as the evolutionary cause underlying convergent evolution. This is based on the premise that repeated evolution of similar traits in response to an environmental pressure is unlikely to be a product of genetic drift (Endler 1986). Phenotypic convergence may be achieved via similar or different genetic changes depending if there are few or many paths for selection to act on. For example, genetic biases and constraints, such as pleiotropy or limited available genetic variation may decrease the number of ways in which an animal can evolve in response to an environmental stressor (Chevin et al. 2010, Christin et al. 2010, Losos 2011). In these instances it is more likely that similar genetics underlie convergent phenotypes. In turn, if genetic constraint is minimal and genetic variation high, more paths are available for evolution to proceed along, leading to greater instances of differing genetic basis of convergent phenotypes. As phenotypic similarity among taxa can be achieved in multiple ways, there has been a substantial effort in identifying instances of trait convergence and the underlying genetic and molecular mechanisms in order to understand the principles governing the evolution of phenotypic convergence (Hoekstra et al. 2006, Gross et al. 2009, O’Quin et al. 2010, Rosenblum et al. 2010, Conte et al. 2012).

Studies on ecological and physiological genomics have begun to uncover how genomes function and respond to produce complex phenotypes in response to environmental challenges. Analysis of transcriptomics is important as it provides an understanding of the variation in genome regulation in response to the environment, ultimately providing an important link in elucidating genotype-environment interactions, phenotypic plasticity and phenotypic evolution (Aubin-
Horth and Renn 2009, Whitehead 2012, Alvarez et al. 2015). Previous transcriptomic studies, such as those conducted using microarray platforms in lake whitefish (*Coregonus clupeaformis*; Derome et al. 2006) and Atlantic salmon (*Salmo salar*; Roberge et al. 2006) have examined whether similar transcriptional patterns underlie convergent phenotypes. Sympatric dwarf (limnetic) and normal (benthic) whitefish ecotypes have diverged in many traits, including bioenergetics and growth, in response to ecological pressures and competitive interactions (reviewed in Bernatchez et al. 2010). These phenotypic differences were found to be consistent across independent, isolated populations and are reflected by parallel differences in transcription of genes associated with swimming activity and energy metabolism between the whitefish ecotype pairs (Derome et al. 2006). Likewise, differences in the transcription of metabolic genes were found to underlie phenotypic differences in growth rate and age to sexual maturity between wild and farmed Atlantic salmon and the transcriptional patterns were consistent among multiple strains (Roberge et al. 2006). These transcriptomic studies provide instances of evolution of parallel molecular mechanisms in different populations or strains of a species in response to similar selective pressures.

Hypoxia (low O\(_2\) levels) is an environmental factor that has significant ecological and physiological consequences in animals, particularly in aquatic habitats due to the relatively low capacitance of water for O\(_2\) and the added pressure of anthropogenic eutrophication (Diaz and Breitburg 2009). As a result, there have been multiple, independent origins of hypoxia tolerance among diverse teleost lineages including perciformes, cypriniformes and characiformes (Hochachka and Somero 2002). Numerous researchers have attempted to identify the underlying traits contributing to whole animal hypoxia tolerance across different levels of biological organization, from the role of specific genes (Rytkonen et al. 2008, Andersen et al. 2009, Rytkonen et al. 2012), gene transcription (Gracey et al. 2001, Ton et al. 2003), biochemical pathways (van den Thillart et al. 1980, Martinez et al. 2006, Davis and Moyes 2007), physiological traits (Sollid et al. 2003, Rutjes et al. 2007) to behavioural responses (Sloman et al. 2006). However, although many traits have been implicated in the evolution of hypoxia tolerance, no
study has directly examined whether species with similar levels of hypoxia
tolerance exhibit convergent transcriptional patterns.

Marine coastal systems are highly heterogeneous environments with areas
prone to frequent O\textsubscript{2} fluctuations, such as dense marshes and isolated tidepools, in
close proximity to the O\textsubscript{2} stable, deeper near-shore waters. As a result, there is great
diversity in hypoxia tolerance among coastal species, such as in the group of fishes
belonging to the superfamily Cottoidea. These fishes, known as sculpins, show a
large range of hypoxia tolerance that matches the likelihood of hypoxia exposure in
their habitat (Mandic et al. 2009, Chapter 2). We recently demonstrated that sculpin
species that differ in hypoxia tolerance show highly divergent patterns of gene
transcription in both the timing of the hypoxia-induced transcriptional change
(initial time-frame of hypoxia exposure for the intolerant silverspotted sculpin,
*Blesias cirrhosis*, and later time-frame of hypoxia exposure for the tolerant tidepool
sculpin, *Oligocottus maculosus*) as well as the induction or repression patterns of the
transcriptional response (Chapter 3). In order to determine if species with similar
hypoxia tolerance exhibited convergent transcription patterns, we examined three
species of sculpin: smoothhead sculpin (*Artedius lateralis*), sailfin sculpin
(*Nautichthys oculofasciatus*) and Pacific staghorn sculpin (*Leptocottus armatus*). All
three species inhabit the high subtidal, which is susceptible to periodic decreases in
O\textsubscript{2}, and exhibit intermediate levels of hypoxia tolerance in relation to other sculpin
species. The species were chosen because of their similar level of hypoxia tolerance,
their location on separate clades of the sculpin phylogenetic tree, which suggests
independent evolution of intermediate-tolerance (Fig. 4.1) and the relative
abundance of individuals of each species. The three sculpin species were exposed to
the same environmental hypoxia level for 72 hours and the liver was sampled for a
microarray analysis of changes in the transcriptome. Overall, the goal of this study
was to determine if three closely-related species of sculpin with similar hypoxia
tolerance showed convergent transcriptional responses to hypoxia exposure.
4.3 Materials and Methods

4.3.1 Experimental animals

Individuals of *Artedius lateralis* (smoothhead sculpin) and *Leptocottus armatus* (Pacific staghorn sculpin) were collected in July and August of 2010 near the Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia. Smoothhead sculpins were captured at Ross Islets (48°52’24.0”N 125°09’42.0”W) using seine nets during low tide and Pacific staghorn sculpins in the Bamfield Inlet (48°49’06.1”N 125°08’30.9”W) using baited minnow traps. The sculpins were collected from habitats typical of these species (Marliave 1997), with the smoothhead sculpin collected from a shallow, sand and pebble beach and the Pacific staghorn from a beach with a muddy substrate. The two species were transported to The University of British Columbia and a third species of sculpin, *Nautichthys oculofasciatus* (sailfin sculpin) was acquired from the Vancouver Aquarium, British Columbia (BC), Canada. The sailfin sculpins were first generation offspring from wild fish caught in the waters around Stanley Park, Vancouver, BC. All three species of sculpin where held in fully aerated recirculating seawater at 12°C and 30 ppt salinity for at least 2 months prior to experimentation. Fish were fed daily with prawns and krill, except 24 hours before the experiments.

4.3.2 Critical O₂ tension

Critical O₂ tension (*P*<sub>crit</sub>; water PO<sub>2</sub> at which an animal no longer maintains routine O₂ consumption rate and the rate decreases with a decline in water PO<sub>2</sub>) can be considered a proxy for hypoxia tolerance in sculpins (Chapter 2). The smoothhead sculpin and the Pacific staghorn sculpin *P*<sub>crit</sub> values of 35.7±6.9 Torr and 37.4±1.2 Torr respectively were obtained from Mandic et al. (2009). Following protocols described in detail in Henriksson et al. (2008), we determined that the *P*<sub>crit</sub> for the sailfin sculpin was 35.5±2.3 Torr (n=6) at 12°C and 30 ppt salinity. The *P*<sub>crit</sub> values among the species were not statistically different (ANOVA, *p* = 0.900).
4.3.3 Hypoxia exposure

For each species, individuals were transferred into four separate, 136 litre tanks of a custom built recirculating system, the Low Oxygen Control and Monitoring Aquatic Research System (Integrated Aqua Systems, Inc.). Fish were allowed to recover in air-saturated water for 24 hours, after which O$_2$ levels were decreased to 23 Torr, a level of hypoxia corresponding to 65% P$_{crit}$ of all three species of sculpin. Fish were sampled for liver, muscle, heart, brain and gill at 3, 8, 24, 48 and 72 hours of hypoxia and two normoxic fish were also sampled at each of the hypoxic time points. All tissues were flash frozen in liquid N$_2$ and stored in a -80°C freezer until analysis, but only liver samples were used in the present microarray experiments.

4.3.4 Microarray experiments

The protocols used for RNA extraction, reverse-transcription of RNA to cDNA, fluorescent labeling of the cDNA and hybridization of the microarrays are described in detail in Chapter 3. Briefly, RNA was extracted from the liver of each of the species using TRlzol (Invitrogen). Total RNA was then purified using glass-fibre filter columns (Qiagen) and reverse transcribed using MMLV-reverse transcriptase (Epicentre). The cDNA samples were purified using QIAquick PCR purification kits (Qiagen) and each sample was labeled separately with Cy5 and Cy3 dyes. Fluorescently labeled cDNA samples were hybridized to a custom cDNA array from woolly sculpin (Clinocottus analis). A total of 25 samples per species were used: 5 individuals sampled at normoxia and 4 individuals sampled at each hypoxic time-point. Hybridizations were only performed between 2 samples within the same species hybridized on the woolly sculpin array. We used an interwoven loop design (Kerr and Churchill 2001), where each sample was hybridized to either 2 or 4 arrays with fluor-reversal, creating a separate loop for each species.

4.3.5 Statistical analysis

The custom woolly sculpin array is comprised of approximately 9600 cDNA clones (array elements; Chapter 3). A total of 1700 clones were sequenced and the
resulting non-redundant contigs were annotated with the gene name only if the matched sequences yielded a hit with an E-value of \(<1 \times 10^{-4}\) in SwissProt (for details see Chapter 3). For clarification, the term ‘clones’ refers to all (sequenced and non-sequenced) array elements, while the term ‘genes’ strictly refers to the sequenced and identified clones. Sequence divergence between the woolly sculpin used to create the microarray platform and the sculpin species under investigation may result in poor hybridization at certain clones. To minimize the effect of poor hybridization, clones with expression levels <2 standard deviations above background for any species were removed leaving 3294 clones for further analysis.

Raw data were log₂-transformed and lowess normalized using the MAANOVA package (Wu et al. 2013) in R (R Core Team 2013). To test for the effect of time in hypoxia, differential expression for each clone across time-points was detected using linear mixed models (‘array’ as a random effect and ‘dye’ and ‘time’ as fixed effects) with 100 permutations and the F-test statistic. False discovery rate (Storey 2002) in the qvalue package (Dabney and Storey 2013) in R was used to determine significance level (set at \(q<0.01\)). Clones showing a main effect of time in hypoxia in at least one of the species of sculpin (2734 clones in total; 1670 clones for smoothhead sculpin, 1183 clones for sailfin sculpin and 1778 clones for Pacific staghorn sculpin) were further analyzed to determine if the transcription patterns differed among the species.

Specifically, we tested whether the slope of the transcription response for short-term hypoxia response (0, 3hr, 8hr) and long-term hypoxia response (0, 24hr, 48hr, 72hr) differed among the species. The time-course was partitioned into short- and long-term responses because: 1) daily tidal events are the primary cause of hypoxic exposure in the environments inhabited by these species, resulting in ecologically relevant hypoxic exposure of up to 8 hours; 2) shallow coastal environments are increasingly influenced by anthropogenic eutrophication resulting in longer-term, physiologically challenging hypoxia exposures (Diaz and Breitburg 2009); and 3) our previous work in hypoxia-tolerant and -intolerant sculpins demonstrated a strong effect of time on transcription with differences between the early and the late hypoxia exposure (Chapter 3). A maximum-likelihood
model was applied to the data to determine the best fit intercept and linear slope for each species over short- and long-term hypoxia, using the subplex method of optim function, as implemented in the find.mle routine of the diversitree package (FitzJohn 2009). For each species pair (smoothhead sculpin vs. sailfin sculpin, smoothhead sculpin vs. Pacific staghorn sculpin, and sailfin sculpin vs. Pacific staghorn sculpin), we then used a likelihood-ratio test to determine whether the slopes were significantly different by comparing whether the full likelihood model (which allowed both species to have different slopes) to a constrained model with a single slope. If the drop in log-likelihood between the full and the constrained model was > 1.92 (the critical value for a chi-square distribution with 1 degree of freedom and an alpha level of 0.05) then we rejected the hypothesis that the slopes are the same for the two species. From this analysis, we broadly classified the data into 4 categories of transcriptional response: differences among the species during short-term hypoxia exposure, differences among the species during long-term hypoxia exposure, differences among the species in both the short- and long-term hypoxia exposure, and no differences among the species. For data presentation, we focused exclusively on annotated genes, which were assigned to gene ontology biological process categories using searches in EMBL-EBI QuickGO (www.ebi.ac.uk/QuickGo/) and Uniprot (http://www.uniprot.org).

4.4 Results and Discussion

To date, very few studies have examined whether phenotypic similarity among species corresponds to transcriptional similarities or differences in wild populations. Therefore, in this study we compared the transcriptional response to hypoxia (i.e., changes in gene transcription over the time in hypoxia) among three closely-related species of sculpin that show a similar level of hypoxia tolerance. Of the total number of clones on the microarray whose transcript levels significantly changed in at least one species over time in hypoxia (2556 clones), the patterns of transcription did not differ among the species in 64% of the clones, suggesting an overall similar biological response to hypoxia (Table 1). However, certain biological processes were enriched for genes that differed in transcription patterns among the
species, including metabolism, protein production, cell count (apoptosis and cell proliferation) and immune response (Table 1). Here, we focus specifically on biological processes considered extremely relevant to hypoxic survival, mainly metabolism and protein production, for which transcription of 59% and 73% of the genes respectively differed among the three species (Table 1, Fig. 4.2-4.3). These differences in transcription patterns of genes involved in biological processes considered essential to hypoxic survival indicate that the underlying molecular basis of the phenotype differs among the species.

Hypoxic survival is critically linked to the maintenance of energy supply and demand (Boutilier 2001). Hypoxia-tolerant animals are able to maintain cellular energy balance during hypoxia exposure by augmenting energy supply through a greater reliance on O$_2$-independent ATP production (e.g. glycolysis) and higher concentrations of substrates to maintain these pathways (e.g. glycogen), as well decreasing energy demand through a coordinated suppression of energetically expensive processes, such as protein production, in order to conserve limited ATP stores (Hochachka et al. 1986, Hochachka et al. 1996, Bickler and Buck 2007). These hypoxia defense strategies have been noted in diverse vertebrate groups including sharks (Routley et al. 2002), various teleost fishes (van den Thillart et al. 1980, Via et al. 1994, Smith et al. 1996), reptiles (Land et al. 1993) and some mammals (Preedy et al. 1985). Mechanisms underlying the hypoxia defense strategies include changes in post-translational modifications (e.g. decrease in protein synthesis via phosphorylation of initiation factor eIF2a; Koumenis et al. 2002) and gene transcription (increase in glycolytic genes and decrease in protein synthesis genes during hypoxic exposure; Gracey et al. 2001, van der Meer et al. 2005).

4.4.1 Energy metabolism

Transcription of metabolic genes varied among the three sculpin species, suggesting that the species may rely on different metabolic pathways to generate ATP during hypoxia exposure. In the smoothhead sculpin there was a decrease in the transcript levels of glycolytic genes (e.g. fructose-bisphosphate aldolase B [ALDOB] and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) and genes
associated with glycogen degradation (glycogen debranching enzyme [GDE]; Fig. 4.4) compared to the other two species. These changes in transcript levels suggest that the smoothhead sculpin shifts glycogen turnover toward synthesis rather than degradation and decreases reliance on glycolysis during hypoxia. There was also a decrease in transcript levels for genes associated with fatty acid oxidation during short- and long-term hypoxia exposure (e.g. hydroxyacyl-coenzyme A dehydrogenase [HADH]; Fig. 4.4). Thus, it would appear that the smoothhead sculpin relies on enhanced amino acid degradation (rather than glycolysis or fatty acid oxidation) to generate substrates for the tricarboxylic acid (TCA), and thereby support ATP generation by oxidative phosphorylation (Fig. 4.2). Indeed, transcript levels of genes associated with amino acid degradation and the TCA cycle showed an increase in transcript levels during long-term hypoxia, while transcript levels of genes associated with amino acid synthesis decreased (Fig. 4.4-4.6). If gene transcript levels relate to biological function, these data suggest that in addition to the typical hypoxia-induced reliance on glycolysis for ATP generation, prolonged hypoxia exposure in smoothhead sculpins induces a greater reliance on amino acid catabolism as a source of ATP. At first glance, this increased reliance on O$_2$-dependent ATP production via amino acid catabolism may seem like a surprising response for an organism to have in an O$_2$ deprived environment, but previous studies have shown enhanced amino acid catabolism during hypoxia in at least two other fish species (Tilapia mossambica; Kutty 1972 and Oncorhynchus mykiss; Medale et al. 1987).

In contrast to the smoothhead sculpin, both the sailfin sculpin and the Pacific staghorn sculpin showed a general increase in the transcription of glycolytic genes during short- and long-term hypoxia exposure (e.g. GAPDH; Fig. 4.4) and both species showed a short-term increase in transcript levels of genes associated with fatty acid oxidation (e.g. HADH) followed by a decrease in transcript level during long-term hypoxia (e.g. medium-chain specific acyl-CoA dehydrogenase [ACADM]; Fig. 4.4). However, these two species diverged in their response for other metabolic processes. Transcription patterns of genes associated with amino acid synthesis and degradation in the sailfin sculpin were similar to those of the smoothhead sculpin,
with a decrease in beta-ureidopropionase [UPB1] and an increase in 4-hydroxyphenylpyruvate dioxygenase [HPD] (Fig. 4.4). The transcriptional response of amino acid-related genes in the sailfin and smoothhead sculpin differed from the Pacific staghorn sculpin, which showed transcriptional patterns that suggest both amino acid synthesis and degradation increased (Fig. 4.4). We also identified a significant increase in genes involved in glycogen degradation in the sailfin sculpin while in the Pacific staghorn sculpin there was shift from glycogen synthesis in the short-term to glycogen degradation during long-term hypoxia exposure. The transcriptional data strongly suggests that the three sculpin species utilize different metabolic pathways for ATP generation during exposure to low O\textsubscript{2} (Fig 4.2).

### 4.4.2 Protein production, protein localization and protein folding

Multiple steps are involved in cellular protein production, including gene transcription, RNA processing and translation (Fig. 4.3). Differences in the transcriptional patterns of genes associated with these processes suggest that the smoothhead sculpin increased protein production during hypoxia exposure, while the sailfin sculpin decreased protein production and the Pacific staghorn had a variable response (Fig. 4.7). In the smoothhead sculpin, this was demonstrated by increased transcript levels of genes associated with translation (e.g. eukaryotic translation initiation factor 4 gamma 2 [EIF4G2] and eukaryotic translation initiation factor 4E-1A [EIF4E1A]; Fig. 4.7). In contrast, the sailfin sculpin showed a decrease in transcription of genes associated with translation, while the Pacific staghorn’s response was variable, as highlighted by transcriptional patterns of both the genes associated with RNA processing (e.g. ribonuclease inhibitor [RNHI] versus RNA-binding motif, single-stranded-interacting protein 2 [RBMS2]) and genes associated with translation (e.g. EIF4G2 versus EIF4E1A; Fig. 4.7). To fully examine protein production, it is important to not only include transcription, RNA processing and translation, but also consider steps such as protein folding and protein targeting or localization. While for large proportion of genes associated with transcription, RNA processing and translation patterns of transcription differed among the species, a majority of genes associated with protein localization and protein folding
were transcriptionally similar among the species (Fig. 4.3). In particular, all three species showed an increase in transcription of genes associated with protein folding, especially during long-term hypoxia exposure. This is not surprising since hypoxia exposure has been shown to increase protein-folding chaperones, such as the heat shock protein 70, in the liver of rats (Aoe et al. 1997) and heat shock protein 90 in the Chinese shrimp (*Fenneropenaeus chinensis*; Li et al 2009).

**4.4.3 Transcription patterns of hypoxia intermediate-tolerant species versus a hypoxia-tolerant species**

We previously showed that the transcription patterns in the very hypoxia-tolerant tidepool sculpin aligned with the characteristics attributed to tolerant animals, such as an increase in transcript levels of glycolytic genes and a decrease in transcripts of genes associated with energetically expensive processes such as cell proliferation (Chapter 3). Unlike the tidepool sculpin, the three intermediate-tolerant sculpin species in this study showed only some of the characteristics considered important to hypoxia tolerance, and these characteristics differed among the species. For example, the smoothhead sculpin showed a decrease in transcription of genes involved in some of the pathways that require O$_2$, such as fatty acid oxidation, while sailfin sculpins showed a decrease in protein production, and both the sailfin sculpin and Pacific staghorn sculpin showed an increase in glycolysis (Fig. 4.2 and 4.3). However, despite the considerable variation in transcriptional patterns for genes that were *a priori* predicted to be important in hypoxia, the three sculpin species exhibit a very similar overall tolerance to hypoxia. Species differences in transcription of some of the genes involved in metabolism and protein production may be offset by additional variation in other traits leading to a similar ability to tolerate hypoxia. The transcriptional patterns thus suggest that the three species of sculpin have evolved similar levels of hypoxia tolerance *via* different underlying mechanisms.
4.4.4 Wild caught versus lab-reared species

In this study, individuals of the smoothhead sculpin and the Pacific staghorn sculpin were wild-caught, while individuals of the sailfin sculpin were first generation lab-reared. This may complicate analyses as the wild-caught species, unlike the sailfin sculpin, may have been exposed to hypoxia during development, which can have consequences on the adult hypoxic phenotype (Robertson et al. 2014). However, an examination of the patterns of all transcriptionally-responsive clones using heat maps across the three species produced no clear indication to suggest that the overall transcriptional response of the sailfin sculpin differed to a great degree from the other two species (data not shown). Of course, this does not exclude the possibility that some of the patterns described in this study are a result of differences in developmental plasticity. One can get around the effects of developmental plasticity by examining first generation lab-reared individuals for all species, although epigenetic effects can persist across many generations increasing the difficulty of unraveling the genetic mechanisms of survival in hypoxia.

4.4.5 Short-term versus long-term responses

Time is an important factor to consider when examining changes in gene transcription (Aubin-Horth and Renn 2009). In our previous work (Chapter 3), we showed that time in hypoxia had a strong effect on gene transcription and that hypoxia-tolerant and -intolerant sculpins showed different temporal patterns of gene transcript levels. In general, the hypoxia-tolerant tidepool sculpin showed changes in gene transcript levels during longer-term exposure (24 to 72 hr) versus the hypoxia-intolerant silverspotted sculpin, which changed gene transcript levels during early (short-term hypoxia; 3 to 8 hr) hypoxia exposure (Chapter 3) and sometimes in opposite directions of the hypoxia-tolerant sculpin. The initial 8 hr hypoxia exposure is an ecologically relevant time-frame for species inhabiting the intertidal, while the longer duration of hypoxia lasting for several days is a physiological challenge testing the species’ capacities for hypoxic survival. Therefore, it is not surprising that different mechanisms may shape the hypoxic response between the short and long exposures to hypoxia and we predicted that in
the current study differences in transcription patterns among the species would occur in both the short- and long-term hypoxia exposure. Indeed, there was an effect of time on transcript levels, with roughly equal number of clones showing differences in transcription patterns among species during short-term hypoxia exposure as during long-term hypoxia exposure (41% and 43% respectively), while a small proportion of clones (16%) showed transcriptional differences across both time scales. Overall, transcription of glycolytic genes, as an example, differed in the species both in the short- and long-term hypoxia, although for most genes the differences occurred only in one time-frame or the other. It would appear that the underlying aspects of hypoxia tolerance in sculpins are influenced by divergent transcriptional responses as well as strong temporal effects. Taken together, this suggests that the capacity to acclimate may differ among the species and despite a similar starting level of hypoxia tolerance the reaction norms may be remarkably different among the three sculpin species when comparing the trajectory from normoxia, through short-term to long-term hypoxia exposure.

4.4.6 Conclusion

In this study, we found that three species of sculpin with similar hypoxia tolerance had very different patterns of transcription in metabolism and protein production genes. These biological processes are thought to influence hypoxia tolerance, suggesting that these species use alternate mechanisms to achieve a similar overall phenotype. Our results argue against convergence as a result of constraint, and decrease the likelihood that similarities in hypoxia tolerance are a result of species relatedness. The divergent transcriptional patterns indicate that different genetic changes were selected upon during the evolution of intermediate hypoxia tolerance in the three species of sculpin.
Figure 4.1 A sculpin phylogenetic tree based on cytochrome b sequences. The data are unpublished courtesy of M.L. Ramon. Species names in red represent hypoxia-tolerant sculpins, in blue represent intermediate hypoxia tolerant sculpins, in green represent hypoxia-intolerant sculpins and in black represent sculpins with unknown hypoxia tolerance. Names of the three species of sculpin examined in this study are boxed (Arctedius lateralis or smoothhead sculpin, Nautichthys oculofasciatus or sailfin sculpin and Leptocottus armatus or Pacific staghorn sculpin).
**Figure 4.2** Schematic representation of genes associated with metabolism. Arrows represent gene transcription changes in response to hypoxia in the smoothhead sculpin (black), sailfin sculpin (blue) and Pacific staghorn sculpin (red). Gray arrows represent genes with similar transcription patterns among the species. Short arrows indicate transcription patterns during short-term hypoxia, long arrows indicate transcription patterns during long-term hypoxia, double arrows indicate transcription patterns during short- and long-term hypoxia, a vertical line indicates no change from normoxia and the letter ‘v’ represents a variable response. Full names for each gene abbreviation are listed in either Fig. 4.3-4.5 legends.
Figure 4.3 Schematic representation of genes associated with protein production, localization and folding.

See Fig. 4.4-4.6 legends for more details.

DNA

Gene

Primary RNA

mRNA

Translation

Ribosome biogenesis

Protein Localization

Protein Folding

Cytosol

Nucleus
**Figure 4.4** Transcript levels of genes associated with metabolism in the smoothhead sculpin (black line, square symbol), sailfin sculpin (blue line, diamond symbol) and Pacific staghorn sculpin (red line, inverted triangle symbol) exposed to 72 hours of hypoxia.

Solid lines represent the genes for which transcription significantly changed in response to hypoxia for a given species ($q < 0.01$) and dashed lines represent genes for which transcription did not significantly change in response to hypoxia. Opaque lines represent the portion of the time-course that is the focus of the difference among the species, while transparent lines represent the portion of the time-course for which transcription does not differ among the species. Letters represent significant difference in transcript levels between species (A-B represent difference in the short term hypoxia and X-Z represent differences in the long term hypoxia). Data are represented as mean ± SE. The genes in the panels are classified in the following categories: glycogen metabolic process (protein phosphatase 1 regulatory subunit 3C-B [ppp1r3cb], glycogen debranching enzyme [GDE] and phosphoglucomutase-1 [PGM1]), glycolysis (fructose-bisphosphate aldolase B [ALDOB], alpha-enolase [ENO1] and glyceraldehyde-3-phosphate dehydrogenase [GAPDH], polyol pathway (aldose reductase [AKR1B1]), the pentose-phosphate shunt (GDH/6PGL endoplasmic bifunctional protein [H6PD]), pyruvate metabolic process (pyruvate dehydrogenase kinase isozyme 4 [PDK] and mitochondrial pyruvate carrier 1 [MPC1]), fatty acid oxidation (hydroxyacyl-coenzyme A dehydrogenase [HADH]), tricarboxylic acid cycle (ATP-citrate synthase [ACYL]), oxidative phosphorylation (cytochrome b-c1 complex subunit 6 [UQCRH], ATPase inhibitor [ATPIF1], NADH dehydrogenase iron-sulfur protein 8 [NDUFS8] and HIG1 domain family member 1A [HIGD1A]), amino acid degradation (homogentisate 1,2-dioxygenase [HGD] and 4-hydroxyphenylpyruvate dioxygenase [HPD]) and amino acid biosynthesis (betaine—homocysteine S-methyltransferase 1 [BHMT] and beta-ureidopropionase [UPB1]).
Figure 4.5 Genes for which all three species showed similar significant changes in transcription in response to 72 hours of hypoxia.

The smoothhead sculpin is represented by the black line and square symbol, the sailfin sculpin is represented by the blue line and diamond symbol and the Pacific staghorn sculpin is represented by the red line and inverted triangle symbol. Data are represented as mean ± SE. The genes in the panels are classified in the following categories: pentose-phosphate shunt (ribulose-phosphate 3-epimerase [RPE]), fatty acid oxidation (medium-chain specific acyl-CoA dehydrogenase [ACADM]), oxidative phosphorylation (NADH dehydrogenase 1 alpha subcomplex subunit 1 [NDUFA1] and ATP synthase subunit d [ATP5H]), translational initiation (eukaryotic translation initiation factor 1b [EIF1B]), protein localization (importin-7 [IPO7] and translocon-associated protein subunit gamma [SSR3]) and protein folding (peptidyl-prolyl cis-trans isomerase FKBP1A [FKBP1A], protein disulfide-isomerase A4 [PDIA4] and T-complex protein 1 subunit zeta [CCT6A]).
**Figure 4.6** Similar transcription patterns in genes for which only a subset of species showed a significant effect of time in hypoxia.

The smoothhead sculpin is represented by the black line and square symbol, the sailfin sculpin is represented by the blue line and diamond symbol and the Pacific staghorn sculpin is represented by the red line and inverted triangle symbol. Solid lines represent the genes for which transcription significantly changed in response to hypoxia for a given species ($q < 0.01$) and dashed lines represent genes for which transcription did not significantly change in response to hypoxia. Data are represented as mean ± SE. The genes in the panels are classified in the following categories: glycolysis (glucose-6-phosphate isomerase [GPI]), tricarboxylic acid cycle (glutamate dehydrogenase [GLUD1]), oxidative phosphorylation (cytochrome c oxidase assembly protein COX14 [COX14], ATP synthase subunit e [ATP5I] and ATP synthase subunit O [ATP50]), amino acid degradation (alanine aminotransferase 2 [GPT2], methylmalonate-semialdehyde dehydrogenase, acylating [ALDH6A1] and D-amino-acid oxidase [DAO]), transcriptional repression (nuclear receptor subfamily 1 group D member 2 [NR1D2]), ribosomal biogenesis (60S ribosomal protein L10 [RPL10], 60S ribosomal protein L39 [RPL39], 40S ribosomal protein S21 [RPS21], 40S ribosomal protein S27 [RPS27] and 40S ribosomal protein S7 [RPS7]), translational elongation (elongation factor 1-gamma [EEF1G] and eukaryotic translation initiation factor 5A-1 [EIF5A]), protein folding (10 kDa heat shock protein [HSPE1], heat shock protein HSP 90-alpha [HSP90AA1] and T-complex protein 1 subunit eta [CCT7]) and protein localization (protein transport protein Sec61 subunit alpha [SEC61A]).
**Figure 4.7** Transcript levels of genes associated with protein production, localization and folding in the smoothhead sculpin (black line, square symbol), sailfin sculpin (blue line, diamond symbol) and Pacific staghorn sculpin (red line, inverted triangle symbol) exposed to 72 hours of hypoxia. The genes in the panels are classified in the following categories: transcriptional repression (histone deacetylase complex subunit SAP18 [SAP18] and carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 2 [CTDSP2]), RNA metabolic process (ribonuclease inhibitor [RNHI]), RNA processing (RNA-binding motif, single-stranded-interacting protein 2 [RBMS2] and ribosome biogenesis protein NSA2 homolog [NSA2] and 60S ribosomal protein L30 [RPL30]), translation (signal peptidase complex sunbunit 2 [SPCS2]), translational initiation (eukaryotic translation initiation factor 4E-binding protein 3 [EIF4EBP3], eukaryotic translation initiation factor 4E-1A [EIF4E1A] and eukaryotic translation initiation factor 4 gamma 2 [EIF4G2]), protein localization (ADP-ribosylation factor 5 [ARF5] and translocation protein SEC63 homolog [SEC63]), and protein folding (sulfhydryl oxidase 1 [QSOX1] and heat shock protein HSP 90-beta [HSP90AB1]). See Fig. 4.3 legend for more detail.
Table 4.1 Summary of clones with similar and different transcription patterns among the three species of sculpin.

Clones are organized into three broad categories: non-sequenced clones, sequenced but unidentified clones, and sequenced and identified clones (genes), which are further divided into biological processes.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Similar Transcription Patterns</th>
<th>Different Transcription Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of genes or clones</td>
<td>Percent</td>
</tr>
<tr>
<td>Metabolism</td>
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<td>41%</td>
</tr>
<tr>
<td>Chromosome organization</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>Protein production</td>
<td>3</td>
<td>27%</td>
</tr>
<tr>
<td>Ribosome biogenesis</td>
<td>5</td>
<td>71%</td>
</tr>
<tr>
<td>Protein folding</td>
<td>5</td>
<td>71%</td>
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<td>60%</td>
</tr>
<tr>
<td>Protein modification</td>
<td>10</td>
<td>50%</td>
</tr>
<tr>
<td>Cell count</td>
<td>9</td>
<td>37%</td>
</tr>
<tr>
<td>Response to reactive O₂ species</td>
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<td>50%</td>
</tr>
<tr>
<td>Immune response</td>
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</tr>
<tr>
<td>Unidentified clones</td>
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</tr>
<tr>
<td>Non-sequenced clones</td>
<td>1328</td>
<td>69%</td>
</tr>
<tr>
<td>Total</td>
<td>1659</td>
<td>65%</td>
</tr>
</tbody>
</table>
Chapter 5

General Discussion

Understanding the traits and mechanisms underlying complex phenotypes that have evolved in response to organisms living in heterogeneous environments ultimately aids us in determining the limitations that set species boundaries and niches and is one of the key themes of integrative biology. Combining evolutionary, physiological and ecological understanding of species provides essential information to enable predictions of how anthropogenic-induced environmental challenges (e.g. eutrophication and climate change) will have impacts on biodiversity in the future. For my doctoral research, my overall objective was to explore how phenotypic plasticity and adaptive evolution have contributed to the ability of different sculpin species to survive bouts of hypoxia. To address my overall objective, I examined if variation in fixed or non-plastic biochemical traits in multiple tissues and transcriptional-based plastic responses in the liver explained the variation in hypoxia tolerance in sculpins. Sculpins are a group of closely-related species that experience different \( O_2 \) regimes resulting in variation in tolerance, and as such are a tractable model system to understand what traits underlie the complex phenotype of hypoxia tolerance in wild fishes living in the nearshore marine environment.

5.1 Major Findings and Implications

5.1.1 Measures of hypoxia tolerance

Critical \( O_2 \) tension (\( P_{\text{crit}} \)) has long been used as an indirect measure of hypoxia tolerance (Hagerman 1998, Chapman et al. 2002, Mandic et al. 2009, Seibel 2011, Speers-Roesch et al. 2012). The primary reason for this is that \( P_{\text{crit}} \) is
considered an indicator of \(O_2\) extraction and delivery and an animal with lower \(P_{\text{crit}}\) potentially has greater hypoxia tolerance as a result of greater \(O_2\) uptake and transport to tissues. In sculpin, the correlation between \(P_{\text{crit}}\) and LOE\(_{50}\), a measure of hypoxia tolerance, supports the idea that \(P_{\text{crit}}\) may be used as a proxy for hypoxia tolerance (Chapter 2). This is also reinforced by a study on hypoxia-tolerant epaulette shark (\(\text{Hemiscyllium ocellatum}\)) and hypoxia-intolerant shovelnose ray (\(\text{Aptychotrema rostrata}\)) which found that a comparable percentage of \(P_{\text{crit}}\) produced similar levels of arterial hypoxemia (Speers-Roesch et al. 2012). However, killifish (\(\text{Fundulus heteroclitus}\)), a well known hypoxia-tolerant species (Stierhoff et al. 2003), was found to have high \(P_{\text{crit}}\) values of approximately 63.9 Torr, indicating that \(P_{\text{crit}}\) does not represent an accurate assessment of tolerance in this species (Richards et al. 2008). Therefore, while \(P_{\text{crit}}\) is an appropriate proxy measure of hypoxia tolerance in sculpins, this is not universally applicable to all fish species, indicating that careful quantification of several measures of hypoxia tolerance are necessary to provide an accurate estimation of hypoxia tolerance.

5.1.2 Multiple candidate traits underlie variation in hypoxia tolerance among sculpins

The hypoxic response of an organism is a complex phenotype with many underlying traits that span across different levels of biological organization. As such, I have examined a number of traits in sculpins, summarized in Table 5.1, from behavior (Mandic et al. 2009a), morphology and physiology (Mandic et al. 2009b) to biochemistry (Chapter 2) and transcriptional responses (Chapter 3 and 4). I have previously shown that aquatic surface respiration, aerial emergence, gill surface area and hemoglobin-oxygen (Hb-\(O_2\)) binding affinity are putative adaptive traits in the evolution of hypoxia tolerance in sculpins. These traits are involved with the extraction and delivery of \(O_2\), and therefore partially determine whole-animal performance through their impact on cellular and tissue function. However, organ systems, from the brain, to heart, liver and muscle, perform different roles during hypoxia exposure, likely resulting in tissue-specific selective pressures. Therefore, it
is necessary to examine the effect of hypoxia on different tissues in order to begin to understand the coordination of traits in combating periodic decreases in O₂.

In this thesis, I found a relationship between biochemical traits and hypoxia tolerance in sculpins in the brain. Specifically, maximal activity of brain metabolic enzymes measured under normoxic conditions correlated with hypoxia tolerance. The most tolerant species had higher activity than the less tolerant species in enzymes associated with anaerobic metabolism, particularly lactate dehydrogenase which correlated with both P_{crit} and LOE_{50} in a phylogenetically independent manner (Chapter 2). Brain is a hypoxia-sensitive tissue and O₂ lack in the brain rapidly leads to organismal death (Nilsson 1996, Boutilier 2001, Nilsson 2001). The high constitutive activity of metabolic enzymes in species frequently exposed to hypoxia may help protect the brain from hypoxia-induced energy imbalance when environmental O₂ rapidly decreases in tidepools at night.

Maximal enzyme activity and metabolic substrates (e.g. glycogen, glucose and creatine phosphate) involved in anaerobic metabolism in the liver and in the white muscle differed across the sculpin species. However, the variation in these tissues, unlike the brain, did not correlate with hypoxia tolerance (Chapter 2). In response to hypoxic exposure, fish have been noted to decrease swimming activity (Chapman and McKenzie 2009), lowering the energetic demand of the white muscle to match the hypoxia-induced decrease in energy supply. Lower energetic demand of the white muscle during hypoxia may negate the necessity of increasing capacity of anaerobic pathways and may explain the lack of relationship between maximal activity of metabolic enzymes in sculpins and hypoxia tolerance. Based on a study on Fundulus grandis which showed that there was an increase in maximal activity of glycolytic enzymes in the liver during exposure to long-term hypoxia, in contrast to a decrease seen in the muscle (Martinez et al. 2006), I predicted that liver function may be important to hypoxic survival and therefore the activities of anaerobic enzymes would be higher in the liver of hypoxia-tolerant versus hypoxia-intolerant sculpin species. However, the lack of relationship suggests that liver function may be less important in the evolution of hypoxia tolerance in sculpins than originally predicted. Or, it is possible that while fixed, constitutively-expressed biochemical
traits in the liver do not correspond to hypoxia tolerance, changes in cellular phenotype of the liver in response to low $O_2$ may help contribute to overall hypoxia tolerance. Indeed, in estuarine long-jaw mudsucker (*Gillichthys mirabilis*) exposure to hypoxia induced increases in transcription of many genes in the liver, in contrast to the muscle where there was primarily a decrease in transcription or the brain which showed minimal changes in gene transcription (Gracey et al. 2001). This makes the liver a good candidate to examine if, and how, transcriptional-based plasticity is related to hypoxic survival in sculpins.

I exposed two species of sculpin (tidepool sculpin and silverspotted sculpin; Chapter 3) and three species of sculpin (smoothhead sculpin, sailfin sculpin and Pacific staghorn sculpin; Chapter 4) to hypoxia and found changes in transcription of genes associated with a number of biological processes in the liver. In response to hypoxic exposure, both the tolerant tidepool sculpin and the intolerant silverspotted sculpin increased transcription of glycolytic genes, apoptotic genes, protein localization genes and many genes associated with the immune response. However, many differences were also noted between the species, where the tidepool sculpin decreased transcription of genes belonging to oxidative phosphorylation, fatty acid oxidation, cell proliferation and antioxidant defense, while the silverspotted sculpin either increased transcript levels or showed a variable transcriptional response (Chapter 3). If the transcription patterns are linked to functional changes, the tolerant tidepool sculpin, in contrast to the intolerant silverspotted sculpin, may lower energy expenditure of the liver through a decrease in either metabolically expensive processes or pathways that require $O_2$ if hypoxia persists for longer than 24 hours. The transcriptionally-induced phenotypic change in the liver is a potential mechanism underlying differences in survival of the two species only when, or if, the species are exposed to longer bouts of hypoxia.

In chapter 4, three sculpin species with intermediate hypoxia tolerance were exposed to low $O_2$ in order to assess gene transcription changes in the liver. While it is difficult to predict how transcription patterns in species with intermediate tolerance will compare to the species with high and low tolerance levels, I expected that transcriptional changes would be induced in similar biological processes across
the same timeframes in all species. This was the case as changes in transcription in the intermediate-tolerant species occurred for many of the same processes, such as glycolysis, oxidative phosphorylation, fatty acid oxidation, protein translation, protein localization and protein folding (Table 5.1). While transcription patterns for some of the biological processes in the three intermediate-tolerant species were similar to the hypoxia-tolerant tidepool sculpin (e.g. decrease in transcription of fatty acid oxidation genes in the smoothhead sculpin, decrease in transcription of translational initiation genes in the sailfin sculpin and an increase in transcript levels of glycolytic genes in the Pacific staghorn sculpin), some were more similar to the hypoxia-intolerant silverspotted sculpin (e.g. increase in transcription of fatty acid oxidation genes in the sailfin and the Pacific staghorn sculpin). The intermediate-tolerant species also exhibited a combination of transcriptional response of the two extreme tolerant species in genes belonging to biological processes such as oxidative phosphorylation and ribosome biogenesis. While it is difficult to predict the extent of functional consequence of these transcription patterns, similarities of the molecular responses to both hypoxia-tolerant and -intolerant species may contribute to the intermediate hypoxia tolerance phenotype in the three sculpin species.

5.1.3 Short- versus long-term hypoxia exposure

A focus on ecologically appropriate time-frames allows us to gain an understanding of the mechanisms that may have been selected for in response to hypoxia. A typical hypoxic bout for an intertidal species is dictated by changes in the tidal cycle and therefore the environmentally relevant exposure of sculpins is up to 6 to 8 hours of hypoxia. Yet, the time-course of hypoxic exposure for sculpin species in both Chapter 3 and 4 was 72 hours with sampling occurring at 3, 8, 24, 48 and 72 hours. The ecologically relevant 3 and 8 hour samples are critical in determining if transcriptional-based plasticity plays a role in hypoxic survival that contributes to the distribution patterns observed for the species along the nearshore environment. While the remainder of the time-course is an arbitrary length of time, it is important to examine as it provides insights into the traits that may contribute to the upper
limits of the species’ capacities to survive hypoxia bouts (i.e. deaths of some individuals were noted in different sculpin species by 72 hours of exposure). As anthropogenic eutrophication of the coastal waters has caused a steady increase in the duration and severity of hypoxia bouts, which are predicted to continue to increase, it is critical that we understand how traits change in different species in response to longer bouts of hypoxia.

5.1.4 The role of constitutive differences in phenotype versus phenotypic plasticity in the intertidal sculpins

Heterogeneous environments, especially if the environmental variation is temporal, are considered to be conducive to the evolution of phenotypic plasticity (Moran 1992), suggesting that organisms inhabiting the intertidal zone may evolve plasticity to combat the frequent and severe changes in stresses such as hypoxia. However, in the case of the hypoxia-tolerant tidepool sculpin transcriptionally-induced phenotypic plasticity does not occur during the ecologically relevant time-frame of 6 to 8 hours and transcriptional changes occur only if hypoxia persists for longer than 24 hours (Chapter 3). While it is possible that the phenotype is altered via other mechanisms, e.g. post-translational modification, it is also possible that the tidepool sculpin is a resilient generalist and expresses a single phenotype that is adequate across alternative environments. This phenotype includes a low routine metabolic rate, high Hb-O₂ binding and high maximal activity of brain glycolytic enzymes (Table 5.1). These constitutive or fixed traits (traits expressed in the absence of hypoxia) contribute to hypoxic survival and have likely evolved in response to hypoxic pressure. While there may be cost associated with maintaining these traits across the different intertidal conditions, such as the trade-off associated with high O₂ extraction and delivery during hypoxic versus hyperoxic exposures, it may be less than the cost associated with phenotypic plasticity. Factors contributing to cost of phenotypic plasticity include energetic cost of alternating phenotype in changing environmental conditions or the cost that comes from a mismatch between phenotype and environment as a result of lag time between cue for environmental change and production of new phenotype. This would favor the
evolution of fixed traits over plastic traits in the tidepool sculpin, suggesting that the evolution of fixed traits may be the primary mechanism of survival during short exposures to hypoxia in the tidepool sculpin. However, if hypoxic conditions persist, phenotypic plasticity may play a role in combating low O$_2$ stress.

The speciation trajectory of sculpins has been thought to be from the deeper subtidal waters to the intertidal zone, suggesting that the deeper subtidal species represent an ancestral state and the intertidal species a derived state (Ramon and Knope 2008). In contrast to the tidepool sculpin, the deeper subtidal silverspotted sculpin (Chapter 3) and the shallow subtidal smoothhead sculpin, sailfin sculpin and Pacific staghorn sculpin (Chapter 4) alter transcription patterns within the initial 8 hours of hypoxia exposure, suggesting that phenotypic plasticity occurs in these species in response to short-term hypoxia. This would raise the possibility that the ancestor to the tidepool sculpin, which likely inhabited the subtidal, also exhibited phenotypic changes in the initial stages of hypoxia. If this was the case, then how and why do the present-day tidepool sculpins lack short-term phenotypic plasticity in response to hypoxia? Plasticity is considered an important first step in establishment of a species in new environment and may lead to the second step of canalization of the plastic traits into non-plastic constitutive or fixed traits, termed genetic assimilation (reviewed in Ghalambor et al. 2007). It is therefore possible that phenotypic plasticity allowed the ancestral intertidal sculpin to initially colonize the new environment and over time, perhaps as a result of high cost of maintenance of plastic traits, there was a genetic assimilation of the environmentally-induced phenotypic changes. While this is very speculative and there is very little evidence to support this, it is interesting to note that most of the subtidal and deeper water species, unlike the tidepool sculpin, showed an increase in transcription of glycolytic genes during short-term exposure to hypoxia (see Table 5.1). However, maximal activity of glycolytic enzymes (considered a good proxy for enzyme concentration) were found to be higher in the liver of the tidepool sculpin than in the other subtidal and deeper water species (Table 5.1), suggesting a tentative link that higher concentrations of glycolytic enzymes may be a result of
genetic assimilation from environmentally-induced phenotypes to constitutively expressed traits.

5.1.5 Convergent evolution of hypoxia tolerance

Species that inhabit the high subtidal occasionally experience periods of low $O_2$ and these species have evolved similar levels of hypoxia tolerance. Hypoxia tolerance is a complex phenotype comprised of many interrelated traits and in Chapter 4 I investigated whether the underlying transcriptional response was similar among species with similar levels of hypoxia tolerance including the smoothhead sculpin, sailfin sculpin and Pacific staghorn sculpin. Transcription patterns of many of the genes associated with energy metabolism and protein production differed among the three sculpin species (Chapter 4). There was also a difference in the maximal activities of enzymes associated with energy metabolism in the brain and liver, where the enzyme activities of the Pacific staghorn sculpin reflected those of the hypoxia-tolerant tidepool sculpin, while the enzyme activities of the smoothhead sculpin were more closely aligned with the hypoxia-intolerant silverspotted sculpin (Chapter 2, Table 5.1). Together, this would suggest that among the closely related sculpin species convergence of hypoxia tolerance phenotype was likely achieved via multiple evolutionary paths, possibly indicating lower genetic constraint. These species may become increasingly exposed to hypoxic conditions, which are predicted to become longer and more frequent in the coastal waters (Diaz and Breitburg 2009). Lower genetic constraint associated with genes deemed important in hypoxia tolerance may be beneficial as it may allow greater evolutionary flexibility for the adaptation of the species to the increasing hypoxic conditions.

5.2 Future Directions

In my dissertation, I studied traits underlying hypoxia tolerance in a number of wild-caught species of sculpin. Examining species from the wild can be advantageous as it provides ecological context that can better inform the mechanistic and evolutionary study of how animals function. However, the
drawback of not using laboratory-reared animals is the unknown contribution of developmental plasticity and transgenerational epigenetic effects to the variation in traits in sculpins. Therefore, to understand if the differences in traits are genetically-based, experiments will need to be performed on individuals that have been reared in the laboratory under common garden conditions. While this may disentangle the effect of developmental plasticity, epigenetic effects can persist for many generations. Therefore one avenue of research is the use of bioinformatics tools, such as methylation arrays, methylation sequencing or chromatin immunoprecipitation followed by sequencing (Ritchie et al. 2015), to examine how epigenetics contributes to the complex phenotype of hypoxia tolerance in sculpins.

In this thesis I examined molecular response to environmental hypoxia in a number of species using transcriptomics, a powerful tool that can provide information on potential mechanisms underlying complex organismal responses to the environment. A major benefit of this approach is that it is not limited to only studying traits that have previously been shown to be important in hypoxia tolerance. Instead transcriptomics allows for a broad examination of changes in many biological processes, which can lead to the uncovering of other potentially important mechanisms. However, gene transcription is not the only determinant of phenotype and as the relationship between transcription and function is complex, further examination is required. One set of approaches to providing connection between gene transcription and phenotype is to study the subsequent steps of the pathway leading from gene to phenotype, such as proteomes and metabolomes or by using techniques to manipulate transcription, such as transgenics and RNAi, to determine the effect on phenotype (Alvarez et al. 2015). Another avenue of research is to examine trans-regulatory elements, such as transcription factors, that affect gene transcription and may explain the variation in patterns and ultimately phenotype. I am currently looking at the hypoxia inducible factor (HIF), an important transcription factor regulating transcription of many genes in hypoxia and prolyl hydroxylase (PHD), an important regulator of HIF (Semenza and Wang 1992, Semenza 1998, Bruick and McKnight 2001, Ivan et al. 2001, Jaakkola et al. 2001), in a number of sculpin species to determine if there is variation in these
trans-regulatory elements that may explain, at least in part, the variation in gene transcription patterns in sculpins. Overall, I have listed a few of many more approaches that can be taken to further explore how variation in hypoxia tolerance evolved in the closely-related species of sculpin.

5.3 Concluding Thoughts

My doctoral dissertation as well as previous research have focused on examining how species that experience different environmental O$_2$ profiles have evolved to cope with hypoxic stress. I have assessed hypoxia tolerance in wild species of fish and correlated morphological, physiological and biochemical traits using phylogenetically-corrected methods in order to determine candidate traits that may be adaptations to hypoxia in sculpins. I have also examined the role of transcriptionally-based phenotypic plasticity in hypoxic response of the intertidal and subtidal fishes and found complex transcriptional patterns with a strong effect of exposure time to environmental challenge. Diverse responses of species to hypoxia pose a significant challenge in predicting the effect of anthropogenic-induced increases in hypoxic events along the coastal waterways. This task can be accomplished, however, by utilizing a multidisciplinary, integrative approach to examine organismal stress response to environmental challenges in the context of physiology, evolution and ecology.
Table 5.1  Summary of traits examined in 5 species.
Information taken from Mandic et al. 2009a, 2009b, Chapter 2, Chapter 3 and Chapter 4. Red represents the hypoxia-tolerant species, blue the intermediate-tolerant species and green hypoxia-intolerant species. The unit for maximal enzyme activity is \( \mu \text{mol/min/g wet tissue} \) and the unit for metabolite concentration is \( \mu \text{mol/g wet tissue} \). Up arrows represent increase in transcript levels, down arrows represent decrease in transcript levels, dashes represent no change in transcription and ‘v’ represents a variable response. Abbreviations in the table: \( P_{\text{crit}} \) (critical oxygen tension), \( \text{LOE}_{50} \) (loss of equilibrium in 50% of the individuals), \( \text{ASR Freq.} \) (aquatic surface respiration frequency), \( \text{Air Emerge Freq.} \) (air emergence frequency), \( \text{Gill S.A.} \) (gill surface area), \( \text{Hb-O}_2 \) (hemoglobin-oxygen), \( \text{PK} \) (pyruvate kinase), \( \text{LDH} \) (lactate dehydrogenase), \( \text{CPK} \) (creatine phosphokinase), \( \text{CS} \) (citrate synthase), \( \text{ATP} \) (adenosine triphosphate), \( \text{CrP} \) (creatine phosphokinase), \( \text{OxPhos} \) (oxidative phosphorylation), \( \text{FAO} \) (fatty acid oxidation), \( \text{N/O} \) (not observed) and \( \text{N/A} \) (data not available).
<table>
<thead>
<tr>
<th>Hypoxia Tolerance</th>
<th>Tidepool</th>
<th>Smoothhead</th>
<th>Pacific staghorn</th>
<th>Sailfin</th>
<th>Silverspotted</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P^{\text{res}}$</td>
<td>25 Torr</td>
<td>36 Torr</td>
<td>37 Torr</td>
<td>36 Torr</td>
<td>44 Torr</td>
</tr>
<tr>
<td>$\text{LOE}^{\text{ela}}$</td>
<td>538 min</td>
<td>224 min</td>
<td>281 min</td>
<td>N/A</td>
<td>25 min</td>
</tr>
<tr>
<td>ARS Freq.</td>
<td>100%</td>
<td>67%</td>
<td>50%</td>
<td>N/A</td>
<td>0%</td>
</tr>
<tr>
<td>ARS $P_{O_2}$</td>
<td>15 Torr</td>
<td>16 Torr</td>
<td>11 Torr</td>
<td>N/A</td>
<td>N/O</td>
</tr>
<tr>
<td>Air Emerge Freq.</td>
<td>100%</td>
<td>0%</td>
<td>100%</td>
<td>N/A</td>
<td>0%</td>
</tr>
<tr>
<td>Air Emerge $P_{O_2}$</td>
<td>9 Torr</td>
<td>N/O</td>
<td>10 Torr</td>
<td>N/A</td>
<td>N/O</td>
</tr>
<tr>
<td>Gill S.A.</td>
<td>77 mm$^3$g$^{-1}$</td>
<td>105 mm$^3$g$^{-1}$</td>
<td>131 mm$^3$g$^{-1}$</td>
<td>N/A</td>
<td>95 mm$^3$g$^{-1}$</td>
</tr>
<tr>
<td>Hb-O$_2$ binding affinity</td>
<td>23 Torr</td>
<td>45 Torr</td>
<td>35 Torr</td>
<td>N/A</td>
<td>57 Torr</td>
</tr>
<tr>
<td>Routine metabolic rate</td>
<td>2.75 μmolg$^{-1}$h$^{-1}$</td>
<td>2.23 μmolg$^{-1}$h$^{-1}$</td>
<td>2.48 μmolg$^{-1}$h$^{-1}$</td>
<td>N/A</td>
<td>3.70 μmolg$^{-1}$h$^{-1}$</td>
</tr>
<tr>
<td>PK/LDH/CPK/CS ATP/CrP Brain</td>
<td>102/169/1008/4.1</td>
<td>90/128/821/3.4</td>
<td>106/169/1094/5.1</td>
<td>N/A</td>
<td>70/100/769/4.0</td>
</tr>
<tr>
<td>0.7/1.7</td>
<td>0.8/3.3</td>
<td>1.2/4.1</td>
<td>N/A</td>
<td>0.6/0.8</td>
<td></td>
</tr>
<tr>
<td>PK/LDH/CPK/CS ATP/CrP/Glycogen Liver</td>
<td>5.6/3.1/31.7/0.8</td>
<td>3.5/2.0/28.7/0.5</td>
<td>4.1/3.9/34.6/0.7</td>
<td>N/A</td>
<td>4.9/1.8/14.3/1.3</td>
</tr>
<tr>
<td>1.5/3.5/250.4</td>
<td>1.4/2.2/329</td>
<td>1.0/0.6/90</td>
<td>N/A</td>
<td>0.8/1.3/126</td>
<td></td>
</tr>
<tr>
<td>PK/LDH/CPK/CS ATP/CrP/Glycogen Muscle</td>
<td>32/305/3150/0.8</td>
<td>41/344/3443/0.4</td>
<td>30/328/2453/0.7</td>
<td>N/A</td>
<td>12/64/1112/0.4</td>
</tr>
<tr>
<td>2.8/232.6/6.4</td>
<td>4.2/21.1/10.4</td>
<td>2.9/21.7/6.2</td>
<td>N/A</td>
<td>0.7/19.7/0.3</td>
<td></td>
</tr>
<tr>
<td>Hypoxia Exposure</td>
<td>Short</td>
<td>Long</td>
<td>Short</td>
<td>Long</td>
<td>Short</td>
</tr>
<tr>
<td>OXPhos</td>
<td>-</td>
<td>↓</td>
<td>v</td>
<td>v</td>
<td>v</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>-</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>FAO</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Translational initiation</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>v</td>
</tr>
<tr>
<td>Translational elongation</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Protein localization</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>v</td>
</tr>
<tr>
<td>Protein folding</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>↑</td>
<td>-</td>
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</tbody>
</table>
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Vinciotti V., R. Khanin, D. D’Alimonte, X. Liu, N. Cattini, G. Hotchkiss, G. Bucca, O.

127


Appendix A

Tissue enzymes in 12 species of sculpin. Tissue enzymes are presented in μmol/min/mg protein except brain CS, liver LDH, liver PK, liver CS and muscle CS are presented in nmol/min/mg protein. Numbers in parentheses represent sample size. Data are means ± SE.

<table>
<thead>
<tr>
<th>Species</th>
<th>Brain</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDH PK CPK CS</td>
<td>LDH PK CPK CS</td>
<td>LDH PK CPK CS</td>
</tr>
<tr>
<td>O. maculosus</td>
<td>1.3±0.1 0.8±0.1</td>
<td>7.2±0.2 27.2±0.1</td>
<td>3.3±0.3 0.4±0.1</td>
</tr>
<tr>
<td>C. globiceps</td>
<td>1.3±0.1 0.8±0.1</td>
<td>9.1±0.4 33.5±0.2</td>
<td>52.2±15.1 30.4±3.3</td>
</tr>
<tr>
<td></td>
<td>(8) (8) (8) (8)</td>
<td>(8) (10) (10) (10)</td>
<td>(8) (8) (8) (8)</td>
</tr>
<tr>
<td>O. snyderi</td>
<td>1.2±0.1 0.7±0.1</td>
<td>8.7±0.5 25.4±0.1</td>
<td>35.1±8.5 17.2±3.1</td>
</tr>
<tr>
<td>M. polyacanthocephalus</td>
<td>1.1±0.1 0.6±0.1</td>
<td>6.0±0.7 32.8±0.4</td>
<td>43.0±10.6 40.3±8.2</td>
</tr>
<tr>
<td></td>
<td>(9) (9) (9) (9)</td>
<td>(8) (11) (11) (11)</td>
<td>(8) (8) (8) (8)</td>
</tr>
<tr>
<td>E. bison</td>
<td>1.0±0.1 0.6±0.1</td>
<td>6.4±0.3 32.4±0.2</td>
<td>11.5±1.5 27.2±4.5</td>
</tr>
<tr>
<td></td>
<td>(9) (9) (9) (9)</td>
<td>(8) (8) (8) (8)</td>
<td>(8) (8) (8) (8)</td>
</tr>
<tr>
<td>A. fenestralis</td>
<td>1.1±0.1 0.6±0.1</td>
<td>6.9±0.4 33.3±0.2</td>
<td>15.5±1.8 25.5±3.9</td>
</tr>
<tr>
<td></td>
<td>(9) (9) (9) (9)</td>
<td>(8) (8) (8) (8)</td>
<td>(8) (8) (8) (8)</td>
</tr>
<tr>
<td>A. lateralis</td>
<td>0.9±0.1 0.7±0.1</td>
<td>7.2±1.0 28.0±0.2</td>
<td>13.5±1.8 25.5±3.9</td>
</tr>
<tr>
<td>L. armatus</td>
<td>1.3±0.1 0.8±0.1</td>
<td>8.1±0.4 37.7±0.2</td>
<td>28.0±4.8 30.0±2.5</td>
</tr>
<tr>
<td>S. marmoratus</td>
<td>1.1±0.1 0.7±0.1</td>
<td>7.0±0.3 36.7±0.2</td>
<td>19.7±1.9 38.8±4.8</td>
</tr>
<tr>
<td></td>
<td>(9) (9) (9) (9)</td>
<td>(8) (12) (12) (12)</td>
<td>(8) (8) (8) (8)</td>
</tr>
<tr>
<td>B. cirrhosis</td>
<td>0.8±0.1 0.6±0.1</td>
<td>6.0±0.3 32.0±0.1</td>
<td>9.0±1.0 24.1±1.3</td>
</tr>
<tr>
<td></td>
<td>(10) (10) (10)</td>
<td>(10) (12) (12) (12)</td>
<td>(10) (10) (10) (10)</td>
</tr>
<tr>
<td>M. scorpius</td>
<td>0.9±0.1 0.6±0.1</td>
<td>5.4±0.2 34.5±0.1</td>
<td>19.8±1.9 33.8±4.1</td>
</tr>
<tr>
<td>C. asper</td>
<td>1.1±0.1 0.9±0.1</td>
<td>7.0±0.9 28.5±0.5</td>
<td>11.1±1.1 15.2±2.9</td>
</tr>
</tbody>
</table>
Appendix B

A hybridization loop for each species of sculpin. The 29 individuals per species are indicated by the number that is next to every time-point.
**Appendix C**

Euclidean distance for the remainder of the genes associated with biological processes presented in figures 3.3 and 3.4.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Ontology: Biological Process</th>
<th>UniProt</th>
<th>Euclidean Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial</td>
<td>GO:0019395~fatty acid oxidation</td>
<td>DDT4</td>
<td>1.251</td>
</tr>
<tr>
<td>Importin-7</td>
<td>GO:0008104~protein localization</td>
<td>DBLOH</td>
<td>1.246</td>
</tr>
<tr>
<td>Heat shock protein HSP 90-alpha</td>
<td>GO:0006457~protein folding</td>
<td>FABPH</td>
<td>1.227</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4E-binding protein 3-like</td>
<td>GO:0006446~regulation of translational initiation</td>
<td>SGK1</td>
<td>1.219</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor</td>
<td>GO:006955~immune response</td>
<td>IF4G2</td>
<td>1.193</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor</td>
<td>GO:0043066~negative regulation of apoptosis</td>
<td>IF4G2</td>
<td>1.193</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor</td>
<td>GO:0008284~positive regulation of cell proliferation</td>
<td>UBE2A</td>
<td>1.193</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>GO:0008284~positive regulation of cell proliferation</td>
<td>K6PL</td>
<td>1.145</td>
</tr>
<tr>
<td>Keratin, type I cytoskeletal 18</td>
<td>GO:0008104~protein localization</td>
<td>CGL</td>
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</tr>
<tr>
<td>Keratin, type I cytoskeletal 18</td>
<td>GO:0043066~negative regulation of apoptosis</td>
<td>EIF1B</td>
<td>1.116</td>
</tr>
<tr>
<td>C-C motif chemokine 25</td>
<td>GO:0006955~immune response</td>
<td>K6PF</td>
<td>1.079</td>
</tr>
<tr>
<td>Protein DJ-1</td>
<td>GO:0000302~response to reactive oxygen species</td>
<td>CFAH</td>
<td>1.052</td>
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<tr>
<td>Enoyl-CoA hydratase, mitochondrial</td>
<td>GO:0019395~fatty acid oxidation</td>
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<td>Annexin A5</td>
<td>GO:0043066~negative regulation of apoptosis</td>
<td>CIDEB</td>
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<tr>
<td>Phospholipid hydroperoxide glutathione peroxidase, mitochondrial</td>
<td>GO:0051276~chromosome organization</td>
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<td>Phospholipid hydroperoxide glutathione peroxidase, mitochondrial</td>
<td>GO:0000302~response to reactive oxygen species</td>
<td>MYD88</td>
<td>1.034</td>
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<tr>
<td>Peptidyl-prolyl cis-trans isomerase F</td>
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