ANOXIC SURVIVAL AND CARDIOVASCULAR RESPONSES OF THE PACIFIC HAGFISH, *EPTATRETUS STOUTII*

by

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ABSTRACT

To determine if anoxic survival in the Pacific hagfish (Eptatretus stoutii L) is aided by the suppression of metabolic rate, excess post-anoxic oxygen consumption (EPAOC) and key metabolites in the glycolytic pathway were analyzed following anoxic exposures of different durations. As the cardiovascular system reflects the needs of the tissue and thus whole animal metabolic rate, cardiac performance during prolonged anoxia was also examined to gain insight into the anoxic cardiac ATP turnover rate.

Hagfish survived 36-h exposure of complete anoxia at 10°C but showed 50% mortality if exposed to anoxia for 48 h. In order to determine if there had been metabolic rate suppression, changes in tissue metabolites were measured during 36 h anoxia exposure and EPAOC was monitored using respirometry. Analysis of EPAOC measurements suggest that hagfish use metabolic rate suppression to enhance anoxia survival during bouts of anoxia greater than 24 h and that metabolic rate was halved during the final third of a 36-h anoxic period. However, analysis of tissue metabolites in the liver, heart, tongue and skeletal muscle showed that glycogen levels were rapidly depleted over the first 6 h, but then stabilized for the duration of the anoxic exposure. Taken together, the results of the respirometry study and metabolic analysis suggest that anoxia survival in E. stoutii is enhanced by metabolic suppression, but that this suppression may occur earlier in the anoxic period than revealed by EPAOC measurements alone.

To gain a better understanding of the use of metabolic rate suppression as a means for surviving anoxic exposures, cardiovascular function was examined during a
36-h anoxic exposure. Cardiac output and ventral aortic blood pressure were measured for 36 h of anoxia and through full recovery. Anoxic bradycardia that halved heart rate within 3 h, which then remained stable at 5 bpm for 33 h of anoxia. Cardiac output, however, was reduced by only ~33%, suggesting metabolite, hormone and waste transport remain important during anoxia. Furthermore, cardiac power output remained unchanged during anoxia. Thus, cardiac metabolic rate is not suppressed and its routine cardiac ATP demand is met through glycolysis and circulating blood glucose.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ix</td>
</tr>
<tr>
<td>CO-AUTHORSHIP STATEMENT</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER 1: OVERALL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The Importance of Oxygen</td>
<td>1</td>
</tr>
<tr>
<td>Adaptations to Decreasing Levels of Oxygen Availability</td>
<td>3</td>
</tr>
<tr>
<td>Hypoxic Adaptations</td>
<td>4</td>
</tr>
<tr>
<td>Hypoxic Survival Guide</td>
<td>4</td>
</tr>
<tr>
<td>Anoxic Adaptations</td>
<td>6</td>
</tr>
<tr>
<td>Anoxic Survival Guide</td>
<td>6</td>
</tr>
<tr>
<td>Anoxia-Tolerant Vertebrates</td>
<td>6</td>
</tr>
<tr>
<td>Determining Anoxic Metabolic Rate</td>
<td>8</td>
</tr>
<tr>
<td>Cardiovascular Function in Anoxia</td>
<td>10</td>
</tr>
<tr>
<td>Study Species</td>
<td>11</td>
</tr>
<tr>
<td>Ancient Fishes: Agnathans</td>
<td>11</td>
</tr>
<tr>
<td>Hagfishes</td>
<td>11</td>
</tr>
<tr>
<td>Thesis Objectives</td>
<td>13</td>
</tr>
<tr>
<td>CHAPTER 2: ANOXIC SURVIVAL AND METABOLIC RATE SUPPRESSION IN THE PACIFIC HAGFISH (EPTATRETUS STOUTII)</td>
<td>20</td>
</tr>
<tr>
<td>Introduction</td>
<td>20</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>23</td>
</tr>
<tr>
<td>Animals</td>
<td>23</td>
</tr>
<tr>
<td>Experimental Protocols</td>
<td>23</td>
</tr>
<tr>
<td>Respirometry</td>
<td>24</td>
</tr>
<tr>
<td>Tissue Metabolites</td>
<td>25</td>
</tr>
<tr>
<td>Measurements of Blood Variables</td>
<td>26</td>
</tr>
<tr>
<td>Percent Body Mass</td>
<td>27</td>
</tr>
<tr>
<td>Calculations and Statistics</td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td>28</td>
</tr>
<tr>
<td>Pre-anoxic Status</td>
<td>28</td>
</tr>
<tr>
<td>Post-anoxic Oxygen Consumption</td>
<td>29</td>
</tr>
<tr>
<td>Post-anoxic Metabolic Status</td>
<td>30</td>
</tr>
<tr>
<td>Discussion</td>
<td>31</td>
</tr>
<tr>
<td>References</td>
<td>48</td>
</tr>
<tr>
<td>CHAPTER 3: CARDIAC RESPONSES TO ANOXIA IN THE PACIFIC HAGFISH, EPTATRETUS STOUTII</td>
<td>53</td>
</tr>
<tr>
<td>Introduction</td>
<td>53</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>55</td>
</tr>
<tr>
<td>Animals</td>
<td>55</td>
</tr>
<tr>
<td>Surgical Procedure</td>
<td>55</td>
</tr>
<tr>
<td>Data Acquisition</td>
<td>56</td>
</tr>
<tr>
<td>Anoxic Exposure and Recovery</td>
<td>56</td>
</tr>
<tr>
<td>Calculations and Statistics</td>
<td>57</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Results</td>
<td>58</td>
</tr>
<tr>
<td>Anoxia</td>
<td>58</td>
</tr>
<tr>
<td>Normoxic Recovery</td>
<td>59</td>
</tr>
<tr>
<td>Discussion</td>
<td>60</td>
</tr>
<tr>
<td>Routine and Maximum Cardiovascular Variables</td>
<td>60</td>
</tr>
<tr>
<td>Cardiovascular Responses to Severe Hypoxia and Anoxia</td>
<td>62</td>
</tr>
<tr>
<td>Cardiac Control in Hagfish</td>
<td>65</td>
</tr>
<tr>
<td>References</td>
<td>73</td>
</tr>
<tr>
<td>CHAPTER 4: GENERAL DISCUSSION</td>
<td>77</td>
</tr>
<tr>
<td>Objectives</td>
<td>77</td>
</tr>
<tr>
<td>Objective 1</td>
<td>77</td>
</tr>
<tr>
<td>Objective 2</td>
<td>77</td>
</tr>
<tr>
<td>Objective 3</td>
<td>78</td>
</tr>
<tr>
<td>Perspectives</td>
<td>78</td>
</tr>
<tr>
<td>References</td>
<td>81</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>82</td>
</tr>
<tr>
<td>Appendix A</td>
<td>82</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.1: Mean pre-anoxic routine metabolic rate and post-anoxic recovery variables..........................................................................................................................40
Table 2.2: Mean ATP equivalents calculated from measured and estimated EPAOC’s......................................................................................................................................40
Table 2.3: Tissue glucose, CrP, ATP and blood glucose following exposure to normoxia (control) and for varying durations of anoxia........................................41
Table 2.4: Percent body mass of tissues........................................................................41
Table 2.5: Total depletion of glycogen and accumulation of lactate between control values and following 36 h of anoxia. .................................................................41
Table 3.1: Comparison of routine and maximal cardiovascular variables in vivo for hagfish and species-specific responses to either hypoxia or anoxia............68
LIST OF FIGURES

Figure 2.1: Representative oxygen consumption trace of a hagfish taken from normoxia, exposed to 6 h of anoxia and then allowed to recover in normoxia. * indicates periods of spontaneous activity. ..................................................................................42

Figure 2.2: Average O2 during the 24-h pre-anoxic acclimation period (N = 22). Shading indicates dark, night time period. ..................................................................................43

Figure 2.3: Average measured EPAOC for the 3 anoxic treatment groups. Individual fish underwent one of the three anoxic treatments. Time 0 represents transfer of fish from normoxia into anoxia. Normoxia was restored at 6 h (N=8), 24 h (N=7), and 36 h (N=7), and EPAOC was measured. Average routine metabolic rate (RMR) was calculated from the preceding normoxic period (see Materials and Methods). .................................................................................................44

Figure 2.4: Mean anoxic ATP turnover rates during 6 h (N = 8), 24 h (N = 7) and 36 h (N = 7) of anoxia as calculated from measured EPAOC. The dashed line represents normoxic ATP turnover calculated from RMR with s.e.m. indicated by the dotted lines (N = 22). * indicates significant difference between exposure times (p≤0.05). ..........................................................................................................................45

Figure 2.5: Glycogen concentrations in (A) tongue (F) and skeletal muscle (O), (B) heart (▽) and liver (◆), and (C) blood pH (●). Dissimilar letters denote statistical differences (p ≤ 0.05) between time points within a specific tissue. N values are 6 to 10 and s.e.m. is indicated by vertical bars. .............................................................................................................46

Figure 2.6: Lactate concentrations in (A) skeletal muscle (O) and tongue (F), (B) heart (▽) and liver (◆), and (C) blood (●). Dissimilar letters denote statistical differences (p ≤ 0.05) between time points within a specific tissue. N values are 6 to 10 and s.e.m. is indicated by vertical bars. .............................................................................................................47

Figure 3.1: Representative ventral aortic blood flow recordings taken from one hagfish after 6 h, 24 h and 36 h of anoxia, and during the 1.5 h and 36 h of normoxic recovery from anoxia. Each trace is for 2 min. Note the different y-axis scale for 1.5 h recovery. ........................................................................................................69

Figure 3.2: Simultaneous recorded cardiovascular variables from hagfish during a 36-h anoxic exposure. Time 0 indicates the start of anoxia. N values are 9 to 10 and s.e.m. is indicated by vertical bars. Statistical differences (p < 0.05) are indicated by dissimilar letters. .............................................................................................................70

Figure 3.3: Simultaneously recorded cardiovascular variables for cannulated fish during a 36-h anoxic exposure. Time 0 indicates the start of anoxia. R indicates routine values. N value is 3 and s.e.m. is indicated by vertical bars. .........................................71

Figure 3.4: Simultaneously recorded cardiovascular variables during a 36-h normoxic recovery from a 36-h anoxic exposure. Time 0 indicates the start of the normoxic flush period. N values are 7 and s.e.m. is indicated by vertical bars. Statistical differences (p< 0.05) are indicated by dissimilar letters. ..............................................72
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CrP</td>
<td>creatine phosphate</td>
</tr>
<tr>
<td>EPOC</td>
<td>excess post-exercise oxygen consumption</td>
</tr>
<tr>
<td>EPAOC</td>
<td>excess post-anoxic oxygen consumption</td>
</tr>
<tr>
<td>fₕ</td>
<td>heart rate</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>H⁺</td>
<td>proton</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>kPa</td>
<td>kilopascal</td>
</tr>
<tr>
<td>RMR</td>
<td>routine metabolic rate</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mO₂</td>
<td>oxygen consumption rate</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>P₅₀</td>
<td>PO₂ at which haemoglobin is 50% saturated with O₂</td>
</tr>
<tr>
<td>PO₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>PO</td>
<td>power output</td>
</tr>
<tr>
<td>Pva</td>
<td>ventral aortic pressure</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Vₙ</td>
<td>cardiac output</td>
</tr>
<tr>
<td>Vₛ</td>
<td>stroke volume</td>
</tr>
</tbody>
</table>
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CO-AUTHORSHIP STATEMENT

This thesis has been written in manuscript format according to the guidelines of the Faculty of Graduate Studies, University of British Columbia. The below listed, co-authored research articles comprise chapters 2 and 3. The vast majority of the research and all data analysis presented in these research articles were conducted by me. Listed co-authors either assisted with portions of the research, provided lab space and equipment and/or contributed towards design of experiments and interpretation of obtained results. All manuscripts were written by me in consultation with my supervisor (Dr. Anthony Farrell) as well as the co-authors.

**Chapter Two:** Anoxic Survival and Metabolic Rate Suppression in the Pacific Hagfish (*Eptatretus stoutii*)

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**Chapter Three:** Cardiovascular Responses to Chronic Anoxia in the Pacific Hagfish, *Eptatretus stoutii*

**Authors:** Georgina K. Cox, Erik Sandblom and Anthony P. Farrell
CHAPTER 1: OVERALL INTRODUCTION

The Importance of Oxygen

Life on earth is thought to have originated during a time when there was little to no oxygen (O$_2$) in the atmosphere. Over the millennia, organisms have evolved and adapted to the increased presence of O$_2$ in such a way that most species have become dependent upon it’s availability within their environments. This dependence resulted from the evolution of an efficient energy producing pathway (oxidative phosphorylation) in which O$_2$ is consumed as a reactant along with organic fuels such as carbohydrate, lipids and proteins to produce metabolic energy, namely adenosine triphosphate (ATP) (Hochachka and Somero, 2002). Although energy production is possible in the absence of O$_2$, the formation of ATP is most efficient with O$_2$ present (Hochachka and Somero, 2002). For example, in the absence of O$_2$, the partial catabolism of 1 mol of glucose via anaerobic glycolysis produces 2 to 3 mol of ATP, whereas the complete oxidation of 1 mol of glucose via oxidative phosphorylation produces ~ 29 mol of ATP (Brand, 2003). In addition, there is the advantage of using a variety of substrates with oxidative phosphorylation versus only two substrates (glucose and glycogen) with anaerobic glycolysis. Furthermore, wastes from oxidative phosphorylation (H$_2$O and CO$_2$) can be easily excreted from the body unlike anaerobic metabolic waste (H$^+$ and lactate), which cannot be passively excreted from the body and can have detrimental effects on cell function with increasing concentration.
When environmental \( O_2 \) decreases to levels that limit \( O_2 \) delivery to the mitochondria, energy production through oxidative phosphorylation first relies on finite cellular stores of \( O_2 \) (Hochachka et al., 1996). Most cells, especially muscles cells, have \( O_2 \) bound to intracellular haemoglobin or myoglobin, which release \( O_2 \) in response to a decreased cellular ATP production (Hochachka, 1980). Up-regulation of \( O_2 \) independent substrate-level phosphorylation via creatine phosphate (CrP) hydrolysis and glycolysis can also serve to augment ATP production. CrP is regarded mostly as a high energy phosphate store that can be utilized during times of decreased \( O_2 \) availability or increased metabolic rate. Large amounts of glucose, commonly stored as glycogen, serve as a fermentable fuel source for an increased glycolytic rate. Although stores of \( O_2 \), CrP and glucose can serve as an energy buffer, these stores are finite and cannot function to augment ATP production indefinitely.

Energy is necessary to fuel cellular function and is derived from splitting the high energy phosphate bonds of ATP (Hochachka and Somero, 2002). It is crucial to maintain a balance between the production and consumption of ATP so that energy demand does not exceed what can be supplied by energy producing pathways. ATP demand cannot be supplied by glycolysis or phosphate stores indefinitely because the replenishment of fixed stores can only be accomplished in the presence of \( O_2 \) or by ATP formed through oxidative phosphorylation (Hochachka and Somero, 2002). Additionally, most vertebrates have routine metabolic rates (RMR) in which ATP demand far exceeds what can be supplied by anaerobic glycolysis. Thus, an inadequate supply of \( O_2 \) to cells will eventually result in an energy deficit which leads to the disruption of cellular processes.
Even with an up-regulation of glycolysis in combination with the utilization of O$_2$ and phosphagen stores, the vast majority of vertebrates can only survive a few minutes of O$_2$ deprivation before ATP demand begins to exceed supply and the disruption of cellular processes lead to death (Boutilier and St-Pierre, 2000). Because a low O$_2$ concentration in the blood (hypoxemia) can lead to an insufficient supply of O$_2$ to the tissues (ischemia), which can cause tissue failure, organ system failure and even death, O$_2$ deprivation can impact animal survival. However, there are a several species that can survive prolonged exposure to decreased O$_2$ levels through a suite of compensatory responses.

**Adaptations to Decreasing Levels of Oxygen Availability**

The major concern during exposure to both hypoxia (low O$_2$) and anoxia (no O$_2$) is the reduced capacity for producing ATP via mitochondrial oxidative phosphorylation. Because of this, vertebrates have developed a suite of behavioural, morphological, physiological and biochemical adaptations to increase survival time during O$_2$ deprivation. Low levels of environmental O$_2$ are common in aquatic habitats, due to the low capacitance of O$_2$ in water. Thus, hypoxic and anoxic adaptations can be observed in most aquatic vertebrates. Aquatic hypoxia can be defined as a partial pressure of oxygen (PO$_2$) below that which first compromises physiological function (Farrell and Richards, 2009). During hypoxia survival becomes dependant on: 1) the ability to increase O$_2$ extraction or acquisition; and 2) the ability to change metabolic phenotypes to maintain energy balance (Richards, 2009). While some hypoxic adaptations can aid
in anoxic survival, those that function to augment uptake and delivery of O\textsubscript{2} become irrelevant and thus anoxic survival is solely dependent upon an ability to change metabolic phenotypes to maintain energy balance.

**Hypoxic Adaptations**

**Hypoxic Survival Guide**

Upon exposure to hypoxia some species employ behavioural adaptations that include avoidance, aquatic surface respiration and aerial emergence (Brauner et al., 1995; Glass et al., 1986; Kramer and McClure, 1982; Yoshiyama et al., 1995). In some vertebrates these adaptations are aided by morphological changes such as either the development of lungs, which aid in efficient uptake of atmospheric O\textsubscript{2}, or increases in gill surface area, which serve to maximize O\textsubscript{2} uptake in the water (Brauner et al., 1995; Nilsson, 2007). Animals that cannot move to the water surface, due to predation pressure or ice cover, rely on physiological and biochemical adaptations to augment O\textsubscript{2} uptake and delivery. In addition to changes in cardiovascular performance and ventilatory frequency, physiological modifications to the oxygen cascade can be made that increase efficiency of O\textsubscript{2} extraction from water at the gill, increase haemoglobin-O\textsubscript{2} affinity, elevate the number of circulating red blood cells and increase the amount of functional haemoglobin within those cells (Lutz and Storey, 1997; Nikinmaa, 2001).

When environmental O\textsubscript{2} levels drop to the point at which O\textsubscript{2} extraction from the water is no longer able to maintain oxidative phosphorylation at a level necessary to
meet routine energy demand, and O\textsubscript{2} and phosphagen stores have been depleted, the only other source of ATP is from anaerobic glycolysis. Thus, there are only two possible biochemical solutions to prolong hypoxic survival: 1) Aerobic ATP production must be augmented by an up-regulation of anaerobic glycolysis (Pasteur effect); and 2) Metabolic rate must decrease (metabolic suppression) to a level that can be supplied by the current rate of ATP production (Hochachka et al., 1996). If an up-regulation in glycolytic rate occurs, tissues must either be able to tolerate the metabolic wastes or there must be a coincident up-regulation of buffering capability or waste removal in order reduce the negative effects of increased anaerobic waste. Similarly, without enhanced stores of fermentable substrate there would be a rapid depletion of available glycolytic fuel.

The ability to depress metabolic rate in response to an oxygen limitation has come to be regarded as the “hallmark” characteristic of hypoxia-tolerant vertebrates as it serves to conserve fuel and decrease the production of harmful wastes (Hochachka et al., 1996). These adaptations can also be employed when oxygen levels decrease to a level where there is no appreciable amount of oxygen available to drive metabolic processes (functional anoxia).
Anoxic Adaptations

Anoxic Survival Guide

During the initial onset of anoxia, anoxia-tolerant species may utilize many of the traits that hypoxia-tolerant species depend on for survival (Glass et al., 1990; Glass et al., 1991; Hylland et al., 1994). However, during protracted anoxic periods (hours, days or months) anoxia-tolerant vertebrates undergo a biochemical reorganization which usually results in the suppression of metabolic rate (Hochachka et al., 1996; Hochachka and Somero, 2002). Thus, to survive prolonged exposure to anoxia animals must possess: 1) anaerobic pathways capable of functioning at a level that can supply ATP demand; 2) fermentable stores to fuel anaerobic metabolism; 3) an ability to minimize the adverse affects of increased metabolic wastes; 4) a cardiovascular system functioning at a level that can transport fuel and wastes; and 5) an ability to fully recover routine metabolic rate and repair damage following anoxic exposure (Hochachka, 1980; Hochachka and Lutz, 2001; Hochachka and Somero, 2002).

Anoxia-Tolerant Vertebrates

Tolerance to anoxia varies across the vertebrate phylum and, while most species can only endure a few minutes of oxygen deprivation, a few species of cyprinid fish and freshwater turtles demonstrate a profound tolerance to anoxia (Ultsch and Jackson, 1982; van den Thillart, 1982; van den Thillart and van Waarde, 1985). In particular, the
crucian carp (*Carassius carassius*) and freshwater turtles (*Chrysemys picta belli* and *Traschemys scripta*) can survive months of anoxia at near freezing temperatures (Lutz and Nilsson, 1997; Ultsch and Jackson, 1982). While all species over-winter in ice-covered ponds, and possess large brain, heart and liver glycogen stores, they utilize different anoxic survival strategies (Clark and Miller, 1973; Farrell and Stecyk, 2007; Hyvärinen et al., 1985; Lutz and Nilsson, 1997; Warren et al., 2006).

Freshwater turtles initially up-regulate glycolytic rate and there is a subsequent rapid depletion in tissue glycogen (Daw et al., 1967; Lutz et al., 2005; Wasser et al., 1991). Within 1-2 h in anoxia, however, the turtle starts to suppress the Pasteur effect and whole body metabolic rate is suppressed to between 5 and 10% of the routine rate at 3°C (Herbert and Jackson, 1985). The metabolic wastes are buffered by calcium bicarbonate sequestered in the shell and skeleton (Jackson, 2000a). In contrast to freshwater turtles, crucian carp remain active during anoxia and as such their brains and cardiovascular system function at routine levels in order to coordinate locomotion, and transport of glycogen and anaerobic waste (Nilsson and Lutz, 2004; Nilsson et al., 1993; Stecyk et al., 2004). Crucian carp accomplish this through the up-regulation of glycolysis to meet a decreased ATP demand (Johansson et al., 1995; Storey, 1985). Metabolic waste produced by the sustained increase in glycolytic rate is converted to ethanol and CO₂ and excreted across the gills (Shoubridge and Hochachka, 1980).

Recovery in both freshwater turtles and cyprinid fish is marked by a period of increased (above routine) metabolic rate (Herbert and Jackson, 1985; van den Thillart and Verbeek, 1991). This increase in metabolic rate is accompanied by an increase in oxygen consumption that serves to increase ATP production through oxidative
phosphorylation in order to produce sufficient energy necessary to restore energetic homeostasis (Gaesser and Brooks, 1984).

Determining Anoxic Metabolic Rate

Metabolic rate can be indirectly measured from either heat production from the combustion of fuel or O$_2$ consumption rates (Weibel, 1984). In the absence of a calorimeter that measures heat production or during anoxia, when measurements of O$_2$ consumption are impossible, another method of determining anoxic metabolic rate must be used. As ATP production in anoxia (anaerobic glycolysis) is limited by fermentable stores that cannot be replenished without O$_2$, the costs incurred through anaerobic metabolism must eventually be paid by oxidative phosphorylation. Consequently following periods where anaerobic glycolysis is used to either augment or exclusively supply ATP, there is a period of elevated oxygen consumption (Gaesser and Brooks, 1984; Hancock and Gleeson, 2008; van den Thillart and Verbeek, 1991). The additional (above routine) oxygen consumption following periods of anoxia reflects anaerobic energy turnover and substrate utilization during an anoxic period and therefore can be used to determine the energetic cost of anoxic survival (van den Thillart and Verbeek, 1991). Thus, in the absence of direct calorimetric measurements, whole animal metabolic rate can be estimated from the excess post-anoxic oxygen consumption EPAOC.

Hill and Lupton (1923) first described excess post-exercise oxygen consumption (EPOC) as an “oxygen debt” that needed to be repaid following intense exercise. The
oxygen debt was thought to reflect the metabolism of lactate following intense exercise (Hill and Lupton, 1923). Subsequent studies found that the EPOC was neither temporally nor causally related to lactate removal (Gaesser and Brooks, 1984). Current studies have discovered that this elevated $O_2$ consumption following intense exercise is not solely a payment of the debt accrued by utilizing anaerobic metabolism (Gaesser and Brooks, 1984; Hancock and Gleeson, 2008). EPOC has been shown to result from the replenishment of fuel, $O_2$ and phosphagen stores, removal of wastes, restoring blood pH, catecholamine stores and hormones to routine levels, and increases in ventilation and cardiovascular work (Gaesser and Brooks, 1984; Hancock and Gleeson, 2008; Mandic et al., 2008; Scarabello et al., 1991; van den Thillart and Verbeek, 1991). By analogy, therefore, EPAOC should also exceed the quantity of oxygen required to maintain a given metabolic rate in anoxia, but to what degree is unknown.

In order determine if metabolic rate suppression occurs during an anoxic exposure and to what degree this suppression aids in conserving fuel stores, EPAOC can be converted to an ATP turnover rate and compared with estimates of anoxic ATP requirements (estimated ATP turnover) calculated from the product of routine metabolic rate (RMR) and time. Thus, EPAOC can be compared to the quantity of oxygen needed to maintain RMR during a given anoxic exposure. Consequently, if metabolic suppression had occurred and anoxic ATP turnover was suppressed, EPAOC would be less than estimated ATP turnover. Additionally, analysis of substrates and products of substrate-level phosphorylation can also be used to determine if there is metabolic suppression within specific tissues during anoxia. Without metabolic suppression, glycogen depletion should occur at a stable rate, as would lactate appearance. A
stabilization of glycogen stores and lactate appearance during prolonged anoxia, therefore, would indicate a reduction in ATP turnover rate associated with metabolic suppression.

*Cardiovascular Function in Anoxia*

Vertebrates rely on the cardiovascular system’s ability to transport respiratory gases, nutrients and metabolic wastes. The level at which the cardiovascular system operates is dictated by the needs of tissues and thus reflects whole animal metabolic rate (Jackson, 2000b). Similarly, during anoxia, anoxia-tolerant vertebrates rely on cardiovascular support to transport metabolites and anaerobic waste among tissues at a rate dictated by metabolic demand. Species that remain active during anoxia, such as the crucian carp, maintain cardiovascular function at routine levels in order to supply active tissues with fuel and transport metabolic wastes (ethanol) to the gills for excretion (Stecyk et al., 2004). Conversely, species that enter a hypometabolic state during anoxia suppress cardiac performance in conjunction with metabolic demand (Arthur et al., 1997; Hicks and Farrell, 2000; Jackson and Ultsch, 1982). Thus, examining cardiovascular function during anoxia can provide insight into the degree of metabolic suppression of the whole animal, as well as the heart as a functioning organ.
Study Species

Ancient Fishes: Agnathans

The agnathans (the lampreys and the hagfish) occupy the a basal position in vertebrate phylogeny (Ota and Kuratani, 2007). They are thought to have diverged from the predominant evolutionary path of vertebrates more than 500 million years ago and during this time have survived large variations (between 15 and 30%) in atmospheric O$_2$ (Berner, 1999; Shu et al., 1999). Because of the low capacitance of O$_2$ in water, these drastic changes in atmospheric O$_2$ were reflected in extremely hypoxic seas. The survival of the extant lineage of hagfishes suggests that the agnathan hypoxic and anoxic responses are conceivably very ancient (Nikinmaa, 2001). As terrestrial animals have ancestral aquatic relatives, it is likely that the origins of hypoxic and anoxic tolerance developed in ancient fishes. Studying the physiological responses to anoxia in extant representatives of so called primitive fish may lead to a greater understanding of the basic mechanisms underlying anoxia tolerance and its evolution.

Hagfishes

Hagfishes are known to inhabit hypoxic sediments and their method of feeding mainly involves burrowing into the coelomic cavities of dead or moribund organisms that have fallen to the sea floor, both of which preclude gill ventilation with oxygenated water (Foss, 1963; Jørgensen, 1998). This life history not only exposes them to extremely
hypoxic, if not anoxic conditions, but it also requires them to swim and feed in these environments. The ability of hagfish to thrive in conditions of limited O₂ availability makes them an ideal species in which to investigate physiological adaptations to anoxia. Their life history, in combination with a variety of morphological, physiological and biochemical characteristics, indicates an ability to withstand prolonged oxygen deprivation.

Hagfish possess many traits common with hypoxia- and anoxia-tolerant vertebrates. In addition to their low metabolic rate, their ability to tolerate decreased blood pH during intense exercise, hypoxia and anoxia, paired the ability to rapidly deplete glycogen stores and increase lactate, indicates a high glycolytic potential (Baldwin et al., 1991; Davison et al., 1990; Forster, 1990; Forster, 1991; Foster et al., 1993; Hansen and Sidell, 1983; Korneliussen and Nicolaysen, 1973; Mellgren and Mathisen, 1966; Munz and Morris, 1965; Perry et al., 2009; Ruben and Bennett, 1980; Steffensen et al., 1984). Although the buffering capability of the blood is low, their comparatively large blood volume may aid in the dilution of anaerobic wastes to a concentration that is not harmful during prolonged anoxia (Forster et al., 2001; Satchell, 1991; Wells et al., 1986). Additionally, the routine myocardial output of the hagfish heart lies within the proposed maximum cardiac glycolytic potential of fish hearts and thus the heart may be able to function at routine levels even in the absence of myocardial O₂ (Farrell, 2007; Farrell and Stecyk, 2007; Hansen and Sidell, 1983). The only remaining characteristic common to anoxia-tolerant vertebrates is the ability to suppress metabolic rate. No study has yet explored the possibility that hagfish suppress metabolic rate during hypoxia or anoxia.
Thesis Objectives

Despite the evolutionary significance of this group of fishes only a handful of studies have attempted to characterize the hypoxia/anoxia tolerance of hagfishes (Axelsson et al., 1990; Forster, 1991; Forster et al., 1992; Hansen and Sidell, 1983; Perry et al., 2009) and only two studies that have explored their physiological responses to acute anoxia (Hansen and Sidell, 1983; Perry et al., 1993). Surprisingly there have been no previous studies have examined prolonged (days) anoxia in hagfish. Thus, the three main objectives of my thesis research were:

1) Determine the maximum duration of anoxic survival in the Pacific hagfish, *Eptatretus stoutii* (Lockington 1878) at 10°C;
2) Determine if this survival involves metabolic suppression; and
3) Characterize the cardiovascular responses of hagfish to chronic anoxia.
References


CHAPTER 2: ANOXIC SURVIVAL AND METABOLIC RATE SUPPRESSION IN THE PACIFIC HAGFISH (EPTATRETUS STOUTII)

Introduction

Hagfishes (Family: Myxinidae) belong to an evolutionary ancient fish lineage that first appeared around 300 million years ago (Bardack, 1991). There are close to 60 extant species of hagfish and many of these inhabit deep seas where hypoxia/anoxia is common. Some species burrow into hypoxic sediments (Lesser et al., 1997; Martini, 1998) and their feeding strategy of burrowing into the body cavity of dead or moribund animals exposes them to severely hypoxic, if not anoxic conditions.

Despite the hagfish’s propensity for inhabiting potentially anoxic conditions, only two previous studies have examined their physiological responses to anoxia (Hansen and Sidell, 1983; Perry et al., 1993). Hansen and Sidell (1983) showed that in situ cardiac performance of Myxine glutinosa was maintained for up to 3 h after cyanide poisoning and azide poisoning. Although there has been no study to date that has investigated anoxic survival in hagfish, other studies have examined hagfish physiology during severe hypoxia (Axelsson et al., 1990; Bernier et al., 1996a; Bernier et al., 1996b; Forster, 1990; Forster, 1991; Forster et al., 1991; Forster et al., 1992; Foster and Forster, 2007; Hansen and Sidell, 1983; Perry et al., 1993) and have investigated anaerobic metabolism in certain tissues (Baldwin et al., 1991; Davison et al., 1990; Forster, 1990; Forster, 1991; Forster et al., 1991; Forster et al., 1992; Foster and Forster, 2007; Hansen and Sidell, 1983; Perry et al., 1993) and have investigated anaerobic metabolism in certain tissues (Baldwin et al., 1991; Davison et al., 1990; Forster, 1990; Forster, 1991; Forster et al., 1991; Forster et al., 1992; Foster and Forster, 2007; Hansen and Sidell, 1983; Perry et al., 1993) and have investigated anaerobic metabolism in certain tissues (Baldwin et al., 1991; Davison et al., 1990; Forster, 1990; Forster, 1991; Forster et al., 1991; Forster et al., 1992; Foster and Forster, 2007; Hansen and Sidell, 1983; Perry et al., 1993).
Foster et al., 1993; Hanson and Sidell 1983). Combined, these studies suggest that the hagfishes are quite remarkable in their tolerance to conditions where oxygen is scarce or absent.

The most important biochemical trait allowing anoxia survival in animals is the ability to maintain cellular energy balance. During anoxia exposure, ATP supply is limited because of a lack of oxidative phosphorylation, therefore ATP can only be generated through substrate-level phosphorylation (primarily glycolysis). The length of anoxic survival will then be determined by substrate availability and/or the accumulation of glycolytic wastes. A down-regulation in ATP turnover conserves glycogen stores crucial for anaerobic ATP production and slows the rate of metabolic waste accumulation. In fact, it has been suggested that the ability to suppress metabolic rate is paramount for anoxic survival (Hochachka et al., 1996; Hochachka and Lutz, 2001). For example, freshwater turtles suppress routine metabolic rate (RMR) by up to 95% during anoxia (Arthur et al., 1997). Alternatively, crucian carp (Carassius carassius) survive by maintaining glycolytic energy production for prolonged periods of anoxia; in part due to large glycogen stores, a high glycolytic potential and an ability to excrete glycolytic wastes by generating ethanol (Hyvärinen et al., 1985; Nilsson, 1990; Nilsson, 2001; Shoubridge and Hochachka, 1980; Stecyk et al., 2004). Additionally, some studies suggest having an inherently low RMR can be functionally adaptive for hypoxic and anoxic survival (Mandic et al., 2009; Stecyk et al., 2004), because if whole animal RMR lies below the maximum glycolytic potential, ATP demand can be met anaerobically. RMR in hagfish is among the lowest recorded values for vertebrates (Brauner and Berenbrink, 2007), and it has been suggested that the low normoxic ATP
demand of the heart can be met anaerobically (Farrell, 1991; Forster et al, 1991). Given
the life history of hagfish it would be advantageous for them to have the ability to
maintain RMR in anoxia and perhaps even increase metabolic rate during feeding in
such conditions. Regardless of whether hagfish maintain RMR or suppress their
metabolism, the maximum glycolytic potential sets the upper limit for the ATP flux to
fuel basic cellular functions during anoxia, as has been demonstrated for cardiac
function (Farrell and Stacey, 2007).

In the absence of direct calorimetric measurements, metabolic rate of whole
animals during anoxia can be estimated from their oxygen uptake during normoxic post-
anoxic recovery because glycolytic ATP production incurs an oxygen debt, as first
described by Hill and Lupton (1923). For example, following intense anaerobic exercise
the amount of excess (above routine) post-exercise oxygen consumed (EPOC)
represents the replenishment of fixed oxygen stores, ATP turnover and substrate
utilization by anaerobic pathways during intense exercise (Hill and Lupton, 1923,
Gaesser and Brooks, 1984). This principle was adapted to anoxic hagfish by measuring
excess post-anoxic oxygen consumption (EPAOC) for anoxic periods of different
durations to determine if hagfish maintain RMR during anoxia or utilize metabolic rate
suppression to enhance anoxia survival. The specific objectives of the present study on
Pacific hagfish, *Eptatretus stoutii* (Lockington 1878) were therefore to determine 1) the
duration of anoxic survival, and 2) whether anoxic survival involves metabolic
suppression.
Materials and Methods

Animals

Hagfish were captured at ~100 m depth in Barkley Sound, British Columbia, Canada (48° 50’N, 125° 08’W) and transported to the Department of Fisheries and Oceans – University of British Columbia Centre for Aquaculture and Environmental Research (CAER), West Vancouver, British Columbia, Canada. They were housed year round in 1,100 L tanks with aerated, flow-through seawater (temperature 10°C ± 1°C; salinity ~30 ppt). A total of 22 animals (153 ± 7 g) were used in the respirometry study, 64 animals (84 ± 33 g) were used to measure metabolites and a further 8 animals were used to determine the percent body mass of tissues analysed for metabolites. Food was withheld for a minimum of two weeks prior to experimentation. All of the following procedures were approved by the University of British Columbia Animal Care Committee (A07-0680; Appendix A) and conducted in accordance with their guidelines.

Experimental Protocols

Two separate sets of experiments were run to fully characterize the metabolic responses of hagfish to anoxia exposure. Briefly, in the first experiment, hagfish were exposed to 6, 24, 36, and 48 h of anoxia and then returned to normoxia where EPAOC was monitored. In the second experiment, fish were terminally sampled immediately following anoxic exposures of 6, 24 and 36 h and blood and tissues were analyzed for various metabolites.
**Respirometry**

Individual hagfish were weighed and placed in 2.5 L Loligo respirometer chambers (Loligo Systems, Tjele, Denmark) at least 24 h before experimentation. Typically, hagfish had recovered from the handling stress by 3 h as evidenced from a plateau in consumption (p O$_2$) following this period. The experimental protocol was as follows. Following recovery from handling stress routine O$_2$ was recorded during a 24-h period, which started around 11 am daily. The following day, the respirometer was made progressively hypoxic by flushing (1 L min$^{-1}$) nitrogen-saturated seawater from a gas exchange column into the chamber until complete anoxia was achieved (0.0 kPa, after ~1 h). Although PO$_2$ in the chamber was continuously measured during this transition period, O$_2$ measurements were suspended. Four anoxic durations were tested: 6 h, 24 h, 36 h and 48 h (each N = 8 fish). Following the anoxic period (see below), normoxia was restored within ~10 min by introducing air-saturated seawater.

O$_2$ measurements continued throughout the recovery period until RMR was restored (fig. 1).

Oxygen consumption was measured using intermittent flow respirometry (Steffensen, 1989). The intermittent-flow cycle was set up such that the respirometers were flushed with aerated seawater (10 ± 1°C, 30 ppt) at a flow rate of 1 L min$^{-1}$ for 5 min. This was followed by a 22-min measurement period during which the chamber was closed and water was recirculated inside the respirometer to ensure proper mixing. Due to a response lag for the Loligo system, the first two minutes of this period represented a wait period. In order to prevent a nonlinear oxygen depletion curve, the water PO$_2$ during the wait period was not used to calculate o O$_2$. Water PO$_2$ was
measured once every second using MINI-DO probes (Loligo Systems, Tjele, Denmark). The oxygen probes were calibrated using aerated sea water and sodium sulfite derived oxygen-free distilled water prior to each trial. The rate of oxygen decline in the closed respirometer was used to calculate $O_2$ using LoliResp6 software (Loligo Systems Tjele, Denmark). The 20-min measurement period resulted in at least a 10% decrease in water $PO_2$ (Steffensen, 1989). The relatively large volume of the respirometer allowed the hagfish to adopt their relaxed, curled position in normoxia and their typical uncurled position during anoxia.

**Tissue Metabolites**

To examine metabolic fuel usage during anoxia, hagfish were terminally sampled during normoxia and after 6-h, 24-h and 36-h anoxic periods (10 hagfish per treatment group). Briefly, individual fish were placed in respirometers as described above and allowed to recover after transfer for 12 h into normoxic water. After the recovery period, fish were either sampled or exposed to anoxia as described above. During sampling, fish were rapidly sacrificed, a blood sample was first collected from the caudal sinus and samples of heart, liver, tongue, and dorsal muscle were thereafter promptly extracted and flash frozen in liquid nitrogen. All tissue samples were stored at -80°C until analysis.

Heart, liver, tongue, and dorsal muscle samples were ground into a fine powder using a mortar and pestle cooled with liquid nitrogen and subsequently stored at -80°C until metabolite extraction. Tissue samples were analysed for glycogen, glucose and lactate. Approximately 100 mg of ground tissue was weighed into pre-cooled micro
centrifuge tubes and ice-cold 1 M HClO₄ was added. Samples were immediately homogenized at the highest speed of a Polytron homogenizer for 15 s at 0°C and then stored on ice. Each sample was vortexed and 200 µl of the homogenate slurry was transferred into a microcentrifuge tube, placed in liquid nitrogen and stored at -80°C for later determination of glycogen content. The remainder of homogenate was centrifuged for 5 min at 4°C at 20,000 g and then the supernatant was neutralized with 3 M K₂CO₃. Neutralized tissue extracts were assayed for lactate and glucose using methods described by Bergmeyer (1983). Tissue homogenate slurries were thawed on ice and partially neutralized with 3 M K₂CO₃. The glycogen content was measured as free glucose following digestion with amyloglucosidase (Bergmeyer, 1983).

For analysis of tissue ATP and CrP (in heart, liver, tongue and dorsal muscle), hagfish were sampled using a modified, faster protocol. Briefly, a separate group of hagfish were sampled during normoxia and immediately following 6-h, 24-h and 36-h anoxia exposure by freeze-clamping the whole fish. The entire sampling protocol took <5 s. Tissues were then extracted from the frozen fish submerged in liquid nitrogen and processed as described above for glycogen, glucose and lactate (Bergmeyer, 1983). This modified protocol was used because even under anaesthesia hagfish continue to move. Preliminary experiments indicated that this movement had dramatic effects of tissue ATP and CrP, but little or no effect on tissue glycogen, glucose or lactate.

**Measurements of Blood Variables**

Blood samples that were collected from the caudal sinus directly prior to tissue extraction were immediately analysed for pH, glucose and lactate. Whole blood pH
measurements were made in triplicate using a Radiometer micro-capillary electrode (G299A) thermostated (10°C) in a Radiometer BMS-2 system and displayed on a Radiometer PHM 73 (Radiometer, Copenhagen, Denmark). Whole blood was subsequently centrifuged in micro centrifuge tubes at 7,000 g for 5 min and plasma was collected and stored on ice for immediate analysis of glucose and lactate using a YSI 2300 stat plus analyzer (YSI Life Sciences, Yellow Springs, Ohio, USA).

**Percent Body Mass**

Fish (N=8) were rapidly sacrificed and blotted dry with paper towel and weighed. The heart, liver, tongue and parietal muscle were dissected out and weighed individually.

**Calculations and Statistics**

The estimated EPAOC assumed that *E. stoutii* maintained RMR throughout anoxia and that there was no additional cost to replenish energy and oxygen stores. Estimated EPAOC’s were calculated from the product of RMR and time in anoxia. Measured EPAOC refer to the actual amount of excess oxygen consumed following anoxic exposure and was calculated for each individual fish as the area between the O$_2$ recovery curve and the calculated RMR (fig. 1; see results). Average anoxic metabolic rate was calculated from the measured EPAOC and anoxic duration. Because of minor activity during recovery (see results), this measurement technique may have lead to a slight overestimate of EPAOC in some individuals. ATP equivalents for the
measured and estimated EPAOC’s were calculated using 4.5 moles of ATP to 1 mol of O2 (Busk and Boutilier, 2005; Rolfe and Brand, 1996).

Statistical differences in EPAOC and measurements of tissue metabolites among different anoxic exposures were tested using one way ANOVA followed by a Holm-Sidak post-hoc test. Comparisons between measured and estimated EPAOC values for a given anoxic exposure were tested using t-tests. Statistical significance was set at P≤ 0.05 and all values are reported as means ± s.e.m. unless otherwise stated.

Results

Pre-anoxic Status

The maximal O2 measured directly following the transfer of fish in to the respiration chambers ranged from 40 to 80 mg O2 kg⁻¹ h⁻¹. Measurements of oxygen consumption and visual observations during the 24 h acclimation period indicate that hagfish are nocturnally active particularly around 22:00 and 4:00, with O2 values reaching ~40 mg O2 kg⁻¹ h⁻¹ (fig. 2.2 at 4:00 during night time). Hagfish were particularly quiescent during the day and O2 values declined to their lowest levels between 16:00 and 20:00 (fig. 2.2 at 16:00 and during daylight). Therefore, RMR was taken as the mean 2 O2 observed during daylight when spontaneous activity was rare. Hagfish RMR was 20.6 ± 1.0 mg O2 kg⁻¹ h⁻¹ (N=22) at 10°C. Spontaneous activity tripled routine O2 to a maximum value of 64.7 ± 3.0 mg O2 kg⁻¹ h⁻¹ (N=22).
Post-anoxic Oxygen Consumption

All hagfish recovered from an anoxic exposure lasting up to 36 h. However, when fish were exposed to a further 12 h of anoxia hagfish survival decreased to 50%. Therefore, EPAOC is graphically reported only for the 6-h, 24-h and 36-h anoxic periods and not for the 48-h period (Table 2.1). During the post-anoxic recovery period, $O_2$ peaked typically within the first 20 min and was approximately 4-times higher than RMR (Table 2.1). Thereafter, $fO_2$ declined exponentially to RMR over 8-36 h (figs. 2.1 and 2.3). While the peak, post-anoxic $O_2$ was not significantly different among anoxic treatment groups (Table 2.1), the duration of EPAOC increased significantly with the duration of the anoxic period (fig. 2.3; Table 2.1). Spontaneous activity occasionally occurred after several hours into recovery, creating minor spikes in the $O_2$ trace (e.g. fig. 2.1), particularly when recovery extended into the night time. As a result, activity during recovery was most common following the 6-h anoxic period (fig. 2.3) and least common following the 36-h anoxic period. Thus, the recovery from the 6-h anoxic exposure appears to last over 18 h, although calculations of mean EPAOC duration averaged only 8 h (fig. 2.3; Table 2.1).

While EPAOC’s for the 24-h and 36-h anoxic periods were approximately 3-fold larger than the 6-h EPAOC, the 24-h and 36-h EPAOC’s were not significantly different from each other (Table 2.1), suggesting that suppression of ATP turnover had occurred. Thus, to estimate when and to what degree metabolic rate suppression had occurred, the ATP equivalents of estimated and measured EPAOC’s were compared (Table 2.2). The 6-h measured EPAOC exceeded the estimated EPAOC by 7%, and the 24-h measured EPAOC was 18% below the estimated EPAOC, however, these
differences were not statistically significant (Table 2.2). The 36-h measured EPAOC was significantly different and was almost half the estimated EPAOC (Table 2.2), suggesting that RMR was maintained for 6 h and perhaps nearly for 24 h of anoxia, but not for the final third of a 36-h anoxic period (fig. 2.4).

Post-anoxic Metabolic Status

All tissues had significantly less glycogen and significantly more lactate following 36 h of anoxia when compared to normoxic controls (figs. 2.5 and 2.6). Even so, there were tissue-specific differences in the rates of glycogen consumption and lactate production (figs. 2.5 and 2.6).

Skeletal muscle and tongue, which together compose roughly 50% of the hagfish’s body mass, showed no significant decrease in glycogen level until after 24 h (fig. 2.5A; Table 2.4). Changes in lactate concentrations in the muscle mirrored changes in glycogen concentrations remaining constant for 24 h of anoxia and increasing significantly after 36 h (fig. 2.6 A). Lactate concentrations in the tongue, however, did not mirror changes in glycogen. Tongue lactate remained constant for 6 h and then significantly increased thereafter (fig. 2.6 A).

In the liver and heart, which together compose roughly 3% of the hagfish’s body mass, glycogen decreased significantly during the initial 6 h of anoxia but not thereafter (fig. 2.5B; Table 2.4). Even so, there was a constant significant rise in blood lactate and liver lactate concentrations, a continuous rise in heart lactate and a significant decrease in blood pH and over 36 h in anoxia (Figs. 2.5C and 2.6C). Glucose concentrations
were maintained in heart, liver and skeletal muscle, but increased significantly in the tongue and blood after 36 h of anoxia (Table 2.3).

With the exception of a single significant decrease in cardiac ATP after 6 h, ATP and CrP levels were maintained in all tissues during anoxia (Table 2.3).

**Discussion**

Hagfish not only inhabit hypoxic environments, but they also willingly enter decaying and severely hypoxic carcasses in order to feed. As a result of their feeding strategy they most likely cease to ventilate under these conditions and therefore experience anoxia (Coolidge et al., 2007; Martini, 1998). Given these behavioural characteristics, the ability of *E. stoutii* to survive anoxia at 10°C for up to 36 h and beyond is therefore perhaps not surprising. Even so, this duration of anoxia tolerance likely approaches the limit for this species at this temperature given the 50% mortality after a 48-h anoxia exposure. This survival time is over 40 hours (8 times) longer than that of the common carp, which survived 5-6 h at 15°C, but shorter than the crucian carp which survived over 2 weeks in anoxia at 8°C (Johnston and Bernard 1983; Nilsson 1990; Stecyk et al., 2004).

Oxygen consumption values for hagfishes are comparable to the lowest recorded values for teleosts of similar size at this temperature (Brauner and Berenbrink, 2007). Among the hagfishes, *Myxine glutinosa* has the highest reported RMR of 48 mg O$_2$ kg$^{-1}$ h$^{-1}$ at 7°C (Steffensen et al., 1984), while the New Zealand hagfish, *Eptatretus cirrhatus*, has the lowest reported RMR of 12 mg O$_2$ kg$^{-1}$ h$^{-1}$ at 11°C (Forster, 1990;
Steffensen et al., 1984). Munz and Morris (1965) calculated RMR for *E. stoutii* to be 11 to 14 mg O$_2$ kg$^{-1}$ h$^{-1}$ at 10°C. Thus, the present RMR measurement of 21 mg O$_2$ kg$^{-1}$ h$^{-1}$ at 10°C for *E. stoutii* falls in the middle of the range of previously reported RMR values for hagfishes. Since the RMR’s measured in this study were taken during periods when spontaneous activity was minimal, and the lowest O$_2$ was reported (fig. 2.2), we are confident that our estimates of RMR are accurate and therefore reliable for estimating EPAOC.

Consistent with our finding that spontaneous activity increased r O$_2$ by 3-fold in *E. stoutii*, similar increases in h O$_2$ (2 to 3-fold) have been observed in *E. cirrhatus* during periods of activity (Forster, 1990). Regardless of the duration of the preceding anoxia, the peak post-anoxic o O$_2$ was 4-fold higher than RMR, suggesting that this may represent the maximum possible t O$_2$ for these fish under these conditions. The maximum e O$_2$ values for hagfish reported here are comparable to the RMR of relatively sedentary fish species like 64 mg O$_2$ kg$^{-1}$ h$^{-1}$ in the lamprey *Geotria australis* (Macey et al., 1991) and 76 mg O$_2$ kg$^{-1}$ h$^{-1}$ in the Atlantic cod (*Gadus morhua*) (Bushnell et al., 1994).

Several lines of evidence from the present study indicate that hagfish depress RMR during prolonged anoxia. Tissue glycogen stores did not steadily decrease and lactate did not steadily increase. Furthermore, EPAOC was unchanged when the anoxic exposure was extended from 24 h to 36 h. This means that metabolic rate suppression occurred at least after 24 h of anoxia, while the glycolytic metabolic rate was very close to being equivalent to that of normoxic RMR for at least the first 6 h of anoxia and possibly for almost 24 h.
However, using EPAOC to evaluate glycolytic rate requires several assumptions, the most important being that there is no net cost to replenishing energy and oxygen stores beyond that equivalent to RMR. This is unlikely to be the case, and so the estimated EPAOC should have had an associated “replenishment cost”, something which is difficult to estimate without additional studies using whole body calorimetry. Indeed, various studies on oxygen consumption following intense exercise indicate that EPOC must exceed estimates using just RMR and time (Borsheim et al., 1998; Gaesser and Brooks, 1984; Hancock and Gleeson, 2008). Thus, a lack of difference between measured and estimated EPAOC values does not rule out metabolic suppression prior to 24 h of anoxia, but suggests that any suppression was small relative to the replenishment cost itself. On the other hand, hagfish did carry oxygen stores into the anoxic period, and these need to be quantified and discounted from the 6 h measurement of EPAOC. If we assume that blood oxygen carrying capacity of *E. stoutii* is equal to that of *E. cirrhatus* (2.22 ml/dL; Wells et al., 1986), then the oxygen stored in the haemoglobin of a 150 g hagfish (assuming a 15% blood volume; Forster et al., 2001) would support the RMR measured here for ~15 minutes. Furthermore, if we assume that myoglobin binds 1.34 ml of oxygen per gram of myoglobin (as is the case for haemoglobin) and that *E. stoutii* have a myoglobin concentration equal to that of *E. cirrhatus* (0.78 mg of myoglobin per g of muscle tissue; Davison et al. 1990; Hedrick et al., 1986; Wells and Forster, 1989), the hagfish oxygen stores can maintain RMR for an additional 5 minutes of anoxia. Thus, the hagfish were most likely relying entirely on anaerobic glycolysis to supply ATP well within the first hour of anoxia and these
oxygen stores would have had a small effect on the measured EPAOC after 6 h, and a negligible effect after 36 h.

Other potential energy stores that could be used in anoxia include CrP and ATP. CrP did not decrease significantly during chronic anoxia, which could be an indication that CrP may play a larger role in cellular phosphate transport as apposed to a phosphorylation buffer. Similarly, ATP levels were also defended and did not decrease. Constant ATP levels paired with the stabilization of glycogen suggest a decrease in ATP consumption and metabolic rate (Bickler and Buck, 2007; Hochachka et al., 1996; Richards et al., 2008).

Measurements of glycogen depletion provided evidence for metabolic rate suppression in major tissues (50% percent of the body mass) between 6 and 24 h of anoxia. During this period, and perhaps even earlier, tissue glycogen stores stopped declining significantly. Consistent with metabolic rate suppression, hagfish rarely moved during any phase of anoxia and even uncoiled their body. This means that our estimate of glycogen depletion during anoxia was not confounded by activity. Two distinct patterns of glycogen usage emerged among the four tissues sampled. While the tongue and parietal (skeletal) muscle conserved glycogen stores at a high level for the first 24 h of anoxia, the heart and liver significantly depleted glycogen stores within 6 h (fig. 2.5).

The skeletal muscle of hagfish has been shown to produce considerable amounts of lactate when exhaustively exercised (Davison et al. 1990; Ruben and Bennett, 1980). As skeletal muscle composes ~ 35% of total hagfish body mass, it is likely that the metabolic rate in this tissue is similar to whole animal metabolic rate, which translates
to an ATP turnover of 2.9 µmol g\(^{-1}\) h\(^{-1}\) (fig. 2.4). Furthermore, if one glycogen molecule produced 3 ATP, and there was no metabolic suppression during 36 h of anoxia, glycogen would drop from 10 µmol g\(^{-1}\) wet tissue to zero within ten hours (10 µmol g\(^{-1}\) wet tissue / (2.9 µmol g\(^{-1}\) h\(^{-1}\) x 0.35)). Glycogen levels did not drop to zero but were maintained at 40% of the initial levels, ie. 4 µmol g\(^{-1}\) wet tissue (fig. 2.5A). As glycogen levels were found to be higher than expected in the skeletal muscle, it is concluded that metabolic suppression occurred.

Had the tongue maintained whole body RMR, glycogen would not have been depleted for approximately 177 h and would have dropped from 36 µmol g\(^{-1}\) wet tissue to 29 µmol g\(^{-1}\) in 36 h. Following 36 h of anoxia glycogen levels were 27 µmol g\(^{-1}\) wet tissue. Based on glycogen depletion, this suggests that there was no metabolic suppression in the tongue if RMR of the tongue is equal to that of the whole body.

When actively feeding, the tongue, which is ~7% of total body mass, has been shown to have high glycolytic potential, even when compared to the white muscle of burst swimming tuna (Table 2.4) (Baldwin et al. 1991; Guppy et al. 1979). Baldwin et al. (1991) measured lactate concentrations produced in hagfish retractor muscles of *E. cirratus* following 1.5- to 6.5-min bursts of continuous feeding to be among the highest ever reported for muscle work by unrestrained animals. This high active metabolic rate suggests that the tongue may also have a higher RMR in comparison to the whole body. This would also be consistent with the above measurement for tongue glycogen levels. If this is the case then metabolic suppression may have occurred.

In contrast to tongue and muscle, the depleted state of glycogen stores in the heart and liver prior to 6 h suggests that metabolic suppression in these tissues is slower.
to take effect during prolonged anoxia, but ultimately, may occur before 6 h (fig. 2.5B). Normoxic control values for cardiac glycogen, glucose and lactate measured here are comparable to those measured by Hansen and Sidell (1983) in the ventricle of *M. glutinosa*. The cardiac glycolytic capacity of hagfish has been demonstrated by Hansen and Sidell (1983) and Forster (1991). Furthermore, 20 h of severe hypoxia resulted in comparable levels of cardiac glycogen and lactate to those measured here following 24 h of anoxia (Hansen and Sidell, 1983). As the liver in fish is the major source of glucose (Nilsson and Östlund-Nilsson, 2008), the significant decrease in glycogen stores in the liver during the initial 6 h of anoxia may be linked to supporting other tissues.

Although no previous study has measured liver glycogen following anaerobic work, the normoxic liver glycogen levels measured here are comparable to those measured in *M. glutinosa* (Foster et al. 1993). Of the hagfish species studied to date, *E. cirrhatus* appears to have substantially more glycogen stores when compared to other hagfish species. For example, glycogen in the heart of *E. cirrhatus*, measured to be ~60 µmol g⁻¹ wet tissue, is nearly triple that previously measured in the *M. glutinosa* ventricle (~22 µmol g⁻¹ wet tissue) (Forster, 1991; Hansen and Sidell, 1983). Furthermore, glycogen in the tongue of *E. cirrhatus* (~85 µmol g⁻¹ wet tissue) is over double values we measured for *E. stoutii* (Baldwin et al. 1991). In comparison with other hypoxia- and anoxia-tolerant vertebrates, glycogen stores in hagfish are extremely low. Liver glycogen stores during the winter in the crucian carp are over 170 times greater than reported hagfish values (Hyvärinen et al, 1985). Anoxia-tolerant freshwater turtles that substantially suppress metabolic rate still have between 9 and 47-times more liver glycogen than hagfish (Daw et al, 1967; Packard and Packard, 2005, Warren and
Jackson, 2008). Glycogen stores in the heart are comparable to those found in rainbow trout, but again are substantially less than the stores of the anoxia-tolerant crucian carp and freshwater turtles (Beall and Privitera, 1973; Gesser, 2002; Vornanen, 1984; Warren and Jackson, 2008). Regardless of the amount of glycogen stored, *E. stoutii* have a metabolic profile consistent with a glycolytic potential capable of suppling ATP demand during 36 h of anoxia. Also, blood glucose remained constant in all tissues except the tongue (Table 2.3). The significant increase in lactate production and decrease in glycogen in the tongue following anaerobic metabolism in both *E. cirrhatus* and *E. stoutii* corresponds with a significant increase in glucose content in the tongue (Baldwin et al. 1991).

Theoretically, glycogen depletion should quantitatively match lactate appearance. This was not the case for all tissues (Table 2.5). This apparent discrepancy can be easily explained by the translocation of cellular lactate (and H⁺) into extracellular spaces, the blood particularly. This was indicated by a progressive appearance of blood lactate and a decrease in blood pH (fig. 2.5C; fig. 2.6C). These results are consistent with the findings of Hansen and Sidell (1983) who measured the increase in lactate in the heart to be roughly half of what is expected from the decreases in glycogen. Also, Forster (1991) observed high concentrations of lactate in the perfusate of hypoxic hearts. This suggests that hagfish use their uniquely large blood volume to dilute metabolic tissue wastes during anoxia. Davison et al. (1990) showed that there was no significant difference in the lactate levels or pH between subcutaneous sinus blood and blood from the ventral aorta, indicating that our blood samples, taken from the caudal sinus, are likely reliable indicators of total blood lactate and pH.
Our calculations from glycogen depletion suggest that the tongue and muscle retained 80% and 70% of the lactate produced whereas the liver and heart only retained 37% and 32% of the lactate produced (Table 2.5). The possibility that hagfish excrete lactate seems unlikely as lactate was undetectable in water samples taken at the end of the 36-h anoxic period (data not shown). Davidson et al. (1990) also observed that while increased blood lactate and decreased blood pH corresponded with increase myotomal lactate levels following exhaustive exercise, pH recovered faster than lactate. This is consistent with the findings that lactate removal is not tightly coupled with EPOC in lower vertebrates (Gesser and Brooks, 1984). Additionally, it is likely that during anoxia the heart and liver were more metabolically active than muscle (tongue), had a greater blood flow and thus released more lactate.

Although tissue specific differences were observed, all tissues showed a stabilization of glycogen stores following 6 h of anoxia. As glycogen content in tissues did not differ significantly between 6 h and 36 h in approximately 50% of the hagfish’s tissues, it is unlikely that the significant difference in EPAOC’s in this study was due to a metabolic cost of restoring glycogen stores. Thus, re-oxidation of haemoglobin and myoglobin, the restoration of phosphate stores, glycogen stores, glucose levels and the removal of lactate are unlikely to significantly affect EPAOC between anoxic exposures.

In summary, the present study provided evidence for metabolic rate suppression during prolonged anoxia in hagfish and a uniquely large blood volume of 15% for buffering metabolic wastes may be the key physiological adaptations allowing hagfish
to withstand prolonged periods of complete anoxia (Baldwin et al., 1991; Fänge, 1985; Forster et al., 2001; Hansen and Sidell, 1983).
TABLES

Table 2.1: Mean pre-anoxic routine metabolic rate and post-anoxic recovery variables.

<table>
<thead>
<tr>
<th>Anoxic Period (h)</th>
<th>Pre-anoxic RMR (mg O_2 kg(^{-1}) h(^{-1}))</th>
<th>Peak Post-anoxic s O2 (mg O_2 kg(^{-1}) h(^{-1}))</th>
<th>Duration of EPAOC (h)</th>
<th>EPAOC (mg O_2 kg(^{-1}))</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>20.8 ± 1.8</td>
<td>82.7 ± 8.2(^a)</td>
<td>8.6 ± 1.7(^a)</td>
<td>133.2 ± 31.1(^a)</td>
<td>8</td>
</tr>
<tr>
<td>24</td>
<td>21.3 ± 2.5</td>
<td>83.1 ± 6.9(^a)</td>
<td>22.0 ± 3.5(^b)</td>
<td>418.5 ± 97.0(^b)</td>
<td>7</td>
</tr>
<tr>
<td>36</td>
<td>19.7 ± 2.3</td>
<td>73.3 ± 4.0(^a)</td>
<td>36.6 ± 5.7(^c)</td>
<td>371.7 ± 41.4(^b)</td>
<td>7</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences within columns (p≤0.05).

Table 2.2: Mean ATP equivalents calculated from measured and estimated EPAOC’s.

<table>
<thead>
<tr>
<th>Time in Anoxia (h)</th>
<th>Estimated EPAOC: ATP equivalents (mmol kg(^{-1}))</th>
<th>Measured EPAOC: ATP equivalents (mmol kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>17.5 ± 1.5</td>
<td>18.7 ± 4.4(^a)</td>
</tr>
<tr>
<td>24</td>
<td>71.7 ± 8.6</td>
<td>58.9 ± 9.4(^b)</td>
</tr>
<tr>
<td>36</td>
<td>99.9 ± 11.6</td>
<td>52.3 ± 5.8(^b)</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences within columns (p≤0.05).
Table 2.3: Tissue glucose, CrP, ATP and blood glucose following exposure to normoxia (control) and for varying durations of anoxia.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Metabolite (µmol g⁻¹ wet tissue)</th>
<th>Control</th>
<th>Time in Anoxia (h)</th>
<th>6</th>
<th>24</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>Glucose</td>
<td>0.31 ± 0.12 (8)</td>
<td>0.56 ± 0.22 (9)</td>
<td>0.54 ± 0.07 (9)</td>
<td>0.29 ± 0.15 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CrP</td>
<td>1.14 ± 0.05 (6)</td>
<td>0.81 ± 0.36 (3)</td>
<td>0.66 ± 0.31 (3)</td>
<td>0.71 ± 0.07 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>6.93 ± 0.43a (6)</td>
<td>3.45 ± 1.51b (3)</td>
<td>2.47 ± 1.13b (3)</td>
<td>4.04 ± 0.47ab (4)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Glucose</td>
<td>0.95 ± 0.32 (10)</td>
<td>1.43 ± 0.38 (9)</td>
<td>1.33 ± 0.11 (9)</td>
<td>1.33 ± 0.32 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CrP</td>
<td>0.74 ± 0.09 (6)</td>
<td>5.33 ± 3.28 (6)</td>
<td>7.26 ± 3.88 (6)</td>
<td>2.97 ± 1.89 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>2.25 ± 0.58 (6)</td>
<td>2.71 ± 0.31 (6)</td>
<td>2.79 ± 0.11 (6)</td>
<td>2.40 ± 1.08 (7)</td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>Glucose</td>
<td>0.003 ± 0.03a (10)</td>
<td>0.15 ± 0.06a (9)</td>
<td>0.75 ± 0.12a (9)</td>
<td>1.73 ± 0.20a (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CrP</td>
<td>5.07 ± 0.79 (6)</td>
<td>8.59 ± 1.68 (6)</td>
<td>7.01 ± 1.08 (6)</td>
<td>5.27 ± 1.23 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>10.10 ± 1.35 (5)</td>
<td>11.95 ± 3.33 (6)</td>
<td>11.00 ± 1.71 (7)</td>
<td>14.82 ± 1.56 (7)</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>Glucose</td>
<td>0.21 ± 0.04 (9)</td>
<td>0.42 ± 0.04 (10)</td>
<td>0.51 ± 0.06 (10)</td>
<td>0.58 ± 0.17 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CrP</td>
<td>5.58 ± 0.52 (6)</td>
<td>7.88 ± 1.17 (5)</td>
<td>7.22 ± 0.56 (7)</td>
<td>5.63 ± 0.86 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>9.37 ± 0.96 (6)</td>
<td>10.87 ± 1.63 (6)</td>
<td>14.08 ± 1.48 (7)</td>
<td>13.95 ± 2.82 (7)</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>Glucose (µmol ml⁻¹)</td>
<td>0.55 ± 0.05a (10)</td>
<td>0.61 ± 0.06a (10)</td>
<td>1.36 ± 0.07b (10)</td>
<td>1.16 ± 0.22b (10)</td>
<td></td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (p≤0.05) between time points within a specific tissue.

Table 2.4: Percent body mass of tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Body mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>3.0</td>
</tr>
<tr>
<td>Tongue</td>
<td>6.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>35.5</td>
</tr>
</tbody>
</table>

Table 2.5: Total depletion of glycogen and accumulation of lactate between control values and following 36 h of anoxia.

<table>
<thead>
<tr>
<th>Delta</th>
<th>Liver</th>
<th>Heart</th>
<th>Muscle</th>
<th>Tongue</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen (µmol g⁻¹ glycosyl units g⁻¹ wet tissue)</td>
<td>11.1</td>
<td>26.9</td>
<td>6.59</td>
<td>11.8</td>
<td>N/A</td>
</tr>
<tr>
<td>Lactate (µmol g⁻¹ wet tissue or µmol ml⁻¹ blood)</td>
<td>8.13</td>
<td>17.1</td>
<td>11.0</td>
<td>16.3</td>
<td>24.7</td>
</tr>
</tbody>
</table>
Figure 2.1: Representative oxygen consumption trace of a hagfish taken from normoxia, exposed to 6 h of anoxia and then allowed to recover in normoxia. * indicates periods of spontaneous activity.
Figure 2.2: Average \( \text{O}_2 \) during the 24-h pre-anoxic acclimation period (\( N = 22 \)).
Shading indicates dark, night time period.
Figure 2.3: Average measured EPAOC for the 3 anoxic treatment groups. Individual fish underwent one of the three anoxic treatments. Time 0 represents transfer of fish from normoxia into anoxia. Normoxia was restored at 6 h (N=8), 24 h (N=7), and 36 h (N=7), and EPAOC was measured. Average routine metabolic rate (RMR) was calculated from the preceding normoxic period (see Materials and Methods).
Figure 2.4: Mean anoxic ATP turnover rates during 6 h (N = 8), 24 h (N = 7) and 36 h (N = 7) of anoxia as calculated from measured EPAOC. The dashed line represents normoxic ATP turnover calculated from RMR with s.e.m. indicated by the dotted lines (N = 22). * indicates significant difference between exposure times (p ≤ 0.05).
Figure 2.5: Glycogen concentrations in (A) tongue (F) and skeletal muscle (O), (B) heart (▼) and liver (●), and (C) blood pH (●). Dissimilar letters denote statistical differences (p ≤ 0.05) between time points within a specific tissue. N values are 6 to 10 and s.e.m. is indicated by vertical bars.
Figure 2.6: Lactate concentrations in (A) skeletal muscle (O) and tongue (F), (B) heart (▽) and liver (●), and (C) blood (●). Dissimilar letters denote statistical differences (p ≤ 0.05) between time points within a specific tissue. N values are 6 to 10 and s.e.m. is indicated by vertical bars.
References


CHAPTER 3: CARDIAC RESPONSES TO ANOXIA IN THE PACIFIC
HAGFISH, EPTATRETUS STOUTII

Introduction

Hagfishes routinely inhabit oxygen-depleted environments. The ability of the branchial heart of hagfish to maintain or slightly increase cardiac function under severe hypoxia and chemical anoxia has been repeatedly demonstrated (Axelsson et al., 1990; Christensen et al., 1994; Forster, 1991; Forster et al., 1992; Hansen and Sidell, 1983; Perry et al., 1993; Perry et al., 2009). However, the longest duration for these studies is for only 3-h (Hansen and Sidell, 1983). Chapter 2 shows that hagfish can survive 36 h at 10°C and some up to 48 h. Most hypoxia-tolerant vertebrates respond to long periods (weeks) of severe hypoxia by decreasing cardiac power output (Farrell and Stecyk, 2007), the only exception being crucian carp (Carassius carassius), which maintained cardiac power output for 5 days (Stecyk et al., 2004b). This feat is likely related to the fact that crucian carp have a low routine cardiac ATP demand that can be met by its high glycolytic capacity, large glycogen stores and an ability to deal with glycolytic wastes by generating ethanol (Hyvärinen et al., 1985; Nilsson, 1990; Nilsson, 2001; Shoubridge and Hochachka, 1980; Stecyk et al., 2004b). Routine cardiac power output in hagfish is even lower than that of the crucian carp because of a remarkably low pressure generating ability rather than a low routine cardiac output (Axelsson et al., 1990; Farrell, 2007a; Forster et al., 1991; Forster et al., 1992). As power output is an indirect measure of cardiac ATP demand, it has been hypothesised that the hagfish’s

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2 A version of this chapter will be submitted for publication. Cox, G.C., Sandblom, E. and Farrell, A.P. (2010) Cardiac Responses to Chronic Anoxia in the Pacific Hagfish (Eptatretus stoutii).
cardiac ATP demand could potentially be met by the glycolytic capacity of the heart (Axelsson et al., 1990; Farrell, 1991a; Forster, 1991). Thus, the ability of the hagfish to maintain cardiac performance during short-term hypoxia has long been attributed to the low routine ATP demand of the heart, a high cardiac glycolytic potential and a high blood volume in which to dilute anaerobic wastes (Farrell, 1991b; Farrell, 2007a; Farrell and Stecyk, 2007; Forster, 1991; Hansen and Sidell, 1983). These conditions lead to the suggestion that hagfish can maintain cardiac performance during prolonged anoxia. By comparison, the hypometabolic state of cold-acclimated anoxic freshwater turtles reduce metabolic rate and cardiac power output to about 5% of routine, which greatly slows the build up of harmful anaerobic wastes (Arthur et al., 1997; Farrell and Stecyk, 2007; Herbert and Jackson, 1985; Hicks and Farrell, 2000a; Hicks and Farrell, 2000b; Jackson and Ultsch, 1982).

By using measurements of excess post-anoxic oxygen consumption (EPAOC) and glycolytic metabolites, Chapter 2 showed that, like freshwater turtles, the Pacific hagfish’s, *Eptatretus stoutii*, survival of a 36-h anoxic period was aided by metabolic rate suppression starting as early as 6 h and with a >50% suppression by 36 h. Furthermore, during anoxia the circulatory system is no longer needed for O$_2$ transport, which is regarded as its major function in normoxia. Thus, it seems unlikely that cardiac power output is maintained in hagfish during prolonged anoxia, as previously suspected based on studies of a much shorter duration. Therefore, the purpose of this study was to test the hypothesis that the Pacific hagfish, like all other hypoxia- or anoxia-tolerant vertebrates studied to date, with the exception of *Carassius carassius*, profoundly depress cardiac activity during prolonged severe hypoxia.
Materials and Methods

Animals

Hagfish (*Eptatretus stoutii*) were captured at ~100 m depth in Barkley Sound, British Columbia, Canada (48° 50’N, 125° 08’W) and transported to the DFO-UBC Centre for Aquaculture and Environmental Research, West Vancouver, British Columbia, Canada. They were housed year round in 1,100 L tanks with aerated, flow-through seawater (~30 ppt at 10°C ± 1°C). Fish were fed squid once every 3 weeks. Ten fish (0.16 ± 0.04 kg) were used in this study and food was withheld for a minimum of two weeks prior to experimentation. All of the following procedures were approved by the University of British Columbia Animal Care Committee (A08-0312; Appendix A) and conducted in accordance with their guidelines.

Surgical Procedure

Fish were anaesthetized in 10°C sea water and tricaine methanesulfonate (MS-222; 0.4 g L⁻¹; Sigma, St. Louis, MO) for approximately 45 min. Fish were weighed, transferred to a surgery table and placed ventral side up on water-soaked foam. In order to measure ventral aortic pressure (Pva), cardiac output (Vb) and heart rate (fH), a ventral midline incision was made to gain access to the ventral aorta. A cannula (PE 50, with a PE 10 tip) was occlusively implanted in the second or third afferent gill artery to measure Pva, as described in Axelsson et al. (1990). The cannula, filled with heparinized saline (200 IU ml⁻¹), was advanced into the ventral aorta. Following this a Transonic transit-time
blood flow probe (2.5 mm SB, Transonic systems, Ithaca, NY, USA) was positioned around the ventral aorta immediately anterior to the heart to measure $V_b$. The cannula and lead of the flow probe were secured to the body wall with silk sutures.

Following surgery fish were revived in a 2.5 L plexi-glass Loligo respirometer chamber (Loligo Systems, Tjele, Denmark). The chamber was flushed continuously with fresh aerated sea water at a flow rate of 1 L min$^{-1}$. The relatively large volume of the respirometer allowed to fish to adopt their relaxed curled position during normoxia and their uncurled position during anoxia.

Data Acquisition

The following day, the cannula was connected to a pressure transducer (model DPT-6100, pvbMedizintechnik, Kirchseeon, Germany) calibrated against a static column of seawater multiple times during the anoxic exposures. A 4chAmp amplifier (Somedic, Hörby, Sweden) was used to amplify signals from the transducer. The flow probe was connected to a T206 Transonic flow meter (Transonic Systems Inc., Ithaca, NY, USA). Data were recorded for subsequent analysis using Power Lab unit (ADInstruments Pty Ltd, Castle Hill, Australia) connected to a laptop computer running LabChart Pro software (v.6.0; ADInstruments Pty Ltd, Castle Hill, Australia). Routine $V_b$, $f_{hr}$ and $P_{va}$ were recorded for several hours prior to and during the anoxic exposure.

Anoxic Exposure and Recovery

The chamber was made progressively hypoxic by flushing (1 L min$^{-1}$) nitrogen-saturated seawater directly from a gas exchange column into the chamber until anoxia
was achieved (0.0 kPa, typically after ~1 h). \( V_b, f_H \) and Pva were recorded continuously. Water PO\(_2\) in the chamber was measured every second using a MINI-DO probe (Loligo Systems, Tjele, Denmark). The oxygen probes were calibrated using fully aerated seawater and sodium sulphite derived oxygen-free distilled water prior to each trial. The chamber remained anoxic for 36 h (see Chapter 2). Following the anoxic period, normoxia was restored within 5 min by introducing air-saturated seawater at a flow rate of 1 ml min\(^{-1}\). Fish were monitored for a further 36 h under normoxic conditions.

**Calculations and Statistics**

Cardiac output through the ventral aorta was measured by the Transonic flow probe and \( f_H \) was determined by counting the number of systolic peaks over a 4 minute period. Cardiac stroke volume \((V_s)\) was calculated by dividing \( V_b \) by \( f_H \). Cardiac power output \((PO)\) was calculated as the product of \( V_b \) and Pva. Reported values are mean values (± s.e.m) typically for 10 fish. Many hagfish managed to tie a knot in the cannula reducing the number of fish with viable Pva measurements. Consequently, PO was measured in 3 fish for the 36-h anoxic exposure. Only one cannula had remained functional during recovery from anoxia. In order to test for statistical differences in cardiovascular variables, comparisons among control (routine), anoxic and recovery values were tested using a one-way, repeated measures ANOVA followed by a Holm-Sidak post-hoc test. Statistical significance was set at \( P \leq 0.05 \).
Results

Examples of the raw cardiovascular trace from a single hagfish at selected time periods throughout an experiment are presented in figure 3.1. As can be seen from these traces, the cardiovascular status was extremely stable over time and the changes that took place did so gradually. In both normoxia and anoxia, there was a period of zero ventral aortic flow during diastole. Cardiovascular responses to prolonged anoxia and recovery are summarized in figures 3.2, 3.3 and 3.4.

Anoxia

Hagfish often became active when water PO\textsubscript{2} fell below 3 kPa during the first 30 min of the anoxic period. There was minor tachycardia during the first 30 min of anoxia, likely related to this activity. Heart rate significantly decreased from normoxic routine values (10.4 ± 1.3 beats per min) to 8.1 ± 0.8 beats per min by 1 h into anoxia. A new steady state for f\textsubscript{H} was reached after 2 h of anoxia and f\textsubscript{H} remained stable at about 50% of the normoxic rate for the remainder of the 36-h anoxic exposure (fig. 2).

Routine V\textsubscript{b} was 12.3 ± 0.9 ml min\textsuperscript{-1} kg\textsuperscript{-1} (fig. 2). V\textsubscript{b} was unchanged for the first 3 h of anoxia despite the significant bradycardia that occurred after 1 h of anoxia. However, after 6 h of anoxia, V\textsubscript{b} was significantly reduced by ~30% (fig. 3.2) and remained unchanged for the remaining 30 h of the anoxic period.

Mean normoxic V\textsubscript{s} was 1.3 ± 0.1 ml kg\textsuperscript{-1}. V\textsubscript{s} increased to 2.7 ml kg\textsuperscript{-1} during the first 3 h of anoxia to offset the bradycardia and maintain V\textsubscript{b}. By 36 h of anoxia, V\textsubscript{s} was double routine V\textsubscript{s} (fig 3.2, 3.3).
Mean normoxic Pva was 0.89 ± 0.04 kPa (N= 3). The initial period of anoxia triggered a 60% increase in pressure but routine Pva was restored by 3 h and remained stable for the remaining 33 h of anoxia. In fish where Pva was measured along with $V_b$ (N=3) changes in $f_{ht}$, $V_b$ and $V_s$ showed similar patterns as seen in figures 1 and 2 (fig. 4). Although $V_b$ declined during anoxia in these three fish, the only significant decrease was at 24 h, perhaps due to a low statistical power.

Routine cardiac PO was 0.26 ± 0.02 mW g$^{-1}$ Mv$^{-1}$ (N=3). Cardiac PO mirrored changes in Pva and became stable at ~ 0.2 mW g$^{-1}$ Mv$^{-1}$ for the duration of the 36-h anoxic exposure. There was no significant decrease in cardiac PO.

**Normoxic Recovery**

Following 10 min of normoxic recovery, $f_{ht}$ had increased significantly when compared with 36-h anoxic value. Also within 1 h, $f_{ht}$ had increased significantly by 50% compared with the routine $f_{ht}$ (fig. 3.4). $f_{ht}$ reached its maximum value after 1.5 h into recovery before decreasing back to the routine normoxic value after 6 h of recovery (fig 4).

Similar to $f_{ht}$, $V_b$ increased significantly by 10 min into normoxic exposure compared with the 36-h anoxic value, and was almost twice routine $V_b$ (P<0.05) after 1 h of recovery (24.6 ± 3.6 ml min$^{-1}$ kg$^{-1}$). By 3 h into recovery, routine $V_b$ was restored. $V_s$ briefly remained significantly higher (P<0.05) than routine $V_s$ during recovery but by 1 h into recovery, $V_s$ was significantly lower than the 36-h anoxic value and was similar to routine $V_s$ (fig. 3.4). There was only one fish that had a functioning cannula during recovery and in this fish Pva increased to 1.2 kPa following 10 min of normoxia,
decreasing back to 1.0 kPa by 20 min and remaining constant until 6 h of recovery. By 24 h, Pva had returned to a routine value of 0.9 kPa.

**Discussion**

These are the first in vivo measurements of cardiovascular variables in a hagfish exposed to prolonged anoxia. Table 3.1 compares in vivo routine and maximum cardiovascular variables for *E. stoutii* data obtained during this study with cardiovascular responses among other hagfish species to varying degrees of short-term severe hypoxia.

**Routine and Maximum Cardiovascular Variables**

Routine $V_b$ for *E. stoutii*, like its routine oxygen consumption (chap. 1), was found to lie between routine values for *M. glutinosa* and *E. cirratus* (Table 1; Axelsson et al., 1990; Forster et al., 1992). Similar to other hagfish species, a period of zero flow was observed during diastole (Axelsson et al., 1990; Davie et al., 1987; Satchell, 1986). Zero flow in the ventral aorta results from a relatively inelastic outflow tract from the ventricle and contrasts with the continuous diastolic flow seen in both elasmobranchs and teleosts (Farrell and Jones, 1992). During the second hour of recovery from anoxia, *E. stoutii* achieved a maximum $V_b$ of 26 ml min$^{-1}$ kg$^{-1}$. As this value compares well with the observed maxima for both *M. glutinosa* and *E. cirratus*, this value may approach the maximum $V_b$ for this species at this temperature.
As is the case for many fish species, hagfish can increase \( V_s \) by increasing \( V_s \), up to a 3-fold (Axelsson et al., 1990; Farrell, 1991b; Forster et al., 1992). A 2-fold increase was observed here (fig. 3.2; table 3.1). While routine \( V_s \) in \textit{E. stoutii} was double that found for other hagfish species, \( f_{hi} \) was correspondingly 50% lower than routine \( f_{hi} \) for \textit{M. glutinosa} and \textit{E. cirrhatus} (Axelsson et al., 1990; Forster et al., 1992) (Table 3.1).

Anesthetised \textit{E. stoutii} increased \( f_{hi} \) 3-fold (15-42 bpm) at 8 to 10°C (Chapman et al., 1963; Johnsson and Axelsson, 1996). A similar 3-fold increase in routine \( f_{hi} \) occurred during recovery from anoxia. Again this may be the maximum \( f_{hi} \) for this species, but if so, it is lower than maximal \( f_{hi} \) observed in other hagfish species (Table 3.1).

Blood pressure measurements are difficult to maintain over prolonged periods because of the ease with which hagfish can tie themselves, and any attached catheters, in knots. Regardless, the hagfish heart is well-known for generating the lowest ventral aortic blood pressure among fishes based on \textit{in vivo} measurements and with perfused heart preparations (Table 3.1) (Farrell, 1991b; Forster et al., 1988; Johnsson and Axelsson, 1996). One previous study reported Pva for \textit{E. stoutii} to be 0.5 kPa higher than the values attained here (Reite, 1969). The measurements of routine Pva during normoxia in this study were found to be the lowest among hagfish values (Table 3.1).

As cardiac PO is estimated from \( V_b \) and Pva, the comparatively low Pva of hagfishes results in a cardiac PO that is lower than any vertebrate (Farrell, 1991b). Routine cardiac PO for \textit{E. stoutii} was found to be intermediate between \textit{M. glutinosa} and \textit{E. cirrhatus} values (Table 3.1). Maximal cardiac PO (0.4 mW g\(^{-1}\)) attained during this
study was upon entry into anoxia (fig. 2; table 1). No other study has reported a maximum cardiac PO greater than 0.8 mW g⁻¹ for any hagfish species to date. This is an important finding because it has been estimated that the maximum glycolytic potential of the hagfish heart could support an anoxic cardiac PO of 0.8 mW g⁻¹, provided sufficient glucose was available and glycolytic waste products were not damaging (Farrell, 1991a; Farrell, 2007a; Hansen and Sidell, 1983).

Cardiovascular Responses to Severe Hypoxia and Anoxia

Previous studies report that $V_b$ was slightly increased or maintained during short-term hypoxia in hagfish (Table 3.1). Axelsson et al. (1990) reported that exposure of $M. glutinosa$ to a water $P_O_2$ of 4 kPa had no cardiovascular effect and $V_b$ was maintained during a 15-min exposure to a $P_O_2$ of 1.5-2.2 kPa. Forster et al. (1992) reported a 40% increase in $V_b$ in $E. cirrhatus$ when exposed to a $P_O_2$ of 5.3 kPa, mostly through an increase in $V_s$. Since the hagfish heart has no coronary circulation and its oxygen supply comes from venous blood, the low venous oxygen content reported in this earlier study probably means that the heart had become functionally anoxic (Forster et al., 1992). The observation that hagfish become active in response to hypoxia is common among studies where hagfish have been subjected to water $P_O_2$’s below 3 kPa (Forster, 1990; Forster et al., 1992; Perry et al., 1993; chap. 2). The brief tachycardia was observed within the first 30 min of anoxia, but could not be specifically assigned to either hypoxic exposure or activity.
The ability of hagfish to maintain $V_b$ during functional anoxia for 15 and 35 min does not necessarily speak to the glycolytic capacity of the heart. In fact, the low $P_{50}$ of hagfish blood (≈ 1 kPa; Forster et al., 1992 to 1.64; Wells et al., 1986) and a constant blood pH (Forster et al., 1992) could mean that the length of hypoxic exposure in previous studies may have been insufficient to elicit an anoxic response. Furthermore, calculations made in chapter 2 suggest that RMR of *E. stoutii* could be easily maintained for 20 min using oxygen stores. Hansen and Sidell (1983) observed no change in relative cardiac performance for 3 h of anoxia. However, *E. stoutii* in the current study did not decrease $V_b$ significantly until after 3 h of anoxia. Nevertheless, in the present study, $V_b$ decreased by only one-third after 6 h of anoxia and was maintained unchanged at this level for a further 30 h of anoxia. In fact, neither Pva nor cardiac PO decreased during anoxia (fig. 3.4). This result clearly suggests that anaerobic pathways maintained a near routine cardiac ATP demand during prolonged anoxia. Thus, similar to the crucian carp but unlike other fishes studied to date, hagfish do not decrease cardiac PO during prolonged anoxia. As hagfish presumably utilize their large blood volume to buffer metabolic wastes, perhaps a reduction in their cardiac output would be likely to negatively impact their buffering ability.

The depression of cardiac performance in *E. soutii* during prolonged anoxia is clearly minor compared with other hypoxia- and anoxia-tolerant vertebrate species. Warm-acclimated turtles at 22°C reduce cardiac PO by 5-fold during anoxia, while cold-acclimated turtles (5°C) display a remarkable 22-fold reduction (Hicks and Farrell, 2000a). Even hypoxia tolerant common carp (*Cyprinus carpio*) reduce cardiac PO by 3.2-, 4.1- and 7.7-fold during severe hypoxia at 5, 10 and 15°C, respectively (Stecyk and
Farrell, 2002). In anoxic hagfish, $V_b$ decreased on average by only 33% during final 30 h of anoxia and cardiac PO was unchanged. During anoxia, cold-acclimated turtles decreased $V_b$ 13-fold and common carp decreased $V_b$ by 3.5- to 4.9-fold (Hicks and Farrell, 2000a; Stecyk and Farrell, 2002).

Others have previously suggested that the cardiac ATP requirement of hagfish lies within the glycolytic ATP generating capacity of the heart largely due to its low pressure generating ability (Farrell, 1991a; Farrell, 1991b; Farrell, 2007a; Forster, 1991; Hansen and Sidell, 1983). The present study emphasizes the long-term nature of this glycolytic potential. Cardiac ATP turnover rate during a 20 h exposure to hypoxia had been previously calculated from the depletion of cardiac glycogen stores ($PO_2 < 0.3$ kPa) (Hansen and Sidell, 1983), but this glycogen depletion rate could be sustained only if there was either a 100-fold depression in routine cardiac PO or an increase in the extracellular glucose supply (Farrell and Stecyk, 2007). The present study suggests that the second scenario is the more likely. Indeed, Chapter 2 showed a significant decrease in cardiac glycogen following 6 h and an increase in blood glucose by 24 h into anoxia. The source of the blood glucose in hagfish is unknown, but could be the liver, as in crucian carp (Hyvärinen et al., 1985), especially since live glycogen was shown to decrease early into anoxia (<6 h) and prior to the suggested metabolic depression of major tissues.

Consequently, I reject the hypothesis that hagfish undergo a profound suppression of cardiovascular function to survive prolonged anoxia. Hagfish are more similar to crucian carp, which are the only known vertebrate to maintain cardiac PO during prolonged anoxia (Stecyk et al., 2004b).
Cardiac Control in Hagfish

Although one study has reported nerves and ganglion cells adjacent to and within the epicardium of *E. stoutii* (Hirsch et al., 1964), the hagfish heart is considered functionally aneural (Augustinsson et al., 1956; Greene, 1902; Jensen, 1961; Jensen, 1965; Nilsson, 1983). Thus, changes in cardiac function are necessarily slow. Most fishes respond to environmental hypoxia with reflex bradycardia, mediated mainly by vagal innervation of the heart (Farrell, 2007b). In fact, the anoxia-tolerant crucian carp, the common carp and the warm-acclimated (but not cold-acclimated) freshwater turtle maintain cholinergic and adrenergic control of the heart during anoxia (Farrell and Stecyk, 2007; Stecyk et al., 2004a). Given the hagfish’s apparent lack of autonomic cardiac regulation and the ability to meet routine cardiac ATP demand anaerobically (Farrell, 2007a; Nilsson, 1983), it is surprising that hagfish halved their $f_H$ during anoxia, albeit over 6 h. Equally surprising is that $f_H$ remained unchanged for a further 30 h of anoxia. There remains an unexplained question of how this bradycardia is regulated.

Previous studies with hagfishes have revealed an adrenergic control of $f_H$ and contractility. Indeed the heart has its own intrinsic stores of catecholamines (Augustinsson et al., 1956; Bloom et al., 1961; Johnels and Palmgren, 1960; Ostlund et al., 1960; Perry et al., 1993; von Euler and Fänge, 1961). Furthermore, reserpine injection to deplete cardiac catecholamine stores inhibited cardiac contractility and produced bradycardia in both *E. stoutii* and *M. glutinosa* (Chapman et al., 1963; Bloom...
et al., 1961). In addition, Axelsson et al. (1990) found that the β-adrenoreceptor antagonist sotalol significantly lowered $f_{hi}$ in *M. glutinosa*, while Forster et al. (1992) showed that propranolol injection decreased $f_{hi}$ and that injection of catecholamines increased $f_{hi}$ in *E. cirrhatus*. Furthermore, all hagfish species show an increase in $f_{hi}$ following adrenaline injection (Axelsson et al., 1990; Chapman et al., 1963; Forster et al., 1992). These results have led to the suggestion that cardiovascular control may involve catecholamine release into the circulation (Axelsson et al., 1990; Forster et al., 1992; Perry et al., 1993), or even paracrine actions of cardiac catecholamine stores. Indeed, Perry et al. (1993) observed elevated humoral catecholamine levels in response to acute hypoxia, anoxia and air exposure. Thus, the changes in blood pressure and branchial vascular resistance in response to hypoxia reported in previous studies for hagfish could be humoral given the apparent absence of autonomic innervation of the vasculature.

Even so, the level of circulating catecholamines is relatively low compared with teleosts (Perry et al. 1993). Thus, in order for catecholamines to elicit cardiovascular responses the heart must either have high catecholamine affinity or a paracrine signaling by endogenous catecholamine stores (Farrell, 2007a). Also, constant release of catecholamines in the *in situ* preparation may indicate a tonic adrenergic control of intrinsic $f_{hi}$ (Farrell, 2007a). As catecholamines cannot be synthesized in the absence of oxygen, the steady anoxic $f_{hi}$ may be the intrinsic pacemaker rate in the absence of tonic catecholamine stimulation. Once oxygen becomes available after prolonged anoxia, catecholamine synthesis can resume, restoring and even increasing $f_{hi}$. The rate of change could then be related to the rate of catecholamine re-synthesis.
The findings that increased potassium, hypoxemia and decreased blood pH do not elicit the release of catecholamines in hagfish (Perry et al., 1993), coupled with the observation that hagfish become active during hypoxic exposures, may indicate that initial increases in blood catecholamine levels are in response to confinement stress. Conversely, increased catecholamines in the blood may also be involved in the mobilization of glucose stores within the liver (Reid et al., 1998). Freshwater turtles up-regulate glycolysis during the initial stages of anoxia which leads to a small depletion in liver glycogen stores (Jackson, 2000). If hagfish also employ this strategy, the increased catecholamines may be responsible for the observed drop in liver glycogen prior to 6 h in anoxia (chapter 2). Regardless of the function of this initial catecholamine release, what triggers their release in hagfish during anoxia remains a mystery.

In summary, despite the presence of an anoxic bradycardia within the first hour, hagfish appear to maintain $V_b$ for 3 h of anoxia followed by a 33% reduction of $V_b$ during the final 30 h of a 36-h anoxia exposure when compared with routine $V_b$. This reduction in cardiovascular function is relatively minor in comparison with other hypoxia- and anoxia-tolerant vertebrates. Additionally, cardiac PO and Pva appear to be maintained during the anoxia, suggesting that routine cardiac ATP demand is met by the glycolytic capacity of the heart in combination with circulating glucose during 36 h of anoxia (Hicks and Farrell, 2000a; Stecyk and Farrell, 2002). The ability of the hagfish heart to recover and increase in performance following anoxic exposure further illustrates anoxic tolerance in the hagfish cardiovascular system.
Table 3.1: Comparison of routine and maximal cardiovascular variables in vivo for hagfish and species-specific responses to either hypoxia or anoxia.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cardiac output (ml min⁻¹ kg⁻¹)</th>
<th>Stroke volume (ml kg⁻¹)</th>
<th>Heart rate (min⁻¹)</th>
<th>Ventral aortic pressure (kPa)</th>
<th>Power output (mW g⁻¹ MV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Routine</td>
<td>Max</td>
<td>Hypoxic/Anoxic response</td>
<td>Routine</td>
<td>Max</td>
</tr>
<tr>
<td>M. glutinosa</td>
<td>8</td>
<td>24*</td>
<td>=</td>
<td>0.4</td>
<td>0.7*</td>
</tr>
<tr>
<td>E. stoutii</td>
<td>12</td>
<td>26</td>
<td>↓26%</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>E. cirrhatus</td>
<td>16</td>
<td>25*</td>
<td>↑40%</td>
<td>0.7</td>
<td>1.3^ 1.0*</td>
</tr>
</tbody>
</table>

Values indicated with * are maximal values following injection of adrenaline. Values indicated with ^ are maximal values following injection of propranolol. = means that the hypoxic anoxic response was to maintain routine values. Test condition for M. glutinosa: Temperature of 8-10°C with a hypoxic exposure of 15-35 min at PO₂ of 1.5 to 2.2 kPa. Test conditions for E. cirrhatus: temperature of 17°C with a hypoxic exposure of 15 min at PO₂ 5.3 kPa.

Myxine glutinosa data taken from Axelsson et al. (1990) and Farrell (2007).

Eptatretus stoutii data taken from current study.

Figure 3.1: Representative ventral aortic blood flow recordings taken from one hagfish after 6 h, 24 h and 36 h of anoxia, and during the 1.5 h and 36 h of normoxic recovery from anoxia. Each trace is for 2 min. Note the different y-axis scale for 1.5 h recovery.
Figure 3.2: Simultaneous recorded cardiovascular variables from hagfish during a 36-h anoxic exposure. Time 0 indicates the start of anoxia. N values are 9 to 10 and s.e.m. is indicated by vertical bars. Statistical differences (p ≤ 0.05) are indicated by dissimilar letters.
Figure 3.3: Simultaneously recorded cardiovascular variables for cannulated fish during a 36-h anoxic exposure. Time 0 indicates the start of anoxia. R indicates routine values. N value is 3 and s.e.m. is indicated by vertical bars.
Figure 3.4: Simultaneously recorded cardiovascular variables during a 36-h normoxic recovery from a 36-h anoxic exposure. Time 0 indicates the start of the normoxic flush period. N values are 7 and s.e.m. is indicated by vertical bars. Statistical differences ($p \leq 0.05$) are indicated by dissimilar letters.
References


CHAPTER 4: GENERAL DISCUSSION

Objectives

The purpose of this thesis was to:

1) Determine the maximum duration of anoxic survival in the Pacific hagfish, *Eptatretus stoutii*;
2) Determine if this survival involves metabolic rate suppression; and
3) Characterize the cardiovascular responses of hagfish to chronic anoxia.

**Objective 1**

The limit of anoxic survival was shown to be approximately 36 h with a 50% mortality following a 48 h anoxic exposure at 10°C. Changes in cardiovascular function and glycogen stores may also be used to indicate the end of the anoxic survival period. Common and crucian carp increased cardiac performance towards the end of the anoxic survival period (Farrell and Stecyk, 2007; Stecyk and Farrell, 2002). In addition, mortality in anoxic freshwater turtles is preceded by a mobilization of remaining glycogen stores that increases plasma glucose levels (Ultsch and Jackson, 1982). Similarly, in the present study, hagfish elevated blood glucose at 24 h and $V_b$ tended to increase between 24 h and 36 h.

**Objective 2**

Previous studies have shown hagfish possess morphological, physiological and biochemical traits vital to survival during O$_2$ deprivation (see chapter 1; Hagfish).
But it was unclear whether this survival was aided by metabolic rate suppression or whether they maintained their low metabolic rate with anaerobic glycolysis.

Analysis of anoxic ATP turnover rates calculated from EPAOC suggested a 50% suppression of metabolic rate after 36 h, but possibly not before 24 h of anoxia. Analysis of tissue glycogen depletion in the liver, heart, tongue and skeletal muscle, which together comprise roughly 50% of hagfish body mass, however, revealed that metabolic suppression occurred within 6 h of anoxia. The implication of this difference is likely that there is a net cost above RMR of recovering from anoxia but it is not totally related to the restoration of high energy phosphates, glycogen, and glucose or lactate removal.

**Objective 3**

The potential for metabolic depression was also examined in a working organ – the heart. Despite anoxic bradycardia, previously unreported for any hagfish species, $V_b$ remained initially unchanged for 3 h due to an increase in $V_s$. $V_b$ was maintained at 67% of routine levels for the final 33 h of the 36-h anoxic period, but cardiac PO, and hence its ATP turnover, remained constant. Cardiac recovery from anoxia saw $V_b$ return to a routine value after 3 h and $f_i$ after 6 h.

**Perspectives**

Both hypoxia- and anoxia-tolerant vertebrates employ a suite of adaptations to survive periods of decreased O$_2$ availability (Hochachka et al., 1996). Many of these adaptations are observed in the present study on hagfish and include the most effective
behavioural response of avoidance, indicated by my observation of “escape” attempts in response to decreasing water PO$_2$. Morphological adaptations include a large blood volume in which to dilute anaerobic wastes and to store O$_2$. Physiological and biochemical adaptations to decreasing O$_2$ levels included: metabolic suppression, a cardiovascular system functioning at a low routine PO (and hence ATP demand) so that it can remain close to routine levels despite an anoxic bradycardia; and an anaerobic metabolism capable of supplying ATP demand through the depletion of fermentable stores.

An anoxic survival time of 36 h of complete anoxia at 10°C is impressive compared with most vertebrates that survive only minutes of complete anoxia. However, this survival time pales in comparison to vertebrate anoxia-tolerant champions such as the crucian carp or freshwater turtles. Crucian carp and freshwater turtles have been identified as principal examples of anoxia-tolerant vertebrates and each utilize very different anoxic survival strategies, yet a trait common to both is the presence of large liver glycogen stores to fuel anaerobic glycolysis. Although hagfish possess a number of traits common with these species, hagfish glycogen stores were lower. As a result not only do they straddle the two anoxic survival strategies, they also straddle the classic definitions of hypoxia- vs. anoxia-tolerant species.

Though many of the adaptations observed in the present thesis aid in anoxic survival, the combination of behavioural, morphological, physiological and biochemical traits described here and in hagfish literature (see chapter 1; Hagfish) indicate an extreme hypoxia tolerance as opposed to a true anoxia-tolerance. This is consistent with their life history that subjects them to routine hypoxia but only brief periods of anoxia
during feeding. This conclusion is substantiated by high glycolytic capacity of both the tongue and heart. The tongue was found to have the highest glycogen stores among the tissues and is capable of rapidly depleting them during feeding (Baldwin et al., 1991). The heart has comparatively low glycogen stores and they are almost completely depleted prior to 6 h, thus its ability to function at routine levels during anoxia suggests a high glycolytic capacity aided by circulating blood glucose. The depletion of cardiac glycogen stores prior to 6 h maybe correlated with the initiation of an anoxic bradycardia. Similarly to the bradycardia observed in many hypoxia-tolerant vertebrates, the observed decrease in $f_{11}$ in chapter 3 increases the time blood remains in the lumen of the heart. This allows more time for both glucose extraction by and waste excretion from myocytes. As hagfish are known to inhabit waters that routinely experience hypoxia, the heart’s ability to maintain routine performance may also be a hypoxic adaptation as it is the tissue most prone to becoming functionally anoxic during sever hypoxia. Additionally, while a low RMR and ability to suppress metabolic rate is functionally adaptive during anoxia, it is more likely that, given the low glycogen stores, these are adaptations to surviving prolonged periods of low environmental $O_2$ and thus reduced ATP supply through oxidative phosphorylation. Thus, the ability of the hagfish to survive 36 h of anoxia likely results from a combination of adaptations that serve to drastically increase ATP supply during brief periods of activity in hypoxia/anoxia or augment ATP production when $O_2$ is scarce.
References


APPENDICES

Appendix A

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0680

Investigator or Course Director: Anthony (Tony) P. Farrell

Department: Zoology

Animals:

Hagfish 70
Hagfish

Start Date: November 26, 2007
Approval Date: December 10, 2007

Funding Sources:

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)

Funding Title: Comparative Cardiovascular Physiology

Unfunded Title: Cardiovascular responses to anoxia in Hagfish
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A08-0312

Investigator or Course Director: Anthony (Tony) P. Farrell

Department: Aqua and Enviro Research

Animals:

Hagfish 70

Start Date: May 21, 2008

Approval Date: June 23, 2009

Funding Sources:

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)

Funding Title: Comparative and integrative cardiorespiratory physiology

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.
This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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