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Effects of Acute and Chronic Hypoxia on Respiratory Physiology of Paddlefish (Polyodon Spathula)

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Effects of acute and chronic hypoxia on respiratory physiology of paddlefish

(*Polyodon spathula*)

By

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Among the basal bony fishes, the American paddlefish (*Polyodon spathula*) has a unique respiratory strategy of ram-ventilation. However, despite the increasing problems caused by hypoxia in natural habitats occupied by this species, little information exists about their response to hypoxia. Four studies were conducted to examine the physiological and biochemical responses of juvenile paddlefish (150-181 g) to acute and chronic hypoxia. Acute hypoxia tolerance, aerobic metabolic rates and swimming capabilities of paddlefish in an intermittent respirometer or swim flume were evaluated under normoxic (partial pressures of oxygen [pO_2] =140 mm Hg) and hypoxic (pO_2 =62 mm Hg) conditions at 18 ºC and 26 °C. Additionally, blood oxygen transport, blood acid-base balance and metabolic stress were evaluated in paddlefish independently exposed to 4 different pO_2s: normoxia =148 mm Hg, mild hypoxia =89 mm Hg, moderate hypoxia =59 mm Hg and extreme hypoxia =36 mm Hg, at 21ºC. Blood samples were collected from paddlefish after they had been exposed to treatment pO_2's for 0.25, 2, 6, 24 and 72 hours, and analyzed for hematocrit, pO_2, total oxygen content, pCO_2, pH, hemoglobin, Na^+, K^+, Ca^{2+}, Cl^-, glucose, lactate, etc. A third study used 1-D and 2-D J-resolved ^1H

NMR to analyze metabolite changes in muscle tissue of paddlefish exposed to normoxia (148 mm Hg), or acute (0.25 h) or chronic (72 h) moderate hypoxia (59 mm Hg). The last study examined the effect of moderate hypoxia (pO_2: 59 mm Hg) and subsequent recovery in normoxia (pO_2: 148 mm Hg) on plasma cortisol, blood oxygen transport, blood acid-base balance, metabolic, ion-osmoregulation and enzyme parameters in paddlefish. The results indicate that paddlefish have a critical pO_2 of 74 mm Hg at 18 ^oC and 89 mm Hg at 26 ^oC and a lethal oxygen threshold of ~2 mg/ L. Sensitive to moderate hypoxia, death occured after 3-8 hours of extreme hypoxia. Paddlefish have reduced capacity for metabolic depression and, as a result, survival in hypoxia is limited due to a reduction in both aerobic and anaerobic (glycogen and glucose) energy stores as well as the accumulations of toxic H^+ and lactate. Nonetheless recovery is possible.

DEDICATION

This dissertation is dedicated to my mother (Akua Adubea), twin brother (Johnny), wife (Judith) and kids (Jannis and Jadon). Furthermore, my work in graduate school would not have been fulfilling without the love and encouragement of Paafi, Caro, Emma and their families. I am grateful to you all.

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CHAPTER I

INTRODUCTION

1.1 Study background

Biodiversity is rapidly declining in freshwater ecosystems all over the world (Dudgeon et al. 2006; Jelks et al. 2008). Migratory freshwater fishes, which rely on natural flow regimes to complete their life cycles, are among the species experiencing the greatest population declines. These fishes have been impacted by impoundments and water control structures, resulting in loss of spawning cues, spawning and nursery habitat, and blocking of seasonal migration routes (Poff et al. 1997). Among these fish species are evolutionarily primitive fishes of the order Acipenseriformes (paddlefishes and sturgeons). Acipenseriforms are found in the Northern Hemisphere and reproduce in freshwater. Using different migratory patterns, they migrate principally for reproduction and feeding (Billard and Lecointre 2001). Among the most vulnerable of the acipenseriforms is the North American paddlefish (*Polyodon spathula*), considered primarily potamodromous (migrate only within freshwater systems) although they have the ability to survive in brackish waters (Billard and Lecointre 2001).

1.2 Paddlefish life history

Paddlefish are among the most ancient freshwater fishes, having been in existence for about 150 million years (Romer 1967; Moy-Thomas 1971). They belong to the family

Polyodontidae (Bond 1979), which contains only one other species, the Chinese paddlefish (*Psephurus gladius*) native to the Yangtze-Kiang River (Pflieger et al. 1975). Paddlefish are considered one of the largest of the freshwater fishes, growing to a length of over 2 m and weighing over 90 kg (Allardyce 1992; Epifanio et al. 1996; Mims and Shelton 2005). They can live as long as 50 years (Russell 1986; Scarnecchia et al. 1996). Paddlefish are filter feeders, feeding primarily on zooplankton and other aquatic invertebrates (Rosen and Hales 1981; Kozfkay and Scarnecchia 2002). Paddlefish migrate great distances to spawn, relying on water temperature, photoperiod, and discharge to cue spawning activities (Purkett 1961; Russell 1986). They spawn in areas with gravel or rock substrate and enough current to prevent sedimentation (Purkett 1961). Mature females produce a large number of eggs, and need about 2-3 years between spawns to develop mature ova (Purkett 1961). Paddlefish are characterized by a long paddle-shaped snout, a large mouth, very small eyes, numerous slender gill rakers, and a large, tapering operculum flap that extends to the pelvic fins (Jennings and Zigler 2000). They are dull in color and often mottled; color ranges from blueish-gray to black dorsally, and grades to lighter on the sides and white ventrally. The skin is smooth, except for a small patch of rhomboid scales on the deeply forked heterocercal caudal fin (Lagler et al. 1977). Paddlefish are known predators of zooplankton (Onders et al. 2008) and serve as hosts to the silver lamprey, *Ichthyomyzon unicuspis* (Cochran and Lyons 2004).

1.2.1 Paddlefish conservation status

Paddlefish are found in 22 US states within the Mississippi River drainage basin, and occupy large streams, rivers and impoundments (Mims 2001; Firehammer and

Scarnecchia 2006). There is evidence that some populations did occur in Lake Erie (Trautman 1981) and in other Great Lakes around the turn of the 20th century (Hubbs and Lagler 1964; McAllister et al. 1981); however, all these populations appear to have been extirpated (Gengerke 1986). Characteristic features of these habitats are the existence of natural variation in the seasonal and annual physicochemical regimes which have existed for many years and to which the fish have adapted to complete their life cycle. Physiochemical changes in these riverine conditions have resulted in a loss of spawning and rearing habitats, resulting in the current listing of paddlefish under category 2 of the Endangered Species Act of 1973 in the US (Graham 1997). Fishes within category 2 are those that US Fish and Wildlife believe are possibly threatened, but conclusive data on biological vulnerability and threat to support such a listing are lacking. Paddlefish are a high economic value item when fished as a source of black caviar (Onders et al. 2008) and this characteristic has resulted in overfishing in states such as Alabama and Tennessee, contributing to stock reductions (Hoxmeier and DeVries 1996). By 1994, the number of states listing paddlefish as endangered, threatened, or a species of concern had increased from 5 in 1983 to 11 (Graham 1997). Although current records indicate that paddlefish population in most states within the Mississippi River basin are stable (Bettoli et al., 2009), there are still concerns about the sustainability of such stocks because of increased fishing pressure and habitat destruction. In 1992, because of concern for declining paddlefish populations, the species was added to the Appendix II list of the United Nations' Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). This CITES listing prevents the import or export of paddlefish and their products into or out of the United States unless a CITES permit is obtained through

the U.S. Fish and Wildlife Service. Paddlefish are currently listed on the International Union for Conservation of Nature (IUCN) Red List of threatened species as a vulnerable fish species, meaning that they face a high risk of extinction in the wild. To protect paddlefish through restocking and habitat restoration, an understanding of the ecology and physiology of this species is needed.

1.3 Hypoxia in aquatic environments

Hypoxia, or low oxygen concentration, is an increasing problem in aquatic ecosystems (Diaz and Rosenburg 1995). According to the Committee on Environment and Natural Resources (CENR 2010), hypoxia incidence has increased almost 30 fold in the US since 1960. There are a number of factors that affect dissolved oxygen (DO) concentrations and can lead to hypoxia, such as: increasing temperature (Carpenter 1996; Matear et al. 2000; Conley et al. 2007; Rabalais et al. 2009), flow, light and phytoplankton biomass (Kemp et al. 2009). The pervasive nature of these factors indicates that DO fluctuations are a common natural occurrence in most aquatic ecosystems (Caduto 1990; Pihl et al. 1992). However, anthropogenic influences have increased the prevalence of hypoxia in aquatic ecosystems. Some of the main influences include temperature increases from global warming, which increases the respiratory oxygen demand of fish (Harris et al. 2006) and reduces oxygen solubility (Carpenter 1966), and excessive nutrient inputs from agriculture lands (Shields et al. 2009; Moore et al. 2010), which increase the biological oxygen demand of aquatic ecosystems (Service 2004; Vaquer-Sunyer and Duarte 2008; Kemp et al. 2009). Nutrient inputs have resulted in increasing rates of eutrophication in many aquatic ecosystems (Boesch et al. 2001) and chronic hypoxia in many ecosystems all over the world (Bonsdorff et al. 1997). Nutrient

inputs result in hypoxia as a result of biological processes such as nitrification and the decomposition of organic matter (Abeliovich 1992).

Hypoxia in most freshwater systems is now recognized as a large-scale threat to ecosystem health and can result in a reshaping of fish assemblages through changes in species composition, population decline, and a decrease in fish biomass (Diaz and Rosenberg 1995; Alexander et al. 2000; Charette and Prepas 2003). Within a given aquatic ecosystem, the combination of temperature and hypoxia may influence the habitat used by fish as they try to avoid extremes. Coutant (1985) called this concept "temperature-oxygen squeeze" and it was the prevailing hypothesis to explain the decline of striped bass during the 1980s in Chesapeake Bay (Coutant and Benson 1990; Secor and Niklitschek 2001).

The availability of oxygen is one of the major abiotic factors that can exert a strong species-selective force, because the need for oxygen is among the most pressing physiological demands on fishes (Hughes 1973). Anoxia or hypoxia in aquatic environments may lead to physiological stress and sometimes death in most fish species (Diaz and Rosenberg 1995, 2008; Breitburg et al. 1997; Diaz and Breitburg 2009). The ability to adapt to a particular environment is an important factor in setting species distribution among heterogeneous environments (Mandic et al. 2009). Organisms that have a greater capability to take up oxygen are able to maintain a routine metabolic rate at lower oxygen tensions and have a greater chance of surviving in hypoxic environments (Mandic et al. 2009). Those fish species which do not have the capabilities to tolerate reduced oxygen concentrations may be extirpated from an area as a result of hypoxia (Doudoroff and Shumway 1970). Similarly, the threat of hypoxia-induced biodiversity

loss is linked in part to the effect of hypoxia on fish metabolism and its implications on fish reproduction and development (Wu 2002, 2009).

1.3.1 Physiological responses of fishes to hypoxia

Hypoxia imposes physiological constraints on fishes, particularly when the level of oxygen is insufficient to meet internal demands. Richards (2011) defines hypoxia relative to fishes as the partial pressure of oxygen (pO_2) when physiological functions are first compromised and metabolic rate can no longer be maintained at a specific temperature. Because of the frequency and pervasiveness of hypoxia in aquatic ecosystems, resulting both from natural and anthropogenic causes, fishes will develop different responses to hypoxia during their ontogeny (Barry et al. 1995; Ishibashi et al. 2005; Ishibashi et al. 2007). Most fishes initially respond to hypoxia by increasing their ability to maintain oxygen delivery for fulfillment of respiratory needs (Wu 2002). In teleosts this is usually accomplished through activities that ensure an increase in the rate of water flow over the gills and an increase in the diffusional capacity of the gills (Randall 1970; Wu and Woo 1985). It can also be achieved by increasing the number of red blood cells and oxygen binding capacity of hemoglobin as seen in European flounder, *Platichthys flesus* (Soldatov 1996) and European eel, *Anguilla anguilla* (Wood and Johansen 1972). Other hypoxia response mechanisms include a reduction in locomotor activities and depression of overall energy metabolism as demonstrated by common carp, *Cyprinus carpio*, (Zhou et al. 2000), common sole, *Solea solea* (Dalla Via et al. 1994) and Atlantic cod, *Gadus morhua* (Schurmann and Steffensen 1994). These responses conserve energy (Wu 2002) and indicate that locomotor activities such as exercise performance could be used as an integrated measure of a fish's physiological suitability

to an environment (Nelson 1989; Nelson et al. 1994). Unfortunately, these adaptive behaviors are relatively slow and are insufficient to meet the energy demands of fishes in hypoxic environments, and thus result in stress to the organism (Van den Thillart and van Waarde 1985). To compensate for this lack of sufficient response, most fishes respond to acute hypoxia by a short-term increase in anaerobic metabolism to supply energy usually supplied by aerobic metabolism, thus preventing a drastic drop in cellular energy status (Lutz and Nilsson 1997; Lutz et al. 2003; Nilsson and Renshaw 2004). For example, blood lactate concentrations increased by a factor of three when rainbow trout, *Oncorhynchus mykiss,* were exposed to hypoxia, indicating an increase in anaerobic metabolism caused by an increase in oxygen demand by the branchial muscles (Holton and Randall 1967). Although DO concentrations < 5-6 mg/ L are generally considered hypoxic in freshwater systems (Diaz and Breitburg 2009), a wide range of behavioral and physiological adaptations to hypoxia exist in fish, indicating that no single universal threshold for hypoxia exist. Thus different taxa and life stages may be affected by hypoxia differently (Hagerman 1998; Vaquer-Sunyer and Duarte 2008; Richards 2011) and as such, may manifest different oxygen tolerance thresholds (Gray et al. 2002).

1.3.2 Developmental changes in hypoxic regulatory abilities

Juvenile fish need a relatively large portion of energy for somatic growth; therefore, age and maturity are important influences on energy utilization. Short-term hypoxia studies suggest that tolerance to hypoxia does not change as size in adult fishes increases (Smale and Rabeni 1995). However, information is lacking relative to the effect of hypoxia on early life stages of fish and is due to the rapid changes in body structure and physiology during early development. As a result, quantification of the effects of

hypoxia for specific life stages in fish (Rombough 1988) isdifficult. Generally, exposure to chronic and intermittent hypoxia can result in an overall reduction in the amount of energy available for both somatic growth and reproduction, leading to reduced juvenile growth rates, smaller size at maturity, smaller adult body size, lower fecundity per brood, delayed spawning, and sometimes mortality (Wu 2002, 2009; Richmond et al. 2006). Effects of low DO concentrations, however, differ with different developmental stages and species (Rombough 1988). Although it is commonly believed that the larval stage is the most vulnerable (Doudoroff and Shumway 1970; Davis 1975), the ontogenic stage of greatest vulnerability to hypoxia is different for different fishes (Siefert and Spoor 1973; Spoor 1977). In the case of paddlefish, successful long-term recovery efforts will require an understanding of how early life history stages survive in hypoxic conditions. Paddlefish are likely to be more sensitive to hypoxia during early life stages (Barton 2002), because they have less capacity for movement, are undergoing rapid growth requiring oxygen for aerobic metabolism and have less stored energy reserves. The magnitude of the primary stress response is typically greatest during these stages in fishes, and usually decreases with sexual maturity due to a reduced threshold for adrenocorticotropic hormone feedback (Pottinger et al. 1995; Barcellos et al. 2012; Koakoski et al. 2012). Thus, the understanding of the stress response of juvenile paddlefish allows fisheries managers and aquaculturists to manage for the most vulnerable members of the population which, in effect, may protect the tolerant population against a worst case scenerio.

1.3.3 Metabolic responses of fishes to hypoxia

Metabolism is the life-sustaining process by which an organism transforms consumed food into heat and available energy for physiological functions (Gillooly et al. 2001). There are two types of metabolism: aerobic, which requires oxygen, and anaerobic, which does not. Aerobic metabolism utilizes oxygen to derive energy and is very efficient and sustainable. In contrast, anaerobic metabolism occurs during periods of hypoxia and is a highly inefficient mode of energy production. Anaerobic metabolism results in the accumulation of lactate (Dunn and Hochachka 1986, 1987; Dalla Via et al. 1994), nitric oxide (Hansen and Jensen 2010), adenosine (Renshaw et al. 2002), fatty acids and amino acids (Affonso et al. 2002). Various experimental methods provide important insight into the form-function-environment relationships of teleost metabolic systems and how they affect a fish's ability to tolerate different environmental stressors (Horodysky et al. 2011). Some of these methods include measurement of metabolic rate (Beamish 1970; Burggren and Bemis 1992), swimming performance (Brett 1964; Beamish 1978; Nelson 1989; Richards 2009) and metabolic scope for activity (Fry 1947).

A relatively new investigative tool is metabolomics, which allows for an increased understanding of metabolic pathways through the measurement of small, low molecular weight metabolites, which are the intermediate and end products of metabolism (Rochfort 2005; Nicholson and Lindon 2008). Metabolomics has its roots in early metabolite profiling studies and is currently one of the rapidly expanding areas of scientific research that improve the understanding of biological systems. The discipline of metabolomics offers a reliable approach to understanding total changes within the complex biochemical matrix of living organisms (Holmes 2010). For example, Pincetich

et al. (2005) found that under hypoxia, concentrations of adenosine triphosphatase (ATP), inorganic phosphate (Pi) and phosphocreatine (PCr) decreased in the Japanese medaka, *Oryzias latipes*. However, all phosphometabolites returned to pre-hypoxia levels by 1.3 hours (h). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the most frequently used techniques for metabolomics. NMR analysis has been shown to be sufficiently robust to be of use in metabolomic studies (Lenz et al. 2003, 2004), having the capability to detect variations in factors such as age, sex and diet (Rochfort 2005). NMR metabolomics studies offer the advantage of sample recovery post analysis because analytes are not separated as is in the case of MS metabolomics. While one dimensional (1D) ^{1}H NMR is the most commonly used method in NMR metabolomics, a number of studies have used two dimensional (2D) techniques (Rochfort 2005; Viant et al. 2003). There are also studies where NMR and MS techniques are used to complement one another (Karakach et al. 2009). Metabolomics has been used in aquaculture and fisheries sciences to assess the stress response of many fish species living in disturbed environments (Lin et al. 2009; Savorani et al. 2010). Metabolomic analyses rely on multivariate statistics such as principal component analysis (PCA) and least squares regression analysis to analyze data generated from NMR analysis. PCA is used to extract and display any systematic variation in the data. A PCA model currently provides the best method to identify groupings, trends, and outliers in the data (Trygg et al. 2006).

1.3.4 Response of paddlefish to hypoxia

Paddlefish are phylogenetically primitive; therefore, an understanding of their biochemical and metabolic responses to hypoxia may provide insight into the evolutionary tolerance to hypoxia. Acipenseriforms are the only Actinopterygian

ancestors of the teleosts with a total dependence on gas exchange within the aquatic medium (Burggren and Randall 1978). Further, paddlefish are the only members of this group that ram ventilate. Ram ventilation is a passive means of ventilating the gills by relying on the velocity of water moving past the gills to supply oxygen. In comparison to typical active ventilation, which relies on buccal and opercular pumping to move water across the gills, ram ventilation has an energetic advantage (Freadman 1981; Steffensen 1985; Burggren and Bemis 1992). In fishes, the transition from active to ram ventilation depends on swimming speed which in turn may depend on ambient pO_2. At low pO_2s, however, fish may be forced to swim at increased speeds to obtain sufficient oxygen (Wood et al. 1992). For paddlefish, Burggren and Bemis (1992) found that the blood pO_2 of freely swimming fish did not change significantly when pO_2 was decreased from 150 mm Hg to 90 mm Hg. However, paddlefish died quickly when the pO_2 dropped below 90 mm Hg (Burggren and Bemis 1992). Thus, for paddlefish, it appears that the critical pO_2 (pO_{2crit}), which denotes the onset of anaerobic metabolism (Seibel 2011), is very close to the lethal pO_2. Burggren and Bemis (1992) also found that paddlefish usually have a resting metabolic rate that is about twice that of other acipenseriforms under normoxia due to their constant swimming behavior, which was typically about 70-80% of their maximum sustainable speed. Unfortunately, little scientific research has been done with regard to the tolerance of paddlefish to hypoxia, despite the fact that these fish experience variations in DO concentrations due to life history and as a result of eutrophication and temperature changes in their environment (Rabelais et al. 1999). Of note, most of the locations where paddlefish no longer exist are known to have problems with extreme hypoxia (Rosa and Burns 1987; Arend et al. 2011). Therefore, it is necessary to

determine whether hypoxia may be a causal or contributing factor to their disappearance from these systems. Through an understanding of the physiological response of paddlefish to hypoxia, conservation biologists and resource managers can develop improved management strategies to address their population decline.

1.4 Project overview

The main goal of this project is to gain an understanding of the tolerance and regulatory strategies of paddlefish to hypoxia. Experiments were conducted to compare the effects of acute and chronic hypoxia on tolerance limits, metabolic responses (i.e., oxygen consumption rates and metabolomics), physiological performance (i.e., swimming), biochemical responses (i.e., acid-base status, ion-osmoregulation, enzymes, etc.), blood oxygen transport and recovery capacity of juvenile paddlefish.

1.4.1 Expected results and benefits

Results of this study will be beneficial for understanding paddlefish responses to hypoxia and providing fisheries managers with needed information to conserve paddlefish populations and manage paddlefish fisheries. Results will also provide tolerable DO concentrations at different temperatures, which will be a useful ecological parameter for paddlefish researchers and aquaculturists for determining suitable habitats and maximizing survival in culture ponds. The use of the ABL80 FLEX CO-OX blood gas analyzer offers the opportunity to identify the effects of hypoxia on specific biochemical regulatory systems such as acid-base regulation, ion-osmoregulation and blood gas regulation with just one experiment. This study also utilizes metabolomics to better understand physiological responses to hypoxia and provide a comparison with

more classical physiological approaches. Unlike classical metabolite detection methods which concentrate on single pathways with minor interactions between them, metabolomics focuses on the complex interactions of the various components of the whole system. In addition, this information is obtained during the exact time the organism is responding to the stressor, thus, providing the most recent view of the organism's phenotype. Thus, for an organism like a paddlefish in which there is little known about the stress response mechanism, metabolomics offers the opportunity to simultaneously identify and quantify all metabolic pathways responding to a particular stressor at the same time. Thus, more information can be obtained about the stress response of paddlefish with fewer experiments. This experimental approach will save time and reduce the number of paddlefish needed for experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University (Protocol approval number: 11-058).

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CHAPTER II

PHYSIOLOGICAL AND METABOLIC RESPONSES OF JUVENILE PADDLEFISH *POLYODON SPATHULA* TO HYPOXIA AND TEMPERATURE

2.1 Abstract

The American paddlefish (*Polyodon spathula*) is a declining, primitive, migratory fish, and a potential aquaculture species. Hypoxia is an increasing problem in the natural habitats that the paddlefish has historically inhabited, including the Mississippi River system and nearby drainage systems, and is a potential problem under managed culture conditions. However, an understanding of the effects of hypoxia on paddlefish are lacking. To understand the response of juvenile paddlefish to hypoxia, acute hypoxia tolerance was measured. Aerobic metabolic rates and swimming capabilities were also measured in an intermittent respirometer or swim flume under normoxic (pO_2 = 140-155 mm Hg) and hypoxic (pO_2 = 62-70 mm Hg) conditions at 18 ºC and 26 °C. Paddlefish acclimated to 18 ºC and 26 ºC had routine metabolic rates of 211 mg/kg/h and 294 mg/kg/h, respectively, with a corresponding Q_{10} of 1.5. At 18 ºC, paddlefish had a critical partial pressure of oxygen (pO_{2crit}) of 74 mm Hg (4.70 mg/ L) and at 26ºC, a pO_{2crit} of 89 mm Hg (4.84 mg/ L). Paddlefish had a lethal oxygen threshold of 31.0 mm Hg (~2 mg/ L) and 37.0 mm Hg (~2.03 mg/ L) at 18ºC and 26ºC, respectively. Swimming capability decreased when exposed to hypoxia with a 24% and 41% reduction in U_{crit} at 18ºC and 26ºC, respectively. Therefore, paddlefish are relatively sensitive to hypoxia, and at

temperatures from 18-26°C require a dissolved oxygen concentration ≥ 4.7 mg/ L to maintain basal aerobic metabolism and ≥ 2.0 mg/ L to survive under acute hypoxia.

Keywords: Hypoxia, normoxia, pO_{2crit}, paddlefish, respirometry, temperature.

2.2 Introduction

Hypoxia, or low oxygen concentration, is an increasing problem in aquatic ecosystems with reported incidence in the U.S. increasing almost 30 fold in the past 50 years (Diaz and Rosenberg 2008; CENR, 2010). Dissolved oxygen (DO) fluctuations are a common natural occurrence in most aquatic ecosystems (Caduto 1990; Pihl et al. 1992); however, anthropogenic influences have contributed to an increase in hypoxic episodes.. Two of the main causes of hypoxia include continual rising of mean global temperatures and excessive phosphorus inputs from agriculture lands (Shields et al. 2009; Moore et al. 2010). Elevated temperatures increase the metabolic oxygen demand of fish (Harris et al. 2006) and reduce oxygen solubility (Carpenter 1966). Phosphorus inputs cause eutrophication and thereby increase the biological oxygen demand of aquatic ecosystems (Service 2004; Vaquer-Sunyer and Duarte 2008; Kemp et al. 2009).

The availability of oxygen can exert a strong influence on species diversity, because the physiological need for oxygen is critical for fishes (Hughes 1973). Hypoxia in aquatic environments may lead to physiological stress and potentially death in most fish species (Diaz and Rosenberg 1995: Diaz and Breitburg 2009). The ability to adapt to a particular environment is an important factor in establishing species distribution among heterogeneous environments (Mandic et al. 2009). Organisms that have a greater capability to uptake oxygen per unit time are able to satisfy metabolic oxygen requirements at relatively lower partial pressures of oxygen (pO_2) and correspondingly

have a greater chance of surviving in hypoxic environments (Mandic et al. 2009). Those fish species which do not have the capabilities to tolerate reduced oxygen concentrations may no longer inhabit an area as a result of hypoxia (Doudoroff and Shumway 1970).

Hypoxia imposes physiological constraints on fishes, particularly when the level of oxygen is insufficient to meet internal demands. Because of the frequency and pervasiveness of hypoxia in aquatic ecosystems, resulting both from natural and anthropogenic causes, fishes have developed different responses to hypoxia during their ontogeny (Barry et al. 1995; Ishibashi et al. 2005; Ishibashi et al. 2007). Most fishes initially respond to hypoxia by attempting to increase their ability to deliver oxygen to fulfill respiratory needs (Wu 2002). In teleosts, this response is usually accomplished by increases in the rate of water flow over the gills and in the diffusional efficiency of the gills (Randall 1970; Wu and Woo 1985). Increase in oxygen delivery can also be achieved by increasing the number of red blood cells and/ or the oxygen binding capacity of hemoglobin as seen in the European flounder, *Platichthys flesus* (Soldatov 1996) and the eel, *Anguilla anguilla* (Wood and Johansen 1972). Other hypoxia response mechanisms include a reduction in locomotor activities and the depression of overall energy metabolism as demonstrated by the common carp, *Cyprinus carpio* (Zhou et al. 2000), common sole, *Solea solea* (Dalla Via et al. 1994) and Atlantic cod, *Gadus morhua* (Schurmann and Steffensen 1994). Unfortunately, for many species, little is known about their specific responses to hypoxia. Some of these fish species are of both ecological and economic importance and as such, information about their response to hypoxia is needed to ensure their proper management. One such species is the American paddlefish, *Polyodon spathula*.

The American paddlefish is among the most primitive freshwater fishes, having been in existence for approximately 150 million years (Romer 1967; Moy-Thomas 1971). They belong to the family Polyodontidae (Bond 1979), which contains only one other species, the Chinese paddlefish, *Psephurus gladius*, which is native to the Yangtze-Kiang River (Pflieger 1975). Paddlefish are found in 22 US states within the Mississippi River drainage basin and nearby drainage basins, and occupy large streams, rivers and impoundments (Mims 2001; Firehammer and Scarnecchia 2005). Because acipenseriform fishes are the only Actinopterygian ancestors of the teleosts that depend exclusively on DO in the water (Burggren and Randall 1978), an understanding of their physiological and metabolic responses to hypoxia may provide insight into the evolution of hypoxia tolerance. Most of the information available about the response of acipenseriform fishes to hypoxia is from studies on sturgeons. Because of their primitive morphological and physiological traits, such as low cardiac performance and relatively poor gill ventilation, sturgeons generally are less efficient in oxygen respiration compared to other fishes (Klyashtorin 1982; Agnisola et al. 1999). As a result, sturgeons generally show characteristic sensitivity to hypoxia in terms of metabolic responses, where routine metabolism decreases in hypoxic environments (Secor and Niklitschek 2001). One of the main ways fish decrease their metabolic rate is by reducing energy consumption (Sloman et al. 2006) through reduced feeding and activity. Swimming is considered as one of the most metabolically energy demanding activities (Fry 1971). In a hypoxic environment when the supply of oxygen is insufficient to meet ATP requirements needed to supply energy demands, acipenseriforms will reduce their swimming activity in an effort to reduce their metabolic demands. For example, Crocker and Cech (1997) showed that at

all temperatures and sizes, juvenile white sturgeon, *Acipenser transmontanus*, decreased MO_2 by about 57% and swimming activity by 70% in response to hypoxia (pO_2= 80 ± mm Hg).

Although paddlefish and sturgeon are related, there are significant anatomical and ecological distinctions between them. The most important of these distinctions is that paddlefish are obligate ram ventilators and thus swim constantly to actively move water over their gills to remove oxygen. This difference in respiratory strategy suggests that these two taxa may employ different responses to hypoxia. Most of what is known about paddlefish responses to hypoxia is based on Burggren and Bemis (1992). The authors showed that paddlefish are able to regulate oxygen when subjected to a pO_2 range of 150 mm Hg to 90 mm Hg. However, with a further 5- 10 mm Hg reduction of pO_2 they lose equilibrium very quickly. Unfortunately, apart from the Burggren and Bemis study, little is known regarding the responses of paddlefish to hypoxia. As an obligate ram ventilator, paddlefish are expected to have a high oxygen requirement, which is expected to limit their tolerance to hypoxia. These fish experience variations in DO concentrations due to life history movements and as a result of eutrophication and temperature changes in their environment (Rabelais et al. 1999). Therefore an investigation into the response of paddlefish to hypoxia and how this response is influenced by the environmental temperature will provide more comprehensive information about the evolution of hypoxia tolerance in these primitive acipenseriforms.

Also, the decline in the meat and caviar supplies from wild sturgeon and paddlefish populations has created a demand for farm-raised paddlefish products (Van Eenennaam et al. 2005). The sustainability of paddlefish aquaculture, however, depends

on the successful development of specific culture techniques that take the environmental requirements of this species into consideration. Because of the importance of oxygen in aerobic respiration, the availability of oxygen is often the biggest limiting factor in fish ponds after the food requirement needs of a fish have been met (Boyd and Tucker 1998). The direct effect of oxygen availability on the feed consumption and metabolism of a fish influences growth and survival in a fish pond. Thus, an understanding of the response of paddlefish to hypoxia will help in the development of a profitable paddlefish aquaculture industry.

The first objective of this study was to determine paddlefish tolerance to acute hypoxia exposure. The second objective was to determine the effect of acute hypoxic stress at 18 ^{o}C and 26 ^{o}C on juvenile paddlefish metabolic rate (MO_2; mg/kg/hr) and swimming performance. It was hypothesized that there would be no significant difference in the mean MO_2 and maximum sustainable swimming speed (U_{crit}) between juvenile paddlefish exposed to normoxia compared to those exposed to hypoxia. It was also hypothesized that temperature would have no effect on these variables.

2.3 Materials and Methods

2.3.1 Fish source and care

Wild-caught paddlefish broodstock were obtained from the Noxubee River System within the Noxubee National Wildlife Refuge, MS and artificially spawned at the Private John Allen National Fish Hatchery in Tupelo, Mississippi. In March 2012, paddlefish fry (4 days post hatch) were transferred from the hatchery to 458-l circular recirculating tanks supplied with aerated well-water with a pH of 8, located at the Mississippi Agriculture and Forestry Experimental Station's South Farm Warmwater

Aquaculture Research Facility at Mississippi State University, Starkville, MS. Fish were initially held at a density of 1 per L and later reduced to 0.2 per L one month later to avoid overcrowding. Temperature within the tanks was maintained at 22 ºC using an in-line water heat pump (Titans® HP-7, Aqualogic, San Diego, CA, USA). Fish were fed with cultured *Daphnia* spp. for the first 30 days at a density of 70-140 individuals/ L. *Daphnia* spp. were collected from ponds fertilized with rice bran and inorganic fertilizer (10-43-0) following recommendations by Mims et al. (1999) and Rosen and Hales (1981). *Daphnia* spp. were supplemented with cultured *Artemia* sp. (Great Salt Lake Artemia cysts; Artemia International LLC, Fairview, Tx, USA) at a density of 1000 naupli/ L every day. After 30 days, fish were fed a mixture of *Artemia* sp. at a density of 2000 nauplii/ L and spirulina algae powder (Artemia International LLC, Fairview, TX, USA) at 10 g/ L. As the fish grew larger (8-20 g), they were fed floating catfish pellets (0.6-1.6 mm; Rangen EXTR 450; Rangen, Inc., Angleton, TX, USA) containing 44% crude protein and 15% crude fat at 10% of body weight per day. Water pH in the holding tanks was maintained at 6.8-7.2 and ammonia was never reached detectable levels.

2.3.2 Hypoxia tolerance

Three weeks before the start of the experiment, fish were acclimated to either 18 ºC or 26 ºC by a daily increase or decrease of 1 ºC. These water temperatures were selected as representative of spring and summer conditions in one of their natal river systems, the Noxubee River System, based on weekly measurements of water temperature during a 7-month period. Once fish reached treatment temperatures, they were acclimated for an additional two weeks prior to the start of the experiment. To determine the acute hypoxia threshold of juvenile paddlefish, fish were placed into 265-L

flow-through tanks (containing 120-L of water per tank) at their acclimation temperature (18 ºC or 26 ºC) and a pO_2 of 149 mm Hg. Fish were placed into each tank 36 h before the start of experiment, and food was then withheld. Flow was ceased immediately prior to oxygen manipulation. There were 8 fish per tank, and 6 replicate tanks for each temperature treatment. The pO_2 of the water in each tank was lowered stepwise by bubbling nitrogen gas through an airstone at a rate of 15 mm Hg every 5 minutes from 149 mm Hg to a pO_2 at which 50 % of fish lost equilibrium. After each 15 mm Hg drop in pO_2, fish were held for an additional 20 minutes prior to reducing to the next pO_2 level. Experiments were carried out 2 tanks at a time (one for each temperature treatment). At the end of each trial, weight and length were determined for each fish within each replicate of each treatment after recovery in normoxic water.

2.3.3 Routine metabolic rate

Juvenile paddlefish (10.13 ±0.30 g; Table 1) were acclimated for 20 days to either 18 ºC or 26 ºC following a protocol similar to that described previously. Following acclimation, metabolic rate was measured for individual fish at each temperature (n=10/ temp) at seven successive water oxygen tensions (149, 134, 119, 104, 89, 74 and 59 mm Hg). Each paddlefish was weighed and placed into an 8-L acrylic, intermittent respirometer with a fiber-optic oxygen probe (DAQ-PAC-F1X; Loligo Systems, Tjele, Denmark), and controlled by computer software (AutoResp version 1.0.0). The respirometer was housed in a glass tank (105-L) at the same treatment temperature. Fish were acclimated for 1 h within the respirometer, while oxygen consumption rates were monitored to ensure fish reached a steady rate of consumption. In the respirometer, fish were able to move freely.

Table 2.1 Mean (±SE) lengths (cm) and weights (g) of American paddlefish.

	Length (cm)		Weight (g)	
	18 ºC	26 ºC	18 ºC	26 ºC
Treatment group				
Hypoxia Tolerance	12.61 ±0.4	12.73 ±0.2	10.11±0.3	10.15±0.5
Routine MR-Normoxia	12.82 ±0.3	12.71 ±0.4	10.16 ±0.4	10.09 ±0.4
Active MR - Normoxia	19.13 ±0.4	19.12 ±0.4	20.38 ±0.4	20.42 ±0.5
Active MR - Hypoxia	19.23 ±0.4	19.12 ±0.3	20.50 ±0.4	20.42 ±0.5

Table 2.1 represents mean (± standard error) lengths (cm) and weights (g) of American paddlefish, *Polyodon spathula* exposed to normoxia and hypoxia at two different temperatures (18ºC and 26ºC). MR = Metabolic Rate.

Routine metabolic rate measurements commenced 1 h after the acclimation period. A pilot study organized 4 days earlier determined that within 15-30 minutes of being placed in the respirometer, fish maintained a baseline level of activity, determined by tail-beat frequency of 40-50 tail-beats/ minutes. A 1 h acclimation period was used based on MO_2 measurements showing that baseline levels were reached by 30-50 minutes (Fig. 2.1). After the 1 h acclimation period, routine MO_2 was measured for three 420 sec periods at each oxygen tension in order to effect measurement of a drop in water oxygen tension. After measuring MO_2 at each oxygen tension, a flow of nitrogen gas followed, introduced into the tank surrounding the respirometer via an air-stone to reduce pO_2 in the water to the next treatment concentration. To compensate for any microbial respiration, a blank was run each day, before and after fish trials, under the same conditions as the actual trials. No reduction in oxygen was measured in the blanks so no adjustment was made for oxygen consumption by fish in the trials. Fish length was measured after each experiment.

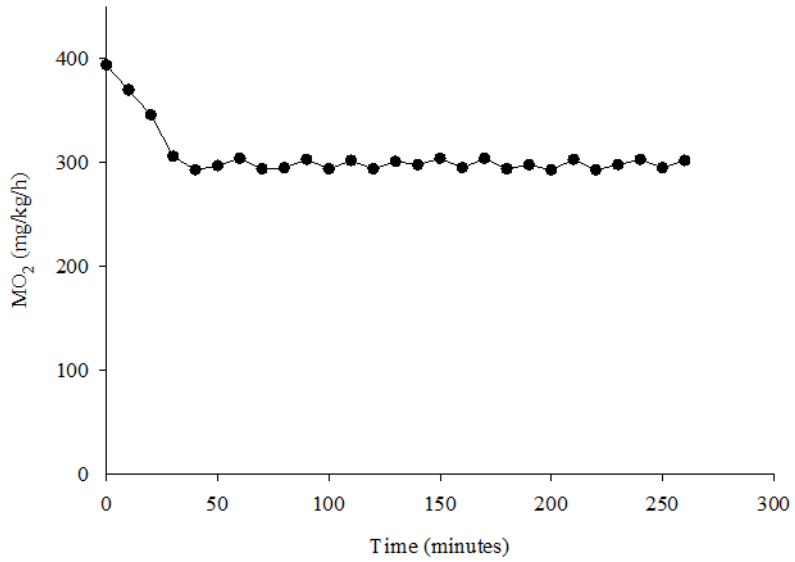

Figure 2.1 An example of the time course of routine metabolic rate of juvenile American paddlefish.

Figure 2.1 is an example of the time course of routine metabolic rate (MO_2) of juvenile American paddlefish, *Polyodon spathula* in an intermittent respirometer at 26 °C. Each data point represents the mean of three repeated MO_2 measurements.

The rate of increase in the metabolic rate of paddlefish for each 10 °C rise in temperature (Q_{10}) was determined for each fish. This is an index of thermal sensitivity, which expresses the effect of temperature change on an organism's overall metabolism (Di Santo and Bennett 2011). Q_{10} was determined using the equation used by Schmidt-Nielsen (1997):

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{\frac{10}{T_2 - T_1}} \quad \text{(Eq. 2.1)}$$

Where, K_2 is the metabolic rate at the higher temperature (T_2) and K_1 is the metabolic rate at the lower temperature (T_1).

2.3.4 Swimming performance

Paddlefish were acclimated to either 18 ºC or 26 ºC for 20 days using the same protocol described previously. For this experiment, there were four treatments with two pO_2 levels (149 ±3 mm Hg; normoxia and 59 ±3 mm Hg; hypoxia) for each temperature (18 ºC and 26 ºC). Swimming trials were conducted in a modified 100-L swimming flume based on the design of Blazka et al (1960) and described by Beecham (2004) using methods similar to Allen (2006). Individual fish (n=12 per treatment; 20.39 ±0.21 g; Table 1) were weighed and placed in the flume and acclimated to a velocity of 10 cm/ s for 1 h. Afterwards, swimming performance (U_{crit}) was measured by increasing water velocity by 5 cm/ s every 30 minutes until fish fatigued. Fatigue was defined as impingement on the screen at the rear of the flume for 15 seconds. Half-way (15 minutes) into each velocity increment, tail beat frequency was recorded for 30 sec. This procedure was followed for all fish until the fish became fatigued. When a fish became impinged, measurement time was paused and water velocity reduced to 0 cm/ s for 10 seconds, allowing the fish to move off the rear screen. Once off the screen, the water velocity was resumed and the measurement time continued. If impingement occurred three consecutive times at the same water velocity, the trial was ended, and the length of the fish was determined. The fish was then returned to a holding tank with water at its acclimation temperature. Impingement can be a behavioral choice to refuse to swim rather than physiological exhaustion. The use of three repeated impingements at the same water velocity introduced a higher probability that the swimming endpoint was physiological exhaustion rather than a refusal to swim.

2.3.5 Active metabolic rate

Active metabolic rate, which is the energy required to perform different levels of activity, was also measured for each fish for velocities of 30 cm/ s and above by measuring the drop in pO_2 in the water for the 30 minute period using a fiber-optic oxygen meter with a dipping probe (FIBOX 3, PreSens-Precision Sensing GmbH, Regensburg, Germany**)** connected to the flume. After each 30 minute velocity period, the water in the chamber was exchanged for 15 seconds, increasing the pO_2 to the treatment level. Active metabolic rate was recorded for velocities of 30 cm/ s and above because data collected at lower velocities in all treatments were characterized by a high variance. The MO_2 was calculated similar to Reidy et al. (2000):

$$MO_2 = \frac{\left[\left(\frac{\Delta PO_2}{\Delta T}\right)(V-M)\alpha O_2\right]}{M} \qquad \text{(Eq. 2.2)}$$

Where, ΔpO_2 is the change in the partial pressure of oxygen in the water (mm Hg), ΔT is the time interval (minutes), V is the flume volume (L), M is the mass of the fish (kg) and αO_2 is the solubility coefficient of oxygen at the experimental temperature and salinity taken from Boutilier et al. (1984).

It has been demonstrated that when fish are not stressed, the standard metabolic rate, which is the energy required to maintain basic biological functions, can be estimated by extrapolating active MO_2 to zero (Beamish 1970; Beamish and Mookherjii 1964). Thus, the lowest MO_2 are believed to occur at zero activity (Van den Thillart et al. 1994). Standard metabolic rates in this experiment were calculated by extrapolating the values obtained from active MO_2 for the last three swimming velocities (40 cm/ s, 35 cm/ s and 30 cm/ s) for both experimental temperatures to zero activity in the fish. The resulting

values obtained were within the range of error of MO_2 recorded for routine MO_2 in the respirometer. This suggests that paddlefish in the respirometer were as close to inactivity as possible.

2.3.6 Metabolic scope for activity

Metabolic scope for activity (Fry 1947), which is the energy available to support growth and swimming, was determined for fish in normoxic conditions. Standard metabolic rates generated by the extrapolation method above were used to calculate the metabolic scope for activity. It was calculated by subtracting the standard metabolic rate from the maximum active metabolic rate, which is the rate of oxygen consumption at the highest swimming velocity. Active MO_2 was recorded only for velocities $\geq$ 30 cm/ s due to limitations in measurement sensitivity below this water velocity. As a result, the metabolic scope for activity was not calculated for fish in hypoxia because active MO_2 was determined for only two velocities (30 cm/ s and 35 cm/ s) for paddlefish in hypoxia and, as such, these could not be extrapolated. A flow chart of materials and methods can be found in the appendix. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University (Protocol approval number: 11-058).

2.4 Statistical analyses

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) at a significance level of $p < 0.05$. Normality and equality of variance were tested with Shapiro-Wilk and Levene's tests, respectively. Data are represented as mean ± standard error (SE). A student's *t*-test was used to compare acute

hypoxia threshold concentrations and metabolic scope for activity between the two treatment temperatures, 18 ºC and 26 ºC. One-way repeated measures analysis of variance (ANOVA) was used to compare routine MO_2 between pO_2s in each temperature treatment. Student's *t*-tests were also used to compare routine MO_2 between treatment temperatures at each pO_2. For active MO_2 and tail-beat frequency, one-way repeated measures ANOVAs were used to compare either MO_2 or tail-beat frequency between water velocities within each temperature and oxygen treatment group. One-way ANOVAs were also used to compare active MO_2 or tail beat frequency between temperature and oxygen treatment groups at each water velocity. Two-way ANOVAs were used to compare mean U_{crit} between temperature and oxygen treatments. When the results showed significant differences, a *post-hoc* Holm-Sidak multiple comparison test was used to isolate the treatments differences.

2.5 Results

2.5.1 Acute hypoxia tolerance

The pO_2 at which 50% of paddlefish acclimated to 18 ºC lost equilibrium was 31.0 ± 1.1 mm Hg (1.97 mg/ L)and was considered to be the threshold oxygen tension. This oxygen level was different from that of fish acclimated to 26 ºC which lost equilibrium at a pO_2 of 37.0 ± 1.6 mm Hg (2.03 mg/ L; Fig. 2.2). Both pO_2 values corresponded to oxygen concentrations of approximately 2.0 mg/ L. Approximately, 40% of the fish died after they lost equilibrium, suggesting that the threshold oxygen tension is close to the lethal pO_2.

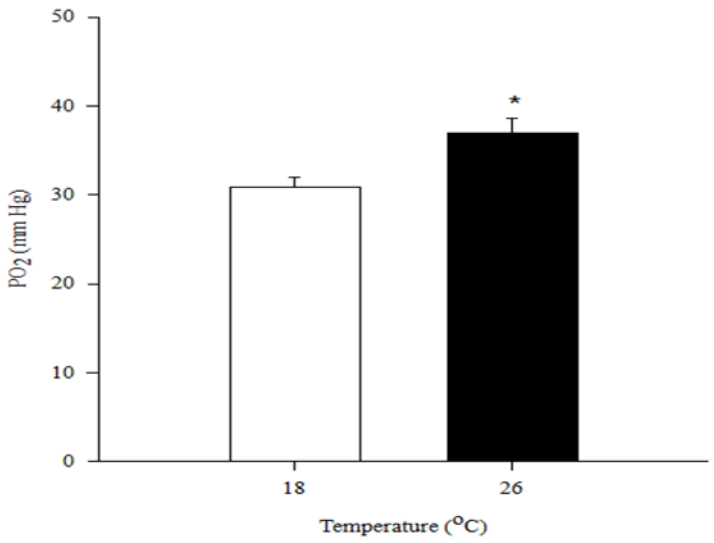

Figure 2.2 Acute hypoxia tolerance of juvenile American paddlefish.

Figure 2.2 represents acute hypoxia tolerance of juvenile American paddlefish, *Polyodon spathula*, at 18 ºC and 26 ºC. Data are presented as mean (±SE) oxygen tension (mm Hg) at which 50% of paddlefish lost equilibrium. Asterisks (*) indicate a significant difference between the temperature treatments (Student's t-test, $p < 0.05$, n= 6/ temperature).

2.5.2 Routine metabolic rate

The MO_2 for paddlefish acclimated to 18ºC remained steady at 211.41 ± 2.11 mg/kg/h between pO_2s from 149 mm Hg to 74 mm Hg (Fig. 2.3). MO_2 then began to drop slightly, becoming different from normoxia (149 mm Hg) at 59 mm Hg. Below 59 mm Hg fish became agitated and began to lose equilibrium. In contrast, MO_2 for paddlefish acclimated to 26 ºC remained steady at 294.12 ± 1.81 mg/kg/h between pO_2s from 149 mm Hg to 89 mm Hg. At 89 mm Hg, MO_2 then began to decrease, becoming different from normoxia (149 mm Hg) at 74 mm Hg. Fish in this treatment became agitated below 74 mm Hg and began to lose equilibrium. Therefore, 74 mm Hg (4.70 mg/ L) and 89 mm Hg (4.84 mg/ L) were considered to be critical pO_2s (pO_{2crit}) for fish acclimated at 18 ºC and 26 ºC, respectively. MO_2 was different between the two temperatures, with fish acclimated to 26 ºC having a higher MO_2 at all pO_2s (except at 74

mm Hg) than fish at 18 ºC. The average Q_{10} value from 18 ºC to 26 ºC over a range of pO_2s from 149 mm Hg to 89 mm Hg was 1.50 (Fig 2.4).

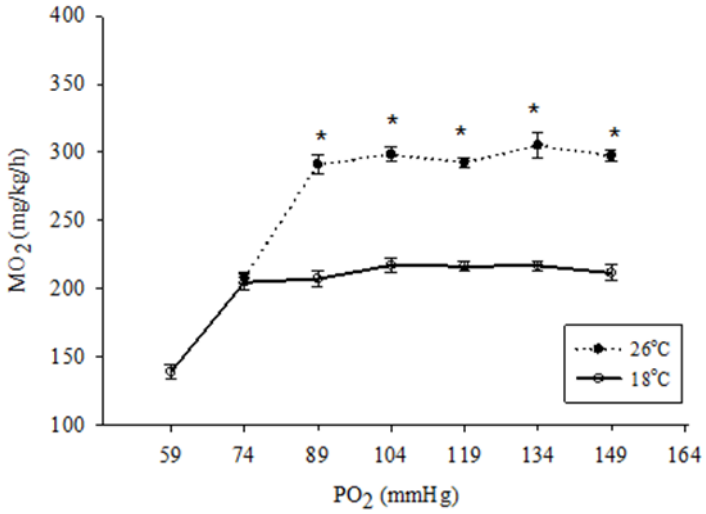

Figure 2.3 Routine metabolic rate (MO_2) of juvenile American paddlefish.

Figure 2.3 represents mean (±SE) routine metabolic rate (MO_2) of juvenile American paddlefish, *Polyodon spathula*, exposed to graded hypoxia at different temperatures (18 ºC or 26 ºC). PO_{2crit} for paddlefish at 26 ºC and 18 ºC was 89 mm Hg and 74 mm Hg, respectively. Asterisks (*) indicate values significantly (Student's *t*-test, $p < 0.05$, n=10) different between the two treatment temperatures at a particular pO_2. Fish at 26 ºC lost equilibrium below 74 mm Hg.

2.5.3 Critical swimming speed and tail beat frequency

When U_{crit} was compared between temperatures at the same water velocity, U_{crit} significantly increased with increasing temperature in normoxia (Fig. 2.5). There was however no effect of temperature on U_{crit} in hypoxia. Hypoxia also decreased U_{crit} at both temperatures. Hypoxia decreased tail beat frequency in both treatment temperatures. Tail-beat frequency also increased as velocity increased in all treatments (Fig. 2.7). Fish in all treatments switched from steady swimming to burst-and-glide swimming as they approached U_{crit}.

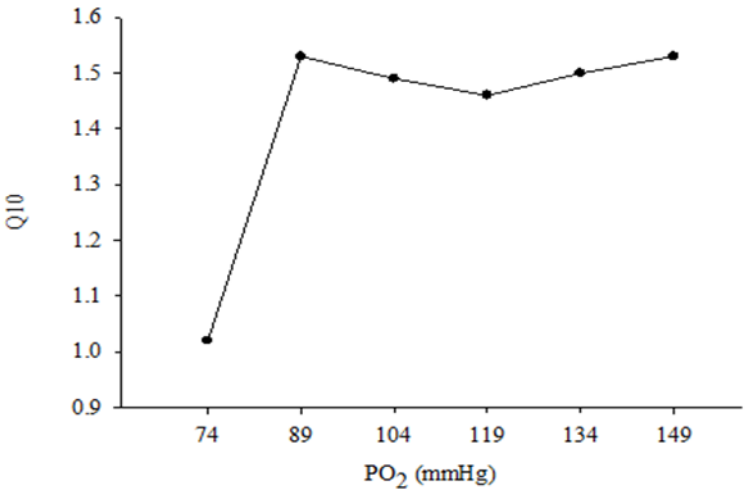

Figure 2.4 Q10 values for routine oxygen consumption in juvenile American paddlefish.

Figure 2.4 represents Q10 values for routine oxygen consumption in juvenile American paddlefish, *Polyodon spathula*, (mean (±SE) wet weight: 10.13 ±0.3 g, n=10) acclimated to two temperatures: 18 ºC or 26 ºC. The pO_{2crit} for paddlefish at 26 ºC is 89 mm Hg.

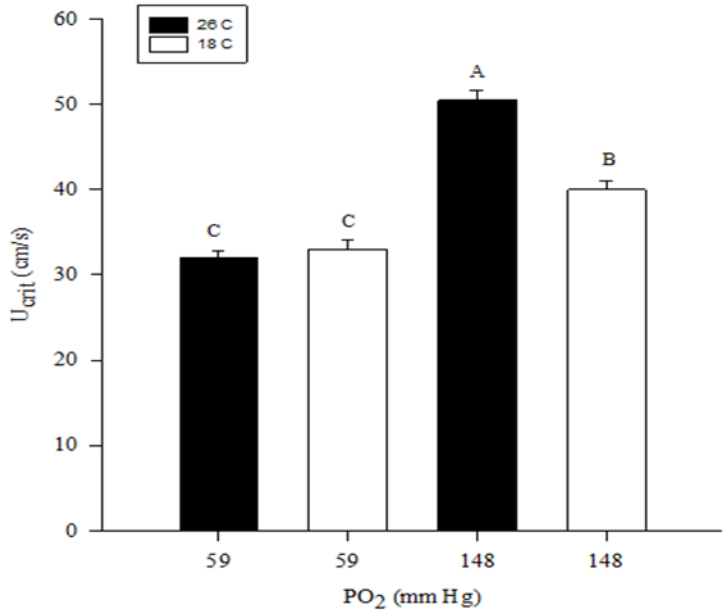

Figure 2.5 Critical swimming speed (U_{crit}) for juvenile American paddlefish.

Figure 2.5 represents mean (±SE) critical swimming speed (U_{crit}) for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg) at two temperatures, 18 ºC or 26 ºC. Treatments with different letters are significantly different (two-way ANOVA, Holm-Sidak multiple comparison test, $p < 0.05$, n=12/ treatment).

2.5.4 Active MO_2, standard MO_2, and metabolic scope for activity

MO_2 increased with increasing velocity for both treatment temperatures in normoxia and hypoxia (Fig. 2.6). MO_2 was compared to temperature at each water velocity. For normoxia, MO_2s at all recorded velocities were higher in fish acclimated to 26 °C as compared to those acclimated 18 °C. In contrast, for hypoxia, there was no effect of temperature on MO_2. For both temperatures, MO_2 was lower in hypoxia. At 30 cm/ s and 35 cm/ s (the only velocities at which MO_2s were recorded for fish in hypoxia), MO_2s for fish in normoxia were greater than those in hypoxia at both temperatures (18 °C and 26 °C).

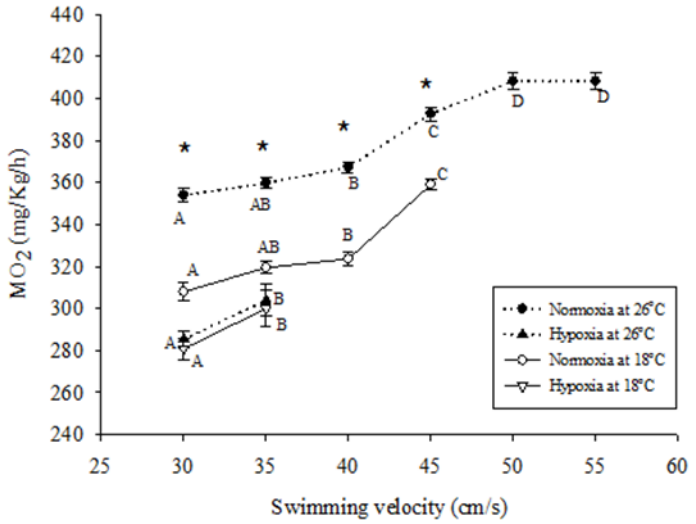

Figure 2.6 Mean active metabolic rate (MO_2) for juvenile American paddlefish.

Figure 2.6 represents mean active metabolic rate (MO_2) for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148±3 mm Hg) or hypoxia (59 ±3 mm Hg) at two temperatures (18°C or 26°C). One-way ANOVA, Holm-Sidak multiple comparison test ($p < 0.05$, n=12/ treatment) was used to compare active MO_2 between all treatments. MO_2 was significantly different between normoxia and hypoxia at each velocity for each temperature. Asterisks (*) indicate velocities at which MO_2 was significantly different between 18 °C and 26 °C-acclimated fish at normoxia. MO_2s with different letters within a particular treatment are significantly different (one-way repeated measures ANOVA, Holm-Sidak multiple comparison test) from each other.

Tail beat frequency differed between temperatures in normoxia, but not in hypoxia (Fig. 2.7). The standard metabolic rate in normoxia was 210 mg/kg/hr and 274 mg/kg/hr for fish acclimated to 18 ºC and 26 ºC, respectively. The metabolic scope for activity for paddlefish in normoxia decreased with increasing temperature with values of 150 mg/kg/hr and 134 mg/kg/hr for fish acclimated to 18 ºC and 26 ºC, respectively.

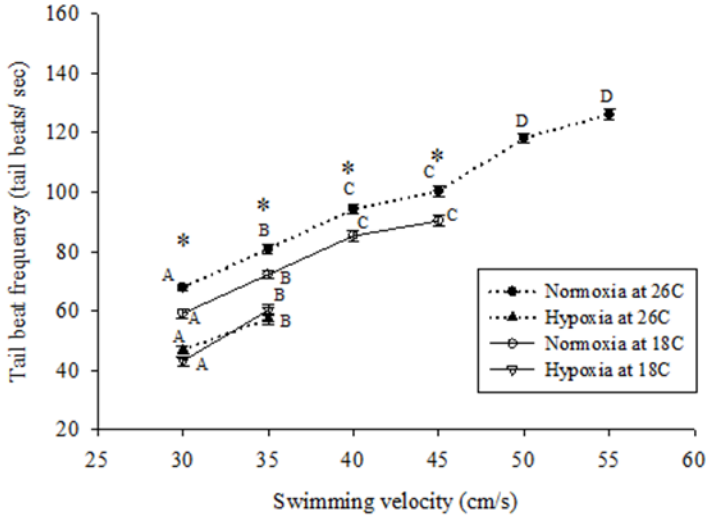

Figure 2.7 Tail-beat frequency for juvenile American paddlefish.

Figure 2.7 represents mean tail-beat frequency (TBF; ±SE) for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg) at two temperatures, 18 ºC or 26 ºC. TBF was significantly different between normoxia and hypoxia at all velocities for each temperature. Asterisks (*) indicate TBF values significantly different (one-way ANOVA, Holm-Sidak multiple comparison test $p < 0.05$, n=12/ treatment) between 18 ºC and 26 ºC-acclimated fish at a particular swimming velocity at normoxia. TBFs with different letters within a particular treatment are significantly (one-way repeated measures ANOVA, Holm-Sidak multiple comparison test) different from each other.

2.6 Discussion

Unlike other primitive ray-finned fishes, the chondrosteans rely exclusively on DO in the water. Paddlefish are unique members of this group because they are obligate ram ventilators. In this study, the unique strategies this species utilizes to adapt to hypoxia and the limitations hypoxia imposes have been investigated. Paddlefish have a

relatively low tolerance to hypoxia and a high pO_{2crit}, which increases with temperature. A primary response of juvenile paddlefish to hypoxia is a decrease in routine metabolic rate, which also results in decreased swimming capabilities (U_{crit}).

2.6.1 Effect of hypoxia on paddlefish metabolism

The pattern of oxygen consumption exhibited by paddlefish in this experiment and in Burggren and Bemis (1992), where paddlefish regulated oxygen until a pO_{2crit} was reached, beyond which they conformed to the environmental oxygen concentration, has also been demonstrated in several species of sturgeon (Nonnotte et al. 1993; Secor and Niklitschek, 2001). Burggren and Bemis (1992) found that paddlefish (2-10 g) acclimated to 24 ºC had a pO_{2crit} of 90 mm Hg, which is similar to the pO_{2crit} of 89 mm Hg measured for paddlefish at 26 ºC in this experiment. It is however higher than some other acipenseriforms such as *A. baeri* (Nonnotte et al. 1993) and Adriatic sturgeon, *A. naccarii* (McKenzie et al. 2007; Table 2.2). It is also higher than fishes such as the Greenland cod, *Gadus ogac* and largemouth bass, *Micropterus salmoides*, which are considered hypoxia intolerant (Steffensen et al. 1994; Cech et al. 1979; Table 2.2). Similarly, the increase in pO_{2crit} with increasing temperature exhibited by paddlefish in this experiment has also been shown in acipenseriforms (Klyashtorin 1976) and other fishes (Fry and Hart 1948; Schurmann and Steffensen 1997). PO_{2crit} increases with temperature due to increasing metabolic demands and therefore increased oxygen demands (Seibel 2011; Richards 2009). PO_{2crit} is often used as an indicator of hypoxia sensitivity in fishes (Mandic et al. 2009; Chapman et al. 2002). Fish with higher pO_{2crits} are considered hypoxia sensitive, whereas those with lower pO_{2crits} are considered hypoxia tolerant (Chapman and

McKenzie, 2009). Therefore, the high pO_{2crits} measured in the present study indicate that paddlefish are relatively intolerent to hypoxia.

2.6.2 Effect of hypoxia on metabolic rate and swimming capacity

The increase in U_{crit} and metabolic rate with increasing temperature demonstrated by paddlefish in normoxia is a relationship typically found in most fishes (Koumoundouros et al. 2002; Claireaux et al. 2006). Adams et al. (2003) demonstrated that as temperature increased from 10 ºC to 20 ºC, both shovelnose sturgeon, *Scaphirhynchus platorynchus* and pallid sturgeon, *S. albus*, increased their U_{crit} from 19.48 cm/ s to 36.98 cm/ s and from 15.05 cm/ s to 35.93 cm/ s, respectively. Juvenile green sturgeon, *Acipenser medirostris*, have also been shown to increase their U_{crit} as temperatures increased from 19 ºC to 24 ºC (Allen et al. 2006).

Table 2.2 PO_{2crit} of selected fish species.

Species	Common name	Weight (g)	Temperature (°C)	PO_{2crit} (mmHg)	References
Polyodon spathula	American paddlefish	10.16±0.4	18	74	This study
Polyodon spathula	American paddlefish	10.09±0.4	26	89	This study
Polyodon spathula	American paddlefish	2-10	24	90	Burggren and Bemis (1992)
Acipenser naccarii	Adriatic sturgeon	565	23	36.8	McKenzie et al. (2007)
Acipenser baeri	Siberian sturgeon			40	Nonnotte et al. (1993)
Gadus ogac	Greenland cod	143-223	4.5	50-60	Steffensen et al. (1994)
Oncorhynchus mykiss	Rainbow trout		20	27	Ott et al. (1980)
Cyprinus carpio	Common carp		25	20	Ott et al. (1980)
Carassius auratus	Goldfish	4.88	12	22.97	Fu et al. (2011)
Lates niloticus	Nile perch	4-28	23	26.4	Chapman et al. (2002)
Gymnocorymbus sp.	Black skirt tetra	2.78-3.39	25	16.8	Kramer and McClure (1982)
Petrocephalus catostoma	Ntachi	1.4-37	23	11.5	Chapman et al. (2002)
Astatotilapia aeneocolor	Yellow belly Albert	3.6-7.1	18-20	14.8	Melnychuk and Chapman (2002)
Astronotus ocellatus	Oscar	310 ±42	28	46	Scott et al. (2008)
Astatotilapia velifer		0.8-12.9	23	12.9	Rosenberger and Chapman (2000)
Pseudocrenilabrus multicolor		2.2-8.9	23	7.9	Rosenberger and Chapman (2000)
Micropterus salmoides	Largemouth bass	230-470	20	<40	Cech et al. (1979)
Micropterus salmoides	Largemouth bass	230-470	25	40-50	Cech et al. (1979)
Micropterus salmoides	Largemouth bass	230-470	30	50-60	Cech et al. (1979)

The standard metabolic rate measured in paddlefish in normoxia is also similar to that of Burggren and Bemis (1992) and is about twice that of most other fishes (Burggren and Bemis 1992; Horodysky et al. 2011). This is likely due to the fact that paddlefish are ram ventilators, maintaining about 70-80% of their maximum sustainable speed during

normal steady swimming (Burggren and Bemis 1992; Careau et al. 2008). The high standard metabolic rate and the sensitivity to hypoxia in paddlefish indicate that to prolong survival in hypoxia, they must adapt sustainable means of conserving energy, such as reduction of metabolic rate as indicated by the results of this study.

This hypoxia-induced reduction in metabolic rate is observed in acipenseriforms and many other fishes as a means to delay the onset of anaerobic glycolysis and thus prevent early acidosis (Muusze et al. 1998). In other acipenseriforms, white sturgeon, *A. transmontanus,* are known to reduce their metabolic rate by 65% and 88% when exposed to hypoxia at 16 ºC and 20 ºC, respectively (Crocker and Cech 1997). In terms of teleosts, goldfish, *Carassius auratus*, also reduce their metabolic rate by 59% when exposed to hypoxia at 20 ºC (Van Waversveld et al. 1988, 1989). Similarly, Cech et al. (1979) reported that the reduction of pO_2 from 130 mm Hg to 40 mm Hg reduced the metabolic rate of largemouth bass, *Micropterus salmoides*, by approximately 54%. Van den Thillart et al. (1994) showed that Common sole, *Solea solea*, significantly reduce their metabolic rate when exposed to hypoxia.

In this experiment, concomitant with a hypoxia-induced reduction in metabolic rate was a reduction in swimming capacity. U_{crit} decreased by 24% and 41% at 18 ºC and 26 ºC, respectively, between normoxic and hypoxic conditions. This reduction in U_{crit} after exposure to hypoxia has also been reported in other fishes such as Atlantic cod, *Gadus morhua*, (Petersen and Gamperl 2009; Dutil et al. 2007) and coho salmon, *Oncorhynchus kisutch*, (Dahlberg et al. 1968). Because swimming consistutes a major proportion of the energy budget of fishes, swimming performance is often used as an integrated measure of a fish's physiological suitability to an environment (Nelson 1989;

Nelson et al. 1994; Richards 2009). Thus, the large reduction in U_{crit} in paddlefish between normoxic and hypoxic conditions indicates that hypoxic environments exert a relatively high energetic cost on paddlefish and may limit the types of habitats that they can occupy.

2.6.3 Effects of temperature on hypoxia tolerance and scope for activity

This study also supported the known dynamic relationship among water temperature, basal metabolism and threshold oxygen tension that has been demonstrated in many other fishes (Beamish and Mookherjii 1964; Crocker and Cech 1997; Gillooly et al. 2001). Q_{10} is often used as a measure of the rate of change of the biological system of a fish in relation to its increased energy requirement when temperature increases. Most fish have a Q_{10} of around 2 (Prosser and Brown 1961; Beamish 1963; Zheng 2008). The Q_{10} obtained in this experiment for paddlefish in normoxia is consistent with that found in the Chinese sturgeon, *Acipenser sinensis* (1.4, 20 ºC to 30 ºC; Liu et al. 2011) but less than that of the green sturgeon, *Acipenser medirostris* (4.1, 19 ºC to 24 ºC; Mayfield and Cech 2004). The Q_{10} value obtained here indicates that little metabolic adjustment occurs in the fish as it moves between 18 ºC and 26 ºC, when there is adequate oxygen supply. This lack of response is an indication that these temperatures fall within the natural range of juvenile paddlefish. A low Q_{10} value may also be advantageous to a migratory fish like the paddlefish, as it moves through different temperatures in its migratory paths.

A decrease in the scope for activity of paddlefish when temperature increased from 18 ºC to 26 ºC in normoxic conditions suggests that the amount of energy available for swimming and growth is reduced when temperature correspondingly increases. This change is due to greater basal metabolic costs at higher temperatures as demonstrated by

higher standard and routine metabolic rates. In addition, aerobic metabolic demands for oxygen at high temperatures are challenged by decreases in oxygen solubility in water. Thus, when oxygen tensions are already low in a hypoxic environment, increasing temperatures likely require paddlefish to utilize anaerobic metabolism earlier so that metabolic demands are met. In this study, such a response was demonstrated by the higher threshold oxygen tension for loss of equilibrium and the higher pO_{2crit} with increasing temperature.

The threshold oxygen tensions measured for paddlefish in this experiment were similar to threshold concentrations for the Russian sturgeon, *Acipenser gueldenstaedtii*, stellate sturgeon, *A. stellatus* and Siberian sturgeon, *A. baeri*, which ranges from 28-36 mm Hg, for temperatures ranging from 18 ºC to 26 ºC. Threshold oxygen tension also showed an increase with increasing temperature in all these sturgeons (Lozinov 1952; Klyashtorin 1976). When the threshold oxygen tensions for paddlefish are converted to DO concentrations they are similar to those measured for bluefin tuna, *Thunnus maccoyii* (1.57 and 2.49 mg/ L; Fitzgibbon et al. 2010), but higher than those of French grunt, *Haemulon flavolineatum*, (1.2 mg/ L), white mullet, *Mugil curema*, (1.5 mg/ L; Fangue et al. 2001), smallmouth bass, *Micropterus dolomieu*, (1.2 mg/ L), largemouth bass, *Micropterus salmoides*, (1.0 mg/ L), fathead minnow, *Pimephales promelas* (0.73 mg/ L), bluegill, *Lepomis macrochirus*, (0.66 mg/ L; Smale and Rabeni 1995) striped bass, *Morone saxatilis* (1.6 mg/ L) and northern pipefish, *Syngnathus fuscus* (1.5 mg/ L; Miller et al. 2002). The results indicate that the threshold oxygen tension is influenced by temperature, which may be valuable for predicting paddlefish threshold oxygen concentrations in different habitats. According to Vasquer-Sunyer and Duarte (2011),

threshold oxygen concentrations may increase by 0.01 mg/ L for every 1 ºC rise in temperature. The effect of temperature on the threshold oxygen tension and the Q_{10} values obtained in this study indicate that there are increased metabolic oxygen demands on the fish as temperatures increase (Fry and Hart 1948; Schmidt-Nielsen 1975; Richards 2009; Seibel 2011; Vasquer-Sunyer and Duarte 2011). However, aerobic metabolic demands for oxygen at high temperatures are challenged by decreasing oxygen solubility in water. Thus, when oxygen tensions are already low in a hypoxic environment, increasing temperatures will likely require paddlefish to utilize anaerobic metabolism earlier to meet metabolic demands.

Paddlefish apparently have a limited adaptive capacity for hypoxia as demonstrated by the lack of change in MO_2, U_{crit} and tail beat frequency under hypoxic conditions with increases in temperature (18 ºC to 26 ºC). Paddlefish are considered a highly aerobic fish with limited anaerobic metabolism capabilities (Burggren and Bemis 1992). Anaerobic metabolism can temporarily meet energy demands, but is generally not sustainable due to induced respiratory acidosis, which may eventually lead to death (Connett et al. 1990; Boutilier 2001). Thus, what may be considered mild hypoxia for paddlefish at lower temperatures may be lethal for fish at higher temperatures. This means that paddlefish and other fishes that experience high temperature-induced metabolic stress will have a better tolerance for hypoxia in relatively colder temperatures than in higher temperatures. According to a review by Vaquer-Sunyer and Duarte (2011), for every 1 ºC increase in water temperature, survival durations of most fishes exposed to hypoxia can be reduced by as much as 3.95 h. With presumed increases in water temperature caused by global warming, the combined effect with hypoxia may reduce not

only the quality of habitats for paddlefish and other acipenseriforms but also the quantity of such habitats. For example, a 1 ^{o}C increase in water temperature during the summer in the Chesapeake Bay, could eliminate much of the habitat of juvenile Atlantic sturgeon, *A. oxyrhynchus*, (Niklitschek and Secor 2005). To better understand the effect of temperature on lethal oxygen threshold, MO_2, and habitats utilized by paddlefish, future studies need to investigate a greater range of temperatures to cover both extreme winter and summer conditions.

2.6.4 Potential hypoxia coping mechanism in paddlefish

Paddlefish are obligate ram ventilators (Burggren and Bemis 1992). In this study they were observed to increase their mouth gape in hypoxia as compared to normoxia. This same pattern was observed in progressive hypoxia challenges, where mouth gape width appeared to increase with decreasing oxygen tension, although these findings were not quantified. Thus, one means for increasing oxygen uptake in paddlefish appears to relate to the regulation of mouth gape as opposed to increasing ventilation frequency. This mechanism of increasing gape width while reducing metabolic rate in hypoxia has been observed in other ram ventilators such as the southern bluefin tuna, *Thunnus maccoyii* (Fitzgibbon 2010). However, this response differs from those mechanisms utilized by other fishes when exposed to hypoxia. Some fish decrease metabolic rates without any noticeable increase in mouth gape (Nilsson et al. 1993; Schurmann and Steffensen 1994; Crocker and Cech 1997). Some fish also increase ventilation frequency and/ or ventilatory volume when exposed to hypoxia (Campagna and Cech 1981; Berschick et al. 1987; Scott et al. 2008). Some fish on the other hand increase swimming velocity, thus metabolic rate, in an attempt to escape hypoxic conditions (Whitmore et al.

1960; Spoor 1990). Herbert and Steffensen (2005) showed that the Atlantic cod, *Gadus morhua*, increase their swimming velocity by 18% when pO_2 declined from 149.6 mm Hg to 99.3 mm Hg. The strategy observed in paddlefish may, however, not be efficient in addressing hypoxia and may be the reason why paddlefish lose equilibrium at pO_2s very close to their pO_{2crit}. Further research is needed to determine by how much paddlefish are able to increase gill ventilation by increasing water flow over the gills, reducing their oxygen consumption, and increasing their mouth gape.

2.7 Conclusion

Paddlefish are sensitive to hypoxia, likely due to their high routine metabolic rate as a consequence of ram ventilation. In response to acute hypoxia, they are able to reduce their metabolic rate, potentially increasing their chances for survival. However, swimming capacity is also reduced in response to hypoxia suggesting that predatory avoidance or the ability to move to a normoxic environment may be affected. Increasing temperature exacerbates these effects, increasing pO_{2crit} and decreasing the lethal oxygen threshold. Thus, the combined effect of temperature and hypoxia may reduce paddlefish habitat quality and quantity. Therefore, the provision of a suitable environment for paddlefish must take into consideration not only the oxygen concentration but also the effect of temperature on the metabolic oxygen demand of this species. The effect of temperature on the swimming capabilities of paddlefish suggest that the establishment of velocities for culverts and fishways must take into consideration the water temperature. It is recommended that maximum velocity for juvenile paddlefish (10-20 g) culverts and fishways should be 40 cm/ s and 51 cm/ s for fish at 18 ^oC and 26 ^oC, respectively. Given the confining nature of respirometers and the reduced potential of confined fish to use all

available energy saving strategies as compared to those in the wild (Peake 2004; Peake and Farrell 2004; Peake 2008), these recommendations can be considered conservative. Paddlefish in both natural and aquaculture systems will require DO concentrations of ≥ 4.7 mg/ L to sustain aerobic metabolism. Given that DO fluctuations are a common occurance in pond aquaculture systems, maximum efficiency in such systems could not be maintained.

2.7.1 References

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CHAPTER III

EFFECTS OF ACUTE AND CHRONIC HYPOXIA ON ACID-BASE REGULATION, HEMATOLOGY, ION AND OSMOREGULATION AND METABOLISM OF JUVENILE AMERICAN PADDLEFISH

3.1 Abstract

Despite the increasing prevalence of hypoxia in natural habitats occupied by the American paddlefish, a basal bony fish and ram ventilator, information about its response to hypoxia is scarce. To understand the physiological and biochemical responses of juvenile paddlefish (~150 g) to acute and chronic hypoxia, blood oxygen transport, blood acid-base balance and metabolic stress were evaluated under four different partial pressures of oxygen [pO_2; normoxia 148 ±3 mm Hg (~100% saturation; 8.92 mg/ L), mild hypoxia = 89 ±3 mm Hg (~60% saturation; 5.35 mg/ L), moderate hypoxia = 59 ±3 mm Hg (~40% saturation; 3.57 mg/ L) and extreme hypoxia = 36 mm Hg (24% saturation; 2.14 mg/ L)], all at 21ºC. Blood samples were collected from paddlefish after they had been exposed to treatment pO_2s for 0.25, 2, 6, 24 and 72 hours, and analyzed for hematocrit, pO_2, total oxygen content, oxygen saturation, pCO_2, pH, hemoglobin, Na^+, K^+, Ca^{2+}, Cl^-, glucose and lactate. Mild hypoxia only caused a reduction in blood pO_2 and blood oxygen saturation. Both short term (acute; <24 hours) and long term (chronic; ≥24 hours) moderate and extreme hypoxia caused a decrease in blood pH, pO_2, total oxygen content, plasma Na^+ and Cl^- at all time points. Acute moderate and extreme hypoxia

resulted in an increase in blood pCO_2, plasma glucose, lactate and hematocrit. Chronic exposure to moderate hypoxia resulted in an increase in plasma lactate, red blood cell count and hemoglobin but no change in plasma pCO_2 and glucose. Paddlefish were able to physiologically compensate for mild hypoxia, but exhibited secondary stress responses and were unable to return to homeostasis when exposed to both acute and chronic moderate hypoxia, and died after 3-8 hours of extreme hypoxia. At 21 ^{o}C, paddlefish require water with a pO_2 > 89 mm Hg (5.35 mg/ L) to maintain aerobic metabolism, and chronic exposure to moderate and extreme hypoxia (< 59 mm Hg; < 3.57 mg/ L) may lead to mortality.

Keywords: acid-base, glucose, stress, paddlefish, hematology, lactate, osmoregulation, pO_2, pH

3.2 Introduction

Hypoxia in aquatic systems is caused by both anthropogenic activities leading to nutrient enrichment and eutrophication, and natural causes such as thermal stratification (Diaz and Breitburg 2009; Speers-Roesch et al. 2012). The incidence of hypoxia has greatly increased in the last 50 years, and is projected to increase further if global climate change and anthropogenic activities contributing to its spread are not curtailed (Diaz and Breitburg 2009). The increasing incidence of hypoxia has led to studies investigating the response of fishes to different levels of hypoxia (Richards 2011).

In fishes, aerobic cellular metabolism is driven by oxygen acquired from the surrounding aqueous environment (Nikinmaa and Salama 1998). Physical and chemical factors affect the concentration of oxygen in the water which is linked to the efficiency of physiological processes in fish. Hypoxia, defined as the partial pressure of oxygen (pO_2)

at which the physiological functions of fish are compromised (Richards 2011), may exert detrimental effects on respiration, swimming and growth (Clayton 1993; Herbert and Steffensen 2005; Wang et al. 2009). Exposure to hypoxia, either acute or chronic, disturbs physiological homeostasis, induces stress responses and may adversely effect health (Pickering 1998). Environmental stressors like hypoxia may lead to significant changes in normal function with eventual consequences such as reduced growth or death unless overcome by adequate adaptive responses (Selye 1950). Adaptive responses involve physiological and biochemical adjustments directed towards achieving homeostasis to ensure survival (Pickering 1981; Wendelaar Bonga 1997; Barton 2002).

Homeostatic responses to hypoxia in fishes include compensatory adjustments designed to maintain oxygen delivery to the tissues to meet ongoing metabolic needs. Increases in ventilation rate and amplitude are some of the earliest responses of fish to hypoxia (Maxime et al. 1995; Wu 2002; Chapter 2). Continued exposure to hypoxic conditions may trigger the release of additional red blood cells (RBCs) from the spleen and kidney into the circulatory system (Lai et al. 2006). Increase in RBC number and hemoglobin (Hb) concentration increases the oxygen carrying capacity of the blood, maintaining sufficient oxygen supply to the tissues during hypoxic exposure (Wells 2009).

Acute and chronic hypoxia induces the release of stress hormones, such as catecholamines and cortisol, into the blood (Herbert and Steffensen 2005; Lai et al. 2006), causing changes in blood chemistry and hematology, which in turn lead to changes in osmoregulation and metabolism (Mazeaud et al. 1977; Barton 1997; 2000). As a result, changes in ventilation, as well as hematological and metabolic parameters such

as RBC count, hematocrit (Hct), Hb concentration, blood glucose, lactate, ions and osmolality are reliable indicators for quantifying the stress response and relate to hypoxia as well (Wedemeyer and McLeay 1981; Lochmiller et al. 1989; Wedemeyer et al. 1990; Barton 1997; Wendelaar Bonga 1997; Barton et al. 2002).

Exposure to hypoxia may also affect acid-base regulation. The efficiency of the fish gill in exchanging respiratory gases from blood to water, such as carbon dioxide (CO_2), helps in the efficient removal of metabolic CO_2 from tissues. Metabolic CO_2 produced by the tissues diffuses into the blood where it reacts with water to produce H^+ and bicarbonate (HCO_3^-), maintaining a low partial pressure of carbon dioxide (pCO_2) and consequently a well-regulated pH level. Hypoxia tends to disrupt acid-base regulation by inducing changes in blood pH, pCO_2 and HCO_3^- concentrations (Heisler 1982; 1984; 1993). These changes are incurred by hypoxia-induced CO_2 and lactic acid accumulation resulting in the release of H^+ into blood, thus changing pH balance. Hypoxia induced hyperventilation also results in increased HCO_3^- concentration, resulting in alkalosis.

Despite the abundant information about responses to hypoxia in fishes, there is a paucity of information on the responses in acipenseriforms. Acipenseriforms are unique in that they are the only actinopterygian ancestors of teleosts with a total dependence on aquatic gas exchange (Burggren and Randall 1978). Therefore, information about the mechanisms controlling their response to hypoxia will improve understanding of the respiratory physiology of teleost ancestors and the evolutionary processes underlying hypoxia responses in teleosts. This information can also be beneficial for evaluating paddlefish management strategies in both natural and culture (aquaculture) fisheries.

Previous studies indicate that fish in this primitive group are oxyregulators, similar to most teleosts (Nonnotte et al. 1993; Maxime et al. 1995; Chapter 2). Known responses by acipenseriforms to hypoxia include increases in blood concentrations of cortisol, glucose and lactate (Baker et al. 2005; Kieffer et al. 2011). Some of the reported hypoxia survival strategies displayed by sturgeons include hyperventilation (Crocker and Cech 2002; Baker et al. 2005) and reduced metabolic rate (Crocker and Cech 1997; Niklitschek and Secor 2009). Hypoxia has also been reported to reduce growth and increase mortality in sturgeon (Secor and Gunderson 1998; Niklitschek and Secor 2009). Among acipenseriforms, less is known about the respiratory physiology of paddlefishes as compared to sturgeons.

The two extant species of paddlefish, American paddlefish, *Polyodon spathula* and the endangered Chinese paddlefish *Psephurus gladius*, belong to the family Polyodontidae (Bond, 1979) which has been in existence at least since the early part of the Cretaceous period (Romer, 1967; Grande et al. 2002). The American and Chinese paddlefish are considered vulnerable to substantial population decline and thus critically endangered by the International Union for the Conservation of Nature (Grady 2004; Qiwei 2010). American paddlefish are found in the Mississippi River drainage basin and nearby Gulf of Mexico drainages (Jennings and Ziegler 2000; Mims 2001; Horvath et al. 2006). Some of the known habitats of paddlefish are associated with high levels of nitrogen and phosphorus inputs (eutrophication) from agriculture and urban nonpoint sources (Harned et al. 2004). Eutrophication has been associated with hypoxia related fish kills and corresponding loss of sensitive species in many aquatic systems around the world (Wu 1982; Diaz and Rosenberg, 1995; Diaz 2001; Diaz and Breitburg 2009;

Chapmn and McKenzie 2009). Available information indicates that American paddlefish are obligatory ram ventilators and oxyregulators with a relatively high metabolic rate and sensitivity to hypoxia, with a minimum oxygen requirement of > 2 mg/ L (Burggren and Bemis 1992; Patterson et al. 2013; Chapter 2). Because paddlefish are obligatory ram-ventilators, they do not undergo metabolic depression to the degree observed in sturgeons when exposed to hypoxia (Burggren and Bemis 1992; Crocker and Cech 1997; Chapter 2).

Similar to other acipenseriforms, paddlefish lack the RBC pH protecting beta adrenergic Na^+/H^+ exchanger (βNHE; Berenbrink et al. 2005; Regan and Brauner 2010). These catecholamine-stimulated exchangers are found on the RBC membrane of more derived fishes, and exchange H^+ for Na^+, increasing RBC pH (Nikinmaa 1992; Thomas and Perry 1992; Pelster and Decker 2004; Regan and Brauner 2010; Rummer et al. 2013). A neutral or slightly increased RBC pH helps to upload oxygen at the gills. A reduced RBC pH, in contrast, reduces the affinity of hemoglobin for oxygen (Bohr effect) and as a result hinders oxygen uptake at the gills (Benesch and Benesch 1961; Riggs 1981; Jensen 2004). The absence of these exchangers suggests that paddlefish may be unable to efficiently regulate pH and transport oxygen during exposure to hypoxia and other stressors that cause acidosis.

The potential challenges of hypoxic environments may limit juvenile paddlefish recruitment within adult populations, thereby destabilizing paddlefish populations and possibly causing population declines. Considering the vulnerability of the American paddlefish to population decline based on their reliance on aquatic gas exchange, their position as basal bony fishes, and their utilization of ram ventilation, an understanding of

the physiological response of paddlefish to hypoxia is critically important to effectively address basic and applied science concerns.

Therefore, the objective of this study was to understand the biochemical and metabolic responses of paddlefish to graded levels of acute and chronic hypoxia.

3.3 Materials and Methods

3.3.1 Fish source and acclimation

Paddlefish were artificially spawned at the Aquaculture Research Center at Kentucky State University, Frankfort, Kentucky and the fry were shipped to the Mississippi State University South Farm Aquaculture Facility approximately 9 days after hatching. Fish were initially held in 450-L circular (1 m diameter) recirculating tanks supplied with air saturated well-water at 21° C and a pH of 8. They were initially held at at a density of 1 fish/ L. To avoid crowding, density was reduced to 0.2 fish/ L one month after initial stocking. For the first month, the fish were fed with *Daphnia* spp. at a density of 70-140 individuals/L/day. The *Daphnia* spp. were cultured in fertilized ponds maintained at the facility, following recommendations by Mims et al. (1999) and Rosen and Hales (1981). *Daphnia* spp. was supplemented with an *Artemia* sp. (Artemia International LLC, Fairview, TX, USA) culture at a density of 1000 naupli/ L every day. Survival at the end of the first month was approximately 61%. After 1 month, fish were fed a mixture of *Artemia* sp. at a density of 2000 nauplii/ L and spirulina algae powder (Artemia International LLC, Fairview, TX, USA) at 10 g/ L. After they were about 1.5 months old, fish were transitioned to trout fry/finger diet (STARTER: 55% protein; #0-#3 crumble) for another month (78% survival) after which they were transitioned to extruded floating pellets (Rangen EXTR 400: 40% crude protein; Rangen, Inc., Angleton, TX,

USA) for the rest of the study (about 95% survival). Feed size for EXTR 400 was gradually increased from 1.6 mm initially to a final size of 3.2 mm to account for fish growth.

Water temperature was maintained with an in-line water heat pump (Titan® HP-7, Aqualogic, San Diego, CA, USA). Water flowing into tanks was mechanically and biologically filtered with fluidized bead filters. To raise fish (1 year old;~14 cm) to the desired size, they were transferred to 3600-L circular (2.4 m diameter) tanks (at a density of 0.04 fish/ L) with the same water supply and maintained at 21° C for 7 months with aquaculture immersion heaters (Process Technology, Mentor, Ohio, USA). Each tank was fitted with a mechanical filter bag (Filter Specialists Inc., Michigan City, IN, USA) and a UV sterilizer (SMART HO, Emperor Aquatics Inc., Pottstown, PA, USA). Dissolved oxygen (DO) levels were maintained near saturation with multiple air stones throughout this period. Protocol for the feeding and maintenance of paddlefish was similar to that described in Chapter 2.

3.3.2 Hypoxia trials

One week before the start of experiments, fish were transferred from the holding tanks into 300-L circular (1.6 m diameter) experimental tanks with the same water quality conditions. Each experimental tank was fitted with a magnetic pump and a canister filter (Red Sea, Houston, TX, USA) containing activated carbon (Pentair Aquatic Eco-Systems, Inc, Apopka, FL, USA) to maintain optimum water quality. Nitrite and ammonia were measured daily with a water quality analysis kit (model: AQ-2; LaMotte Chemical Products, Co., Chestertown, Maryland, USA), and water pH was measured

daily with a pH meter (pH10A, YSI Inc., Yellow Springs, OH, USA). Food was withheld for 24 h before each trial to ensure a post-absorptive state (Barton et al. 1988).

In experimental trials, juvenile paddlefish were exposed to 4 different pO_2s [normoxia 148 ±3 mm Hg (100% saturation; 8.92 mg/ L), mild hypoxia = 89 ±3 mm Hg (60% saturation; 5.35 mg/ L), moderate hypoxia = 59 ±3 mm Hg (40% saturation; 3.57 mg/ L) and extreme hypoxia = 36 mm Hg (24% saturation; 2.14 mg/ L)] at 21 ºC in experimental tanks (300-L circular; 1.6 m diameter) for 72 hours (h). These levels of hypoxia were based on the results of Chapter 2 which showed that the critical partial pressures of oxygen (pO_{2crit}) for paddlefish are 74 mm Hg and 89 mm Hg at 18 ºC and 26 ºC, respectively. They also have a lethal minimum oxygen threshold of 31.0 mm Hg (~2 mg/ L) and 37.0 mm Hg (~2.03 mg/ L) at 18 ºC and 26 ºC, respectively. Thus, the oxygen levels were chosen to reflect paddlefish in environments near saturation, with mild hypoxia, moderate hypoxia and those where oxygen content is at the minimum required. Hypoxia was induced by bubbling nitrogen gas into the water to drop pO_2 at a rate of 3 mm Hg/ minute until experimental water pO_2 was reached. Treatment oxygen levels were reached after approximately 20, 30, and 34 minutes for mild, moderate and extreme hypoxia. Each treatment had 4 randomly assigned replicate tanks with 12 randomly assigned fish per tank. Simple randomization was achieved by assigning different numbers to individuals that were stocked into experimental tanks. Replicate tanks were set up in concrete raceways that served as temperature-controlled water baths.

Two fish were sampled from each tank for blood at 0.25, 2, 6, 24 and 72 h of exposure to hypoxia. Short term (0.25, 2 and 6 h) exposure to treatments was considered acute while long term exposure (24 and 72 h) was considered chronic (Pickering et al.

1991). Blood was collected from the caudal vasculature of fish (immobilized by a quick blow to the head) with a BD Vacutainer® 22-gauge hypodermic needle (Becton, Dickson and Co., Franklin Lakes, New Jersey, USA) into heparinized Monoject® blood collection tubes (Tyco Healthcare Group LP, Mansfield, MA, USA). Blood samples were gently mixed by inversion to ensure adequate mixing with heparin and then placed on ice. Overall sampling time was < 2 minutes for each fish. Blood was immediately divided into 3 aliquots without exposing the blood within the collection tube to air. Withdrawal of blood samples was performed with a needle and syringe through the soft stopper of the collection tube a few seconds after collection. Immediately afterwards, each fish was measured to the nearest millimeter (mm) from the eye to the fork of the tail (eye-fork length) and weighed to the nearest gram (g). Tissue samples were also collected, but the procedure for preparation is described in another experiment (Chapter 4).

Remaining blood in the collection tube (aliquot 1) was placed on ice and later analyzed with a blood gas analyzer (ABL80 FLEX CO-OX, Radiometer Medical, Bronshoj, Denmark) at the United States Department of Agriculture (USDA) Poultry Laboratory in Starkville, MS, within 15 minutes of collection for pH, Hct, hemoglobin, pO_2, pCO_2, HCO_3^-, total oxygen content, osmolalility, sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-). All measured parameters were adjusted to reflect the treatment temperature of 21 ºC. The second aliquot of blood from each fish was used to quantify RBC concentrations using a cell counter (Z1 Coulter Counter**,** Beckman Coulter, Inc., Brea CA, USA). The mean RBC size (mean corpuscular volume [MCV]) was calculated with the formula:

$$\text{MCV} = (\text{Hct} \times 10) \div \text{RBC concentration} \qquad \text{(Eq. 3.1)}$$

The mean Hb content per RBC (mean corpuscular hemoglobin [MCH]) was calculated with the formula:

$$\text{MCH} = (\text{Hb} \times 10) \div \text{RBC concentration} \quad \text{(Eq. 3.2)}$$

The mean concentration of hemoglobin in a given volume of RBCs (mean corpuscular hemoglobin concentration [MCHC]) was also calculated with the formula:

$$\text{MCHC} = (\text{Hb} \times 100) \div \text{Hct} \quad \text{(Eq. 3.3)}$$

The last set of blood samples (aliquot 3) was centrifuged at 10,000xg (model: 59A; Fisher Scientific, Pittsburgh, PA, USA) for 5 minutes (at an air-conditioned room temperature of 21 ºC) and plasma was collected and stored for 2 days at -80 ºC for glucose and lactate analysis. Plasma glucose and lactate was measured with a biochemical analyzer (VITROS DT60 II analyzer, Ortho-Clinical Diagnostics, Inc, Rochester, NY, USA) using glucose and lactate test slides (Infolab Inc., Greensboro, NC, USA). A flow chart of materials and methods can be found in the appendix. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University (Protocol approval number: 11-058).

3.4 Statistical analyses

Statistical analyses were conducted using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) at a significance level of $p < 0.05$. Normality and homogeneity of variance were tested with Shapiro-Wilk and Levene's tests, respectively. Data that did not meet parametric assumptions were log transformed. A two-way analysis of variance (ANOVA) with water pO_2 and time as the factors was used to test for the effects of hypoxia on blood parameters of juvenile paddlefish. Data are presented as mean ±

standard error. When significant differences were present, a *post-hoc* Holm-Sidak multiple comparison test was used to isolate the treatment differences.

3.5 Results

There were no differences among treatments for any of the water quality parameters measured. Mean ±SE values were: pH (7.28 ±0.12), ammonia (0.25 ±0.03 mg/ L), and nitrite (0.07 ±0.01). There were no differences in mean weights (153 ±6.44 g) and eye-fork lengths (33 ±1.03 cm; eye to fork of tail) among treatments. For fish exposed to normoxia, there was no effect of time on any parameter, thereby allowing this treatment group to be used as a control.

All fish exposed to extreme hypoxia either lost equilibrium or died before the 24 h sampling time. Therefore, fish exposed to this treatment were only analyzed for 0.25, 2 and 6 h. No mortalities occurred in any of the other treatments during the experiment. Paddlefish were observed hyperventilating for the first 10-20 minutes of exposure to moderate and extreme hypoxia. This response ceased until about 4 h later when periodic increases in mouth gape and swimming speed in a potential hyperventilation mode were observed for about 2 minutes. This brief and intermittent hyperventilation was observed approximately 2-3 times every day.

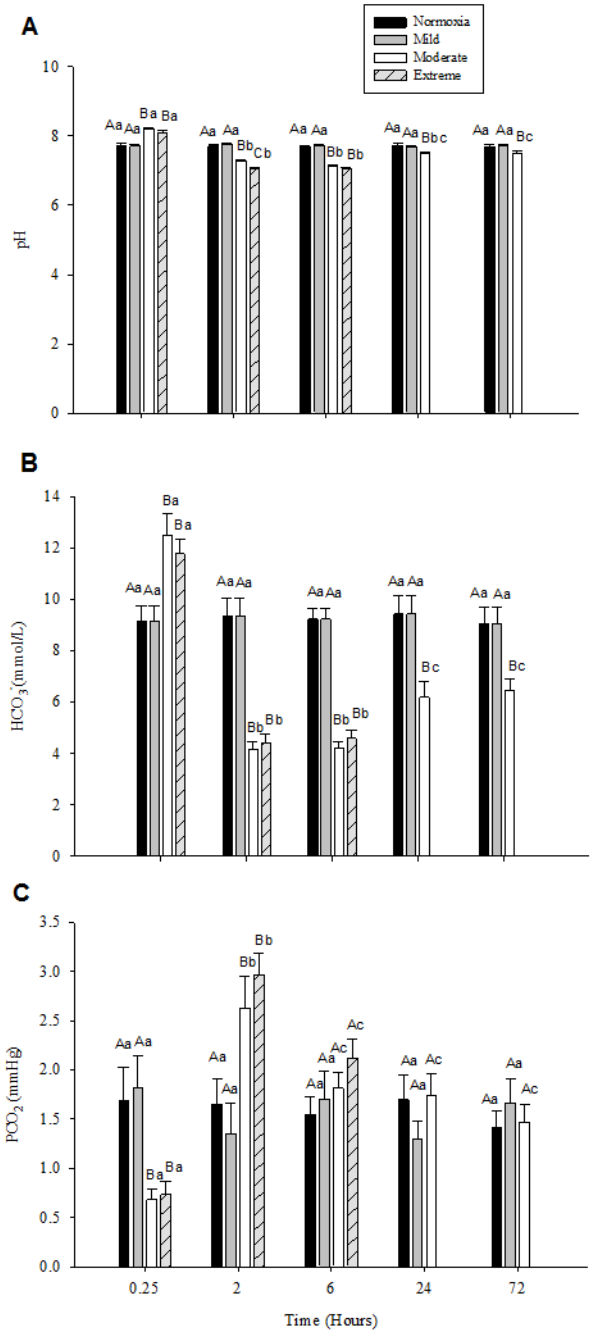

Figure 3.1 Mean plasma pH (A), HCO_3^- (B) and pCO_2 for juvenile American paddlefish.

Figure 3.1 represents mean plasma pH (A), HCO_3^- (B) and pCO_2 for juvenile American paddlefish, *Polyodon spathula*, exposed to different water pO_2s [normoxia (148 ±3 mm Hg) or mild hypoxia (89 ±3 mm Hg) or moderate hypoxia (59 ±3 mm Hg) or extreme hypoxia (36 ±3 mm Hg)] with time at 21 °C. Bars with different capital case letters indicate significant difference between pO_2 treatments (normoxia, mild and moderate hypoxia). Bars with different lower case letters indicate significant difference between exposure time within a particular pO_2 ($p < 0.05$, n=8/ treatment; two-way ANOVA; *post hoc:* Holm-Sidak multiple comparison test).

Exposure of fish to mild hypoxia did not affect blood pH (Fig. 3.1A), plasma HCO_3^- (Fig. 3.1B) or blood pCO_2 (Fig. 3.1C). In comparison, pH and HCO_3^- for fish exposed to moderate and extreme hypoxia increased after 0.25 h, and thereafter decreased below the level of normoxic fish (Fig. 3.1 A and B). Blood pCO_2 decreased after 0.25 h of exposure to moderate and extreme hypoxia, increased beyond normoxic levels after 2 h, and then decreased back to normoxic levels at 6 h and thereafter (Fig 3.1C).

Blood pO_2 of paddlefish decreased with decreasing water pO_2 at 0.25, 24 and 72 h (Fig. 3.2A). At 2 and 6 h, there was no difference between moderate and deep hypoxia. Oxygen saturation of paddlefish blood did not change with exposure to mild hypoxia (Fig. 3.2B), but decreased with exposure to both moderate and extreme hypoxia at all time points (Fig. 3.2B). The total oxygen content of paddlefish blood did not change with exposure to mild hypoxia over time. It decreased with a further decrease in water pO_2 at 0.25, 2 and 6 h (Fig. 3.2C). At 24 h the oxygen content of paddlefish exposed to moderate hypoxia increased to the level of normoxic fish, and then decreased below the level of normoxic fish at 72 h.

RBC count showed no change with exposure to mild hypoxia but increased at 24 and 72 h in fish exposed to moderate hypoxia (Table 3.1). RBC count did not change in fish exposed to extreme hypoxia at 0.25, 2 and 6 h. Blood Hct level did not change under mild hypoxia conditions but increased under moderate and extreme hypoxia conditions throughout the experiment (Table 3.1). Blood Hb concentration increased in fish exposed to moderate hypoxia at 24 h (Table 3.1).

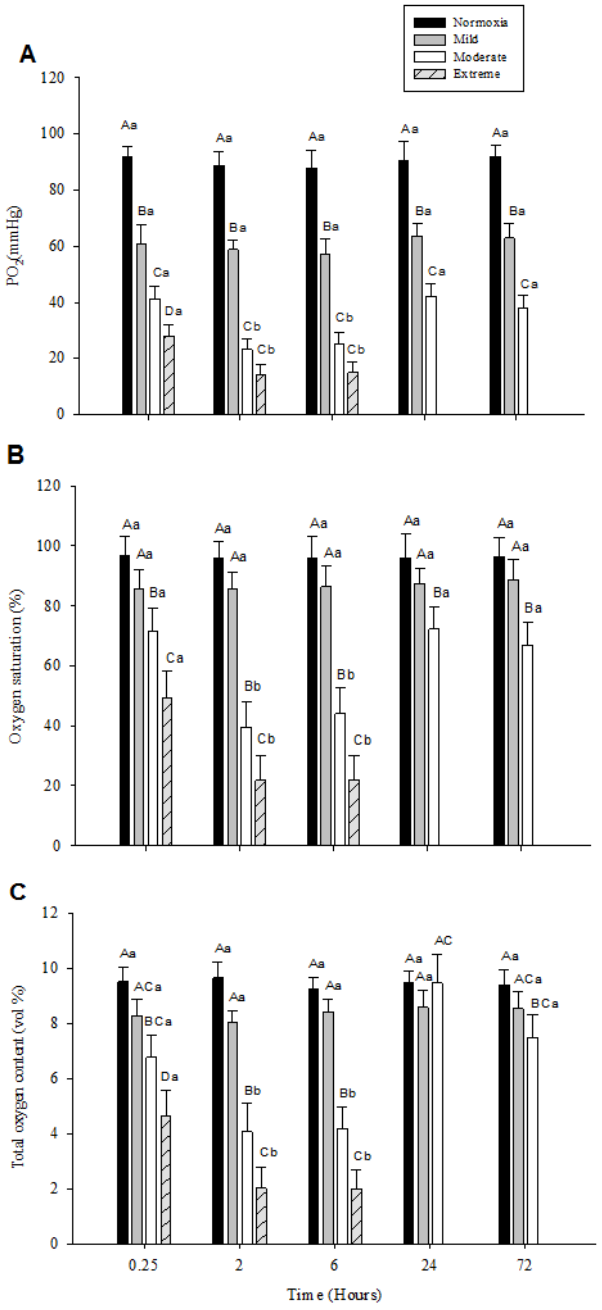

Figure 3.2 Mean blood pO_2 (A), oxygen saturation (B) and content (C) for juvenile American paddlefish.

Figure 3.2 shows mean blood pO_2 (A), oxygen saturation (B) and content (C) for juvenile American paddlefish, *Polyodon spathula*, exposed to different water pO_2 [normoxia (148±3 mm Hg) or mild hypoxia (89 ±3 mm Hg) or moderate hypoxia (59 ±3 mm Hg) or extreme hypoxia (36 ±3 mm Hg)] with time at 21 ºC. Bars with different upper case letters indicate significant difference between pO_2 treatments (normoxia, mild and moderate hypoxia). Bars with different lower case letters indicate significant difference between exposure time within a particular pO_2 ($p < 0.05$, n=8/ treatment; two-way ANOVA; *post hoc:* Holm-Sidak multiple comparison test).

Table 3.1 Mean (±SE) hematological values for juvenile American paddlefish.

Treatment		Red blood cell count	Hematocrit	Hemoglobin
PO_2(mmHg)	Time (H)	(10^6/µl)	(%)	(g/dl)
148	**0.25**	1.60 ±0.09 ^{Aa}	23.88 ±1.97 ^{Aa}	7.03 ±0.37 ^{Aa}
	2	1.55 ±0.11 ^{Aa}	24.13 ±2.00 ^{Aa}	7.20 ±0.40 ^{Aa}
	6	1.53 ±0.08 ^{Aa}	22.63 ±1.38 ^{Aa}	6.89 ±0.30 ^{Aa}
	24	1.58 ±0.09 ^{Aa}	24.38 ±1.66 ^{Aa}	7.09 ±0.38 ^{Aa}
	72	1.48 ±0.13 ^{Aa}	23.25 ±1.83 ^{Aa}	6.98 ±0.44 ^{Aa}
89	**0.25**	1.54 ±0.12 ^{Aa}	24.13 ±2.26 ^{Aa}	6.96 ±0.36 ^{Aa}
	2	1.55 ±0.13 ^{Aa}	24.75 ±2.02 ^{Aa}	6.85 ±0.39 ^{Aa}
	6	1.70 ±0.12 ^{Aa}	24.88 ±1.81 ^{Aa}	7.05 ±0.36 ^{Aa}
	24	1.70 ±0.16 ^{Ab}	25.00 ±2.25 ^{Aa}	7.09 ±0.43 ^{Aa}
	72	1.81 ±0.20 ^{Aa}	24.25 ±2.01 ^{Aa}	6.91 ±0.37 ^{Aa}
59	**0.25**	1.61 ±0.20 ^{Aa}	36.63 ±2.00 ^{Ba}	6.89 ±0.32 ^{Aa}
	2	1.72 ±0.12 ^{Aa}	39.00 ±1.72 $_{Ba}$	7.05 ±0.38 ^{Aa}
	6	1.73 ±0.13 ^{Aa}	38.25 ±1.81 ^{Ba}	6.81 ±0.36 ^{Aa}
	24	2.03 ±0.09 ^{Bab}	39.50 ±1.42 ^{Ba}	9.43 ±0.38 ^{Bb}
	72	2.53 ±0.15 ^{Bb}	41.13 ±1.90 ^{Ba}	8.20 ±0.37 ^{Aa}
36	**0.25**	1.70 ±0.14 ^{Aa}	34.88 ±1.98 ^{Ba}	6.84 ±0.42 ^{Aa}
	2	1.60 ±0.13 ^{Aa}	40.38 ±1.15 ^{Ba}	7.00 ±0.37 ^{Aa}
	6	1.75 ±0.16 ^{Aa}	39.00 ±2.03 ^{Ba}	6.78 ±0.37 ^{Aa}
	24	-	-	-
	72	-	-	-

Table 3.1 represents mean (±SE) hematological values for juvenile American paddlefish, *Polyodon spathula*, exposed to different water pO_2s [normoxia (148±3 mm Hg) or mild hypoxia (89 ±3 mm Hg) or moderate hypoxia (59 ±3 mm Hg) or extreme hypoxia (36 ±3 mm Hg)] with time at 21 ºC. Different upper case letters indicate significant difference between pO_2 treatments (148 mm Hg, 89 mm Hg, 59 mm Hg and 36 mm Hg). Different lower case letters indicate significant difference between exposure time within a particular pO_2 ($p < 0.05$, n=8/ treatment; two-way ANOVA; *post hoc:* Holm-Sidak multiple comparison test).

MCV of fish did not change after exposure to mild hypoxia, but increased at 0.25, 2 and 6 h in moderate and extreme hypoxia (Table 3.2). Thereafter, MCV of fish under moderate hypoxia conditions decreased to normoxic levels (Table 3.2). MCH of fish did

not change after exposure to mild and extreme hypoxia, but decreased in those fish exposed to moderate hypoxia at 72 h (Table 3.2). MCHC of fish did not change after exposure to mild hypoxia, but decreased after exposure to moderate hypoxia at 0.25, 2, 6 and 72 h (Table 3.2). Exposure of paddlefish to extreme hypoxia decreased MCHC of fish at 0.25, 2 and 6 h (Table 3.2).

Plasma glucose did not change in fish exposed to mild hypoxia, but increased at 2 and 6 h and then decreased at 24 and 72 h for fish exposed to moderate hypoxia (Fig. 3.3A). Similarly, plasma glucose increased at 0.25, 2 and 6 h for fish exposed to extreme hypoxia (Fig. 3.3A). Exposure of paddlefish to mild hypoxia did not have an effect on lactate, but exposure to moderate hypoxia resulted in increases at 2, 6, 24 and 72 h (Fig. 3.3B). Exposure of fish to extreme hypoxia increased plasma lactate at 0.25, 2 and 6 h (Fig. 3.3B).

Table 3.2 Mean (±SE) red blood cell indices for juvenile American paddlefish.

Treatment		**Mean corpuscular volume**	**Mean corpuscular hemoglobin**	**Mean corpuscular hemoglobin concentration**
PO_2 (mmHg)	Time (H)	(fL)	(pg)	(g/dl)
148	**0.25**	151.45 ±13.22 ^{Aa}	45.46 ±4.22 ^{Aa}	30.85 ±2.89 ^{Aa}
	2	161.20 ±16.79 ^{Aa}	47.81 ±3.96 ^{Aa}	31.28 ±2.42 ^{Aa}
	6	150.22 ±11.12 ^{Aa}	45.63 ±2.41 ^{Aa}	30.86 ±1.37 ^{Aa}
	24	154.32 ±6.48 ^{Aa}	45.70 ±3.24 ^{Aa}	30.28 ±2.91 ^{Aa}
	72	160.70 ±15.23 ^{Aa}	49.08 ±3.31 ^{Aa}	31.37 ±3.06 ^{Aa}
89	**0.25**	157.88 ±9.83 ^{Aa}	47.53 ±4.62 ^{Aa}	30.77 ±2.59 ^{Aa}
	2	164.66 ±16.41 ^{Aa}	46.26 ±4.18 ^{Aa}	29.40 ±2.46 ^{Aa}
	6	150.28 ±12.48 ^{Aa}	42.24 ±2.23 ^{Aa}	29.17±2.30 ^{Aa}
	24	160.49 ±23.84 ^{Aa}	43.64 ±4.04 ^{Aa}	31.40 ±3.02 ^{Aa}
	72	146.97 ±19.65 ^{Aa}	42.04 ±3.79 ^{ABa}	30.10 ±3.12 ^{Aa}
59	**0.25**	246.29 ±26.63 ^{Ba}	46.11 ±4.62 ^{Aa}	19.00 ±1.23 ^{Ba}
	2	234.98 ±19.75 ^{Ba}	42.97 ±4.56 ^{Aa}	18.57 ±1.91 ^{Ba}
	6	226.62 ±15.19 ^{Bac}	40.20 ±2.53 ^{Aa}	18.16 ±1.53 ^{Ba}
	24	196.92 ±8.65 ^{Aac}	47.13 ±2.82 ^{Aa}	24.03 ±1.27 ^{Aa}
	72	167.60 ±13.18 ^{Abc}	33.50 ±2.95 ^{Ba}	20.09 ±1.88 ^{Ba}
36	**0.25**	214.30 ±16.92 ^{Ba}	42.26 ±4.40 ^{Aa}	20.37 ±2.16 ^{Ba}
	2	264.83 ±17.11 ^{Ba}	46.40 ±3.87 ^{Aa}	17.30 ±1.98 ^{Ba}
	6	234.75 ±16.86 ^{Ba}	41.52 ±4.01 ^{Aa}	17.70 ±2.26 ^{Ba}
	24	-	-	-
	72	-	-	-

Table 3.2 represnts mean (±SE) red blood cell indices for juvenile American paddlefish, *Polyodon spathula*, exposed to different water pO_2 [normoxia (148±3 mm Hg) or mild hypoxia (89 ±3 mm Hg) or moderate hypoxia (59 ±3 mm Hg) or extreme hypoxia (36 ±3 mm Hg)] with time at 21 ºC. Different upper case letters indicate significant difference between pO_2 treatments (148 mm Hg, 89 mm Hg, 59 mm Hg and 36 mm Hg). Different lower case letters indicate significant difference between exposure time within a particular pO_2 ($p < 0.05$, n=8/ treatment).

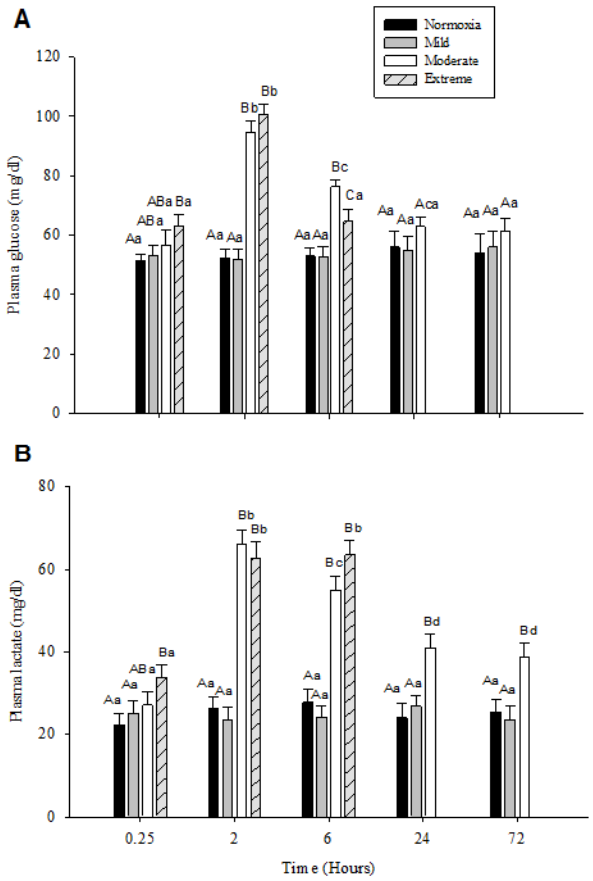

Figure 3.3 Mean plasma glucose (A) and lactate (B) for juvenile American paddlefish

Figure 3.3 shows mean plasma glucose (A) and lactate (B) for juvenile American paddlefish, *Polyodon spathula*, exposed to different water pO_2 [normoxia (148 ±3 mm Hg) or mild hypoxia (89 ±3 mm Hg) or moderate hypoxia (59 ±3 mm Hg)] or deep hypoxia (36 ±3 mm Hg) with time at 21 ºC. Bars with different capital letters indicate significant difference between pO_2 treatments (normoxia, mild and moderate hypoxia). Bars with different small letters indicate significant difference between exposure timewithin a particular pO_2 ($p < 0.05$, n=8/ treatment; two-way ANOVA; *posthoc*: Holm-Sidak multiple comparison test).

Exposure to mild hypoxia had no effect on plasma Na^+, but exposure to moderate and extreme hypoxia, decreased plasma Na^+ at 0.25, 2 and 6 h. Plasma Na^+ of moderate hypoxic fish then increased back to normoxic levels at 24 and 72 h (Table 3.3). Plasma Cl^- for fish exposed to mild, moderate and extreme hypoxia followed the same pattern as Na^+ (Table 3.3). There was no effect of hypoxia on K^+ in fish blood plasma. Exposure of paddlefish to mild hypoxia had no effect on plasma osmolality, but exposure to moderate hypoxia decreased plasma osmolality at 0.25, 2, 6 and 24 h before increasing to the level

of normoxic fish at 72 h (Table 3.3). For fish exposed to extreme hypoxia, plasma osmolality decreased at 0.25, 2 and 6 h (Table 3.3).

Table 3.3 Mean plasma ion concentrations for juvenile American paddlefish.

Treatment		Na^+	Cl^-	K^+	Osmolality
PO_2 (mmHg)	Time (H)	(meq/L)	(meq/L)	(meq/L)	(mOsmol/kg)
148	**0.25**	124.25 ±3.81 ^{Aa}	104.38 ±3.22 ^{Aa}	2.79 ±0.12 ^{Aa}	307.75 ±4.05 ^{Aa}
	2	122.38 ±4.34 ^{Aa}	103.63 ±4.10 ^{Aa}	2.59 ±0.11 ^{Aa}	309.50 ±4.84 ^{Aa}
	6	123.25 ±3.53 ^{Aa}	105.13 ±3.86 ^{Aa}	2.71 ±0.09 ^{Aa}	305.38 ±4.53 ^{Aa}
	24	124.63 ±3.78 ^{Aa}	101.87 ±3.48 ^{Aa}	2.62 ±0.10 ^{Aa}	312.00 ±4.88 ^{Aa}
	72	121.25 ±3.66 ^{Aa}	104.13 ±2.58 ^{Aa}	2.67 ±0.09 ^{Aa}	308.88 ±4.78 ^{Aa}
89	**0.25**	119.00 ±5.52 ^{Aa}	104.13 ±4.41 ^{Aa}	2.64 ±0.15 ^{Aa}	303.25 ±5.90 ^{Aa}
	2	122.38 ±5.71 ^{Aa}	100.75 ±4.71 ^{Aa}	2.70 ±0.16 ^{Aa}	305.63 ±4.57 ^{Aa}
	6	121.63 ±5.18 ^{Aa}	106.00 ±4.97 ^{Aa}	2.70 ±0.13 ^{Aa}	302.63 ±6.19 ^{Aa}
	24	120.00 ±5.97 ^{Aa}	103.50 ±3.78 ^{Aa}	2.75 ±0.10 ^{Aa}	302.63 ±3.58 ^{Aa}
	72	121.38 ±5.22 ^{Aa}	104.25 ±4.75 ^{Aa}	2.68 ±0.11 ^{Aa}	300.25 ±5.27 ^{ABa}
59	**0.25**	84.63 ±4.55 ^{Ba}	78.63 ±6.63 ^{Ba}	2.36 ±0.32 ^{Aa}	277.50 ±4.61 ^{Ba}
	2	95.75 ±6.34 ^{Bab}	82.13 ±5.76 ^{Ba}	2.11 ±0.29 ^{Aa}	279.63 ±4.13 ^{Ba}
	6	97.75 ±5.61 ^{Bab}	83.00 ±6.62 ^{Ba}	2.15 ±0.30 ^{Aa}	281.00 ±3.85 ^{BCa}
	24	110.88 ±6.25 ^{Ab}	102.13 ±6.21 ^{Ab}	2.63 ±0.36 ^{Aa}	281.13 ±4.05 ^{Ba}
	72	115.38 ±5.78 ^{Ab}	108.50 ±4.85 ^{Ab}	2.19 ±0.31 ^{Aa}	294.38 ±4.50 ^{Aa}
36	**0.25**	82.63 ±5.83 ^{Ba}	77.00 ±4.61 ^{Ba}	2.38 ±0.21 ^{Aa}	274.13 ±3.92 ^{Ba}
	2	92.25 ±4.11 ^{Ba}	80.25 ±4.81 ^{Ba}	2.28 ±0.32 ^{Aa}	281.88 ±4.43 ^{Ba}
	6	98.38 ±4.82 ^{Ba}	85.25 ±4.58 ^{Ba}	2.19 ±0.27 ^{Aa}	284.76 ±4.26 ^{BCa}
	24	-	-	-	-
	72	-	-	-	-

Table 3.3 represents mean plasma ion concentrations for juvenile American paddlefish, *Polyodon spathula*, exposed to different water pO_2 [normoxia (148 ±3 mm Hg) or mild hypoxia (89 ±3 mm Hg) or moderate hypoxia (59 ±3 mm Hg) or extreme hypoxia (36 ±3 mm Hg)] with time at 21 °C. Different upper case letters indicate significant difference between pO_2 treatments (148 mm Hg, 89 mm Hg, 59 mm Hg and 36 mm Hg). Different lower letters indicate significant difference between exposure time within a particular pO_2 ($p < 0.05$, n=8/ treatment).

3.6 Discussion

This study describes the physiological changes occurring in juvenile paddlefish during 72 h of exposure to water with different pO_2s (> 145 mm Hg, 89 mm Hg, 59 mm Hg and 36 mm Hg). As a basal bony fish, the unique respiratory strategy of ram ventilation appears to require a reliance on anaerobic metabolism at relatively high pO_2s compared to that of most fishes. Paddlefish respond to acute hypoxia by a brief respiratory alkalosis followed by hypoxemia, hyperglycemia, acidosis, RBC swelling, ion loss, and die if they are unable to compensate (at extreme hypoxia: 36 mm Hg). Survival under hypoxic conditions was presumably related to increases in RBCs and Hb which led to improved oxygen transport and blood oxygen content during chronic hypoxia exposure.

3.6.1 Response to acute (< 24 h) hypoxia

Acute hypoxic exposure altered acid-base regulatory systems. As the maintenance of an internal pH environment is essential for the optimum function of enzymes and stability of proteins (Somero 1969; Shaklee et al. 1977; Brauner and Baker 2009), the survival of paddlefish may depend in part on the ability of its regulatory systems to maintain a constant pH under changing environmental conditions.

The rise and fall in pH and HCO_3^- of fish exposed to acute hypoxia reflect the reduction in oxygen diffusion across the gills (Cech et al. 1979), the subsequent reduction in oxygen saturation and content, and the extent of compensatory ability of paddlefish. In paddlefish exposed to moderate hypoxia for 0.25 h, the increase in pH and HCO_3^- coupled with the reduction of pCO_2, apparently without anaerobic respiration occuring, suggest that hyperventilation is used in an attempt to compensate for reduced oxygen

uploading at the gills. Hyperventilation increases water volume flowing over the gills, and thereby increases the diffusion gradient between the water and blood (Jensen et al. 1993; Brauner and Wang 1997). Hyperventilation is typically accompanied by a respiratory alkalosis (increased HCO_3^- and pH) as CO_2 excretion is enhanced (Gilmour 2001; Perry and Gilmour 2006), similar to what was observed in this study. Increase in ventilation and associated alkalosis in response to hypoxia has been shown in fishes with a range of hypoxia tolerance such as carp, *Cyprinus carpio* (Glass et al. 1990), sea bass, *Dicentrarchus labrax* (Thomas and Hughes 1982a), and rainbow trout, *Oncorhynchus mykiss* (Thomas et al. 1982b, 1986; Iwama et al. 1987). Increased blood pH also increases Hb-oxygen affinity (Bohr effect) allowing for greater oxygen loading (Nikinmaa and Salama 1998).

Sustaining hyperventilation is problematic for paddlefish due to their reliance on ram ventilation. Paddlefish, which constantly swim at 70-80% of their maximum sustainable speed, only use buccal pumps when they are physically restrained and limited to swimming at speeds of ≤0.6 body lengths per second. Under these conditions, they become extremely stressed and sometimes lose equilibrium (Burggren and Bemis 1992). Therefore, active ventilation is inefficient and paddlefish may functionally hyperventilate by increasing ram ventilation through increased swimming activity (Burggren and Bemis 1992; Chapter 2). However, as increased swimming speed increases metabolic rate (Burggren and Bemis 1992; Chapter 2) and therefore energetic demand, this method of hyperventilation is presumed to be energetically inefficient and unsustainable. Paddlefish also increase ventilation when water pO_2 decreases by enlarging mouth gape to increase the volume of water flow over the gills (Chapter 2). This type of hyperventilation has

been observed in other species including: skipjack tuna, *Katsuwonus pelamis* (Brown and Muir 1970), bluefin tuna, *Thunnus maccoyii* (Fitzgibbon 2010), rainbow trout and the live sharksucker, *Echeneis naucrates* (Steffensen 1985).

It has been suggested that hypoxia-induced respiratory alkalosis in fish increases the oxygen capacity and facilitates oxygen off-loading to tissues (Maxime et al. 2000). The increase in pCO_2 with a corresponding increase in lactate concentrations after 2 h in moderate and extreme hypoxia suggest that both respiratory and metabolic acidosis may have countered the effect of respiratory alkalosis at 2 h, leading to a sharp decrease in pH. Increased pCO_2 suggests that respiratory acidosis likely occurred because accumulated CO_2 was not efficiently eliminated (Perry and Gilmour 2006). The elimination of CO_2 is dependent on the carbonic anhydrase-catalyzed reversible-reactions of CO_2 and the acid base relevant ions (H^+ and HCO_3^-; Perry and Gilmour 2006). Through this reaction most of the CO_2 in the blood is in the form of HCO_3^- (Albers 1970; Tufts and Perry 1998). Correspondingly, acid-base regulation in most fishes is achieved at the gills through adjustment of plasma HCO_3^- concentration through the exchange of H^+ and HCO_3^- with Na^+ and Cl^-, respectively (Perry and Gilmour 2006; Brauner and Baker 2009). The decreased HCO_3^- at 2 and 6 h for paddlefish exposed to moderate and extreme hypoxia suggests that a large portion was excreted at the gills as CO_2. The reduction in HCO_3^- concentration below the level of normoxic fish at 2 and 6 h may be due to enhanced Cl^-/HCO_3^- exchange to restore the Cl^- pool (Heisler 1984), which had decreased during the same time period.

Increased glucose and lactate concentrations, as previously observed in sturgeons (Cech and Doroshov 2004) and other fishes (Barton 1997; Pankhurst and Van Der Kraak

1997; Wendelaar Bonga 1997; Falahatkar et al. 2009; Van Landeghem 2010; Rapp et al. 2012), indicate a partial reliance on anaerobic metabolism. The oxygen consumption rate and swimming capabilities of paddlefish are known to decrease when exposed to hypoxia (Chapter 2). Therefore, the decrease in blood pO_2 and the elevation in blood pCO_2, and glucose and lactate concentrations after acute moderate and extreme hypoxia exposure suggest that hyperventilation was not an effective response, causing a switch to anaerobic metabolism to compensate for the loss of aerobic energy production (Holeton and Randall 1967). Paddlefish have been observed to have a pO_{2crit} (pO_2 at which fish switch to anaerobic metabolism because aerobic metabolic demand cannot be sustained by ambient pO_2) of 74-90 mm Hg (Burggren and Bemis 1992; Chapter 2). This pO_{2crit} is higher than those reported for other relatively hypoxia-sensitive species such as Adriatic sturgeon, *Acipenser naccarri* (McKenzie et al. 2007), Siberian sturgeon, *A. baeri* (Nonnotte et al. 1993) and rainbow trout (Ott et al. 1980). The reduction in glucose concentration in moderate and extreme hypoxic fish after 6 h may be an indication that stored energy reserves were quickly exhausted and may explain the mortalities observed in extreme hypoxic fish in this study and that of Burggren and Bemis (1992). Hypoxia-tolerant fishes are known to conserve energy reserves and extend survival in hypoxic environments through metabolic depression, thereby delaying reliance on anaerobic metabolism (Richards 2009). Because paddlefish are obligatory ram ventilators, they have limited capacity to reduce metabolic rate. Thus, the need to utilize anaerobic metabolism exhausts the limited energy reserves and likely reduces the survival time in moderate and extreme hypoxia (Bicker and Buck 2007).

Changes in hematological parameters may signify hemoconcentration or hemodilution of fish blood, and may also be an indication of increased erythropoiesis or epinephrine-induced RBC swelling (McDonald et al. 1991; Morgan and Iwama 1997; Barton et al. 2002; Jensen, 2004). In the present study, during the first 6 h after exposure to moderate and extreme hypoxia, the absence of change in RBC number and increases in MCV indicate that the increases in Hct were due to RBC swelling. The decline in Na^+, Cl^-, and osmolality levels further suggests a rapid hemodilution, presumably due to increased gill permeability (McDonald and Milligan 1997). The overall changes in blood ions (Cl^- and Na^+) and osmolality found in paddlefish are similar to the response of some freshwater fishes to hypoxia (Baldisserotto et al. 2008) and other environmental stressors (McDonald and Milligan 1997), whereby elevation of epinephrine levels correlate with increased ion efflux across the gills (Mazeaud and Mazeaud 1981; McDonald and Rogano 1986). Release of epinephrine into the circulatory system causes an increase in aortic blood pressure, due to vasoconstriction and elevated cardiac output, which leads to increased permeability of the gills to ions (Mazeaud and Mazeaud 1981; McDonald and Milligan 1997). In addition, the attempt by hypoxic fish to increase oxygen uptake by increasing lamellar perfusion, and thereby increasing the functional surface area of the gill, also led to the inadvertent loss of ions such as Cl^- and Na^+ to the environment. This specific loss of ions across the gills, referred to as osmorespiratory compromise (Nilsson, 1986) has been observed in many fishes (Gonzalez and McDonald 1992; McDonald and Milligan 1997). The reduced pH of the blood could also inhibit enzymes and related ion transporters, thereby reducing the influx of associated ions (Evans et al. 2005).

3.6.2 Response to chronic (≥ 24 h) hypoxia

In paddlefish, the response to chronic hypoxia was characterized by compensatory reductions of blood pH and oxygen content and an increase of ion losses. The inability of paddlefish to buffer pH after hypoxic exposure is likely due to the reduction in HCO_3^-, which correspondingly reduces the buffering capacity of HCO_3^-. Also, like many other water breathers, the low blood pCO_2 values of paddlefish (~1.6 mm Hg), limit long-term ventilatory pH regulation because the success of this strategy depends on a high pCO_2 gradient between the water medium and the blood (Rahn 1966; Heisler 1986; Dejours 1988; Evans et al. 2005; Perry and Gilmour 2006). Fishes and other aquatic vertebrates known to adjust blood pH through long-term hyperventilation are reported to have a much higher blood pCO_2 (> 20 mm Hg) (Truchot 1987; Evans et al. 2005). In addition, the metabolic cost of sustaining hyperventilation for long durations may be too high for hypoxic paddlefish (Evans et al. 2005). Like most fishes, paddlefish rely on the net transfer of acid-base relevant ions across the gill, between water and blood, to maintain pH homeostasis during hypoxia-induced acid-base perturbations (Heisler 1984; Evans et al. 2005; Perry and Gilmour 2006; Brauner and Baker 2009). In fishes, the net transport of acid-base relevant ions to maintain blood pH homeostasis is achieved through elevation of plasma HCO_3^-, which is accomplished through exchange of plasma Cl^- with environmental HCO_3^- (Heisler 1984; Claiborne et al. 2002; Evans et al. 2005; Brauner and Baker 2009). This study indicates that recovery from acidosis appears to be slower in paddlefish compared to that of other acipenseriformes. White sturgeon, *Acipenser transmontanus*, recover from severe acidosis within 24 h through increase in HCO_3^- and a corresponding decrease in Cl^- (Baker et al. 2009). The inability of paddlefish, exposed to

moderate hypoxic conditions, to compensate for pH after 72 h may be due to a constant production of H^+ via respiratory and metabolic acidosis, resulting from their inability to depress metabolic activity to the degree of other acipenseriforms (Burggren and Bemis 1992; Crocker and Cech 1997; Chapter 2). The timeline for blood pH recovery has been shown to vary among fishes (Burggren and Cameron 1980; Scott and Rogers 1981; Kakizawa et al. 1997; Brauner and Baker 2009). Generally, the duration of time for pH recovery through the net transfer of acid-base relevant ions ranges from a few h to 4 days in fish (Larsen and Jensen 1997; Brauner and Baker 2009).

The high basal activity level of paddlefish necessitated by obligatory ram ventilation requires a relatively high internal oxygen concentration to maintain routine metabolism compared to other fishes (Burggren and Bemis 1992; Chapter 2). In this study, normoxic fish had relatively high Hb concentrations, similar to that of some facultative ram ventilators like rainbow trout (Lai et al. 2006), epaulette shark, *Hemiscyllium ocellatum* and grey carpet shark, *Chiloscyllium punctatum* (Chapman and Renshaw, 2009), presumably to facilitate this oxygen requirement (Wells 2009). Therefore, the drop in oxygen content due to acute hypoxia exposure reduced the ability of the fish to maintain routine aerobic metabolism. Compensation to maintain adequate oxygen through elevated Hb via RBC release, did not occur until 24 h. The delayed increase in Hb concentrations indicates that oxygen supply was likely insufficient after the initial exposure to moderate and extreme hypoxia, resulting in low oxygen content of the blood at 2 and 6 h. This delayed response to increase blood oxygen carrying capacity may have led to mortalities of fish exposed to extreme hypoxia. For most teleosts, erythropoiesis and corresponding Hb elevation usually occur within a few minutes to

hours in response to hypoxia (Nikinmaa 2006). Although paddlefish appear to have a delayed capacity for Hb increase, they may compensate by maintaining higher baseline concentrations of Hb than most teleosts. When compared to teleosts, basal actinopterygians have higher Hb buffer values (Wood and LeMoigne 1991; Berenbrink et al. 2005; Regan and Brauner 2010; Harter et al. 2014), and similarly high Hb concentrations for paddlefish in this study have been observed in bowfin, *Amia calva* (Weber et al. 1976; Randall et al. 1981), Cuban gar, *Atractosteus tristoechus* (Siret et al. 1976) and spotted gar, *Lepisosteus oculatus* (Smatresk and Cameron 1982). The high Hb concentrations are a mechanism for buffering blood pH through Hb-H^+ binding. The descendants of the basal actinopterygians possess RBC beta adrenergic Na^+/H^+ exchangers (βNHEs) which buffer pH through Na^+-H^+ exchange, and have been suggested as developing in association with *retia mirabilia* to protect oxygen secretion to the eye (Berenbrink et al. 2005).

The reduction in ion losses observed at 24 and 72 h may be due to a reduction in blood epinephrine concentration, which would thereby cause reductions in heart rate and blood pressure as well as gill permeability. Although epinephrine concentration was not determined in this study, ion losses in fish have been shown to be highly correlated with elevated concentrations of epinephrine in the blood (Mazeaud and Mazeaud 1981; McDonald and Milligan 1997). Increase in the Hb concentration and the corresponding increase in oxygen content suggest that less lamellae will be utilized for oxygen uptake, thereby reducing the functional surface area of the gill exposed to water and decreasing the rate of loss of ions. Recovery of ions could also be due to the activation of previously inactive transport sites on the gills (Postlethwaite and McDonald 1995).

3.6.3 Ecological implications of hypoxia

The sensitivity of paddlefish to hypoxia indicates that they may require a high DO concentration to ensure survival in natural habitats. It has been observed that sublethal effects of hypoxia can have a strongly adverse impact on fish habitat quality (Arend et al. 2011). As such, hypoxia-induced migrations have been observed in many fishes (Jones 1952; Schurmann et al. 1998; Breitburg 2002; Arend et al. 2011). Based on the findings of this study, and given the high migratory capabilities of paddlefish (Rosen et al. 1982; Mettee and O'neil 2009), paddlefish probably avoid water with a $pO_2 \leq 59 \pm 3$ mm Hg (3.57 mg/ L) in an attempt to avoid hypoxic stress.

In conclusion, this study shows that paddlefish can compensate for acute and chronic exposure to mild hypoxia, but not moderate and extreme hypoxia. Acute responses to hypoxia involved hyperventilation to increase oxygen supply and the utilization of anaerobic metabolism at relatively high water pO_2s as compared to most fishes. At extreme hypoxia (36 mm Hg), paddlefish died, presumably due to a delayed increase in blood oxygen capacity as well as an inability to compensate for acidosis and ion loss in a timely manner. The ability of paddlefish to survive chronic hypoxic exposure appears dependent on the severity of hypoxia and its ability to effect long-term compensatory adjustments to maintain adequate oxygen supply to the tissues. Therefore, DO concentrations are of primary concern for habitats utilized by paddlefish and should be seriously considered by managers and biologists.

3.6.4 References

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CHAPTER IV

^{1}H-NMR STUDY OF THE METABOLIC RESPONSE OF AMERICAN PADDLEFISH TO ACUTE AND CHRONIC HYPOXIA

4.1 Abstract

Hypoxia is a global problem in aquatic ecosystems, affecting the metabolism and survival of fishes, and is an increasing problem in the natural habitats of the American paddlefish (*Polyodon spathula)*. Exposure of paddlefish to acute and chronic hypoxia is known to cause hyperglycemia, acidosis, hypoxemia and potentially death. To provide a better understanding of the physiological response of paddlefish to hypoxia and characterize associated biomarkers, 1-D and 2-D J-resolved ^{1}H NMR were used to analyze metabolite changes in muscle tissue of paddlefish exposed to either normoxia (148 ±3 mm Hg; 100% saturation; 8.92 mg/ L) or acute (0.25 h) or chronic (72 h) hypoxia (59 ±3 mm Hg; 40% saturation; 3.57 mg/ L) at 21 ºC. In addition, muscle glycogen concentrations were determined to understand the effect of acute and chronic hypoxia on energy reserves. Observed changes in metabolites included increases in inosine (indicative of increased muscle function) and phosphocholine (indicative of immune suppression) and a depletion of both aerobic (glucose, NAD^{+}, and AMP/ADP) and anaerobic (glycogen) energy stores. Related changes included increases in lactate (suggesting the activation of anaerobic metabolism), creatine, myo-inositol, and 3-hydroxybutyrate (indicating the utilization of alternative energy sources during hypoxia).

This study suggests that depletion of energy stores (i.e., glucose and glycogen) and resulting increase in lactate are important factors influencing metabolism and survival of paddlefish during hypoxia.

Keywords: Acipenseriformes, metabolomics, NMR, hypoxia, paddlefish, glucose, stress

4.2 Introduction

The availability of oxygen is a major abiotic variable in aquatic ecosystems, and exerts a strong species-selective force because of its role in providing energy to fuel physiological processes (Hughes 1973). Low dissolved oxygen (DO) or hypoxia may lead to physiological stress and sometimes death in fishes (Diaz and Rosenberg 1995, 2001, 2008; Breitburg 2002; Breitburg et al. 2003; Diaz and Breitburg 2009). In fishes, perceived hypoxia has been described by Richards (2011) as the partial pressure of oxygen (pO_2) at which physiological functions are first compromised and aerobic metabolic rate can no longer be sufficiently maintained.

The ability to adapt to a particular environment is an important factor in establishing species distributions among heterogeneous environments (Mandic et al. 2009). Organisms that have a greater capability to acquire oxygen are able to maintain a routine metabolic rate at lower oxygen tensions and have a greater chance to survive in hypoxic environments (Mandic et al. 2009). Fishes with limited capabilities to tolerate reduced environmental oxygen concentrations may disappear from an area where hypoxia prevails (Doudoroff and Shumway 1970; Kramer 1987).Therefore, the threat of biodiversity loss and the corresponding effect on the assemblages of species are partially

linked to the effect of hypoxia on metabolism, particularly growth, development and survival (Wu 2002, 2009).

Because of the frequency and pervasiveness of hypoxia in aquatic ecosystems, resulting both from natural and anthropogenic causes, fishes have developed various responses to hypoxia. Behavioral responses to hypoxia include avoidance (Jones 1952; Schurmann et al. 1998; Breitburg 2002), the use of various air breathing organs (Graham et al. 1977; Graham 1983) or gill ventilation with the oxygen-rich water from the air-water interface (Gee and Gee 1991; Kramer and McClure 1982). Physiological and biochemical responses generally involve an initial attempt to increase their ability to maintain oxygen delivery to meet respiratory needs (Wu 2002), which is achieved through hyperventilation (Randall 1970; Wu and Woo 1984), increases in the number of red blood cells and increases in the oxygen binding capacity of hemoglobin (Wood and Johansen 1972, Wood et al. 1975; Wood 1980; Soldatov 1996). Other hypoxia response mechanisms include increases in anaerobic metabolism (Hochachka and Somero 1984; Dunn and Hochachka 1986, 1987; Hochachka 1986; Hochachka et al. 1996; Nilsson and Renshaw 2004) and depression of overall energy metabolism (Hochachka and Dunn 1983; Dalla Via et al. 1994; Schurmann and Steffensen 1994; Zhou et al. 2000), so that energy demands during hypoxia can be temporarily met (Bickler and Buck 2007).

Anaerobic metabolism is associated with an inefficient use of substrates and accumulation of lactate (Dunn and Hochachka 1986; 1987; Dalla Via et al. 1994; Bickler and Buck 2007), nitric oxide (Hansen and Jensen 2010) and H^+ (Claiborne et al. 2002). Therefore, the most common strategy for long-term survival of fish in a hypoxic environment is metabolic depression (Bickler and Buck 2007). The question then arises,

how does a fish, such as the American paddlefish (*Polyodon spathula)*, which totally depends on aquatic gas exchange and has limited capacity for metabolic depression contend with hypoxic conditions? Paddlefish are endemic to the Mississippi River drainage basin and nearby Gulf of Mexico drainages (Jennings and Ziegler 2000; Mims 2001; Horvath et al. 2006; Paukert and Scholten 2009). They are obligatory ram ventilators and oxyregulators with a relatively high metabolic rate and high sensitivity to hypoxia; with a minimum oxygen requirement of > 2 mg/ L (Burggren and Bemis 1992; Patterson et al. 2013; Chapter 2).

Various experimental methods offer important insight into the form-function-environmental relationships of fish metabolic systems which affect the ability to tolerate different environmental stressors (Iwama et al. 1997; Wells and Pankhurst 1999; Dahlhoff 2004; Horodysky et al. 2011). Most methods utilize traditional measurement tools targeting specific biomarkers. Metabolomics, a relatively new scientific tool, yields an increased understanding of metabolic pathways through the measurement of small, low molecular weight metabolites, which are the intermediate and end products of metabolism (Rochfort 2005; Nicholson and Lindon 2008). Metabolomics is considered to be a "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" (Daviss 2005) and provides a more holistic characterization of the physiological changes occuring at the time of sampling. Metabolomics has its roots in early metabolite profiling studies and is currently one of the rapidly expanding areas of scientific research, joining genomics, transcriptomics and proteomics as scientific fields that enhance understanding of the components of biological systems. The discipline of metabolomics offers a reliable approach to understanding changes within the complex

biochemical matrix of living organisms (Lindon et al. 2007; Pan and Raftery 2007). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the techniques most frequently used for metabolomics investigations. NMR analysis has been shown to be sufficiently robust for use in metabolomic studies (Lenz et al. 2005; Pelczer 2005; Karakach et al. 2009; Lardon et al. 2013a, b), having the capability to detect variations in factors such as age, sex and diet (Rochfort, 2005). While one dimensional (1D) ^{1}H NMR is the most commonly used method in NMR metabolomics, a number of studies have used two dimensional (2D) techniques (Viant et al. 2003; Rochfort 2005; Lardon et al. 2013a, b). Metabolomics has been used in aquaculture and fisheries sciences to assess the stress response of fishes to disturbed habitats (Bundy et al. 2009; Viant et al. 2003; Karakach et al. 2009; Lin et al. 2009). Metabolomic analyses rely on multivariate statistics, such as principal component analysis (PCA) and least squares regression analysis, to analyze data generated from NMR analyses. PCA is used to extract and display any systematic variation within the data and PCA models provide the best method to identify groupings, trends, and outliers within the data (Trygg et al. 2006).

The use of metabolomics to investigate the response of fish to hypoxia has been valuable, but limited to a few hypoxia-tolerant teleosts (Podrabsky et al. 2007; Hallman et al. 2008; Lardon et al. 2013a, b). Most of these studies have found that the response of fish to hypoxia is characterized by reduction in adenosine triphosphatase (ATP) and phosphocreatine (PCr), reduced concentration of excitatory metabolites such as glutamate and glutamine, increase in inhibitory metabolites such as gamma-amino butyric acid and glycine, increase in branched-chain amino acids such as valine, isoleucine and leucine and accumulation of lactate (Pincetich et al. 2005; Hallman et al. 2008; Lardon et al.

2013a, b). Knowledge about the specific metabolites that characterize the metabolic response of acipenseriforms and other relatively hypoxia-intolerant species is lacking. Therefore, in this study metabolomics are used to investigate metabolic changes occurring in paddlefish during exposure to acute and chronic hypoxia, providing information that can be the basis for a comparative understanding of respiratory physiology of both acipenseriformes and teleosts.

4.3 Materials and Methods

4.3.1 Fish source and acclimation

Paddlefish were bred at the Aquaculture Research Center at Kentucky State University, Frankfort, Kentucky and resulting fry were shipped to the South Farm Aquaculture Facility (South Farm) of Mississippi State University approximately 9 days after hatching. Fish were initially held at a density of 1 fish per L in 450-L circular (1 m diameter) recirculating tanks supplied with air saturated well-water at 21° C and a pH of 8. To prevent crowding, density was reduced to 0.2 fish/ L one month later. Water temperature was maintained with an in-line water heat pump (Titan® HP-7, Aqualogic, San Diego, CA, USA). Water flowing into tanks was mechanically and biologically filtered with fluidized bead filters. To get fish to the desired experimental size, 1 year old fish were transferred to 3600-L circular (2.4 m diameter) tanks (at a density of 0.04 fish/ L) with the same water supply and maintained at 21° C for 7 months with immersion heaters (Process Technology, Mentor, Ohio, USA). Each tank was fitted with a mechanical filter bag (Filter Specialists Inc., Michigan City, IN, USA) a mechanical/ biological fluidized bead filter (PolyGeyser, Aquaculture Systems Technologies, L.L.C., New Orleans, LA, USA) and an ultraviolet (UV) sterilizer (SMART HO, Emperor

Aquatics Inc., Pottstown, PA, USA). DO levels were maintained near saturation with multiple air stones throughout the holding period. Protocol for the feeding and maintenance of paddlefish was described in chapter 3.

4.3.2 Hypoxia trials

One week before the start of experiments, fish were transferred from the holding tanks into 300-L circular (1.6 m diameter) experimental tanks containing water having the previously described properties. Each experimental tank was fitted with a magnetic pump and a canister filter (Red Sea, Houston, TX, USA) containing activated carbon (Pentair Aquatic Eco-Systems, Inc, Apopka, FL, USA) to maintain optimal water quality. Nitrite and ammonia were measured daily using a water quality analysis kit (model: AQ-2; LaMotte Chemical Products, Co., Chestertown, Maryland, USA) and pH was recorded daily with a pH meter (pH10A, YSI Inc., Yellow Springs, OH, USA). Feeding was suspended for 24 h before each trial to ensure a post-absorptive state (Barton et al. 1988). In experimental trials, juvenile paddlefish in experimental tanks were exposed to moderate hypoxia (59 ±3 mm Hg; 40% saturation; 3.57 mg/ L) at 21 ºC for 72 hours (h). Another group of fish held in normoxic conditions (148 ±3 mm Hg; 100% saturation; 8.92 mg/ L) served as controls. The choice of moderate hypoxia was based on the results of Chapter 2 which showed that paddlefish have a critical partial pressure of oxygen (pO_{2crit}) of 74 mm Hg and 89 mm Hg at 18 ºC and 26 ºC, respectively, and a lethal minimum oxygen threshold of 31.0 mm Hg (~2 mg/ L) and 37.0 mm Hg (~2.03 mg/ L) at 18ºC and 26ºC, respectively. Thus, the oxygen levels used in the experiment reflected environments that are nearly saturated and those with hypoxic conditions that are critical but above the lethal limit. Hypoxia was induced by bubbling nitrogen gas into the water

to reduce pO_2 at a rate of 3 mm Hg/ minute until the experimental level of water pO_2 was reached, requiring approximately 30 minutes. Each treatment consisted of 4 randomly assigned replicate tanks containing 12 randomly assigned fish per tank. Simple randomization was achieved by assigning different numbers to individuals that were stocked into respective experimental tanks. The tanks were placed within concrete raceways that served as temperature-controlled water baths.

Two fish were sampled from each tank for epaxial white muscle tissue after 0.25 and 72 h of exposure to moderate hypoxia. The remainder of experimental fish were destined to be used in a separate experiment described elsewhere (Chapter 3). After euthanasia, effected by a quick bow to the head, one gram (g) of tissue was collected from the epaxial muscle in front of the dorsal fin of each fish. Muscle tissue samples were removed using a scalpel and forceps, quickly wrapped in foil, flash frozen in liquid nitrogen, and stored at -80 ºC until NMR analysis. Overall sampling time was < 4 minutes per fish. Fish length was measured to the nearest millimeter (mm), from the eye to the fork of the tail (eye-fork length). Fish were also weighed to the nearest g immediately after tissue collection. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University (Protocol approval number: 11-058).

4.3.3 Metabolite extraction

Muscle samples were extracted following the protocol of Lin et al. (2007) and Wu et al. (2008). Approximately 0.1g of muscle was ground into fine powder with a mortar and pestle cooled in liquid nitrogen, transferred into a pre-weighed 2.0 ml screw-top sample vial, weighed and then lyophilized overnight in a freeze-dryer. The lyophilized

sample was re-weighed to determine the muscle moisture content, after which about 0.04-0.05 g of tissue was transferred into a 2.0 ml Eppendorf snap-cap vial. Metabolites were extracted by adding 10 ml/g of ice cold methanol, 10 ml/g of ice cold chloroform and 9 ml/g of ice cold ultrapure deionized water to the vial at a final ratio of 2:2:1.8. Samples were vortex mixed for 20 sec three separate times, with samples placed on ice for 40 sec between each mixing. After the completion of vortex mixing, samples were placed on ice for 10 minutes to allow the layers to partition and thereafter were centrifuged at 10,000xg for 10 minutes at 4°C. A 500 ml sample of the polar solvent (supernatant) was transferred to a new pre-cooled 2.0 ml snap-cap vial, lyophilized overnight, and stored at -80 ^{o}C.

4.3.4 NMR sample preparation

Immediately prior to NMR analysis, each frozen sample was re-suspended in 300 ul of 0.1M sodium phosphate buffer in deuterized water (D_2O), pH 7.4, and 6 ul of 1 mg/ml trimethylsilyl- 2,2,3,3-d4 propionate (TMSP; internal shift standard) solution in D_2O (final sample concentration of 0.11 mM TMSP). The re-suspended sample was vortexed for 10 sec three times, and then transferred to a 5 mm Shigemi NMR tube (Shigemi Inc, Allison Park, PA).

4.3.5 ^{1}H-NMR data acquisition

Both 1 dimensional (D) and 2D data were acquired at 21 ^{o}C using a 700.12 MHz NMR spectrometer (Bruker Ultrashield Plus 700, Bruker BioSpin 110 Corp., Billerica, MA, USA) fitted with a cryoprobe (Bruker Cryoplatform, Bruker BioSpin Corp.). Commercial software (Topspin 2.1; Bruker BioSpin Corp.) was used to set NMR spectral processing parameters. Each sample was pulse calibrated and shimmed individually

before it was analyzed. To acquire 1-D ^{1}H-NMR spectra, muscle samples were scanned with 32 transients, collecting 64 k data points, using a 90^{o} pulse, covering a spectral width of 8,418 Hz, with a 5 sec recycle time, and a 3 minutes acquisition time. A 1 Hz exponential line broadening was applied, data were Fourier transformed, auto phase corrected, baseline corrected using a 5th order polynomial, and shift referenced to the TMSP peak at 0.0 ppm.

For 2-D data acquisition, samples were scanned with 16 transients and 32 increments, collecting 8 k data points, covering a spectral width of 8,418 Hz in F2 (chemical shift axis), 40 Hz in F1 (spin-spin axis), with a 1.5 s recycle time, and a 15 minute acquisition time. Data set was zero filled to 1,024 points in F1, and 8k in F2 (both dimensions multiplied by sine-bell window functions). Acquired data were double-complex Fourier transformed, tilted, symmetrized about F1, and calibrated to the TMSP peak at 0.0 ppm. A skyline projection of the 2D spectrum was created as recommended by Ludwig and Viant (2010).

Multiple 1-D and 2-D skyline projection spectra were overlayed and visually inspected using MestReNova v. 8.1 (Mestrelab Research S.L., Santiago de Compostela, Spain) to identify areas where peaks were unaligned. Since peaks were aligned with TMSP, no corrections were made to data. Spectra sample baselines were aligned, and the water peak (4.60-5.15 ppm) and the methanol extraction peak (3.31-3.40 ppm) removed from each sample before analysis. Data were binned (0.005 ppm) from 0.80-9.00 ppm and then normalized for total spectral area.

4.3.6 Metabolite identification

Metabolites in muscle tissue of paddlefish exposed to normoxia and hypoxia were identified from both 1-D and 2-D data. Metabolites from 1-D data were identified with Chenomx NMR Suite v.8.0 (Chenomx, Inc., Edmonton, Alberta, Canada). Metabolites from 2-D data were identified and quantified by the Birmingham Metabolite Library (BML) NMR data mining algorithm using partial quotient normalization (PQN) (Ludwig et al. 2012). According to the proposed minimum reporting standards set forth by the Metabolomics Standards Initiative, metabolites were identified to level 2 or below (Sumner et al. 2007).

4.3.7 Glycogen assay

Approximately 10 mg of ground muscle tissue was placed in a pre-weighed 2.0 ml screw top sample vial with 200 µl of ice-cold ultrapure water. This was vortex mixed for 20 sec four separate times, with samples placed on ice between each vortex mixing. The mixture was then centrifuged at 10,000xg for 5 minutes at 4 ^{o}C. Thereafter, the supernatant was transferred to a new pre-cooled snap cap vial and used for determining muscle glycogen content by following a defined procedure stated in a commercial assay kit (ab6520; Abcam, Cambridge, MA, USA). A flow chart of materials and methods can be found in the appendix. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University (Protocol approval number: 11-058).

4.4 Data analysis

Prometab (ProMetab v3.5; University of Birmingham, UK) was used within MATLAB (v. R2014a; The MathWorks, Inc., Natick, MA, USA) to convert 1-D ^{1}H-NMR spectra into chemical shift bins for multivariate chemometric analysis. Multivariate analyses were performed using Partial Least Squares (PLS) Toolbox (v.7.9; Eigenvector Research, Inc., Wenatchee, WA, USA) within MATLAB. Data were pareto-scaled, mean-centered, and inspected for outliers using Hotelling's T^2, Q residuals and plots of studentized residuals vs. residual leverage. PCA was conducted for each sample. A one-way analysis of variance (ANOVA) and a *post-hoc* Holm-Sidak multiple comparison test of PC scores was conducted between normoxia, acute and chronic hypoxia fish with an alpha of < 0.001. PC loadings plots were used to identify buckets important for treatment separation.

Metabolites varying significantly in intensity in response to hypoxia were identified by a univariate analysis of the binned spectra to generate a ^{1}H significant difference spectra (SDS; Goodpaster et al. 2010; Schock et al. 2012, 2013). This was obtained by averaging the binned spectra according to treatment (that is, normoxia, acute hypoxia and chronic hypoxia) and calculating the differences between two treatments at a time. Significantly different bins between treatments were identified by a Student's t-test and plotted similarly to Schock et al. (2013).

For 2-D data, metabolites identified and quantified into chemical shifts and corresponding intensities by the BML PQN algorithm were compared for treatment effects for each sample type using a one-way ANOVA. Normality and equality of variance were tested with Shapiro-Wilk and Levene's tests, respectively. Data were

considered significant at $p < 0.001$ for 2-D metabolites based on the integration of multiple buckets per metabolite. The effect of hypoxia on muscle glycogen concentration of paddlefish was analyzed using a one-way ANOVA and considered significant at $p < 0.05$.

4.5 Results

PC scores for multivariate 1-D data were different between treatments, separating along PC1 (explained variance: 36.22 %) (Fig. 4.1). There was modest separation between acute hypoxia and chronic hypoxia, and greater separation between normoxia and acute hypoxia, and greatest separation between normoxia and chronic hypoxia.

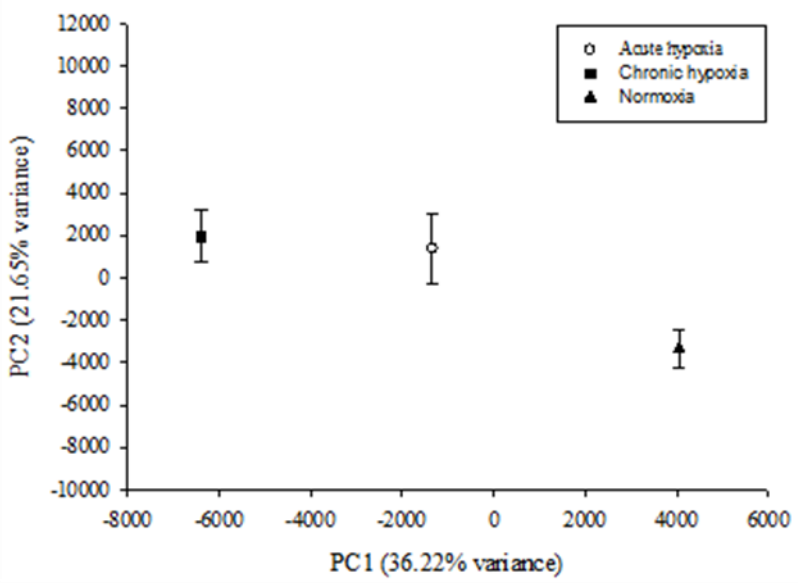

Figure 4.1 Principal component (PC) scores plot of ^{1}H-NMR spectra American paddlefish.

Figure 4.1 represents principal component (PC) scores plot of ^{1}H-NMR spectra from polar portion of extracted muscle tissue of American paddlefish *Polyodon spathula* (n=8 fish/ treatment) exposed to either normoxia, acute (0.25 h) or chronic (72 h) hypoxia. Mean (SE) loadings on PC1 important for data separation.

Samples were clustered more tightly in normoxia, followed by chronic hypoxia and then acute hypoxia (Fig. 4.1). PC loadings on PC1 and PC2 revealed bins that contributed to the separation in treatment groups observed in the PC scores plot (Fig. 4.2). These bins were identified as peaks in 1-D plots of PC1 loadings (Fig. 4.3) versus chemical shift.

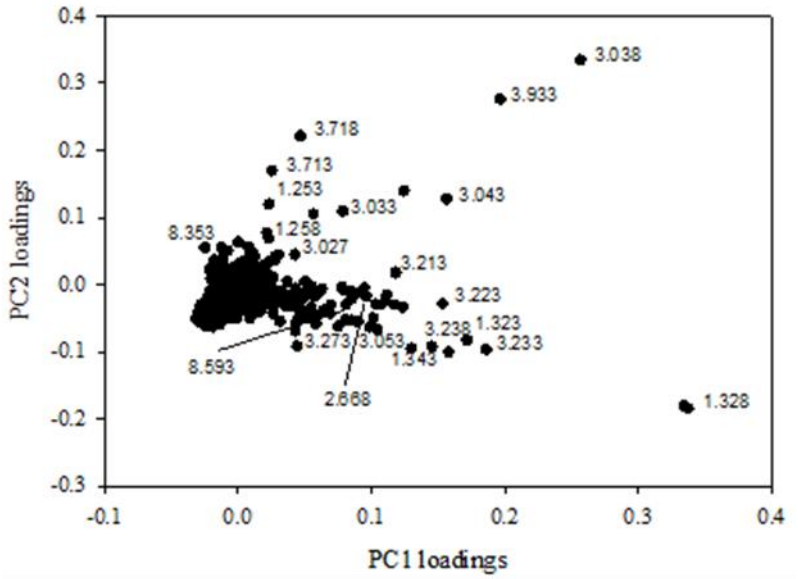

Figure 4.2 Two-dimensional principal component (PC) loadings of juvenile American paddlefish.

Figure 4.2 represents two-dimensional principal component (PC) loadings of buckets from ^{1}H-NMR spectra from polar portion of extracted muscle tissue of American paddlefish *Polyodon spathula* (n=8 fish/treatment). Numbers denote the chemical shift bucket (bucket width: 0.005 ppm), referenced to TMSP at 0.0 ppm.

For univariate 1-D data, those metabolites that contributed significantly to differences in intensity between normoxia and acute hypoxia included: lactate, 3-hydroxyisovalerate, glucose, valine, phosphocreatine, creatine, ATP, anserine and inosine (Fig. 4.4).

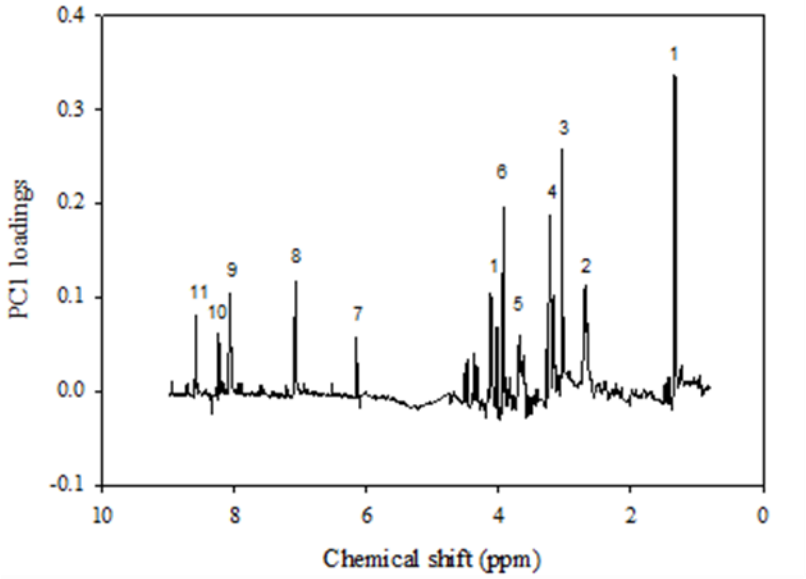

Figure 4.3 Loadings plot of ^{1}H-NMR spectra for juvenile American paddlefish.

Figure 4.3 represents loadings plot of ^{1}H-NMR spectral data from polar portion of extracted muscle tissue of American paddlefish *Polyodon spathula* (n=8 fish/ treatment) on principal component (PC) 1. This PC was important for separating multivariate data. 1 = lactate (1.32-1.34 ppm; 4.11-4.12 ppm); 2 = NADH (2.67 ppm); 3 = creatine (3.04 ppm); 4 = glucose (3.23 ppm); 5= 3-methylhistidine (3.72 ppm); 6= betaine (3.93 ppm); 7 = ATP/ADP (6.15 ppm); 8 = anserine (7.08 ppm); 9 = carnosine (8.07 ppm); 10 = inosine (8.24 ppm, 8.35 ppm); 11= AMP (8.59 ppm).

For difference in intensity between normoxia and chronic hypoxia, valine, lactate, alanine, glucose, 3-methylhistidine, AMP, ATP/ADP, inosine, anserine and phosphocreatine were identified as the major contributing metabolites (Fig. 4.5). Metabolites identified as contributing to the differences in intensity between acute and chronic hypoxia included: 3-hydroxyisovalerate and 3-methylhistidine (Fig.4.6). Similar to 1-D data, 2-D J-resolved analyses identified metabolites that expressed increased muscle activity, anaerobic respiration, protein synthesis, immune suppression and oxidative stress in fish exposed to acute and chronic hypoxia (Table 4.1). Muscle glycogen concentration decreased from normoxia to acute hypoxia, and continued to further decrease in chronic hypoxia (Fig.4.7).

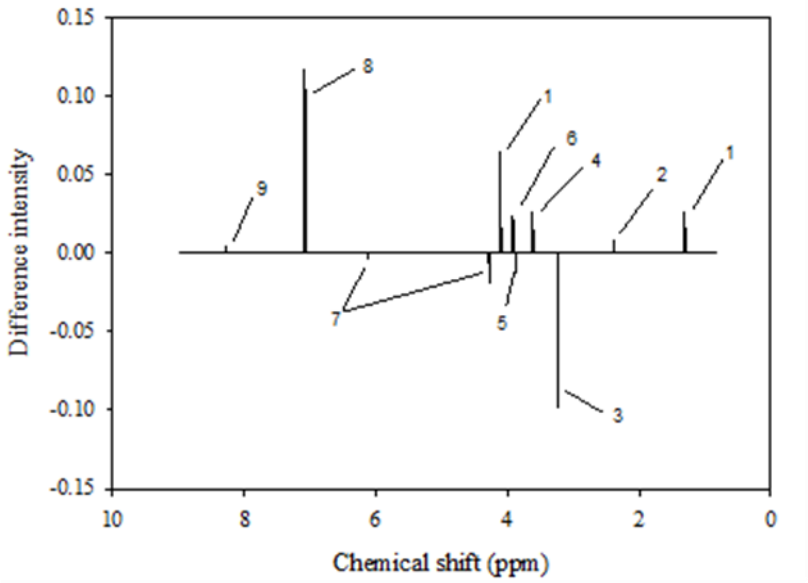

Figure 4.4 Univariate significant difference plot of ^{1}H-NMR spectral data of American paddlefish.

Figure 4.4 represents univariate significant difference plot of ^{1}H-NMR spectral data from polar portion of extracted muscle tissue of normoxic and acute hypoxic American paddlefish *Polyodon spathula* (n=8 fish/ treatment, $p < 0.001$). Positive numbers indicate an increase in hypoxic fish, negative numbers indicate a decrease in hypoxic fish relative to normoxic fish. Numbers indicate metabolites: 1= lactate, 2= 3-hydroxyisovalerate, 3= glucose, 4= valine, 5= phosphocreatine, 6= creatine, 7= ATP, 8= anserine, 9= inosine.

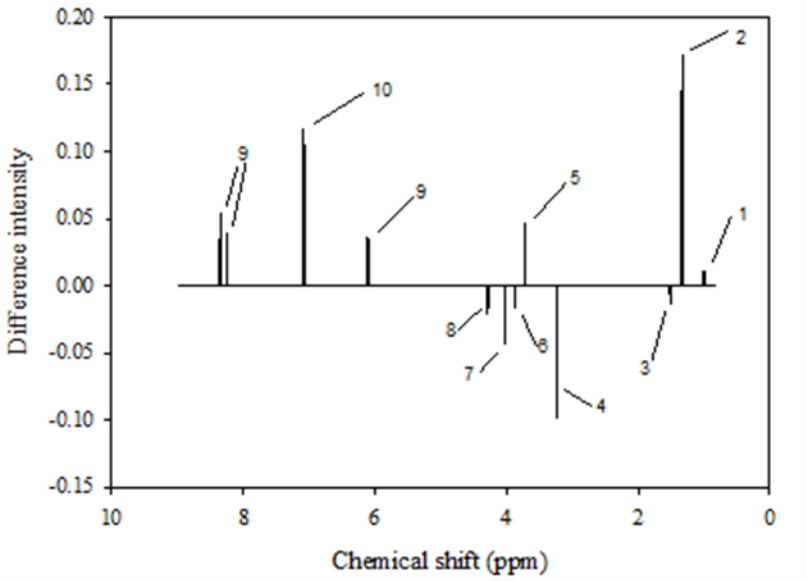

Figure 4.5 Univariate significant difference plot of ^{1}H-NMR spectral data from juvenile American paddlefish.

Figure 4.5 represents univariate significant difference plot of ^{1}H-NMR spectral data from polar portion of extracted muscle tissue of normoxic and chronic hypoxic American paddlefish *Polyodon spathula* (n=8 fish/ treatment, $p < 0.001$). Positive numbers indicate an increase in hypoxic fish, negative numbers indicate a decrease in hypoxic fish relative to normoxic fish. Numbers indicate metabolites: 1= valine, 2= lactate, 3= alanine, 4= glucose, 5= 3-methylhistidine, 6= phosphocreatine, 7= AMP, 8= ATP, 9= inosine, 10= anserine.

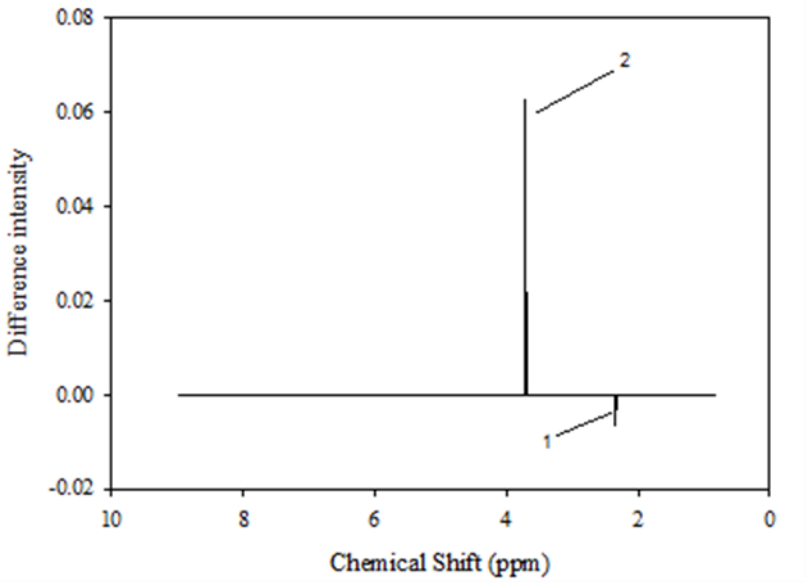

Figure 4.6 Univariate significant difference plot of ^{1}H-NMR spectral data from juvenile American paddlefish.

Figure 4.6 represents univariate significant difference plot of ^{1}H-NMR spectral data from polar portion of extracted muscle tissue of acute and chronic hypoxic American paddlefish *Polyodon spathula* (n=8 fish/ treatment, $p < 0.001$). Positive numbers indicate an increase in hypoxic fish, negative numbers indicate a decrease in hypoxic fish relative to normoxic fish. Numbers indicate metabolites: 1= 3-hydroxyisovalerate, 2= 3-methylhistidine.

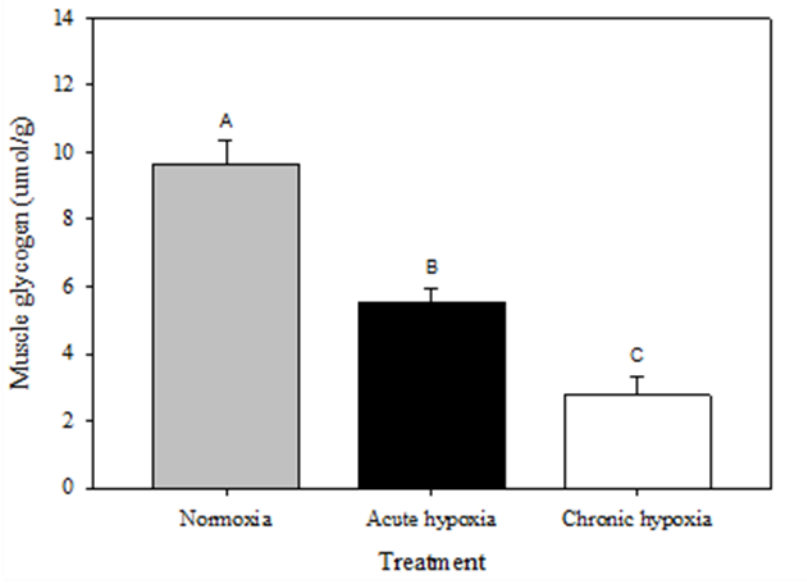

Figure 4.7 Mean (SE) muscle glycogen concentration of American paddlefish.

Mean (SE) muscle glycogen concentration of American paddlefish *Polyodon spathula* exposed to either normoxia (148 ±3 mm Hg), acute (0.25 h) or chronic (72 h) hypoxia (59 ±3 mm Hg). Different letters indicate significant difference between treatments (n=8 fish/ treatment, one-way ANOVA, $p < 0.05$).

Table 4.1 Metabolite changes in the white muscle tissue of juvenile American paddlefish.

	Metabolite	Normoxia vs acute hypoxia	Normoxia vs chronic hypoxia	Acute vs chronic hypoxia
Energy Stores	AMP	NO CHANGE	INCREASED	INCREASED
	ATP	DECREASED	DECREASED	NO CHANGE
	Mannose	NO CHANGE	DECREASED	DECREASED
	Glucose	DECREASED	DECREASED	NO CHANGE
	Glucose-6-phosphate	NO CHANGE	DECREASED	DECREASED
Anaerobic pathway	Creatine	INCREASED	DECREASED	DECREASED
	Lactate	INCREASED	INCREASED	NO CHANGE
	Myo-inositol	NO CHANGE	DECREASED	NO CHANGE
	Phosphocreatine	DECREASED	DECREASED	NO CHANGE
	Alanine	INCREASED	INCREASED	NO CHANGE
	Carnitine	NO CHANGE	INCREASED	INCREASED
Muscle function	Inosine	INCREASED	INCREASED	NO CHANGE
	Taurine	INCREASED	INCREASED	NO CHANGE
Protein synthesis	Lysine	INCREASED	INCREASED	NO CHANGE
	Glutarate	NO CHANGE	INCREASED	NO CHANGE
Oxidative stress	Glutathione	INCREASED	INCREASED	NO CHANGE
Osmotic stress	Betaine	INCREASED	INCREASED	NO CHANGE
Stress pathways	Valine	INCREASED	INCREASED	NO CHANGE
	Isoleucine	INCREASED	INCREASED	NO CHANGE
	Leucine	NO CHANGE	INCREASED	NO CHANGE
Anti-fatigue	Anserine	INCREASED	INCREASED	NO CHANGE
Immune suppression	Phosphocholine	INCREASED	DECREASED	DECREASED

Table 4.1 represents metabolite changes in the white muscle tissue of American paddlefish *Polyodon spathula*, exposed to either normoxia (148 ±3 mm Hg), acute (0.25 h) or chronic (72 h) hypoxia (59 ±3 mm Hg), as indicated by 2-D J-resolved ^{1}H-NMR technique (n= 8/ treatment, one-way ANOVA, $P < 0.001$).

4.6 Discussion

Acute and chronic hypoxia have profound effects on biochemical and physiological processes in fishes (Richards 2009; Wells 2009). However, most hypoxia

studies focus on a few selective metabolites. Therefore, few examples of studies that provide a more holistic coverage of metabolic processes at the time of hypoxic exposure exist. Using a metabolomics approach, this study provides insight into metabolic processes that occur during exposure of paddlefish to both acute and chronic hypoxia. The major patterns observed include increases in inosine (indicative of increased muscle function) and phosphocholine (indicative of immune suppression) and a depletion of both aerobic (glucose, NADH, and AMP/ADP) and anaerobic (glycogen) energy stores. Other changes observed include increases in lactate (suggesting activation of anaerobic metabolism), creatine, myo-inositol, and 3-hydroxybutyrate (suggesting the utilization of alternative energy sources during hypoxia).

4.6.1 Effect of hypoxia on muscle function

In paddlefish exposed to both acute and chronic hypoxia, increases in inosine and taurine are indicative of metabolic stress (Linden 1994; Aldrich et al. 2000; Ramkumar et al. 2001; Schaffer et al. 2010) and increased muscle activity (Sahlin and Katz 1989; Schaffer et al. 2010). Increased muscle activity suggests an increase in swimming activity to maintain the oxygen supply to tissues during exposure to hypoxia. The obligate ram-ventilating life history strategy of paddlefish (Burggren and Bemis 1992) and the lack of a buccal valve to push water over the gills (Burggren and Bemis 1992; Bemis et al. 1997) necessitates open mouth swimming as a means to acquire oxygen. Further, low air volume in the swim bladder reduces buoyancy (Bemis et al. 1997), also requiring continuous swimming. Paddlefish are known to experience respiratory distress and lose equilibrium if they are forced to swim at speeds ≤0.6 body lengths per second (Burggren and Bemis 1992). This spontaneous swimming in hypoxia is however, contradictory to

what has been observed in most teleosts and acipenseriforms exposed to hypoxia. Most fishes rely on metabolic depression, whereby they reduce swimming and other energy demanding processes, to prolong survival in hypoxia (Bickler and Buck 2007). Further, the observed increase in anserine in paddlefish exposed to both acute and chronic hypoxia may relate to maintenance of constant swimming in hypoxia. Anserine has been found in other ram ventilators (Ikeda 1980; Konosu and Yamaguchi 1982) and is known to have an anti-fatigue effect prolonging swimming duration (Kikuchi et al. 2004). In paddlefish exposed to acute hypoxia, the increase in 3-hydroxyisovalerate, a metabolite of the branched-chain amino acid leucine, is likely to suppress muscle protein breakdown and cell damage as fish attempt to maintain spontaneous swimming activity (Nissen et al. 1996). Similarly, the amino acid, 3-methylhistidine, is produced from the breakdown of the skeletal muscle proteins actin and myosin (Asatoor and Armstrong 1967; Young 1970). The increase in 3-methylhistidine in paddlefish exposed to chronic hypoxia is therefore likely due to the reduction of 3-hydroxyisovalerate over time. This may also have led to an increase in protein synthesis as indicated by increases in lysine and glutarate (Aragão et al. 2008, 2010).

4.6.2 Effect of hypoxia on immune response and osmotic stress

Changes observed in phosphocholine suggest that immune function was downregulated in acute hypoxia but was upregulated during chronic hypoxia. Hypoxia has been reported to have an immunosuppressive effect in fish (Hajji et al. 1990; Welker et al. 2007), meaning paddlefish exposed to hypoxia in either aquaculture or natural environments may be susceptible to infections from pathogens and parasites often prevalent in such environments. The observed upregulation in glutathione, an important

antioxidant that protects the cells against oxidative stress (Peña-Llopis et al. 2003), suggests an increased protection of the cells against reactive oxygen species, which are byproducts of aerobic metabolism (Peña-Llopis et al. 2003; Lushchak 2011). Oxidative stress occurs when there is an imbalance of free radical generation versus antioxidant defenses (Rock et al. 1996) and leads to the damage of molecular species such as lipids, proteins, and nucleic acids (McCord 2000). Upregulation of betaine suggests that the exposure of paddlefish to acute and chronic hypoxia resulted in osmotic stress. Betaine is an organic osmolyte that is utilized to maintain cell volume by organisms experiencing hypoxia-induced osmotic stress (Yancey 2005). Another organic osmolyte upregulated in paddlefish during exposure to hypoxia is taurine.

4.6.3 Effect of hypoxia on energy stores

Survival duration of fish exposed to hypoxia depends to a large extent on the amount of stored glycogen and high-energy phosphate substrates, the rate at which ATP is produced and the ability to reduce metabolic requirements (Richards 2011; Svendsen et al. 2011). The reduction in ATP levels in hypoxic fish may result from the inability of the obligate ram-ventilating paddlefish to effect a significant metabolic depression. Paddlefish have to rely on anaerobic ATP production to compensate for the decrease in aerobic metabolism. The present study indicates that anaerobic energy production occurred through the activation of glycolytic pathways with glucose and/ or glycogen as the substrate and lactate as the end product. Reported increases in plasma glucose and lactate in paddlefish exposed to similar hypoxic conditions (Chapters 3) are supported by the changes in muscle glycogen, glucose and lactate observed in the current study. Paddlefish exposed to hypoxia apparently also derived energy from the breakdown of

phosphocreatine to creatine to help sustain muscular exertion. The use of these two energy sources has been reported in acipenseriforms (Kieffer et al. 2001) and many other fishes experiencing metabolic stress (Dobson and Hochachka 1987; Kieffer 2000).

The observed increase in carnitine concentration of paddlefish exposed to chronic hypoxia indicates mobilization of fatty acids to meet the increased energy demand (Costas et al. 2011). Carnitine is required for the transport of long-chain fatty acids into the mitochondria where they can eventually be broken down to acetyl CoA to produce energy via the citric acid cycle (Harpaz 2005; Li et al. 2009). The accumulation of alanine, known to originate from muscle degradation to yield amino acids to produce energy (Nelson and Cox 2005), indicates that this amino acid is being used as an alternate energy source. The mobilization of these alternate energy sources corresponds to a decrease in the concentration of glycogen in the chronic hypoxic fish observed in this study and reduced glucose content reported in Chapter 3. Typically the lack of activation of these glycolytic pathways during hypoxia is inferred to be an indication of metabolic depression (Hochachka and Dunn 1983; Hochachka and Somero 1984). The lower ATP production from these processes, as compared to that from aerobic metabolism, leads to a rather rapid depletion of energy substrates, reducing the duration of survival in hypoxia (Richards 2011). In the present study, this explanation is supported by the reduction in glucose and glycogen in paddlefish exposed to chronic hypoxia. The observed changes in energy turnover differ from those of hypoxia-tolerant organisms that reduce energy turnover through metabolic depression (Hochachka et al. 1996). The inability to significantly reduce the rate of metabolism may account for the reduction in energy stores as well as reduced tolerance of paddlefish to hypoxia. Another factor that may limit

survival of paddlefish exposed to hypoxia is the accumulation of the end products of anaerobic metabolism, such as lactate and associated H^+ (Richards 2011). Thus, the duration of survival of ram ventilating paddlefish under hypoxic conditions may be highly influenced by the amount of available substrates and the ability to excrete the associated metabolic wastes (Richards 2011).

Increases in valine and isoleucine during hypoxia are further indication of the activation of stress response pathways, as these two branched-chain amino acids can be converted into glucose by gluconeogenic tissues during stress to provide additional energy sources (Letto et al. 1986). Usually, stressors leading to increases in levels of plasma cortisol also modify fish amino acid metabolism (Milligan 1997; Costas 2008) due to increased energy demand and the need for synthesis of stress related proteins (Aragão et al. 2008, 2010; Costas et al. 2011).

In conclusion, this study supports the observations presented in Chapter 3 which demontrated that exposure to both acute and chronic hypoxia leads to metabolic stress in paddlefish. This study shows that hypoxia reduces the amount of oxygen available to paddlefish and therefore limits the amount of aerobic ATP that can be produced. Survival of paddlefish exposed to hypoxia, therefore, warrants physiological adjustments designed to increase oxygen acquisition from the water and generate ATP from anaerobic metabolism. Because paddlefish are obligate ram ventilators, part of their response to hypoxia includes increased muscle activity which increases energy demand and consequently may reduce their survival duration. Identified biomarkers for acute hypoxia in paddlefish include increases in muscle lactate, inosine, valine, anserine, creatine, 3-hydroxyisovalerate and decreases in ATP, phosphocreatine and glucose. Identified

biomarkers in paddlefish exposed to chronic hypoxia included increases in muscle lactate, inosine, valine, anserine, 3-methylhistidine and decreased ATP, glucose, and alanine.

The information concerning the glycogen content of paddlefish provides insight into energy reserve status and why observed metabolite changes occurred in each treatment. Most of the metabolite changes observed involved anaerobic metabolism and alternate energy pathway and this study suggests that the depletion of energy stores (i.e., glucose and glycogen) during hypoxia might be a critical factor influencing the metabolic changes observed in paddlefish in other studies (Chapters 2 and 3). This study also indicates the challenges confronting paddlefish under prolonged hypoxia exposure because they are unable to replenish muscle glycogen content. Information about the stress response of paddlefish to hypoxia is also provided, showing that metabolomics can be a valuable investigative tool that complements traditional measurement tools that target specific biomarkers. Analyzing both 1-D univariate and multivariate and 2-D J-resolved data provided complementary data that produced a more holistic understanding of the hypoxia response of paddlefish.

4.6.4 References

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CHAPTER V

RECOVERY FROM HYPOXIA IN JUVENILE AMERICAN PADDLEFISH: BIOCHEMICAL, METABOLIC AND HEMATOLOGICAL ASPECTS

5.1 Abstract

Exposure to hypoxia can lead to severe physiological changes in fish. As a result, recovery metabolism may place limits on survival and overall well-being because the time requirements for restoration of energy stores will influence the ability to survive and resume active metabolism. Unfortunately, there is little information available on hypoxia recovery processes of fish. Due to their high metabolic rate, high critical pO_2 and poor capacity for metabolic depression, American paddlefish (*Polyodon spathula*) are an excellent model to examine hypoxia recovery processes. Blood oxygen transport, blood acid-base balance, ion-osmoregulation and enzyme parameters were compared in juvenile paddlefish (~181 g) exposed to either normoxia (148 mm Hg [8.94 mg/ L]) or moderate hypoxia (59 mm Hg [3.58 mg/ L]) for 96 hours followed by exposure to normoxic water for 4 hours. Exposure to moderate hypoxia resulted in an increase in plasma cortisol and long-term decreases in blood pH, HCO_3^-, pCO_2, pO_2, and oxygen content. Plasma glucose and lactate both increased after 1 hour (acute) hypoxic exposure, with glucose decreasing after 96 h (chronic) of hypoxia. Exposure to chronic hypoxia led to increases in red blood cell numbers, hematocrit and hemoglobin concentration in paddlefish. Plasma Na^+, Cl^-, and osmolality increased after acute hypoxia exposure but recovered

within 96 hours. Plasma lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase also increased after exposure to hypoxia. Subsequent exposure of paddlefish to normoxia resulted in the recovery of cortisol, pH, HCO_3^- and pCO_2, pO_2, and lactate within the first 2 hours. Glucose, hematocrit, red blood cell numbers and hemoglobin did not return to the level of normoxic fish during the 4-hour recovery period. The results indicate that paddlefish are sensitive to moderate hypoxia and survival is limited by a reduction in glucose substrate availability as well as the accumulation of toxic H^+ and lactate. They have the capacity to rapidly recover in normoxia relative to pH, oxygen, metabolic and ionic homeostasis, although restoration of depleted glucose stores is delayed. Therefore, paddlefish survival response to any subsequent stressor requiring anaerobic energy stores (e.g. handling and exercise) may be limited during the recovery period.

Keywords: Hypoxia, glucose, lactate, metabolism, paddlefish, osmolality, recovery.

5.2 Introduction

Hypoxia is an increasingly common occurrence in many aquatic systems inhabited by fish, due to both natural and anthropogenic factors (Diaz and Breitburg 2009). Hypoxia occurs when oxygen demand by fish exceeds the available supply as determined by limitations of solubility and aerial diffusion (Graham 1990; Richards 2011). Further, aquatic hypoxia occurs when the rate of oxygen delivery to the tissues iprovides less than what is needed for oxidative metabolism (Dunn and Hochachka 1986).

Fish survival during hypoxia depends on the capacity for metabolic depression and anaerobic ATP-production (Hochachka and Somero 1984, 2002; Hochachka et al. 1996; Van Ginneken et al. 1998; Boutilier 2001; Richards et al. 2007). Metabolic depression involves decelerating those mechanisms that control physiological processes such as ion movement, protein synthesis and anabolism (Hochachka et al. 1996; Richards et al. 2007; Richards 2009), whereas anaerobic metabolism involves phosphocreatine hydrolysis and the utilization of glycolytic pathways involved in ATP production with lactate as an end product (Dunn and Hochachka 1986, 1987; Boutillier et al. 1988). The amount of ATP produced through anaerobic pathways is about 15 fold lower than that produced via aerobic pathways (Hochachka and Somero 1984). Therefore, anaerobic metabolism is only utilized when metabolic depression is insufficient to compensate for the reduced rates of aerobic ATP production (Hochachka and Somero 1984; Dunn and Hochachka 1986; Richards 2009). Thus, both the severity and the duration of hypoxia influence the reliance on anaerobic metabolism.

Survival duration in hypoxia and subsequent recovery depends to a large extent on the amount of stored glycogen and high-energy phosphate substrates, the rate at which ATP is produced and the ability to reduce metabolic requirements (Richards 2011; Svendsen et al. 2011). Therefore, an important part of the hypoxic response is the release of enzymes controlling rates of anaerobic metabolism used to meet energy requirements (Almeida-Val and Hochachka 1995). Organisms that lack the capacity for long-term metabolic depression during hypoxia, depend more on their ability to maintain ATP supply via anaerobic metabolism to survive hypoxia (Bickler and Buck 2007). Anaerobic metabolism involves glycolysis and phosphocreatine hydrolysis; therefore, these

organisms must immediately replenish their glycogen and phosphocreatine stores when sufficient oxygen becomes available, a process termed oxygen debt repayment (Van den Thillart and Verbeek 1991; Mandic et al. 2008). Oxygen debt repayment is characterized by a period of hyperventilation when the oxygen consumption rate is higher than that of the control fish. Most studies that have investigated oxygen debt repayment are related to exercise recovery. However, the metabolic process for exercise is similar to that of hypoxia (both are oxygen limiting conditions that are characterized by lactic acid production and phosphocreatine hydrolysis), and inference from exercise can be applied to environmental hypoxia. The recovery process in fish is divided into two phases. The initial phase is short and involves restoration of depleted oxygen and phosphocreatine stores (Milligan and Wood 1986; Van den Thillart and Verbeek 1991; Kieffer 2000), whereas the second phase involves converting the accumulated lactate to glucose to restore glycogen stores during a period lasting from several minutes to 10 hours (h) (Brett 1964; Van den Thillart and Verbeek 1991).

Measuring the time course of the hypoxia response as well as the recovery process enhance the understanding of mechanisms underlying the stress response. The time course of the physiological stress response has been well investigated in teleosts (Soivio et al. 1980; Dunn and Hochachka 1986; Powell and Perry 1997; Omlin and Weber 2010). The initial response to hypoxia involves the neuroendocrine system through the release of catecholamines (epinephrine and norepinephrine) from chromaffin tissue (Randall and Perry 1992; Thomas and Perry 1992; Perry and Reid 1994). Then a stimulation of the hypothalamic-pituitary-interrenal (HPI) axis occurs, leading to the release of corticosteroid hormones into the blood (Sumpter 1997; Wendelaar Bonga

1997; Barton 2002). Cortisol is the principal corticosteroid in actinopterygians. Therefore, a measure of its concentration in plasma can be perceived as a useful indicator of the level of stress (Barton 2002), such as in response to hypoxia. Following the initial neuroendocrine response, a series of physiological adjustments (secondary response) aimed at improving oxygen delivery to the tissues and maintaining homeostasis are elicited. These include changes in gill ventilation, oxygen transport (e.g., increases in the number of red blood cells (RBCs) and hemoglobin (Hb) concentration), iono-osmotic parameters and metabolism (Eddy 1974; Barton 2000; Hochachka and Lutz 2001; Pichavant et al. 2003; Kieffer et al. 2011). Quantification of these factors can be useful in understanding the array of mechanisms controlling the hypoxic response in fish. When these secondary responses are insufficient to offset the effects of environmental hypoxia, blood oxygen content decreases, resulting in hypoxemia and reduction in aerobic ATP production (Richards 2009). Although species such as rainbow trout, *Oncorhynchus mykiss,* have been well-studied in this regard, there is relatively little known about the mechanisms that control these responses in acipenseriforms, especially the American paddlefish, *Polyodon spathula*.

Most primitive fishes have anatomical structures for bimodal gas exchange, which increase survival during hypoxia, with a notable exception in the acipenseriforms. The acipenseriforms, including paddlefish, have existed for over 200 million years with a complete dependence on aquatic gas exchange (Burggren and Randall 1978). Paddlefish offer unique insights into hypoxia tolerance and recovery. They are obligatory ram ventilators with a relatively high energy demand for routine metabolism (Patterson et al 2013; Chapter 2), and may not be able to employ some of the successful strategies

utilized by other fishes to survive in hypoxia. They have a high pO_{2crit} coupled with a low capacity for metabolic depression (Chapter 2). The reduced capacity for metabolic depression is due to constant swimming which is necessitated by the absence of a buccal valve to push water across the gills (Burggren and Bemis 1992; Bemis et al. 1997) and low total air volume in the swim bladder, thereby reducing buoyancy (Bemis et al. 1997). As a result, paddlefish response to hypoxia includes a brief respiratory alkalosis, followed by hypoxemia, hyperglycemia, acidosis, ion losses and eventually death when exposed to extreme hypoxia (36 mm Hg; Chapter 3). However, little is known about the recovery capacity and processes after hypoxic exposure in paddlefish. Therefore, the objective of this study was to determine the physiological responses associated with recovery of paddlefish from hypoxia.

5.3 Materials and Methods

Paddlefish were bred at the Private John Allen National Fish Hatchery in Tupelo, Mississippi and juveniles were transported to South Farm Aquaculture Facility at Mississippi State University after 6 months. Fish were held at a density of 0.06 fish/ L in a 3600-L circular (2.4 m diameter) flow-through outdoor tanks (30% water exchange per h) supplied with air saturated well-water for over a year. Water temperature in outdoor tanks was maintained at 18-24 ^{o}C. Fish were fed a formulated diet (floating catfish diet; Rangen EXTR 400; Rangen, Inc., Angleton, TX, USA) with 40% crude protein and 9% crude fat at 3% of body weight per day. Two months prior to the start of experiments, all fish were transferred into two 3600-L circular (2.4 m diameter) recirculating indoor tanks (at a density of 0.03 fish/ L) supplied with air saturated well-water. Fish were fed the same diet at the same rate as they were in the outdoor tanks. Water temperature within

the tanks was maintained at 21 ºC using aquaculture immersion heaters (Process Technology, Mentor, Ohio, USA). Each tank was fitted with a mechanical filter bag (Filter Specialists Inc., Michigan City, IN, USA), a bead filter (PolyGeyser, Aquaculture Systems Technologies, L.L.C., New Orleans, LA, USA) and an ultraviolet (UV) sterilizer (SMART HO, Emperor Aquatics Inc., Pottstown, PA, USA). DO levels in each tank were maintained near saturation with multiple air stones. A 100% water exchange was completed over an 8 h period at the end of every week. Ammonia and nitrite were measured with a commercial water quality analysis kit (model: AQ-2; LaMotte Chemical Products, Co., Chestertown, Maryland, USA) twice weekly. Water pH was measured daily with a pH meter (pH10A, YSI Inc., Yellow Springs, OH, USA). Water DO and temperature were measured twice daily.

5.3.1 Hypoxia experiments

One week before the start of experiments, paddlefish were moved into six 300-L circular (1.6 m diameter) experimental tanks with the same water quality conditions as those in the holding tanks. Each experimental tank was fitted with a magnetic pump and a canister filter (Red Sea, Houston, TX, USA) containing activated carbon (Pentair Aquatic Eco-Systems, Inc, Apopka, FL, USA) to maintain water quality. Water temperature was maintained at 21ºC with thermostatted aquarium heaters (EBO-JAGER TS 200, EHEIM GmbH and Co. KG, Germany). The treatment temperature was chosen to reflect the optimum water temperature for juvenile paddlefish survival and growth (Kuhajda 2014). Food was withheld for 24 h before the start of each trial to ensure a post-absorptive state (Barton et al. 1988).

Two sequential experiments were conducted. In Experiment 1 juvenile paddlefish (mean weight = 181.17 ±5.77 g) were exposed to normoxia (148 mm Hg; 100% saturation; 8.92 mg/ L) for 96 h at 21 ºC. In Experiment 2 the same fish were exposed to moderate hypoxia (59 mm Hg; 40% saturation; 3.57 mg/ L) for 96 h at 21 ºC. These levels of hypoxia were chosen based on the results of previous experiments which showed that water with a pO_2 of 59 mm Hg induced sub-lethal stress (Chapter 3). Thus, the oxygen treatment levels were chosen to reflect paddlefish in normoxic or moderately hypoxic environments.

Experiment 1 started after 1 week of acclimation to experimental tanks. There were 6 randomly assigned replicate circular tanks (300-L) with 5 randomly assigned paddlefish per tank. After acclimation, fish were maintained in normoxic conditions for 96 h. One h into the 96 h treatment period, 1 fish in each replicate tank was sampled for blood. Following the 96 h period, another sample was removed from each replicate and the remaining fish were exposed to a sham recovery treatment. The sham treatment involved placing the magnetic pump associated with the filter into 100% air saturated water and pumping in the water into the experimental tank to simulate increased aeration. At 0.50 h into the sham recovery, 1 fish in each replicate tank was sampled. This procedure was repeated for remaining fish after both 2 and 4 h of recovery. Blood was sampled by collecting a fish with a dip net and anesthetizing it in a bath (same water pO_2 and temperature as treatment) of MS-222 (tricaine methanesulfonate) at a concentration of 150 mg/ L to minimize the effect of handling stress. About 2 ml of blood was collected from the caudal vasculature using a BD Vacutainer® and a 22-gauge hypodermic needle (Becton, Dickson and Co., Franklin Lakes, New Jersey, USA) into heparinized

Monoject® blood collection tubes (Tyco Healthcare Group LP, Mansfield, MA, USA). Blood collection was completed within 2 minutes of netting fish from experimental tanks. Immediately after blood collection, the length of each fish was measured to the nearest millimeter (mm), from the anterior orbit of the eye to the fork of the caudal fin (eye-to-fork length; Hoover et al. 2009), and weighed to the nearest gram (g). In each collection tube, half of the amount of blood was withdrawn using a needle and syringe to prevent exposure to air, and introduced into a 2.0 ml snap cap vial (aliquot 2) and placed on ice. Blood samples remaining in sealed vacutainers were placed on ice and analyzed within 15 minutes of collection using a blood gas analyzer (ABL80 FLEX CO-OX, Radiometer Medical, Bronshoj, Denmark) for pH, HCO_3^-, hematocrit (Hct), oxygen content, pO_2, P_{50}, pCO_2, pH, Hb, Na^+, K^+, Ca^{2+}, Cl^-, and osmolality. Thereafter, a 0.1-0.5 ml sample of the blood remaining in each vacutainer collection tube was used to quantify the number of RBC using a cell counter (Z1 Coulter Counter, Beckman Coulter, Inc., Brea CA, USA). The mean RBC size (mean corpuscular volume [MCV]) was calculated using the formula:

$$\text{MCV} = (\text{Hematocrit x } 10) \div \text{RBC concentration} \qquad \text{(Eq. 5.1)}$$

The mean Hb content per RBC (mean corpuscular hemoglobin [MCH]) was calculated using the formula:

$$\text{MCH} = (\text{Hemoglobin x } 10) \div \text{RBC concentration} \qquad \text{(Eq. 5.2)}$$

The mean concentration of hemoglobin in a given volume of RBCs (mean corpuscular hemoglobin concentration [MCHC]) was calculated using the formula:

$$\text{MCHC} = (\text{Hemoglobin x } 100) \div \text{hematocrit} \qquad \text{(Eq. 5.3)}$$

Blood samples in the snap cap vials (aliquot 2) were centrifuged for 5 minutes at 10,000xg (model: 59A; Fisher Scientific, Pittsburgh, PA, USA) at 21 ºC. For each fish, plasma was split into two 2.0 ml microcentrifuge plastic vials with screw top lids (Eppendorf International, Hamburg, Germany), flash frozen in liquid nitrogen, and stored at -80 ºC. One set of frozen plasma samples were later thawed and analyzed for glucose, lactate, total protein, cholesterol, triglycerides (VITROS DT60II, Ortho-Clinical Diagnostics Inc., Rochester, NY) lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (VITROS DTSC II MODULE, Ortho-Clinical Diagnostics Inc., Rochester, NY). Each sample was run in triplicate. The other set of frozen plasma samples were analyzed for cortisol in duplicate using a commercial kit (Cortisol EIA kit; Oxford Biomedical Research, Rochester Hills, MI, USA).

After sampling, fish were returned to the 3600-L circular (2.4 m diameter) recirculating indoor tanks at 21 ºC and >7.42 mg/ L to recover from Experiment 1. It was demonstrated in a previously conducted pilot study (where the same parameters used in the present study were measured after handling stress) that paddlefish are able to fully recover to control levels 1 week after handling. Based on this result, fish used in Experiment 1 (normoxia) were also used in Experiment 2 (moderate hypoxia) after 3 weeks of recovery.

Experiment 2 was identical to Experiment 1 in acclimation and sampling protocol with the exception of hypoxia. Hypoxia was induced by bubbling nitrogen gas into the water at a rate of 3 mm Hg/ minute until a water pO_2 of 59 ±3 mm Hg was reached. After 1 and 96 h of hypoxic exposure, 1 fish from each replicate tank was sampled for blood as

described previously. Remaining fish were then gradually exposed to normoxic conditions for recovery, achieved in approximately 4 minutes. Normoxic conditions for recovery were obtained by placing the magnetic pump associated with the filter into 100% air saturated water that was pumped in to effect an exchange with the hypoxic water. Time zero was determined as 3 minutes into the water exchange, when recovery water was > 80% saturated with air. Blood samples were collected from fish after 0.50, 2 and 4 h in recovery, as described in Experiment 1. Sampled fish were returned to the 3600-L circular (2.4 m diameter) recirculating indoor tanks at 21 ºC and > 7.42 mg/ L to recover from handling stress. A flow chart of materials and methods can be found in the appendix. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Mississippi State University (protocol approval number: 11-058).

5.4 Statistical analyses

Statistical analyses were performed with SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) at a significance level of $p < 0.05$. Normality and equality of variance were tested with Shapiro-Wilk and Levene's tests, respectively. Data that did not meet parametric assumptions were log transformed. A two-way analysis of variance (ANOVA) with water pO_2 (normoxia and hypoxia) and time as factors was used to evaluate the effects of hypoxia on blood parameters of juvenile paddlefish. Data are presented as mean ± standard error. When significant differences were found, a *post-hoc* Holm-Sidak multiple comparison test was used to isolate treatment differences.

5.5 Results

There were no differences in water quality between the treatments. Mean ±SE values were: pH (7.36 ±0.11), ammonia (0.22 ±0.04 mg/ L) and nitrite (0.07 ±0.03). Water temperature and DO for hypoxia were maintained at 21.0 ±0.1 ºC and 3.58 ±0.06 mg/ L, respectively. Water temperature and DO for fish exposed to normoxia were 21.0 ±0.1 ºC and 8.94 ±0.08 mg/ L, respectively. There were no differences in mean weights (181 ±5.72 g) and eye-to-fork lengths (39 ±1.4 cm; eye to fork of tail) between treatments. Time did not affect any of the variables measured for fish exposed to normoxia (Fig. 5.1-5.3; Tables 5.1-5.3). Exposure to hypoxia decreased blood pH (Fig. 5.1A), HCO_3^- (Fig. 5.1B) and pCO_2 (Fig. 5.1C) after 1 and 96 h, and all increased to the level of normoxic fish within 0.5 h in recovery.

Blood pO_2 (Fig. 5.2A) and oxygen content (Fig. 5.2B) decreased after 1 and 96 h of exposure to hypoxia. After transfer to recovery conditions, blood pO_2 increased to the level of normoxic fish within 2 h, and blood oxygen content increased to the level of normoxic fish within 0.5 h.

Paddlefish Hct increased after 1 h and RBC number and Hb concentration increased after 96 h of hypoxic exposure, but neither returned to normoxic levels after 4 h of recovery (Table 5.1). MCV increased and MCHC decreased within 1 h of hypoxic exposure, but both returned to normoxic levels by 96 h of hypoxic exposure (Table 5.1). There was no effect of hypoxia on MCH (Table 5.1). Plasma glucose increased after 1 h but decreased after 96 h of exposure to hypoxia. It remained lower than in normoxic fish after 0.50, 2 and 4 h in recovery (Fig. 5.3A). Plasma lactate increased after 1 h for fish exposed to hypoxia, and decreased to normoxic levels within 2 h in normoxia (Fig. 5.3B).

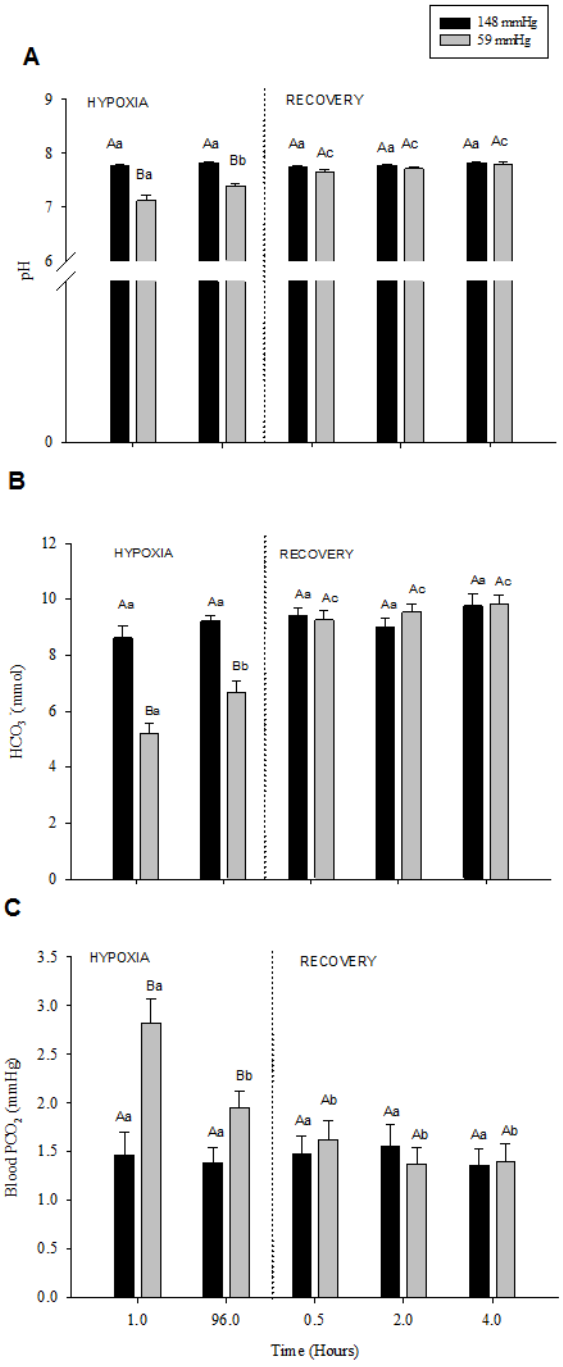

Figure 5.1 Mean (±SE) blood pH (A), HCO3- (B) and pCO2 (C) with time for juvenile American paddlefish.

Figure 5.1 represents mean (±SE) blood pH (A), HCO3- (B) and pCO2 (C) with time for juvenile American paddlefish, Polyodon spathula, exposed to normoxia (148±3 mm Hg) or hypoxia (59 ±3 mm Hg) and recovered in normoxia at 21 oC. Bars with different upper case letters indicate significant differences between normoxia and hypoxia. Bars with different lower case letters indicate significant differences between exposure time within a particular pO2 treatment ($p < 0.05$, n=6/treatment; two-way ANOVA; post hoc: Holm-Sidak multiple comparison test).

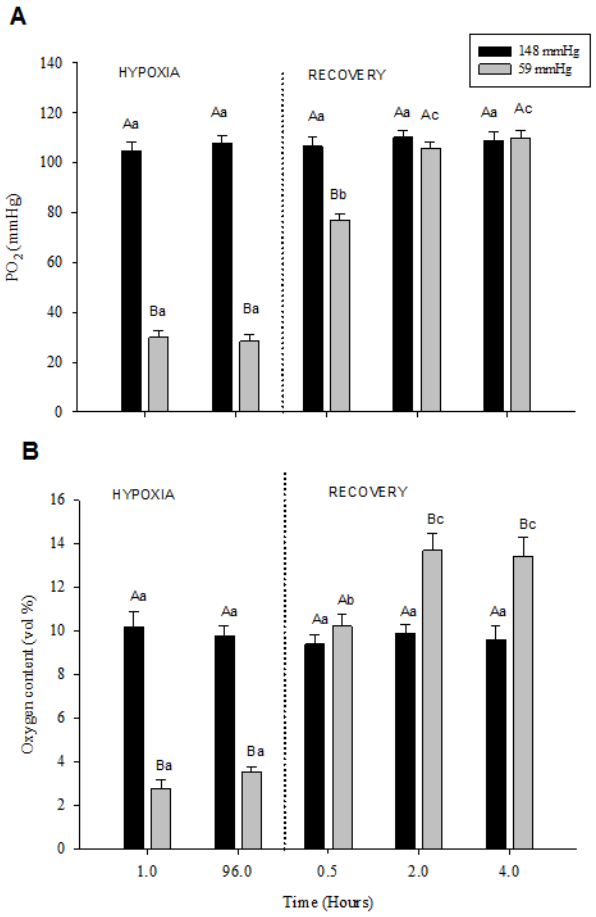

Figure 5.2 Mean (±SE) blood pO_2 (A) and oxygen content (B) with time for juvenile American paddlefish.

Figure 5.2 represents mean (±SE) blood pO_2 (A) and oxygen content (B) with time for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg) and recovered in normoxia at 21 ^{o}C. Bars with different upper case letters indicate significant differences between normoxia and hypoxia. Bars with different lower case letters indicate significant differences between exposure time within a particular pO_2 treatment ($p < 0.05$, n=6/ treatment; two-way ANOVA; *post hoc:* Holm-Sidak multiple comparison test).

Table 5.1 Mean (±SE) hematological values with time for juvenile American paddlefish.

Treatment	Time (Hours)	Hematocrit (%)	[1]RBC (10^6/µl)	Hemoglobin (g/dl)	[2]MCV (fL)	[3]MCHC (g/dl)	[4]MCH (Pg)
Normoxia (148 ±3 mmHg)							
	1	22.38± 1.22 ^{Aa}	1.39± 0.07 ^{Aa}	7.48± 0.51 ^{Aa}	164.55± 11.71 ^{Aa}	32.84± 1.84 ^{Aa}	54.25± 4.65 ^{Aa}
	96	24.13± 1.30 ^{Aa}	1.41± 0.07 ^{Aa}	7.10± 0.33 ^{Aa}	190.22± 12.34 ^{Aa}	27.85± 1.90 ^{Aa}	52.87± 3.94 ^{Aa}
	0.5	22.63± 1.02 ^{Aa}	1.33± 0.08 ^{Aa}	6.86± 0.49 ^{Aa}	167.49± 12.10 ^{Aa}	32.22± 1.95 ^{Aa}	52.55± 3.87 ^{Aa}
	2	23.25± 1.15 ^{Aa}	1.36± 0.09 ^{Aa}	7.25± 0.38 ^{Aa}	183.65± 12.53 ^{Aa}	30.04± 1.75 ^{Aa}	54.33± 3.00 ^{Aa}
	4	24.38± 1.80 ^{Aa}	1.45± 0.06 ^{Aa}	7.10± 0.46 ^{Aa}	167.41± 13.18 ^{Aa}	33.93± 2.61 ^{Aa}	54.29± 4.15 ^{Aa}
Hypoxia (59 ±3 mmHg)							
	1	33.25± 1.64 ^{Ba}	1.41± 0.08 ^{Aa}	7.11± 0.29 ^{Aa}	235.34± 13.52 ^{Bb}	22.94± 2.00 ^{Ba}	52.51± 3.36 ^{Aa}
	96	38.13± 2.00 ^{Ba}	2.24± 0.11 ^{Bb}	10.08± 0.59 ^{Bb}	165.28± 10.90 ^{Aab}	26.14± 2.23 ^{Aa}	42.17± 2.51 ^{Aa}
	0.5	36.38± 1.56 ^{Ba}	2.05± 0.06 ^{Bb}	10.57± 0.51 ^{Bb}	193.26± 12.72 ^{Aab}	27.61± 1.88 ^{Aa}	51.91± 2.98 ^{Aa}
	2	33.50± 1.17 ^{Ba}	2.11± 0.13 ^{Bb}	10.53± 0.39 ^{Bb}	158.95± 11.82 ^{Aa}	31.25± 2.67 ^{Aa}	50.18± 4.80 ^{Aa}
	4	33.38± 1.21 ^{Ba}	2.03± 0.09 ^{Bb}	9.80± 0.61 ^{Bb}	178.02± 11.67 ^{Aa}	29.17± 2.31 ^{Aa}	52.31± 3.73 ^{Aa}

Table 5.1 represents mean (±SE) hematological values with time for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg)] and recovered in normoxia at 21 °C. Different upper case letters indicate significant differences between normoxia and hypoxia. Different lower case letters indicate significant differences between exposure time within a particular pO2 ($p < 0.05$, n=6/ treatment). [1]RBC: Red blood cells; [2]MCV: Mean corpuscular volume; [3]MCHC: Mean corpuscular hemoglobin concentration; [4]MCH: Mean corpuscular hemoglobin.

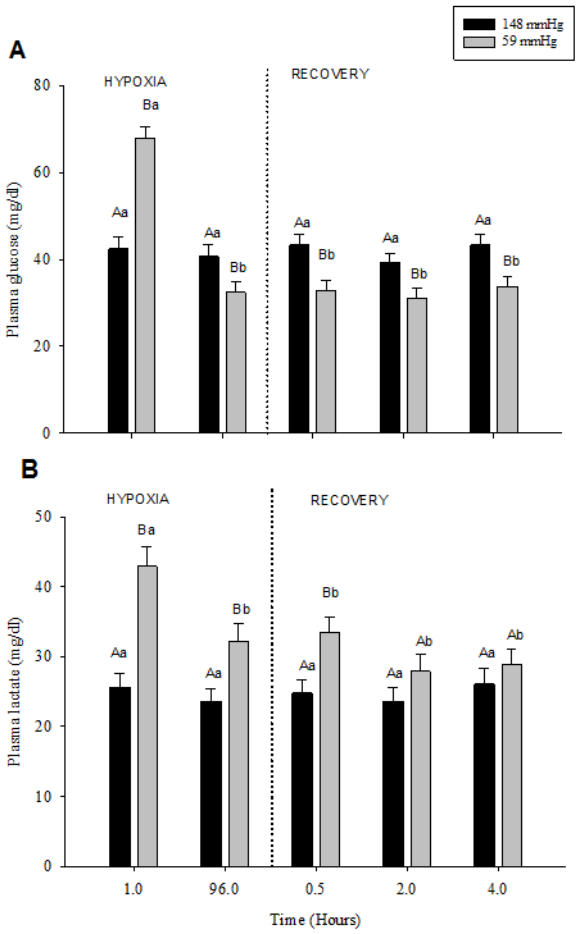

Figure 5.3 Mean (±SE) plasma glucose (A) and lactate (B) with time for juvenile American paddlefish.

Figure 5.3 represents mean (±SE) plasma glucose (A) and lactate (B) with time for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg) and recovered in normoxia at 21 ^{o}C. Bars with different upper case letters indicate significant differences between normoxia and hypoxia. Bars with different lower case letters indicate significant differences between exposure time within a particular pO_2 treatment ($p < 0.05$, n=6/ treatment; two-way ANOVA; *post hoc:* Holm-Sidak multiple comparison test).

Na^+, Cl^- and osmolality decreased after 1 h of exposure to hypoxia and returned to normoxic levels after 96 h of hypoxic exposure (Table 5.2). Plasma Na^+ of fish exposed to hypoxia increased slightly within 2 h in normoxic recovery. There were no effects of hypoxia on K^+ (Table 5.2).

Table 5.2 Mean (±SE) ion concentrations with time for juvenile American paddlefish.

Treatment	Time (Hours)	Na^+ (meq/L)	Cl^- (meq/L)	K^+ (meq/L)	Osmolality (mOsmol/kg)
Normoxia (148 ±3 mmHg)					
	1	127.88± 2.13 ^{Aa}	109.50± 2.96 ^{Aa}	3.73± 0.17 ^{Aa}	262.63± 4.39 ^{Aa}
	96	131.75± 2.97 ^{Aa}	113.63± 3.18 ^{Aa}	3.81± 0.14 ^{Aa}	264.75± 3.70 ^{Aa}
	0.5	127.00± 3.09 ^{Aa}	112.00± 2.91 ^{Aa}	3.65± 0.20 ^{Aa}	260.25± 3.97 ^{Aa}
	2	128.87± 3.19 ^{Aa}	110.75± 3.00 ^{Aa}	3.71± 0.19 ^{Aa}	262.88± 4.29 ^{Aa}
	4	130.00± 3.07 ^{Aa}	109.37± 3.25 ^{Aa}	3.84± 0.20 ^{Aa}	263.75± 4.55 ^{Aa}
Hypoxia (59 ±3 mmHg)					
	1	87.13± 3.11 ^{Ba}	76.50± 3.23 ^{Ba}	3.58± 0.22 ^{Aa}	231.63± 3.90 ^{Ba}
	96	124.13± 2.47 ^{Ab}	113.25± 2.95 ^{Ab}	3.66± 0.17 ^{Aa}	259.68± 3.75 ^{Ab}
	0.5	122.88± 3.11 ^{Ab}	109.50± 2.77 ^{Ab}	3.61± 0.20 ^{Aa}	268.5± 4.11 ^{Ab}
	2	134.50± 2.51 ^{Ac}	114.13± 3.39 ^{Ab}	3.43± 0.25 ^{Aa}	265.63± 4.21 ^{Ab}
	4	127.25± 2.78 ^{Abc}	113.00± 3.29 ^{Ab}	3.76± 0.24 ^{Aa}	267.63± 3.83 ^{Ab}

Table 5.2 represents mean (±SE) ion concentrations with time for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg)] and recovered in normoxia at 21 °C. Different upper case letters indicate significant differences between normoxia and hypoxia. Different lower case letters indicate significant differences between exposure time within a particular pO_2 ($p < 0.05$, n=6/ treatment).

Albumin concentration was below the lower limit of detectability (1 g/ dl) of the measuring instrument, for each of the 3 repeated measurements per sample. Therefore, albumin was either absent in the samples or concentrations were too low to be detected. Total protein concentration decreased after 96 h of hypoxic exposure, but increased to the level of normoxic fish within 2 h in recovery (Table 5.3). Plasma cholesterol and triglycerides did not change after 1 h exposure to mild hypoxia but decreased after 96 h and remained low throughout the recovery period (Table 5.3).

Table 5.3 Mean (±SE) plasma total protein, cholesterol and triglyceride concentrations for juvenile American paddlefish.

Treatment	Time (Hours)	Total proteins g/dL	Cholesterol mmol/L	Triglyceride mmol/L
Normoxia (148 ±3 mmHg)				
	1	1.79± 0.09 ^{Aa}	6.52± 0.20 ^{Aa}	1.37± 0.11 ^{Aa}
	96	1.88± 0.14 ^{Aa}	6.59± 0.09 ^{Aa}	1.32± 0.10 ^{Aa}
	0.5	1.84± 0.12 ^{Aa}	7.46± 0.16 ^{Aa}	1.29± 0.07 ^{Aa}
	2	1.78± 0.11 ^{Aa}	6.61± 0.08 ^{Aa}	1.34± 0.12 ^{Aa}
	4	1.97± 0.12 ^{Aa}	7.41± 0.09 ^{Aa}	1.27± 0.09 ^{Aa}
Hypoxia (59 ±3 mmHg)				
	1	1.74± 0.06 ^{Aa}	6.83± 0.18 ^{Aa}	1.31± 0.07 ^{Aa}
	96	0.69± 0.07 ^{Bb}	3.36± 0.16 ^{Bb}	0.53± 0.13 ^{Bb}
	0.5	0.71± 0.11 ^{Bb}	3.77± 0.22 ^{Bbc}	0.48± 0.09 ^{Bb}
	2	1.68± 0.11 ^{Aa}	3.41± 0.08 ^{Bb}	0.56± 0.11 ^{Bb}
	4	1.70± 0.13 ^{Aa}	3.98± 0.16 ^{Bc}	0.60± 0.11 ^{Bb}

Table 5.3 represents mean (±SE) plasma total protein, cholesterol and triglyceride concentrations with time for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg)] and recovered in normoxia at 21 ºC. Different upper case letters indicate significant differences between normoxia and hypoxia. Different lower case letters indicate significant differences between exposure time within a particular pO_2 ($p< 0.05$, n=6/treatment).

Plasma cortisol increased after 1 h exposure to moderate hypoxia and decreased to the level of normoxic fish within 2 h in recovery, but then increased within 2 h of recovery (Table 5.4). LDH increased in paddlefish exposed to hypoxia after 1 h, and decreased to the level of normoxic fish within 0.50 h of recovery. It then increased at 2 h before decreasing back to the level of normoxic fish at 4 h (Table 5.4). ALT increased after 1 h and AST increased after 96 h of exposure to hypoxia, and both remained elevated throughout the recovery period (Table 5.4).

Table 5.4 Mean (±SE) plasma cortisol and enzyme concentrations for juvenile American paddlefish.

Treatment		Cortisol	[1]LDH	[2]ALT	[3]AST
Normoxia (148 ±3 mmHg)	**Time (Hours)**	**ng/ml**	**U/L**	**U/L**	**U/L**
	1	4.50± 1.63 ^{Aa}	15.63± 2.39 ^{Aa}	2.48± 0.21 ^{Aa}	31.00± 4.40 ^{Aa}
	96	3.67± 1.64 ^{Aa}	16.88± 2.37 ^{Aa}	2.37± 0.26 ^{Aa}	33.86± 4.53 ^{Aa}
	0.5	4.23± 1.91 ^{Aa}	18.25± 2.53 ^{Aa}	2.16± 0.20 ^{Aa}	30.43± 4.47 ^{Aa}
	2	3.83± 1.81 ^{Aa}	17.13± 2.26 ^{Aa}	2.27± 0.21 ^{Aa}	29.29± 4.49 ^{Aa}
	4	4.18± 1.56 ^{Aa}	16.25± 2.03 ^{Aa}	2.25± 0.23 ^{Aa}	26.00± 3.91 ^{Aa}
Hypoxia (59 ±3 mmHg)					
	1	20.50± 1.85 ^{Ba}	41.87± 2.37 ^{Ba}	5.39± 0.32 ^{Ba}	22.00± 4.56 ^{Aa}
	96	13.17± 1.77 ^{Bbc}	37.88± 2.41 ^{Ba}	4.31± 0.23 ^{Bab}	56.14± 3.98 ^{Bb}
	0.5	14.33± 1.46 ^{Bb}	19.00± 2.33 ^{Ab}	4.04± 0.25 ^{Bb}	60.00± 4.45 ^{Bb}
	2	8.00± 1.79 ^{Acd}	33.75± 2.11 ^{Ba}	4.17± 0.25 ^{Bb}	57.51± 4.41 ^{Bb}
	4	6.17± 1.67 ^{Ad}	17.38± 2.36 ^{Ab}	4.49± 0.21 ^{Bab}	66.00± 4.28 ^{Bb}

Table 5.4 represents mean (±SE) plasma cortisol and enzyme concentrations with time for juvenile American paddlefish, *Polyodon spathula*, exposed to normoxia (148 ±3 mm Hg) or hypoxia (59 ±3 mm Hg)] and recovered in normoxia at 21 ºC. Different upper case letters indicate significant differences between normoxia and hypoxia. Different lower case letters indicate significant differences between exposure time within a particular pO_2 ($p< 0.05$, n=6/ treatment). [1]LDH: Lactate dehydrogenase; [2]ALT: Alanine aminotransferase; [3]AST: Aspartate aminotransferase.

5.6 Discussion

In fishes, hypoxia tolerance is characterized by the compensatory ability and relative reliance on aerial respiration, metabolic depression and/ or highly efficient anaerobic metabolism (Dunn and Hochachka 1986; Brauner et al. 1995; Kieffer et al. 2011). Paddlefish, as obligate ram ventilators with a total dependence on aquatic respiration, have limited capacity for metabolic depression when exposed to hypoxia (Burggren and Bemis 1992; Chapter 2). Therefore, efficient physiological adjustments aimed towards maintaining blood oxygen transport as well as mobilizing substrates for anaerobic metabolism and removing the associated wastes (lactate and H^+) are very

critical to paddlefish survival in hypoxia. This study provides evidence that paddlefish are sensitive to both acute and chronic hypoxia, and survival may be dependent on the duration of, or capacity for, anaerobic metabolism. Notably, their recovery, which may be aided by enhanced oxygen transport due to elevated Hb concentration, is relatively rapid.

5.6.1 Response to hypoxia exposure

The increase of cortisol in paddlefish exposed to hypoxia is an indication of the severity of stress. The cortisol concentration of paddlefish exposed to 1 h of moderate hypoxia as determined in the present study, is higher than that observed in paddlefish subjected to acute electroshocking (Davis and Parker 1986) and handling stress (Barton et al. 1998). Plasma cortisol of paddlefish exposed to hypoxia for 1 h was, however, relatively low compared to what has been measured in other fish after acute stress (Barton and Dwyer 1997; Barton et al. 1998). The reduction in cortisol after 96 h of moderate hypoxia exposure may be due to a desensitization of the HPI axis to the chronic hypoxia exposure (Wendelaar Bonga 1997; Barton 2002).

RBC, Hct and Hb of paddlefish exposed to normoxia in this study are either higher or similar to what have been observed in facultative ram ventilators such as rainbow trout (Lai et al. 2006), epaulette shark, *Hemiscyllium ocellatum* and grey carpet shark, *Chiloscyllium punctatum* (Chapman and Renshaw 2009). These high hematological values account for the high blood pO_2 observed in normoxic fish, and may reflect evolutionary adaptations to support their active lifestyles. The high blood pO_2 of fish in normoxia suggests that paddlefish may rely on a large Bohr effect to unload oxygen at the tissues (Jensen et al. 1998; Berenbrink et al. 2005). Thus, a low arterial pO_2 caused by low ambient water pO_2 reduces the pO_2 gradient between blood and the tissues

and as a result hinders efficient oxygen transport and offloading. Therefore, acute hypoxia exposure resulted in an inability of paddlefish to maintain the necessary oxygen supply to the tissues, which resulted in the metabolic disturbances. The lack of increase in RBCs and Hb at 2 h suggests that paddlefish exposed to acute hypoxia do not have the physiological ability to rapidly increase blood oxygen capacity and thereby maintain oxidative metabolism. Increases in glucose and lactate at 1 h support this premise, indicating that the decrease in oxidative metabolism was moderately supplemented by anaerobic metabolism. Thus, glycogen breakdown in the white muscles led to an accumulation of lactic acid which dissociates into lactate and H^+. The reduction in plasma glucose in concert with little change in lactate and LDH at 96 h suggests that glucose stores may have been depleted to support anaerobic metabolism. However, the fact that fish did not eat during the experiment, could account, at least in part, for the rapid depletion of glucose.

The reduction in blood pH in paddlefish is due to hypoxia-induced CO_2 and lactic acid accumulation that results in the release of H^+ into blood. The increases in blood pCO_2 in paddlefish exposed to both acute and chronic hypoxia are similar to those reported in studies that expose fish to oxygen limiting conditions (Piiper et al. 1972; Turner et al. 1983a, b). Blood pCO_2 may have remained elevated throughout the hypoxic exposure period, due to continuous swimming resulting from muscle-produced CO_2 diffusing into the blood. More CO_2 may have been formed through the combination of anaerobically produced H^+ with HCO_3^- (Jones and Randall 1978). Although the fish gill is efficient in CO_2 excretion (Evans et al. 2005), the constant swimming in hypoxia may have reduced efficiency caused by an increase in cardiac output and a consequent

reduction in blood transient time within the gill (Cameron and Polhemus 1974; Turner et al. 1983b).

The increase in MCV with a corresponding decrease in MCHC after 1 h of hypoxia suggest that RBC swelling occurred, likely due to Cl^- entry into RBCs followed by water in exchange for HCO_3^- to buffer blood (McDonald and Milligan 1997). The loss of Na^+ and Cl^- after 1 h of hypoxia could be due to osmorespiratory compromise (Nilsson 1986) caused by increased gill perfusion and permeability (Mcdonald and Milligan 1997). This physiological occurance can adversely affect the fish by decreasing energy reserves, due to the relatively high energetic costs associated with ion and osmoregulation (Rao 1968; Febry and Lutz 1987).

Glucose concentrations in hypoxic fish were lower than those observed in many other fish species exposed to stress (Polakof et al. 2012). Acipenseriforms have been shown to have a low capacity for glucose synthesis (Singer and Ballantyne 2005). However, most acipenseriforms have low aerobic metabolic rates under hypoxia, suggesting metabolic depression as the main mechanism for surviving hypoxia (Burggren and Randall 1978; Crocker and Cech 1997). For fishes exhibiting spontaneous swimming activity in normoxia, relatively high glucose elevation is observed during hypoxia (Polakof et al. 2012). Thus, the relatively small elevation in blood glucose observed in the present study is not consistent with the spontaneous swimming activity observed in paddlefish. A low capacity for anaerobic metabolism may have accounted for the small elevation and the subsequent depletion in glucose concentration.

Increases in plasma ALT and AST activities are considered reliable indicators of liver damage in fish (Casillas et al. 1983; Kpundeh et al. 2013). The increases in ALT

and AST concentrations, associated with exposure to moderate hypoxia, suggest possible liver tissue damage in paddlefish. Decrease in total plasma protein concentration is a potential indication of tissue repair in the liver (Kpundeh et al. 2013; Javed and Usmani 2014). Alternatively, the decrease could be due to either the use of protein as an additional energy source (Clements and Raubenheimer 2006) or reduced synthesis by the liver (Miller et al. 1951).

Albumin, the most abundant plasma protein in vertebrates (more than 50% of total plasma protein content), was below detectable levels, similar to the findings of Grant et al. (1970), suggesting the absence of this protein in paddlefish. Elasmobranchs (Irisawa and Irisawa 1954; Sulya et al. 1961) and gars (Gunter et al. 1961) also lack albumin. Changes in the concentration of plasma cholesterol and triglycerides are also considered reliable indicators of stress in fish (Nelson and Cox 2005). The reduction of these energy metabolites, derived from lipid absorption and fatty acid metabolism (Nelson and Cox 2005), supports the belief that exposure to hypoxia triggered a higher energy demand that aerobic metabolism could not meet. The decrease in the concentration of plasma cholesterol, a precursor molecule in the synthesis of steroid hormones such as cortisol, in hypoxic paddlefish may be the result of inhibition of steroidogenesis pathways (Nelson and Cox 2005) or related to low food intake.

5.6.2 Recovery from hypoxia

Recovery patterns exhibited by fish exposed to hypoxia give valuable insight into the energy metabolites used for coping with hypoxia and their subsequent restoration. Recovery patterns also highlight how toxic metabolites such as lactate, produced from anaerobic respiration, are removed from the blood. The rapid increase in blood pO_2 and

oxygen content shortly after fish were exposed to normoxic recovery conditions suggests that fish were able to rely on aerobic metabolism within 0.5 h and consequently restore the pO_2 gradient. The reduction in lactate concentration also supports this supposition. The rapid restoration of aerobic metabolism was likely aided by the high Hb concentration produced during chronic hypoxia exposure, which apparently led to oxygen content surpassing that of normoxic fish at 2 and 4 h. The lack of change in pCO_2 at the same time both blood pO_2 and oxygen content were increasing suggests that hyperventilation did not play a role in hypoxic recovery, similar to the findings of Burggren and Bemis (1992). The increase in blood pH at 2 and 4 h was likely a manifestation of the retention of metabolically produced HCO_3^- and exchanging the H^+ with Na^+ across the cells (Mcdonald and Milligan 1997). This physiological response is supported by the increase in HCO_3^- and Na^+ without any change in Cl^- at 2 h.

The unfavorable role of lactate in blood acid-base balance suggests that its removal from the blood may be a high priority of hypoxic fish after a return to normoxia. The slight reduction in lactate concentration after 2 h and the subsequent recovery after 4 h suggest that muscle lactate may have been retained and used as a substrate for glycogen re-synthesis or could have been the result of lactate enhanced utilization by oxidative tissues such as cardiac and red muscles (Milligan 1996). Usually, fish exposed to chonic hypoxia accumulate oxygen debt which is repaid during recovery by transporting lactate back to the liver and converting it back to pyruvate via the Cori cycle with the help of LDH (Van den Thillart and Verbeek 1991; Johansson et al. 1995; Lewis et al. 2007). The increase and subsequent decrease in LDH at 2 and 4 h, respectively, suggest that lactate was converted back to pyruvate within 2-4 h in recovery. The recovery of lactate to

normoxic levels is relatively shorter than that observed in teleosts after anaerobic metabolism (Milligan 1996). This difference could be due to either an effective lactate clearance mechanism or a quick repayment of oxygen debt due to the relatively low magnitude of lactate increase (Schulte et al. 1992; Rees et al. 2009). This study also supports the premise of a decreasing cortisol concentration during recovery from anaerobic metabolism positively influencing lactate clearance (Pagnotta et al. 1994; Eros and Milligan 1996; Milligan 1996).

In conclusion, this study demonstrated that exposure to moderate hypoxia is considered a stressor, with the severity or perception being higher during acute exposure as compared to chronic exposure. The duration of survival by paddlefish in moderate hypoxia is likely dependent on how quickly by-products of anaerobic metabolism (lactate and H^+) are cleared and energy reserves (i.e., glucose and glycogen) are replenished. The inability of hypoxic paddlefish to rapidly clear lactate and H^+ or efficiently replenish energy reserves suggests that survival will be compromised if they are exposed to a secondary stressor requiring the use of anaerobic metabolism (e.g., exhaustive exercise and handling). However, hypoxic recovery in paddlefish, as evidenced by changes in lactate concentration, blood pO_2 and oxygen content, occurred rapidly subsequent to exposure to normoxic water. This rate of recovery was likely due to the ability to rapidly increase oxygen content and restore the pO_2 gradient between blood and tissues. Hypoxic recovery is also aided by the ability of paddlefish to rapidly clear lactate and H^+ from the blood, while retaining HCO_3^- after exposure to normoxic water. It is likely that spontaneous swimming activity of paddlefish, which ensures an efficient ventilation of

the gills during the recovery period, enhanced the efficiency of these physiological processes (Milligan et al. 2000; Farrell et al. 2001; Kieffer et al. 2011).

Although paddlefish appear to store metabolic fuels in amounts that will only support short-term anaerobic metabolism to escape hypoxic conditions, they have the capacity for rapid recovery following reintroduction to normoxic conditions. Thus, the most likely response of paddlefish to hypoxia may be avoidance. However, recovery of energy substrates (i.e., glucose) occurred between 2 and 4 h in normoxia. These features indicate that further physiological perturbations from stressors such as handling and aerial exposure may increase mortality for paddlefish exposed to hypoxia. Therefore, environmental or anthropogenic activities that acutely expose paddlefish to hypoxia should be minimized and handling and aerial exposure under these conditions would most probably be lethal. Based on this study and those reported in chapters 2, 3 and 4, it is recommended that paddlefish have access to DO concentrations ≥ 5 mg/ L to maintain aerobic metabolism.

5.6.3 References

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APPENDIX A

A FLOWCHART OF MATERIALS AND METHODS FOR CHAPTER 2

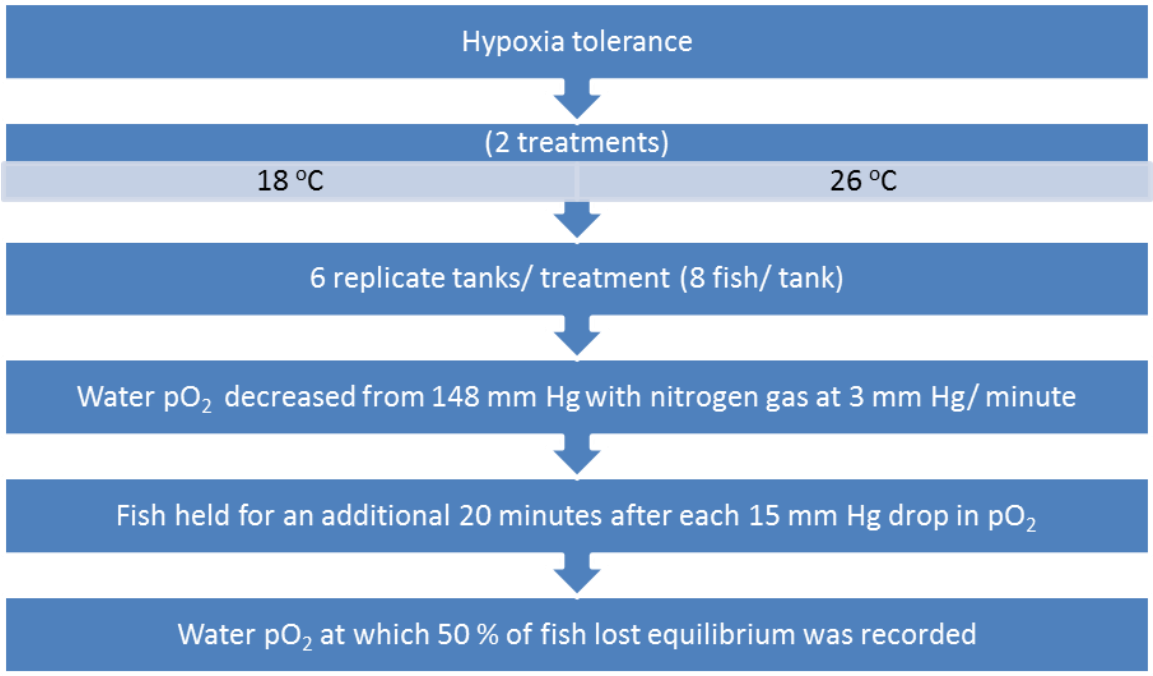

Figure A.1 Acute hypoxia tolerance of juvenile American paddlefish (~10 g) exposed to hypoxia.

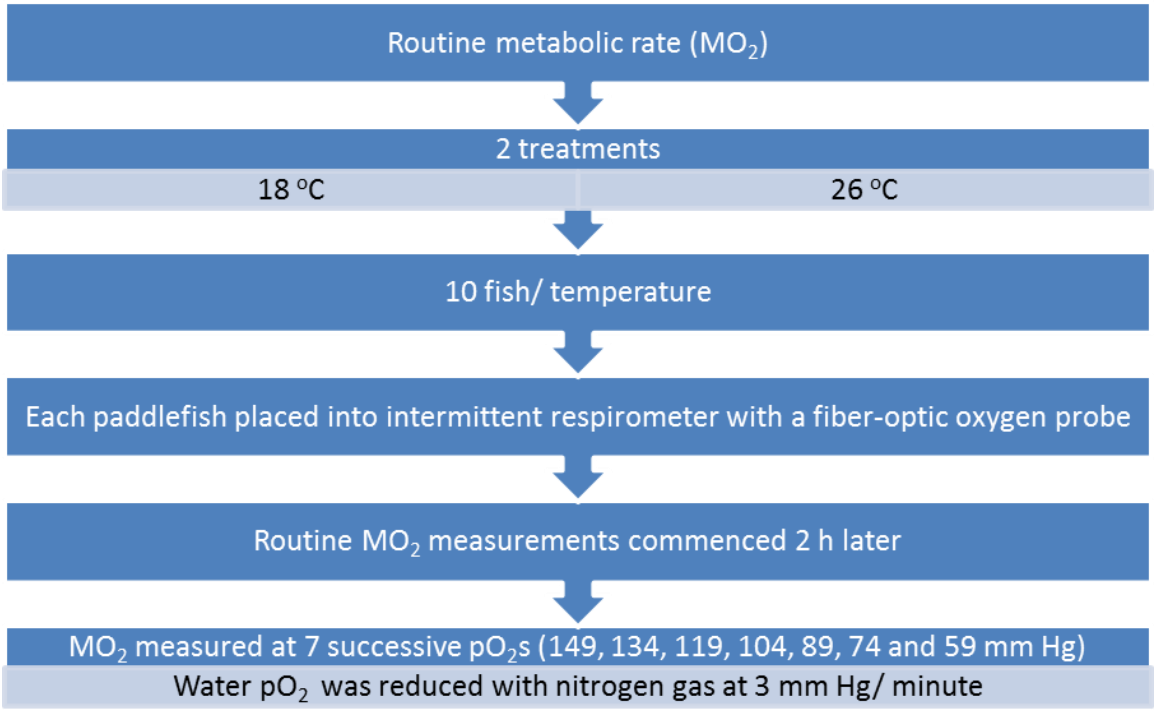

Figure A.2 Routine metabolic rate for juvenile American paddlefish (~10 g) exposed to hypoxia.

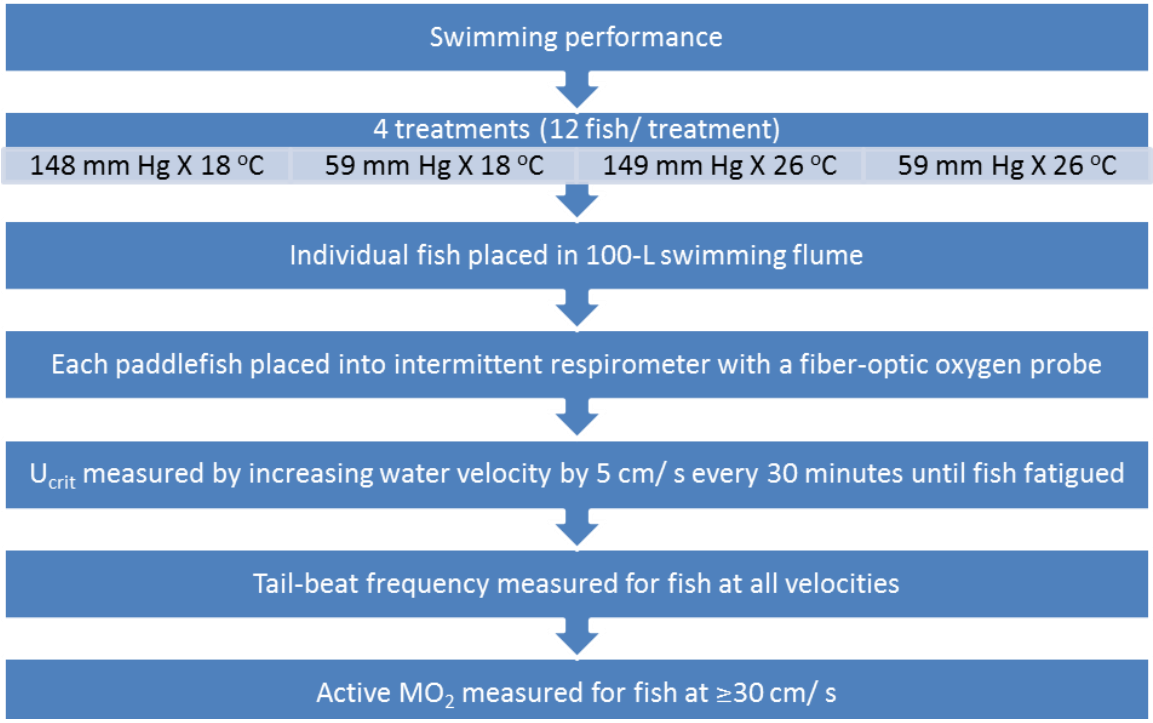

Figure A.3 Critical swimming speed for juvenile American paddlefish (~20 g) expoed to hypoxia.

APPENDIX B

A FLOWCHART OF MATERIALS AND METHODS FOR CHAPTER 3

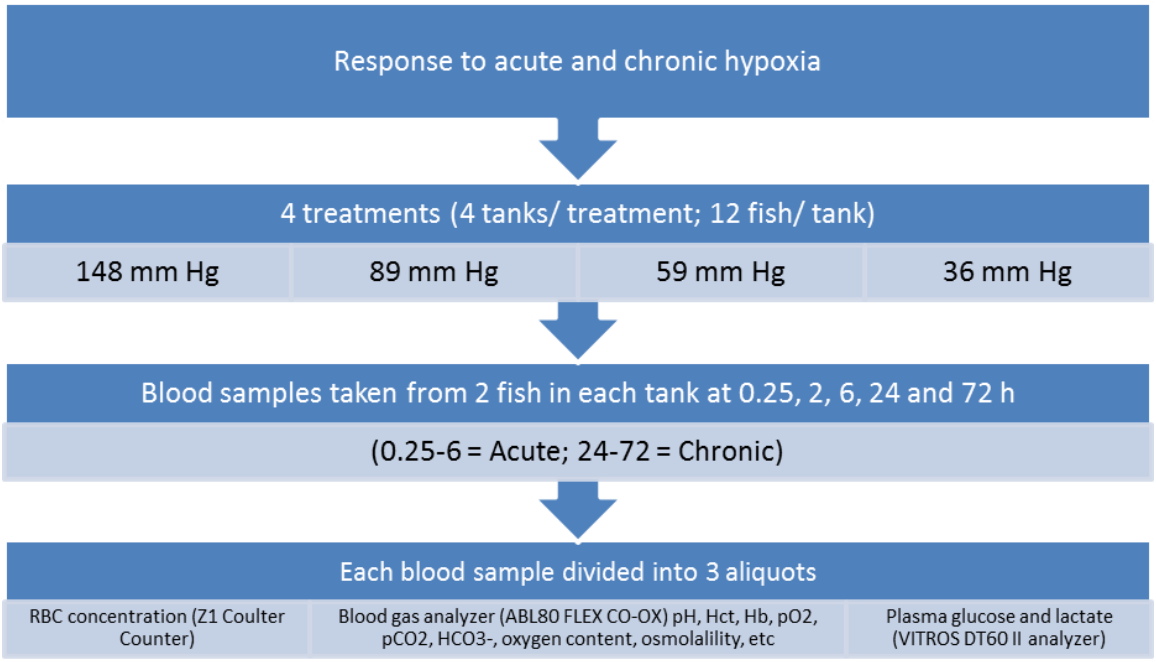

Figure B.1 Physiological response of juvenile American paddlefish (~150 g) to acute and chronic hypoxia.

APPENDIX C

A FLOWCHART OF THE MATERIALS AND METHODS USED IN CHAPTER 4

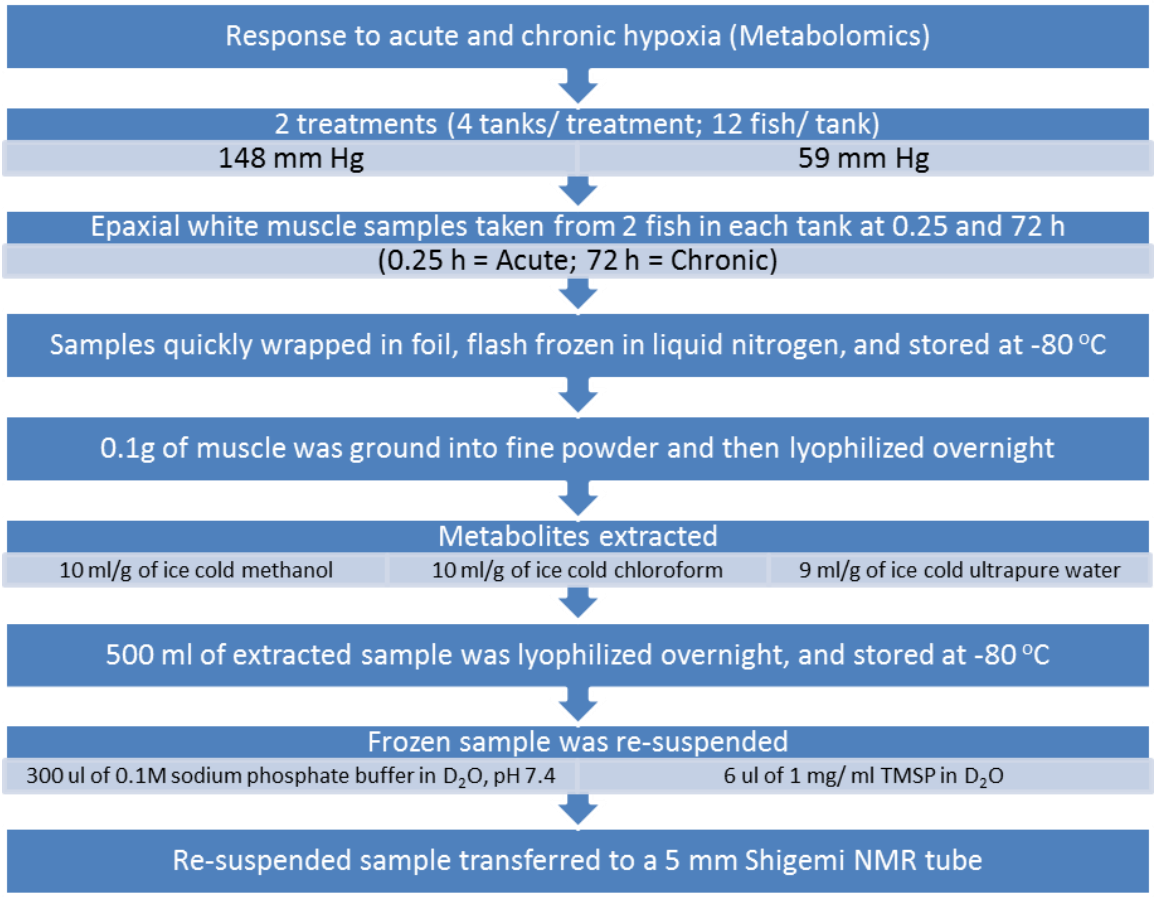

Figure C.1 ^{1}H-NMR study of the metabolic response of juvenile American paddlefish (~150 g) to hypoxia.

APPENDIX D

A FLOWCHART OF THE MATERIALS AND METHODS USED IN CHAPTER 5

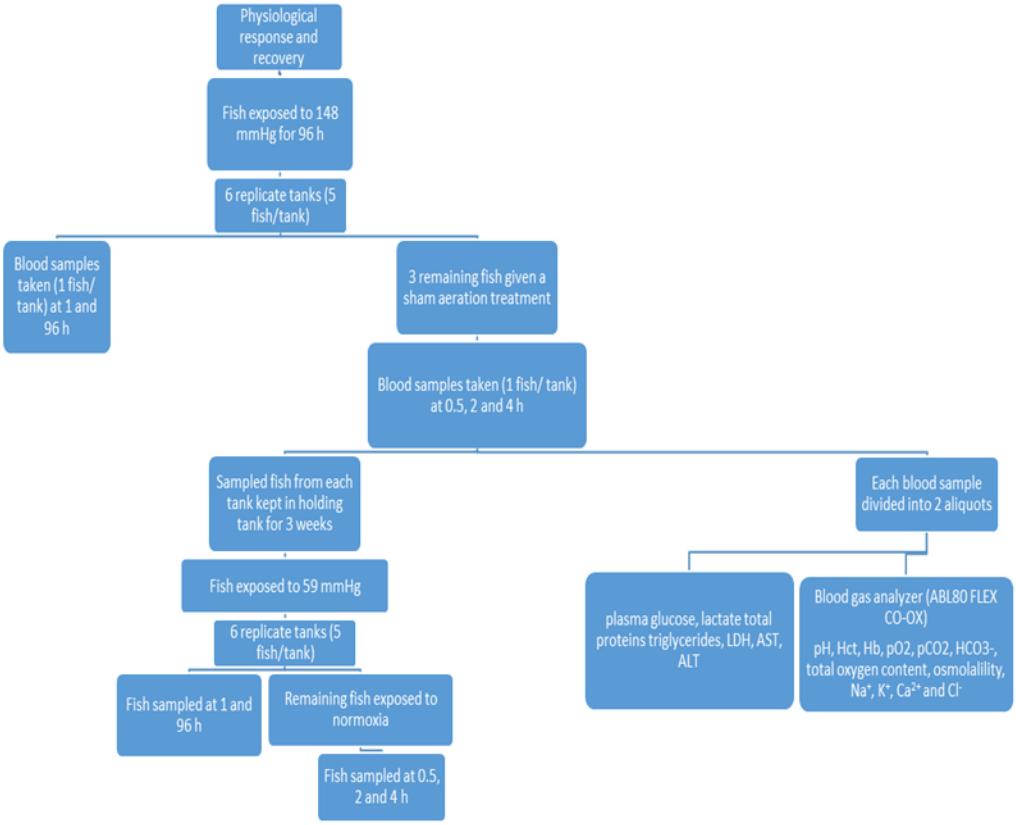

Figure D.1 Physiological response of juvenile American paddlefish (~181 g) to hypoxia recovery.