IMPACTS OF HYPOXIA ON LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*)
BEHAVIOR, PHYSIOLOGY, AND ACCLIMATION POTENTIAL

BY

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THESIS

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ABSTRACT

With urbanization increasing throughout the United States and globally, it is becoming increasingly important to understand the effects of hypoxia (a product of urbanization) on aquatic fauna. This thesis combines two independent, yet complementary, studies to help better understand how hypoxia affects one of the most popular sport fishes, the largemouth bass (*Micropterus salmoides*). The first study, conducted in 2010-2011 in the Chicago Area Waterway System, contained two parts: 1) quantification of largemouth bass movements in relation to dissolved oxygen through field telemetry, and 2) quantification of physiological parameters in largemouth bass within the study site and three reference sites through field sampling, low oxygen shock trials, and P_{crit} analyses (defined as the point at which an animal ceases aerobic respiration and begins anaerobic respiration). Results from this study indicate 1) largemouth bass did not show clear movement patterns out of hypoxic areas, but general avoided these areas altogether, 2) the physiological and nutritional condition of largemouth bass from the study site was similar to reference sites, indicating a lack of chronic stress or limited access to food, and 3) the physiological response of the study site fish to a low oxygen shock of 2.0 mg/L was similar in magnitude to reference sites, as well as similar P_{crit} values during respirometry trials, indicating a lack of an improved tolerance to hypoxia for the largemouth bass within the study site. The second study assessed the acclimation potential of largemouth bass to a low oxygen environment using hatchery-reared fish, to determine if largemouth bass had the ability to induce plastic changes to their phenotype within a hypoxic environment. This study held two groups of fish at differing oxygen levels (3.0 mg/L and 9.0 mg/L) for 50 d, where after they were exposed to a low oxygen shock of 2 mg/L. Results of this study indicate largemouth bass acclimated to a low oxygen environment significantly increased hemoglobin and hematocrit

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levels during an oxygen shock compared to fish not acclimated to low oxygen, indicating

largemouth bass, to some extent, possess the ability to impart advantageous changes to their

phenotype (e.g., increased oxygen uptake capacity) in order to survive in hypoxic conditions.

Together, these two studies help elucidate the potential mechanisms behind which largemouth

bass are able to survive in hypoxic conditions, potentially with little cost to their survival.

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CHAPTER 1:

GENERAL INTRODUCTION

Urbanization is one of the leading factors in the alteration of the structure, function, and dynamics of the planet's terrestrial and aquatic ecosystems, impacting both biotic and abiotic factors (Grimm et al. 2008). Urbanized areas are defined as a place with at least 50,000 people and at least approximately 1550 people per km^2 (US Census Bureau 2010). In the U.S. alone, from the years 1980-2000, the amount of land designated as urban increased by more than 34% (USDA Natural Resources Conservation Service 2001). Globally, this trend has been observed as well, with the percent of the population living in cities larger than 1 million people increased from 13% to almost 20% between the years 1975 and 2000 (UNPD 2001). Because of this substantial increase in people residing in urban environments, irreversible changes to the landscape can occur that can negatively impact biota contained within these systems, especially urban aquatic environments (Andres-Domenech et al. 2010; Casadio et al. 2010; Gasperi et al. 2010; Paul and Meyer 2008).

One way that urbanization can impact aquatic ecosystems is by altering patterns of water runoff following rain events. More specifically, urbanization leads to a reduction in evapotranspiration due to reduced vegetative cover (relative to forested landscapes), coupled with an increase in impervious surfaces (e.g. roads). Together, these two factors prevent infiltration of precipitation during rain events and lead to increased overland runoff (i.e. increased flooding). With over 80% of the U.S. population living in urban areas (US Census Bureau 2010), significant amounts of point and nonpoint source pollution [e.g. total suspended solids (TSS), lead (Pb), copper (Cu)] enter aquatic ecosystems during these overland runoff events (Brombach et al. 2005), affecting watersheds within, surrounding, and distances far-

reaching from the urban landscape (Grimm et al. 2008). Because of these alterations to the landscape, understanding how these factors affect the terrestrial and aquatic environment is essential to successfully guide management practices and regulations designed to protect species inhabiting urban environments. Specifically of interest to researchers are oxygen declines caused by high runoff following wet-weather events.

Due in part to high runoff, a major source of oxygen declines and pollution in the urban environment that can have negative consequences for aquatic species is from combined sewer overflows (CSO). Storm water removal systems have been engineered to collect overland runoff, along with domestic and industrial waste, for streamlined delivery to a sewage treatment plant. In most situations, sewer effluent has minimal impact on the aquatic environment because treatment processes and regulations reduce nutrient levels. However, during large rain events, particularly in large cities, storm water removal systems cannot handle the vast quantities of water entering the combined sewer system due to the combination of impervious surfaces and reduced evapotranspiration. As a result, when the capacity of the combined sewage system has been exceeded during large rain events, pollutant delivery into the aquatic system via CSOs is increased significantly compared to non-urban areas, and untreated sewage and runoff can directly enter the aquatic environment, bypassing treatment processes (Zhu et al. 2008; Baker 2009). Because raw sewage contains high amounts of nutrients (e.g. nitrogen and phosphorous) and oxygen depleting substances (Weibel et al. 1964; Casadio et al. 2010; Gasperi et al. 2010; Andres-Domenech et al. 2010), waters receiving these effluents can be severely impacted. If high levels of oxygen depleting substances are present, hypoxia or even anoxia can occur.

At the state level, Illinois Environmental Protection Agency has set regulations for 'general use' bodies of water with regards to dissolved oxygen. More specifically, Illinois EPA

recommends that, during the months of March-July, dissolved oxygen (DO) should remain above 5.0 mg O_2 L^{-1}, and during August-February, DO should remain above 3.5 mg O_2 L^{-1} (Illinois Environmental Protection Agency 2010). However, for waters labeled by the Illinois EPA as not suitable for general use (i.e. waters where contact with the water should be incidental or accidental, e.g. fishing and boating), Illinois EPA recommends DO never drop below 4.0 mg O_2 L^{-1} (Illinois EPA 2010). The United States Environmental Protection Agency, however, defines hypoxia as DO concentrations below 2-3 mg O_2 L^{-1} (USEPA). For the purposes of this study, hypoxia will be defined as DO concentrations below 2 mg O_2 L^{-1}.

As urbanization increases, the potential for additional aquatic systems to be impacted also increases. Understanding if these impacts (e.g. frequent hypoxia) have negative effects on fish assemblages is important for guiding successful management decisions. Therefore, two potential mechanisms exist that could allow fish communities to persist within areas that experience frequent hypoxia: (1) *behavioral adjustments* that allow fish to avoid areas of hypoxia, (2) physiological adjustments, either through *altered stress responses* or *acclimation*, that allow improved tolerance to hypoxic conditions. Conversely, no adjustments may occur in behavior, resulting in fish persisting, but being in low *health and/or condition*.

Behavioral Adjustments to Challenging Conditions

Rather than inducing plastic changes (which can be energetically expensive and come at a cost to fitness; Moran 1992; Newman 1992; Loman and Claesson 2003), animals may avoid environmental challenges through modifications to behavior to escape challenging conditions (i.e., seek out more favorable conditions), a concept known as the Bogert effect (Bogert 1949). Since the inception of telemetry equipment to track animal movements, major advances in technology have taken place allowing researchers to track animal movements at fine scales in

relation to several environmental factors, including habitat and resource selection, hydroelectric dam passage, migration patterns, and spatial distributions (Jepsen et al 1998; Ortega et al. 2009; Butler and Wahl 2010; Hupp et al. 2010; Rhoads et al. 2010). Specific to the goals of this study, telemetry has been successfully employed to assess habitat and resource selection of animals experiencing environmental stressors. Bell et al. (2003), for example, showed that while the blue crab *Callinectes sapidus* generally avoided hypoxia in the Neuse River Estuary, North Carolina, USA, many blue crabs tended to remain in hypoxic waters, suggesting the detection of hypoxia may be contingent on the magnitude and rapidness of the event. In studies examining how pulsed-flows (i.e. human-controlled releases of water in relation to hydroelectric power generation) affect movement of the rainbow trout *Oncorhynchus mykiss*, researchers have found a propensity of these fish to remain within their home range during non-migratory seasons (Jeffres et al. 2006; Cocherell et al. 2010). Studies such as these suggest sufficient foraging habitat outweighs the energetic effort required to seek more favorable conditions where food source abundance may be limited, motivating individuals to remain in unfavorable conditions rather than spend energy to move (Cocherell et al. 2010). In terms of prey species, however, studies such as these could suggest the cost of an individual moving into an unfamiliar area may expose itself to an increased predation risk. Though DO decreases to levels assumed to be detrimental to aquatic life, behavioral modifications may be affected by multiple factors outside of a specific DO level. More studies are needed to assess potential behavior modifications that allow fish in similar systems to survive.

Health & Condition

The nutritional status of fishes has been previously quantified using a suite of blood-based parameters that include plasma proteins, triglycerides, cholesterol, and ions, such as

chloride (Cl^-), sodium (Na^+), and potassium (K^+) (Wagner and Congelton 2004; Hanson and Cooke 2008). These constituents tend to decrease in fish during periods of low food resource availability (Wagner and Congelton 2004; Congelton and Wagner 2006), possibly due to decreased foraging habitats or lack of food resources. Lactate dehydrogenase (LDH) is responsible for the conversion of pyruvate to lactate (and vice versa) and subsequently is an indicator of tissue damage brought on by cell lysis (i.e. bursting) when found in plasma. In the presence of low oxygen, erythrocyte production tends to increase the capacity for oxygen to bind to hemoglobin. Quantifying mean cell hemoglobin concentrations (MCHC) of an animal experiencing hypoxia and comparing concentrations to one that is not can provide implications as to the severity of the physiological response. In addition to blood-based parameters, relative weight can be used as a metric for determining health of fish in regards to food availability. Relative weight (W_r) is the ratio of the actual weight of a fish to the weight of a normal growing population (Anderson and Neumann 1996). Essentially, fish with a low W_r (i.e. $W_r < 100$, with distance from this value varying by species) are said to be 'thin' and could indicate the presence of decreased feeding conditions (Anderson and Neumann 1996). Together, these blood-based nutritional parameters and whole animal indices can be quantified to identify the health and condition of free-swimming fishes.

Physiological Stress Response

Following the perception of an external stressor, such as low dissolved oxygen, numerous studies have documented a suite of physiological responses common to fishes. Elevating the stress response can cause increased disease susceptibility, decreased growth, and increased metabolism. Quantifying both the *magnitude*, as well as the *kind* of stress response can help us understand how individual fish and fish populations respond to environmental challenges. Of

these responses, the quantification of primary stress responses (i.e. catecholamines and corticosteroid hormone releases) has been the most common (Wendelaar Bonga 1997; Barton 2002). The corticosteroid used in quantifying stress responses in fish is the glucocorticoid, cortisol. Cortisol is responsible for maintaining homeostasis and fueling the 'fight or flight' response upon perception of an external stressor. A concomitant outcome of elevated cortisol concentrations is an inhibited immune response and susceptibility to pathogens (Wendelaar Bonga 1997). The release of cortisol is delayed following the perception of a stressor (approximately 5-10 minutes), making this biochemical response ideal for measuring resting stress levels immediately after capture or when sampled quickly in the field (Barton 2002). However, high cortisol levels can be responsible for stunted growth in fish, suggesting the presence of chronic stress (Wendelaar Bonga 1997). Other metrics used to quantify the stress response in fish are plasma glucose levels, as well as ion fluctuations (Wendelaar Bonga 1997; Caputo et al. 2009). Together, there are a number of blood-based metrics that can be measured to provide an indication of stress and disturbance in fish.

In addition to blood-based indices, it is possible to use whole-animal metrics to quantify disturbance and stress in fish, particularly metrics that relate to oxygen consumption and environmental stressors. Quantifying resting metabolic rates is important for researchers to determine how an animal is responding to an environmental change and its resulting physiological state. In essence, metabolic rate is a measure of energy consumption and tends to increase with exposure to predation, increased foraging activities, burst swimming (Cech 1990; Wang et al. 2009). More recently, researchers have shown metabolic rates increasing in response to a perceived stressor, which subsequently decreased growth rates in largemouth bass (O'Connor et al. 2011). Measuring the oxygen uptake of an animal can give researchers a

quantifiable amount of oxygen being consumed by an animal and subsequently its response to a change in the environment, acclimation potential, and stress level (Cech 1990). An excellent and robust measure of a fish's hypoxia tolerance is critical oxygen tension or P_{crit}. P_{crit} is the oxygen tension (P_{O2}) at which metabolic rate can no longer be maintained aerobically and animals must switch to anaerobic respiration. In essence, it is the threshold at which oxygen levels in the water are so low the animal can no longer utilize ambient oxygen for homeostasis (Fry and Hart 1949; Hughes 1973; Mandic et al. 2009). Theoretically, if an animal has a low P_{crit} value, it can tolerate lower oxygen environments because of an enhanced oxygen extraction capacity (through either a plastic or evolutionary change) and an increased ability to maintain aerobic metabolism (Mandic et al. 2009). Possessing an enhanced oxygen extraction capacity could be related to increased gill surface area, modified tissue oxygen demands, modified hemoglobin binding characteristics, or combinations thereof. These factors may be useful in understanding how fish residing in low oxygen systems are able to survive when conditions are less than favorable.

Acclimation to Challenging Environments

Acclimation, as it relates to prolonged exposure to physiological challenges, is defined as an alteration of a phenotype in response to a single environmental change (Huey et al. 1999) and is typically used in relation to laboratory experiments. This is in contrast to the processes of *acclimatization* and *adaptation*, which are defined as physiological responses to one or more environmental changes in the field (Prosser 1991), and a trait that has been beneficially shaped over a period of time in response to a change (West-Eberhart 1992), respectively. A widely debated explanation related to animals residing in challenging environments is the *beneficial acclimation hypothesis*, first described by Leroi et al. (1994). Beneficial acclimation is defined as an 'acclimation to a particular environment [that] gives an organism a performance advantage

in that environment over another organism that has not had the opportunity to acclimate to that particular environment' (Leroi et al. 1994). Since then, numerous studies have been published challenging the generality of this assumption (i.e. plastic changes do not always lead to increased fitness; Leroi et al. 1994; Huey and Berrigan 1999; Deere and Chown 2006; Lurman et al. 2009), leading researchers to believe that beneficial acclimation is uncommon. However, Wilson and Franklin (2002) argue such studies do not account for the adaptive significance [i.e. fitness consequences (positive, negative, or neutral) of phenotypic plasticity across a range of environments] or test competing hypothesis (Huey et al. 1999) that account for developmental influences that could affect performance and fitness. More recently, studies have successfully demonstrated beneficial thermal acclimation of several animal species residing in different thermal regimes. In one study using Eastern mosquitofish *Gambusia holbrooki*, increased fitness (specifically, increased rate of copulations) was observed in females acclimated to a higher temperature (Wilson et al. 2007); another study used the tardigrade *Macrobiotus harmsworthi* and researchers showed an increased fitness advantage to those tardigrades acclimated to warmer temperatures (Li and Wang 2005). Other studies have demonstrated animals exhibiting increased performance, specifically with the tropical butterfly *Bicyclus anynana* (Geister and Fischer 2007), and the common fruit fly *Drosophila melanogaster* (Frazier et al. 2008) when warm- and cold-acclimated individuals are placed in warm environments. Fish residing within low oxygen environments could experience a form of beneficial acclimation, whereby prolonged, repeated exposure to hypoxic conditions may have resulted in modifications to physiological properties that permit improved performance. To date, relatively little work on beneficial acclimation in relation to dissolved oxygen has been performed, but evidence suggests

that environmental oxygen concentration has the potential to induce physiological changes in physiological properties of fishes (Matschak et al. 1998).

Conclusion

Although numerous studies have been conducted on fish assemblages in large water bodies, rivers, and streams, little work has been done on highly urbanized systems. With the growth of urban areas globally, coupled with the propensity of urbanization to alter the structure and function of aquatic environments, there is a *critical need* to quantify the response of fish (behaviorally and physiologically) to environmental stressors (e.g. hypoxia) in systems such as these. Without this information base, current management practices may be overly conservative or liberal in protecting fish assemblages, or only address symptoms of the problem and not the cause. These potential errors in urban fisheries management may be wasteful of resources, both economic and environmental.

Based on this background, the goals of this thesis are to 1) understand how hypoxia affects movement rates and behavior of fish in a system regularly exposed to hypoxia, 2) compare the health, nutrition, and physiology of fish within an urbanized system to a control site to understand how fish regulate homeostasis internally when faced with periods of hypoxia, and 3) identify potential mechanisms that permit fish to persist within a system that is anthropogenically viewed as degraded. With these goals in mind, we plan to elucidate potential mechanisms fish utilize to regulate homeostasis in the event of an environmental stressor (e.g. hypoxia). Using largemouth bass as a model species, we will use this information to make inferences about broader fish assemblages across urban centers that can be used to help guide management practices and identify regulations that are sufficiently protective of species in these types of environments.

Study Site and Species

The Chicago Area Waterway System (CAWS) was built during the late 19th and early 20th centuries for the purposes of commercial navigation and drainage of urban storm water runoff and municipal wastewater effluent for the city of Chicago (MWRDGC 2008) and is labeled by the Illinois EPA as a body of water not suited for general use activities. Located throughout the CAWS are 225 CSOs that service approximately 970 km^2 of the city of Chicago (MWRDGC 2008). A unique section of the CAWS is the South Fork South Branch Chicago River, commonly known as Bubbly Creek. At the south end of Bubbly Creek is the Racine Avenue Pumping Station, which during large rain events has the capacity to discharge raw sewage and storm water into Bubbly Creek at approximately 89 cubic meters S^{-1} (MWRDGC 2008). Because of this station, and several CSOs located along Bubbly Creek, this area experiences frequent periods of hypoxia and anoxia (anoxia defined as dissolved oxygen concentrations being 0 mg O_2 L^{-1}), both from stagnation and sewage discharge sites. For example, in 2008, 2009, and 2010, the Racine Avenue Pumping Station (located within the CAWS study site) had 15, 23, and 17 respective discharge events with yearly averages of 2768, 1635, and 1601 megaliters (ML; unpublished data MWRDGC). The MRWDGC's continuous dissolved oxygen monitoring (CDOM) program monitors DO concentrations throughout the CAWS on an hourly basis year round. Data published in their reports indicate that in Bubbly Creek minimum DO concentrations in 2008 and 2009 were 0.0 mg O_2 L^{-1} for both years, with mean concentrations of 4.3 mg O_2 L^{-1} in 2008 and 3.1 mg O_2 L^{-1} in 2009. The percentage of time areas of Bubbly Creek experienced DO concentrations below 2 mg O_2 L^{-1} in 2008 and 2009 were 40% and 52%, respectively (MWRDGC 2009; MWRDGC 2010).

Despite the frequent hypoxia/anoxia episodes, the CAWS, and the area around Bubbly Creek in particular, has been shown during recent surveys to contain a rich diversity of fish

species. The Metropolitan Water Reclamation District of Chicago (MWRDGC) has been

performing biological inventory assessments of the CAWS for 32 years (1974-2006), and results

have shown cumulative fish species have increased from 10 to 70 over that time period.

Although the most abundant of these species within the Bubbly Creek area tend to be the gizzard

shad *Dorosoma cepedianum*, the common carp *Cyprinus carpio*, and various *Lepomis* spp., other

species of the family Centrarchidae (*Pomoxis* spp., and *Micropterus* spp.) and Cyprinidae (e.g.

Notropis atherinoides, *Pimephales notatus*, *Cyprinella spiloptera*) are common as well

(unpublished data MWRDGC). Recent surveys in the Bubbly Creek area by the MWRDGC and

Illinois Natural History Survey (INHS) have revealed species diversity within Bubbly Creek is

similar to other areas throughout the CAWS not affected by acute periods of hypoxia

(unpublished data MWRDGC; unpublished data INHS). Based on the prominence of the

hypoxia events, and the strength and stability of the fish populations, the CAWS, and Bubbly

creek in particular, is an ideal location to study the *in situ* responses of individual fish and fish

populations to hypoxia resulting from urbanization.

The study species for the study is the largemouth bass, *Micropterus salmoides*. This

species was chosen because it is abundant throughout the CAWS (unpublished data MWRDGC);

it has well defined home ranges (Gerking 1953; Lewis and Flickinger 1967; Winter 1977), and

does not exhibit large migratory movements over great distances (Winter 1977; Fish and Savitz

1983; Warden and Lorio 1975). Largemouth bass are also sufficiently large to allow for the

successful implantation of telemetry tags, permitting the coupling of field and laboratory

analyses that would not be possible with smaller fish species.

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CHAPTER 2:

BEHAVIORAL AND PHYSIOLOGICAL RESPONSES OF LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) TO RAIN-INDUCED REDUCTIONS IN DISSOLVED OXYGEN IN AN URBAN SYSTEM.

Abstract

Waters in urbanized areas often experience hypoxic events due to combined sewer overflows and urban runoff. Low dissolved oxygen is believed to negatively affect biota in the receiving waters, yet many urban areas have rich and diverse fish species assemblages. This study assessed the behavioral and physiological responses of urban fish to understand how they have adapted to hypoxia. Largemouth bass from the Chicago Area Waterway System (CAWS) were studied using field telemetry and laboratory experiments. Fish movement in relation to dissolved oxygen levels was tracked using acoustic transmitters. Although abundance of largemouth bass before and during hypoxia was low, there was no clear pattern of largemouth bass avoiding these areas altogether. Three physiology components assessed the mechanisms behind patterns observed in the field. Field sampling of largemouth bass at the study site and three reference sites found fish were not in poor nutritional condition and were not suffering from chronic stress. Largemouth bass from the study site had slight improvements in oxygen transport abilities. Fish from the same sites were subjected to an oxygen shock of 2.0 mg·L^{-1} for 6 h and study site fish did not exhibit an enhanced (or reduced) tolerance to low dissolved oxygen when compared to fish in control populations. Results indicate behavioral shifts occurred, reducing time in hypoxic zones, without apparent large physiological costs.

Keywords: Urbanization, hypoxia, physiology, movement, stress

Introduction

Point and nonpoint discharges in urban areas from rain events have long been identified as a significant cause of pollution inputs and poor water quality. In particular, oxygen-depleting substances discharged to, or resuspended within, receiving waters during rain events are considered one of the leading pollutants affecting urban waters (Burton and Pitt 2002). Oxygen depletion problems may occur during a rain event, although reduced oxygen conditions have also been shown to persist for days or even weeks following such events (Burton and Pitt 2002; Alp 2006). The effects of rain events on dissolved oxygen (DO) in urban environments are difficult to assess because of the complex physical and chemical interactions between the discharge events and receiving waters, and site-specific conditions. The assumption is that these events adversely affect the biota within receiving waters, but specific studies to quantify the effect of potential stressors, such as DO, on local biota are lacking.

The adverse effects of hypoxia, (DO concentrations < 2 mg·L^{-1}; Diaz 2001), on aquatic life, such as fishes, have been studied in controlled laboratory settings and have been well documented (Kramer 1987; reviewed in Pollock et al. 2007). When aquatic organisms experience reductions in DO that are severe, they may react by either acquiring additional oxygen from their environment (Timmerman and Chapman 2004), they may move to more oxygen-rich habitat (Bogert 1949; Bell et al. 2003; Craig and Crowder 2005), or otherwise mitigate the potential negative consequences of hypoxia for energy generation and homeostasis (Pollock et al. 2007; Mandic et al. 2009). In addition, fish that have been chronically stressed may have an impaired ability to mount a stress response against subsequent challenges (Barton et al., 1987). Previous work has shown that exposure of fish to repeated and/or prolonged periods of hypoxia can result in a suite of sub-lethal consequences that include reduced swimming speed,

shifts in habitat use, altered availability of prey resources, and, potentially, reduced feeding

opportunities (Pollock et al. 2007; Kramer 1987). In addition, many of the sub-lethal,

physiological consequences of hypoxia exposure are energetically costly to either induce or

correct, potentially resulting in elevated consumption of energy resources for chronically stressed

individuals (Pollock et al., 2007). Chronic elevation of the stress response in fishes has been

linked to an impaired immune system and a loss of ions from plasma, elevated plasma glucose

concentrations, an impaired stress response, and decreased growth (Barton et al. 1987; Pickering

and Pottinger 1989). Exposure to low oxygen environments has also been shown to cause

elevated bacterial/fungal infections, increased parasite loads, elevated levels of oxidative stress,

and possibly endocrine disruption (Pickering and Pottinger 1989; Lushchak and Bagnyukova

2006). While many effects of low DO on fish responses have been investigated in the

laboratory, there is a critical need to examine the impact of rain events in the field, integrating

with laboratory-derived data.

Combined sewer overflow (CSO) discharges can negatively impact aquatic ecosystems

by causing increased nutrient loads, increased levels of sedimentation, biological oxygen demand

(BOD), increased prevalence of bacteria, and elevated concentration of heavy metals (Brombach

et al. 2005; Casadio et al. 2010). However, examinations of the response of aquatic organisms to

hypoxia and CSOs in field situations are lacking, making it difficult to link laboratory-derived

limits to oxygen and the sensitivity of organisms in the wild. Anecdotal observations of the

effects of low oxygen on fishes in the wild are inconclusive, and recent field research suggests

that fishes may not necessarily inhabit the highest oxygenated waters (Hasler et al. 2009).

Based on this background, the first objective of the current study was to quantify the

movement of largemouth bass residing in an urban setting to rain-induced periods of hypoxia.

For this, we deployed a stationary, autonomous telemetry array downstream of a major sewer discharge within the Chicago Area Waterway System (CAWS), and outfitted resident largemouth bass with acoustic telemetry tags. The site has historically been shown to not only experience episodic periods of hypoxia (and anoxia), but also has stable resident fish populations. The second objective was to quantify the physiological response of largemouth bass from the CAWS that frequently experiences prolonged rain-induced hypoxia to an acute hypoxia stressor, and compare this response to reference sites that do not experience episodic bouts of hypoxia. Together, results will elucidate how fish inhabiting an urban environment respond, both behaviorally and physiologically, when exposed to frequent and extended periods of low oxygen.

Materials and Methods

Study site

The study site was located southwest of downtown Chicago, IL, USA, within a formerly natural segment of the CAWS (41º50'N, 87º39'W). The study site spanned approximately 7 km of the South Branch Chicago River (Main Channel) at the confluence of the South Fork of the South Branch Chicago River (Bubbly Creek). Nearly 40 CSOs are located within the study area and contribute to reductions in DO following large rain events. Bubbly Creek itself historically experiences frequent periods of low dissolved oxygen; from 2000-2009, CSOs within the study area discharged sewage an average of 15 times per year, with a minimum of 5 events and a maximum of 23 during a single year (Waterman et al. 2011). Dissolved oxygen was monitored within the study site through the use of three dissolved oxygen sondes (YSI 6600 and YSI 6920, YSI Instruments, Yellow Springs, OH, USA) programmed to log dissolved oxygen data every hour. Sondes were located ~ 1 km downstream of Bubbly Creek, ~ 0.4 km upstream of Bubbly

Creek, and within Bubbly Creek. Start and stop dates and times of CSO discharges were

recorded by the Metropolitan Water District of Greater Chicago (MWRD) to quantify discharges

in relation to DO fluctuations.

Fish movement

The fish species used to relate movement to dissolved oxygen was the largemouth bass

Micropterus salmoides. Largemouth bass were chosen because they previously have been

shown to be abundant throughout the CAWS, as well as being a widely popular sport fish with

ranges that span across the United States. Largemouth bass also have well defined home ranges

(Gerking 1953; Lewis and Flickinger 1967; Winter 1977), do not exhibit large migratory

movements over great distances (Warden and Lorio 1975; Winter 1977; Fish and Savitz 1983),

and can reach sufficient size to permit implantation with long-term telemetry devices (Hasler et

al. 2009). Largemouth bass were collected between July 14th and 16th, 2010, using standard

boat electroshocking (DC) and ranged in size from 224 mm to 350 mm in length ($\bar{x}$ = 274 mm)

and ranged from 180 g to 653 g in weight ($\bar{x}$ = 326 g). Another six largemouth bass were tagged

in April of 2011 using identical procedures to the 2010 tagging to supplement transmitter loss

from 2010. Sex was not determined for any largemouth bass used in this study because fish were

not sampled during the spawning period and sex differences were likely not a factor (Cooke

2004). Fourteen VR2W receivers (Vemco Inc., Halifax, Nova Scotia, Canada) were deployed

within the Main Channel and Bubbly Creek from July, 2010 to October 2011 to record fish

movements. These receivers have a 'listening radius' that logs the presence of a tagged

largemouth bass when in proximity to the receiver, but does not provide positioning data at an

accuracy of more than ± approximately 250 m.

Surgical implantation of telemetry tags

Two tanks were used for surgeries, each filled with 20 L of water taken from the CAWS. Each tank contained two air stones, one bubbling carbon dioxide (CO_2) and the other oxygen (O_2). CO_2 was released at 2.36 $L \cdot min^{-1}$ from a regulator and O_2 at 1 $L \cdot min^{-1}$. Both tanks were buffered with 3.0 $mg \cdot L^{-1}$ of sodium bicarbonate ($NaHCO_3$). Fish were placed ventral side up on a V-cut foam pad and a silicon tube was inserted into the fish mouth to perfuse the gills with oxygenated water. A 15 mm incision was made parallel to and off the midline, anterior to the pelvic girdle, into the peritoneal cavity using a #10 scalpel blade. The transmitter (V7-4L, 22.5 × 7 mm, 1.8 g in air, 1.0 g in water) was placed inside the body cavity and two sutures consisting of four double throws (Mono-Dox absorbable synthetic monofilament 3/0 NFS-1, CP Medical, Portland, OR, USA) were used to close the incision. Surgery times did not exceed 2 min. Fish were then placed in a recovery tank (150 L) for at least 1 h for post-surgical monitoring, following which, they were released into the CAWS.

Physiology

Reference site selection

Basal physiological parameters (i.e., baseline values) were collected to quantify fish stress, health, and condition before experiments were conducted. In addition to collecting baseline physiological samples from fish residing in the study site, we selected three additional reference sites against which fish from the study site could be compared. Requirements for identifying a waterbody as a reference site included (a) being in relative proximity to the study site, and (b) exhibiting higher annual DO profiles than the study site, based on data collected between 2007 and 2009 by the MWRD. Based on these criteria, three locations were chosen as reference sites: (1) the North Shore Channel of the CAWS (42º1'N, 87º42'W) approximately 20 km upstream from the study site (reference site 1), (2) Busse Lake (42º1'N, 88º0'W), a 239 ha

impoundment approximately 35 km northwest of the study site in the Salt Creek Watershed, Ned

Brown Preserve, Chicago, IL, USA (reference site 2), and (3) a section of the Des Plains River

(41º25'N, 88º10'W), south of Joliet IL, USA and approximately 60 km southwest of the study

site (reference site 3). All three sites were contained within the Chicago area. Within each of the

three reference sites, DO data were assessed as the amount of time DO remained above and

below 4.0 mg·L^{-1}. An oxygen concentration 4.0 mg·L^{-1} was used instead of 2.0 mg·L^{-1} to be

confident these sites rarely experienced periods of moderate to low oxygen concentrations. The

three reference sites chosen all remained above 4.0 mg·L^{-1} 95 % of the time during the 3 year

period. During the same 3 year period, DO concentrations in the study site were above 4.0

mg·L^{-1} only 39% of the time.

Field sampling

 Eight largemouth bass (total length between 200 and 300 mm) were collected from each

of the four sites (one study site and 3 reference sites) between June and August, 2011 using

standard, direct current (DC) boat electroshocking. Eight fish from each of the 4 sites were

immediately sacrificed and sampled for blood and tissues to quantify physiological and

nutritional parameters, representing the condition of free-swimming largemouth bass at these

sites. Immediately after being stunned by electrofishing gear, fish were sacrificed by cerebral

concussion and blood was drawn from the caudal vessel with a 21-gauge needle and 1 mL

syringe rinsed with lithium heparin. To quantify hematocrit (Hct, i.e., % packed red cell

volume), a small volume of whole blood was placed in a capillary tube and spun for 2 min using

a hematocrit spinner; due to technical problems in the field, hematocrit data were collected from

3 of the 4 sites only (not from reference site 2). An additional aliquot of whole blood was

transferred to a microcentrifuge tube for subsequent quantification of hemoglobin. The

remaining whole blood was centrifuged for 2 min at 2000 × gravity (*g*), and plasma was separated from erythrocytes and transferred to an additional microcentrifuge tube. Plasma and whole blood were stored in the field in liquid nitrogen, and then transferred to an ultracold freezer (-80 °C) until further laboratory analyses (Suski et al. 2003). A section of white epaxial musculature posterior to the operculum (gill cover) and above the lateral line, was excised with a razor blade and stored in liquid nitrogen until further processing (Suski et al. 2003). To avoid any sampling-induced physiological disturbances, sampling typically took less than 2 minutes to complete (Romero and Reed 2005). Additionally, eight largemouth bass from each site were transported to the Aquatic Research Facility in Champaign, IL, USA for the hypoxia challenge experiment described below, and an additional eight fish from each site were transported to the Kaskaskia Biological Station (KBS; Sullivan, IL, USA) for quantification of critical oxygen tension (P_{crit}), also described below.

Hypoxia challenge experiment

Following transport to the aquatic research facility, largemouth bass from each of the 4 sites were held in aerated tanks to recover from hauling stress for 48-72 hours in outdoor, aerated holding tanks supplied with pond water. Immediately after the recovery period, 16 largemouth bass from each site were placed into opaque, aerated sensory-deprived chambers continuously supplied with fresh water from a central basin in a closed system, with DO maintained at 7.0 mg·L^{-1} (Suski et al. 2006). Following a 24 h acclimation period to these chambers, eight largemouth bass were subjected to a DO shock of 2.0 mg·L^{-1} (± 0.1 SE) for six hours, by bubbling nitrogen gas (N_2) into the chambers to displace oxygen (Suski et al. 2006). Exposure to this DO concentration for this duration of time has previously been shown to elicit a physiological response in largemouth bass (VanLandeghem et al. 2010). A separate group of

eight control largemouth bass remained in the chambers for six hours with no change in DO (6.5 mg·L^{-1} ± 0.1 SE). Following this six-hour period, all largemouth bass were sacrificed with an overdose of tricaine methanesulfonate (MS-222; 250 mg·L^{-1} buffered with 250 mg·L^{-1} sodium bicarbonate), removed from the chambers, and blood and tissues were collected using methods identical to the field sampling described above.

Critical Oxygen Tension (P_{crit})

Largemouth bass from each site (study site and three reference sites) were subjected to decreasing oxygen levels, and both metabolic rates and P_{crit} were quantified to determine if largemouth bass from the study site exhibited lower P_{crit} values compared to populations not exposed to frequent periods of hypoxia. For this, following transport to KBS, largemouth bass were allowed to recover from handling and hauling stressors for 48 h in outdoor, aerated tanks. Fish from at least two study sites were randomly chosen and placed in one of four acrylic experimental chambers (355 mm long × 127 mm inner diameter; 4.5 L volume) outfitted with fiber-optic oxygen probes (calibrated with oxygen-free water and fully aerated water continuously throughout the experiment), immersed in a 500 L tank maintained at 24ºC (± 0.5 º C) and allowed to acclimate for 3 h at ambient temperatures and DO levels (7-8 mg·L^{-1}). Treatments consisted of exposing largemouth bass to declining dissolved oxygen concentrations (6, 4, 3, 2, and 1 mg·L^{-1}) over a 4 h period, during which time metabolic rates and P_{crit} values were measured and recorded continuously using automated software (AutoResp 2.0, Loligo Systems, Tjele, Denmark) based on methods described in Iversen et al. (2010). Briefly, each trial loop during data collection consisted of a 10-minute flush period, followed by a one-minute wait period (to allow for conditions to stabilize), and concluded with a 5-minute measurement period in which the chamber was closed and oxygen decline was quantified every second. Trial

loops were run in triplicate for each dissolved oxygen concentration (Iversen et al. 2010).

Change in oxygen concentration (α) for each chamber was calculated as slope ($\Delta O_{2saturation}/\Delta t$),

and oxygen consumption rate (MO_2, mg $O_2 \cdot kg^{-1} \cdot h^{-1}$) for each fish was calculated by

$$MO_2 = \alpha \cdot V_{resp} \cdot \beta \cdot M_b^{-1}$$

where V_{resp} is the volume of each experimental chamber minus the volume of the fish (L), β is

oxygen solubility (adjusted for temperature and barometric pressure), and M_b is the fish mass

(kg) prior to placing in the chamber. Standard metabolic rate (SMR) was calculated as the

average of the lowest 5% of the MO_2 values and P_{crit} was determined for each individual fish by

the linear regression of the MO_2 values against the oxygen concentration. The P_{crit} was then

determined as the oxygen concentration where the regression line intersected the previously

determined SMR (Schurmann and Steffensen 1997; Iversen et al. 2010). The coefficient of

determination (r^2) for all slope measurements was > 0.95 and oxygen concentration in the

chambers was recorded every 1 second. Chambers were disinfected with iodine between trials to

prevent bacterial growth. Background oxygen consumption of chambers was measured and

subtracted from MO_2 values attained during the trials.

Movement analyses

The movement of largemouth bass in response to hypoxia was quantified using two

separate analyses. For the first analysis, daily mean dissolved oxygen readings recorded by each

sonde were first calculated, with each sonde assumed to be representative of the section of the

study site in which it was deployed. If a sonde (i.e., section of the study site) showed a daily

mean DO of below 2 mg$\cdot$L^{-1} for 4 d or more, these periods were defined as "hypoxia events" and

used for analyses. To quantify movement of the largemouth bass in response to hypoxia events,

the number of unique transmitter IDs (i.e., tagged largemouth bass) located in proximity of a

sonde was summed for a period of 4 d before a hypoxia event and compared to the number of

unique transmitter IDs located in proximity to that same sonde 4 d immediately after the onset a

hypoxia event. A 4 d observation duration was chosen to standardize the duration of

examination, and to exclude transient, brief hypoxia events that can occur at this site.

Physiological analyses

Plasma triglycerides were quantified using a commercially available colorimetric assay

kit (Bioassy Systems, Hayward, CA, USA), and total plasma proteins (TPP) were quantified

using a hand-held refractometer (Reichart VET 360, Reichert Inc., Depew, NY, USA). Plasma

cortisol was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life

Sciences, Kit # 900-071, Farmingdale, NY, USA), and plasma glucose and plasma lactate were

quantified enzymatically using methods described in Lowry and Passonneau (1972). Whole

blood hemoglobin concentration (Hb) was quantified using a commercially available kit (DIHB-

250, BioAssay Systems, Hayward, CA, USA). Mean cell hemoglobin concentration (MCHC)

was calculated by

$$(\text{Hb} \div \% \text{ Hct}) \times 100$$

as per Houston (1990). Plasma sodium (Na^+) and potassium (K^+) were quantified using a flame

photometer (Model 2655-00; Cole-Parmer Instrument Company, Chicago, IL, USA). Plasma

chloride (Cl^-) was quantified using a chloridometer (Model 4435000; Lab-conco Corporation,

Kansas City, MO, USA). Relative weight (W_r) was calculated as [$W/W_s \times 100$], where W_s is a

length-specific weight standard (Anderson and Neumann 1996).

Statistical analyses

A Mann-Whitney U test was used to determine if the numbers of tagged largemouth bass

in an area of the CAWS 4 d prior to a hypoxia event differed significantly from numbers of

tagged largemouth bass observed in the same location 4 d after the onset of a hypoxia event.

A one-way analysis of variance (ANOVA) was used to quantify differences in the

physiological variables, nutritional properties and P_{crit} values between largemouth bass collected

from the study site and the three reference sites. If the main effect was significant, a Tukey-

Kramer HSD *post hoc* test was used to separate means. A two-way ANOVA (main effects:

oxygen exposure, site, and their interaction) was used to test for the impacts of low DO exposure

on the blood and plasma response variables across the four sites. If the interaction was

significant, or if the interaction was not significant but at least one of the main effects was

significant, a Tukey-Kramer HSD *post hoc* test was used to separate means. All tests were

performed using JMP version 7.0 (SAS Institute Inc., Cary, NC, USA). The level of significance

for all tests was assessed at $\alpha = 0.05$.

Results

Movement analyses

During 2010 and 2011, nine time periods were identified as "hypoxia events" as they

experienced daily mean DO concentrations below 2 mg·L^{-1} for 4 d or more (Table 1). Three of

these events occurred downstream of Bubbly Creek and the remaining 6 occurred within Bubbly

Creek, and all but one occurring in conjunction with a CSO discharge due to rain events. There

was no significant difference between largemouth bass abundance in an area of the study site 4 d

prior to the onset of hypoxia compared to the number of largemouth bass in that same area 4 d

after the onset of hypoxia events (Mann-Whitney U = 38.0, $n_1 = n_2 = 9$, $P = 0.85$). More

specifically, during the 9 events, there were only 3 instances where largemouth bass abundance

in an area decreased due to the onset of a low oxygen event (Table 1).

Field sampling

Concentrations of cortisol in plasma did not differ significantly across fish that were

sampled in the field immediately following electroshocking, and all values averaged at or below

approximately 30 ng·mL^{-1} (Table 2). Plasma glucose concentrations were 33-77 % higher for

fish sampled in the field at reference site 1 relative to fish sampled from other locations (Table

2). Concentrations of plasma lactate for all sites did not differ significantly and were below

approximately 3.0 mmol·L^{-1} (Table 2). Hematocrit was significantly higher in largemouth bass

sampled from the study site compared to fish from reference site 1 (Table 2), while hemoglobin

was significantly higher in largemouth bass from the study site compared to references sites 1

and 2 (Table 2). MCHC was similar between largemouth bass from all sites sampled (Table 2).

There were no significant differences across sampling locations for plasma Na$^+$, plasma K$^+$,

plasma Cl$^-$ (Table 2) or nutrition-based parameters (i.e., plasma triglycerides and total plasma

protein; Table 2). Lengths and weights did not differ across sites (Table 2), relative weights of

all fish in the study were greater than 100 (i.e., all fish should be considered an appropriate

weight for their length), and fish from reference site 1 had significantly higher relative weights

than all other sites (Table 2).

Hypoxia challenge experiment

Following exposure of largemouth bass to 2 mg·L^{-1} dissolved oxygen for 6 h, hematocrit

values for reference sites 1 and 2 increased by 25 % and 26 % relative to control values, but

hematocrit concentrations in largemouth bass collected from the study site did not change relative to control fish (Fig. 1*a*, Table 3). A 6 h exposure to 2 mg·L^{-1} dissolved oxygen did not cause any significant change in hemoglobin concentration for any largemouth bass in this study (Fig. 1*b*, Table 3). Similarly, MCHC did not differ significantly following hypoxia exposure or across sites (Fig. 1*c*, Table 3).

Exposure of largemouth bass to 2.0 mg·L^{-1} DO for 6 h did not result in significant increases in plasma cortisol (relative to control values) within an individual site (Fig. 2*a*, Table 3). The plasma cortisol concentration of reference site 2 after low oxygen exposure was almost 3-fold greater than the control value of largemouth bass from reference site 1, however (Fig. 2*a*, Table 3). Concentrations of plasma glucose increased by approximately 41 % across all sites following the low dissolved oxygen exposure, but site-specific increases did not occur (Fig. 2*b*, Table 3). Plasma lactate concentrations for largemouth bass from all sites increased at least 4 fold as a result of a 6 h exposure to a 2.0 mg·L^{-1} low oxygen shock, with the smallest and largest increases observed in reference site 3 and reference site 1 fish, respectively (Fig. 2*c*, Table 3). Plasma ions (Na^+, K^+, Cl^-) did not increase or decrease significantly as a result of any of the main effects within each site (Fig. 3; Table 3). Total lengths and weights of largemouth bass used in the oxygen shock experiment did not differ across sites (Table 3). Relative weight (W_r) differed significantly between sites ($P < 0.001$) and treatments ($P = 0.013$), however, all W_r values were within ideal values for largemouth bass (average range 91-108).

Critical Oxygen Tension (P_{crit})

There was no significant difference in the standard metabolic rate (SMR) between largemouth bass collected from the four sites ($F = 0.3$, $P = 0.83$). Similarly, P_{crit} did not differ significantly across all largemouth bass collected from the four sites ($F = 1.6$, $P = 0.22$; Fig. 4).

Discussion

During this study, we documented nine hypoxic events (defined as dissolved oxygen concentration remaining at or below 2.0 mg·L^{-1} for a minimum of 4 consecutive d), with these events varying in duration from 4 to 52 days; eight of these 9 events were associated with CSO discharges and/or rainfall events. Reductions in DO in urban systems can be caused by the uptake of oxygen from the water column through both biological and chemical processes, particularly during periods of high flow rates generated by pumped sewage, coupled with suspension of highly organic, nutrient-rich bottom sediments (Waterman et al. 2011). Rain-induced reductions of DO within the CAWS did not appear to exert a strong influence on the movement patterns of largemouth bass. More specifically, reductions in DO to 2.0 mg·L^{-1} or lower did not produce a consistent reduction in numbers of largemouth bass residing in locations within the study area. There are three potential explanations for why largemouth bass did not appear to exhibit pronounced shifts in behavior or position following exposure to low dissolved oxygen. First, water bodies can exhibit large variability in dissolved oxygen concentration across small areas, typically as a result of photosynthetic activity of algal populations and algal decay (Wetzel 1983). DO variability is greatest in the optimal zone of sunlight penetration (usually in the top of the water column), which can bring waters in this area to concentrations at or well above saturation (Wetzel 1983). It is possible largemouth bass are spatially seeking and inhabiting waters of higher DO concentration within a limited area (e.g., shifts in swimming depth), eliminating the need to move out of an area entirely despite readings of low oxygen at a sonde. These potential shifts, however, could not be detected by the telemetry gear used for this study. Second, habitat selection by animals typically involves choices and trade-offs (i.e., food vs. shelter vs. predation vs. competition) (Mittelbach 2002; Hasler et al. 2009). Largemouth bass

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within the study area may choose to remain in hypoxic conditions due to the presence of prey items, refuge from flow, etc. (Pyke et al. 1977; Mittelbach 2002; Bartumeus and Catalan 2009), which may be less costly than a movements away from the hypoxic area. Third, previous work has shown that largemouth bass are relatively hypoxia-tolerant. VanLandeghem et al. (2010), for example, showed that, when exposed to hypoxia (2 mg$\cdot$L^{-1}) for up to 6 h, largemouth bass exhibited only minor physiological responses. When subjected to graded hypoxia (90, 60, 45 torr), largemouth bass do not exhibit increased hematocrit levels or a decrease in total blood O_2 content compared to largemouth bass held at normoxic conditions, indicating a tolerance to hypoxia as well as a higher affinity for binding O_2 at lower oxygen tensions (Furimsky et al. 2003). In the current study, largemouth bass did not always inhabit waters with the highest DO available. Thus, largemouth bass appear to make habitat choices based on factors other than low oxygen, suggesting that the choice to remain in these conditions may outweigh costs associated with lower DO levels (Jeffres et al. 2006; Cocherell et al. 2010).

Free-swimming largemouth bass collected in the wild did not appear to be experiencing chronic stress or impaired nutrition relative to free-swimming largemouth bass sampled from the three reference sites. More specifically, concentrations of plasma cortisol, plasma glucose, plasma lactate, or nutrition-based parameters (e.g., triglycerides and total plasma protein) between fish residing in the study site and those from the three reference sites suggests that the study site fish are not experiencing chronic stress and are not nutritionally deprived. Fish that have experienced extended periods of time with little or no feeding can display reductions in concentrations of triglycerides or plasma proteins (Wagner and Congelton 2004; Congelton and Wagner 2006; Hanson and Cooke 2009). Over extended periods of time, this can result in a loss of weight for fish (Gingerich et al. 2010). All largemouth bass sampled in the field had W_r

values that were above an ideal weight of 100, and fish residing in the study site did not have W_r values that were significantly lower than any of the reference sites. Largemouth bass in the study site that reside in areas exposed to frequent and extended periods of rain-induced reductions in dissolved oxygen were therefore not in poor nutritional condition or suffering from chronic stress when compared to fish collected from the three reference sites.

Interestingly, free-swimming largemouth bass collected in the field showed signs of an improved ability to transport oxygen relative to fish from some of the reference sites, possibly resulting from repeated exposures to low dissolved oxygen observed within the study site. More specifically, largemouth bass sampled from the study site had hematocrit proportions that were 26 % greater than reference site 1, and also had hemoglobin concentrations almost 60 % greater than reference site 1 and reference site 2; MCHC of largemouth bass did not vary across sampling sites. Hematocrit is the percentage of packed red cells in a known amount of whole blood, hemoglobin is the total concentration of the oxygen binding protein within the whole blood, while MCHC is the amount of hemoglobin within a known volume of red cells (i.e., the amount of hemoglobin per cell; Houston 1990). Because MCHC was similar in largemouth bass from all sites, the increase in hematocrit for the study site fish relative to reference site 1 was likely due to an increase in the number of red cells within the whole blood of the animals. Increased values of hematocrit and hemoglobin have both been observed in numerous species as a result of extended exposure to low oxygen (Tun and Houston 1986; Petersen and Petersen 1990; Timmerman and Chapman 2004). For example, six weeks of exposure to hypoxia (~40% air saturation) resulted in increases of 11 % and 14 % for hematocrit and hemoglobin (respectively) compared to cod held at normoxia (Petersen and Gamperl 2010). An increase in red cells is often caused by the release of erythropoietin from the renal glands (Jensen et al.

1993), and increases in this hormone have been shown to stimulate splenic releases of stored red cells in rainbow trout *Oncorhynchus mykiss* (Lai et al. 2006). Increases in hematocrit allow for a larger surface area to bind oxygen within the blood, likely conferring a beneficial advantage (either a plastic acclimatization or an adaptation) to the study site fish to survive in a low oxygen environment with little physiological or behavioral cost. Together, results from this study suggest that free-swimming largemouth bass from the CAWS have an improved ability to transport oxygen relative to control sites due to increases in both hematocrit and hemoglobin.

Interestingly, despite differences in oxygen transporting mechanisms observed for fish sampled in the field, following a low oxygen exposure of 2.0 mg·L^{-1} for 6 h in the lab, differences in physiological responses of largemouth bass across sites were minor. More specifically, largemouth bass collected from both the study site and reference sites experienced an increase in both plasma glucose and plasma lactate, indicating an upregulation of the stress response (Wendelaar Bonga 1997), but differences were not detected across sites. Lactate is produced when oxygen delivery to tissues is not sufficient to sustain aerobic respiration forcing fish to respire anaerobically to meet energetic demands, and glucose is produced as part of the stress response and is released to fuel aerobic tissues such as heart or gills (Barton et al. 1986). The increase in both plasma glucose and plasma lactate was similar in magnitude to past studies involving largemouth bass and hypoxia (VanLandeghem et al. 2010), and indicated that the concentration and duration of DO exposure was successful at creating a physiological disturbance for largemouth bass. A lack of a difference in the magnitude of physiological responses observed across sites likely indicates that largemouth bass from the study site did not have an improved or impaired ability to tolerate low DO conditions compared to reference site fish. Cortisol is a hormone that is part of the primary stress response for fish, and is produced

following the onset of a stressor to help maintain homeostasis (Barton et al. 1986). Plasma

cortisol values following hypoxia exposure increased significantly between two different

reference sites, however, there were no site-specific differences in plasma cortisol, suggesting

that this intensity and/or duration of hypoxia did not necessitate production of stress hormones to

assist in maintaining homeostasis. Fishes exposed to hypoxia often experience a loss of ions

from plasma as a result of mechanisms that work to improve oxygen uptake (Gonzalez and

McDonald 1991). Largemouth bass did not experience a loss of either cations or anions from

plasma, providing additional evidence that 6 hour exposure to 2.0 mg$\cdot$L^{-1} DO was not a

significant stressor for these fish.

Critical oxygen tension (P_{crit}) is the point at which an animal can no longer maintain

aerobic respiration and begins to respire anaerobically, as a result of a lack of oxygen (Richards

2009). Hypoxia-tolerant fish have lower P_{crit} values compared to fish that are less hypoxia

tolerant (Iversen et al. 2010), as fish that are hypoxia-tolerant can change to anaerobic respiration

(oxyconformation) at a lower oxygen saturation than fish that are less hypoxia-tolerant. In the

current study, P_{crit} values did not vary between largemouth bass collected from the study site

relative to largemouth bass collected from the other reference sites. Reductions in P_{crit} can occur

through modifications in ventilation rate/amplitude, enhanced blood-oxygen transport, or

modifications in cardiovascular function (Randall 1982; Mandic et al. 2009; Richards 2009).

Largemouth bass from the study site were not regulating metabolic rates or oxygen-carrying

capacity differently than reference site fish, despite residing in an environment where reductions

in DO are frequent and occur for extended periods of time.

While the results from this study did not indicate a strong negative effect of reductions in

DO on the condition or behavior of largemouth bass, but there are three caveats to this

conclusion that must be discussed. First, largemouth bass residing in these areas could experience negative consequences due to their environment that were not quantified in the current study, such as reduced growth, impaired reproduction, reduced immune function, increased parasite loads, endocrine disruption, or truncated life expectancy (Wendelaar Bonga 1997). Increased barge traffic and artificial embankments, for example, were shown to reduce reproductive potential in the Oder-Havel Kanal for young of year (YOY) roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*; Arlinghaus et al. 2002). Similarly, reduced growth rate was observed in Atlantic cod (*Gadus morhua*) exposed to chronic hypoxia (45 % and 56 % O_2 saturation) due to decreased food consumption and decreased activity (Chabot and Dutil 1999). Future studies of hypoxia on wild free-swimming fishes should examine metrics such as these that could change due to exposure to hypoxia and may influence fish. Second, it is often difficult to separate the true effects of hypoxia alone from other ancillary impacts that often occur in concert with hypoxia, (particularly in disturbed habitats) such as elevated carbon dioxide, high hydrogen sulfide, toxic organics, and heavy metals (Kramer 1987). For example, larval fish exposed to varying concentrations of mercury (Hg) and lead (Pb) produce lethal acute effects (Hall 1991). Third, we intentionally used fish from a narrow size range to avoid potential effects of body size on oxygen tolerance. Previous work has shown that large fish may have a metabolic advantage over smaller fish during periods of low oxygen (Nilsson and Östlund-Nilsson 2008), although laboratory studies with largemouth bass showed that smaller fish (23-500 g) can utilize water with lower oxygen concentration than larger fish (1000-3000 g; Burleson et al. 2001). Lack of movement away from hypoxic areas may therefore have been partially driven by our use of small fish. Largemouth bass appear to be relatively hypoxia tolerant and negative

consequences of low dissolved oxygen may be more pronounced for other species that are less hypoxia tolerant.

Despite largemouth bass displaying only minor behavioral and physiological responses to repeated exposure to rain-induced reductions in low oxygen (i.e., no strong pattern of movement away from areas of hypoxia, no loss of ions from plasma during extended hypoxia exposure, etc.), it does not mean that CSO events and rain-induced hypoxia in urban environments should be disregarded or ignored. While absolute fish diversity in the CAWS study site was high (over 30 species recorded; data available at http://www.mwrd.org), the species present at the site are comprised mainly of generalists and/or hypoxia-tolerant species (e.g.,*Lepomis spp., Cyprinus carpio, Ictalurus spp., etc.*) rather than habitat specialists or hypoxia-intolerant species that may exist where oxygen levels are higher (e.g., Salmonidae, *Sander vitreus*) (Nilsson and Östlund-Nilsson 2008). Largemouth bass may not be representative of these other fishes because of their physiological tolerance to hypoxia (Furimsky et al. 2003) and previous tendency to inhabit water with low dissolved oxygen (Hasler et al., 2009). While the history of the CAWS is unique to Chicago and its metropolitan area, sewer systems that use pumping stations and experience CSOs are common, both nationally and internationally. Furthermore, stormwater discharges and nonpoint source runoff can also cause low DO problems in many waterways and the episodic low-DO events that occur in the CAWS are not unique to Chicago (Gray 1997; Brombach et al. 2005; Casadio et al. 2010). Current water quality standards presume that DO must remain above specific thresholds to be protective of resident fish communities, and CSOs are regulated primarily on the basis of effects on DO and other contaminants (Andres-Domenech et al. 2010; Casadio et al. 2010; Gasperi et al. 2010). Results from this study, when combined with previous research on pollutant loads associated with CSO discharges, can enhance regulation decisions

that serve to protect aquatic biota contained within these systems. In addition, this study serves as a model for applying telemetry to free-swimming, wild fish populations to identify movement/activity patterns in urban systems due to anthropogenic stressors.

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Table 1. Largemouth bass present during each low dissolved oxygen event four days before and four days after the onset of the low dissolved oxygen event from July 2010 to October 2011. Delta is the change in fish presence, with a positive number indicating fish movement out of the area, 0 indicating no change, and a negative number indicating fish presence increasing after the low dissolved oxygen event. For reference, the time length (days) for each low oxygen event and average dissolved oxygen concentration during the whole event is included.

Event	Location	Before event	After event	Delta	Event length (d)	Avg. DO (mg·L^{-1})
1	Bubbly Creek	4	2	-2	4	1.04
2	Bubbly Creek	1	1	0	6	1.76
3	Bubbly Creek	1	1	0	7	1.03
4	Downstream	1	2	1	5	1.39
5	Bubbly Creek	1	1	0	52	1.21
6	Downstream	0	3	3	19	0.96
7	Bubbly Creek	2	1	-1	22	0.96
8	Downstream	3	0	-3	9	1.82
9	Bubbly Creek	2	2	0	4	1.43

Table 2. Morphological and physiological parameters of largemouth bass from the study site in the Chicago Area Waterway System, Chicago (CAWS), IL, USA and three reference sites (North Shore Channel of the CAWS, Busse Lake, Des Plaines River) sampled immediately after collection in the field. Data are presented as mean ± SE, significance was assessed at α = 0.05 and indicated by bold text. Levels not connected by the same letter are significantly different.

Variable	Study Site	Reference site 1	Reference site 2	Reference site 3	P-value
Length (mm)	244 ± 6	252 ± 15	248 ± 12	245 ± 10	0.96
Weight (g)	209 ± 20	270 ± 48	241 ± 41	217 ± 31	0.68
Wr	$102 ± 3^b$	$114 ± 3^a$	$106 ± 3^{a,b}$	$103 ± 3^{a,b}$	**0.0321**
Cortisol (ng·mL^{-1})	14.9 ± 10.5	31 ± 16.9	3.6 ± 0.6	7.1 ± 1.6	0.17
Glucose (mmol·L^{-1})	$4.0 ± 0.3^b$	$5.6 ± 0.7^a$	$2.5 ± 0.2^c$	$3.6 ± 0.2^{b,c}$	**<.0001**
Plasma lactate (mmol·/L^{-1})	1.9 ± 0.7	3.1 ± 1.2	1.5 ± 0.3	2.3 ± 0.5	0.42
Hematocrit (%)	$31.1 ± 1.4^a$	$26.1 ± 0.6^b$	n/a	$28.1 ± 0.8^{a,b}$	**0.01**
Hemoglobin (g·dL^{-1})	$10.5 ± 1.5^a$	$6.9 ± 0.4^b$	$6.5 ± 0.4^b$	$7.7 ± 0.1^{a,b}$	**0.01**
MCHC (g·dL^{-1})	34.3 ± 5.6	26.5 ± 1.6	n/a	27.6 ± 0.6	0.30
Water content (%)	78.6 ± 0.1	79.0 ± 0.2	78.6 ± 0.4	79.3 ± 0.1	0.29
Potassium (mEq/L^{-1})	3.78 ± 0.2	2.98 ± 0.4	3.74 ± 0.2	3.27 ± 0.2	0.16
Sodium (mEq·L^{-1})	148 ± 5.3	158 ± 8.0	144 ± 4.4	147 ± 5.7	0.58
Chloride (mEq·L^{-1})	93.4 ± 4.4	93.2 ± 5.7	101.8 ± 2.9	95.1 ± 1.7	0.29
Triglycerides (mmol·L^{-1})	2.6 ± 0.6	4.3 ± 1.1	3.9 ± 0.5	2.2 ± 0.7	0.13
Plasma Protein (g·dL^{-1})	7.0 ± 0.7	9.2 ± 0.9	6.1 ± 0.5	8.1 ± 0.6	0.34

Table 3. Statistical results examining physiological and hematological differences in largemouth bass from the study site in the Chicago Area Waterway System, Chicago (CAWS), IL, USA and three reference sites (North Shore Channel of the CAWS, Busse Lake, Des Plaines River) subjected to an oxygen shock of 2 mg·L^{-1} for 6 hours. Significance was assessed at α = 0.05 and indicated by bold text.

Parameter	Effect	df	F	P
Length	Site	3	1.4	0.24
	Treatment	1	0.0	1.0
	Site*Treatment	3	1.4	0.26
Weight	Site	3	1.1	0.34
	Treatment	1	0.3	0.61
	Site*Treatment	3	1.3	0.30
Wr	Site	3	6.6	**< 0.001**
	Treatment	1	6.6	**0.01**
	Site*Treatment	3	0.2	0.89
% Hematocrit	Site	3	2.4	0. 08
	Treatment	1	20.0	**< 0.001**
	Site*Treatment	3	3.5	**0.02**
Hemoglobin	Site	3	3.9	0.03
	Treatment	1	2.9	0.09
	Site*Treatment	3	0.4	0.75
MCHC	Site	3	2.2	0.1
	Treatment	1	1.5	0.22
	Site*Treatment	3	1.4	0.27
Plasma cortisol	Site	3	3.0	**0.04**
	Treatment	1	1.0	0.31
	Site*Treatment	3	2.8	**0.05**
Plasma glucose	Site	3	1.5	0.23
	Treatment	1	8.0	**0.006**
	Site*Treatment	3	0.7	0.6
Plasma lactate	Site	3	3.4	0.02
	Treatment	1	63.8	**< 0.0001**
	Site*Treatment	3	3.6	**0.02**
Plasma Na^{+}	Site	3	0.7	0.53
	Treatment	1	0.5	0.50
	Site*Treatment	3	1.5	0.24

Table # (cont.)

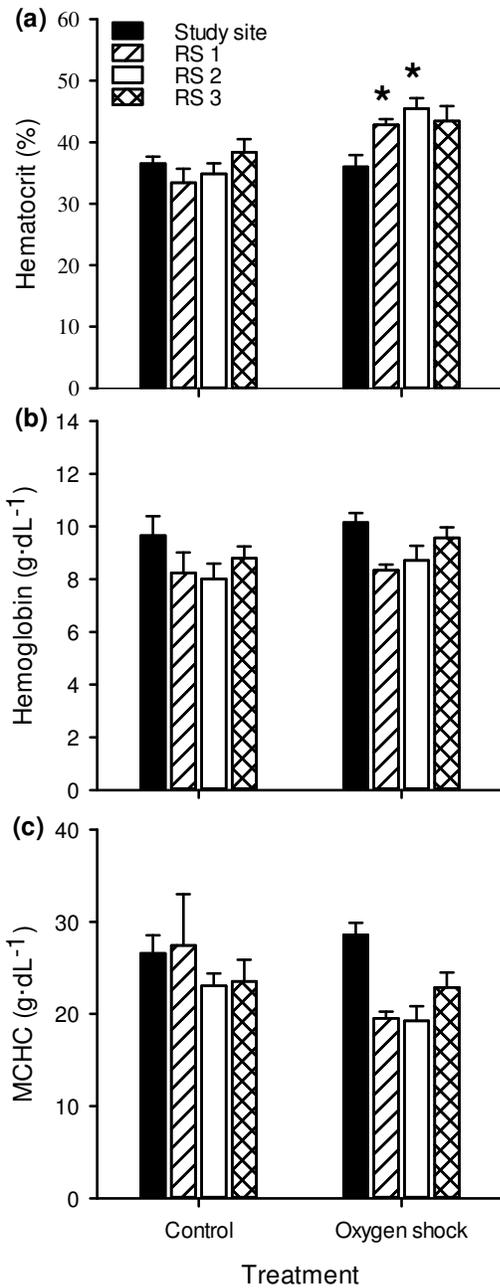|				
Plasma K^+	Site	3	0.5	0.66
	Treatment	1	1.7	0.20
	Site*Treatment	3	1.1	0.34
Plasma Cl^-	Site	3	4.7	**0.005**
	Treatment	1	0.2	0.68
	Site*Treatment	3	4.0	**0.01**
Muscle water content	Site	3	9.0	**< 0.0001**
	Treatment	1	0.08	0.78
	Site*Treatment	3	1.6	0.21

Fig. 1. Hematocrit (a), hemoglobin (b), and MCHC (c) for largemouth bass from the study site in the Chicago Area Waterway System, Chicago (CAWS), IL, USA and three reference sites (RS; North Shore Channel of the CAWS, Busse Lake, Des Plaines River) subjected to a low oxygen shock of 2.0 mg·L^{-1} for 6 h. Control treatment fish were held for 6 h at 6.5 mg·L^{-1}. Error bars show ± 1 SE, and asterisks (*) indicate treatments significantly different from their respective controls. Sample sizes ranged from n = 7 to n = 11.
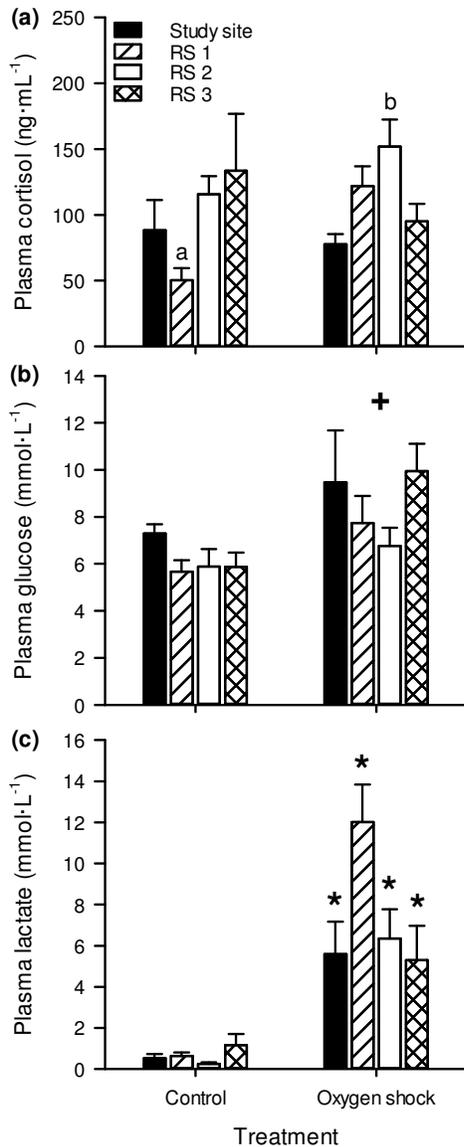
Fig. 2. Concentrations of plasma cortisol (a), plasma glucose (b), and plasma lactate for largemouth bass from the study site in the Chicago Area Waterway System, Chicago (CAWS), IL, USA and three reference sites (RS; North Shore Channel of the CAWS, Busse Lake, Des Plaines River) subjected to low oxygen shock of 2.0 mg·L^{-1} for 6 hours. Control treatment fish were held for 6 h at 6.5 mg·L^{-1}. Error bars show ± 1 SE, letters above bars indicate level of significance and asterisks (*) indicate treatments significantly different from their respective controls and a plus (+) sign indicates a significant treatment effect. Sample sizes ranged from n = 7 to n = 11.
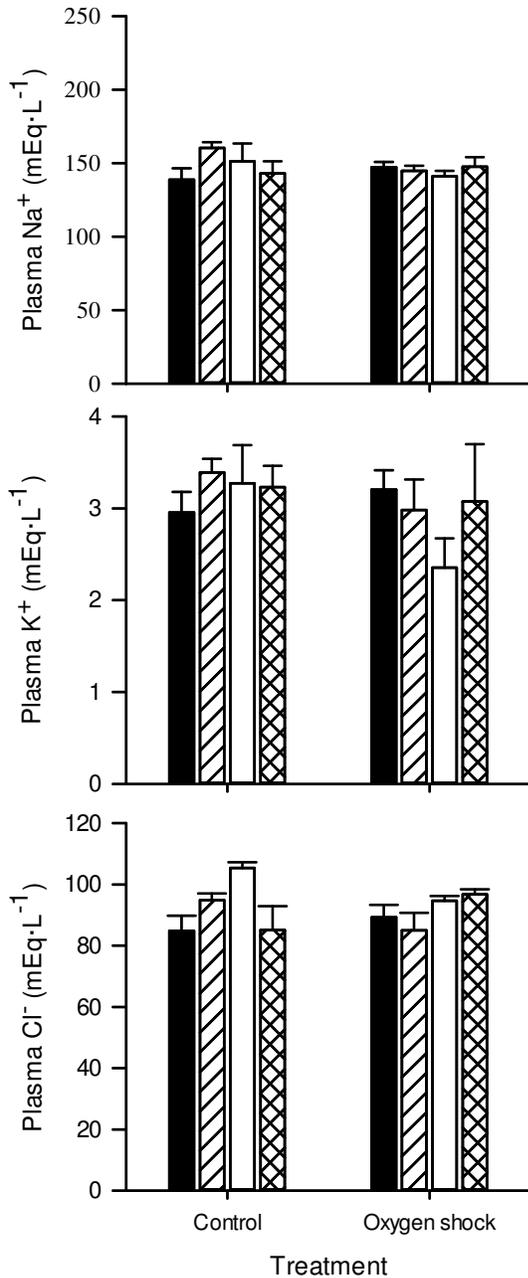
Fig. 3. Concentrations of plasma sodium [Na^+; (a)], plasma potassium [K^+; (b)], and plasma chloride [Cl^-; (c)] for largemouth bass from the study site in the Chicago Area Waterway System, Chicago (CAWS), IL, USA and three reference sites (RS; North Shore Channel of the CAWS, Busse Lake, Des Plaines River) subjected to low oxygen shock of 2.0 $mg \cdot L^{-1}$ for 6 hours. Control treatment fish were held for 6 hours at 6.5 $mg \cdot L^{-1}$. Error bars show ± 1 SE and letters above bars indicate level of significance. Sample sizes ranged from n=7 to n=11.
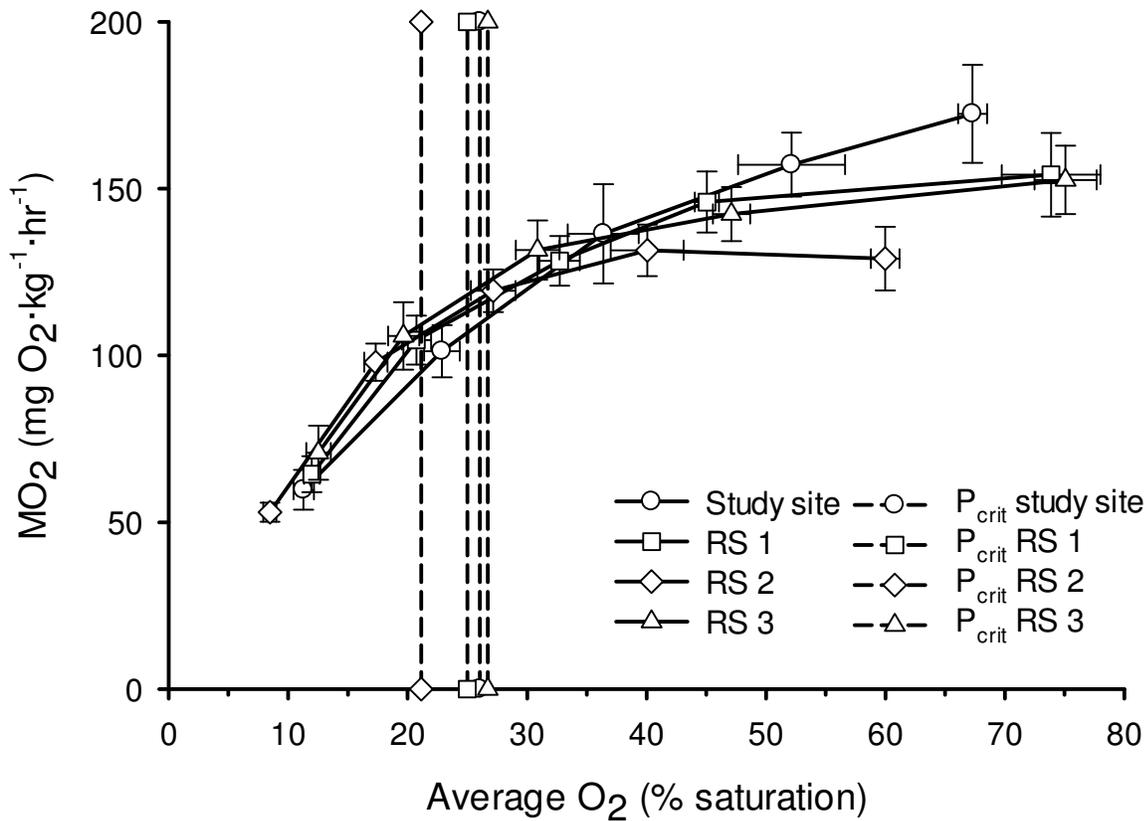
Fig. 4. The relationship between oxygen consumption (MO_2) and % saturation of the water for largemouth bass from the study site in the Chicago Area Waterway System, Chicago (CAWS), IL, USA and three reference sites (RS; North Shore Channel of the CAWS, Busse Lake, Des Plaines River). Mean P_{crit} values (i.e., critical oxygen tension) for each population are indicated by vertical lines with symbols matching consumption line symbols. Error bars show $\pm$ 1 SE for both MO_2 (vertical) and % saturation (horizontal).

CHAPTER 3:

ACCLIMATION TO A LOW OXYGEN ENVIRONMENT ALTERS THE

HEMATOLOGY OF LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*)

Abstract

One of the most severe impacts of urbanization on aquatic systems is the increasing presence of low oxygen environments caused by anthropogenic sources of pollution. These hypoxic environments can have lasting negative impacts on the fish species contained within. As urbanization increases nationally and globally, it is becoming exceedingly important to understand how hypoxia affects aquatic fauna, especially fish species. Largemouth bass were held at 3.0 mg/L and 9.0 mg/L for 50 d, which has previously shown to be temporally sufficient to impart plastic phenotypic changes. Following the holding period, fish from each group were subjected to a low dissolved oxygen challenge of 2.0 mg/L for 6 h and their physiological and hematological parameters were compared to control fish held for 6 h with no change in dissolved oxygen. There was little physiological stress-response differences between the two holding groups, however, the low oxygen holding group had increased hemoglobin and hematocrit levels following the 6 h low oxygen challenge compared to the high oxygen group. These results suggest long-term holding of largemouth bass to low oxygen conditions may confer a beneficial hematological advantage of increased oxygen uptake capacity during periods of low oxygen over fish not acclimated to a low oxygen environment.

Keywords: Stress, physiology, hematology, hypoxia, acclimation.

Introduction

Reversible plastic changes in the physiological response of animals subjected to a change in an environmental variable (e.g., oxygen level) can be classified as beneficial or detrimental. More specifically, animals acclimated to a particular environment are predicted to have an advantage over animals not acclimated to that environment, defined as beneficial acclimation (Leroi et al. 1994). While the majority of studies investigating beneficial acclimation have examined thermal acclimation (Deere and Chown 2006; Geister and Fisher 2007; Wilson et al. 2007; Lurman et al. 2009), few studies have investigated the effect of hypoxia, especially in terms of the physiological and hematological response. Previous research on hypoxia acclimation has investigated the effects of a low oxygen environment on the swimming performance, metabolic rates, and gill morphology of fish (Timmerman and Chapman 2004; Fu et al. 2011; Zhao et al. 2012); however, little is known about the physiological responses of fish exposed to chronic hypoxia.

Aquatic organisms possess a suite of potential mechanisms to overcome environmental challenges, especially related to low oxygen environments. Behavioral shifts, such as avoidance of hypoxic conditions or increased surface respiration (Kramer and Mehegan 1981), are two common mechanisms fish utilize to overcome environmental hypoxia. For example, blue crabs (*Callinectes sapidus*) tend to avoid hypoxic conditions in the Neuse River Estuary, North Carolina, USA during periods of low dissolved oxygen (Bell et al. 2003). Other tolerance mechanisms for hypoxia include changes in physiology, either through the regulation and release of stress hormones, or plastic changes in phenotypes. The release of stress hormones (e.g., cortisol) typically starts a chain reaction of subsequent responses (i.e., secondary and tertiary stress responses), which enable the animal to maintain homeostasis and cope with a hypoxic

disturbance (Barton 2002). However, there are costs associated with both behavioral and physiological coping mechanisms, and these costs can occur at both an individual- or community level. Hypoxia, for example, can make an animal more susceptible to predation (Pollock 2007), and increasing competition in more oxygenated waters where prey resources are less abundant, causing density-dependent reductions of growth rates (Eby et al. 2005). Reproductive behavior and success can also be negatively affected by chronic hypoxia, through decreased egg fanning and nest abandonment (Takegaki and Nakazono 1999), and decreased weights which increase the likelihood of abandoning parental care (Jones and Reynolds 1999).

Although hypoxia can occur naturally in the environment (e.g. stratification, low light, reduced mixing; Wetzel 1983; Officer et al. 1984; Chapman et al. 1998), anthropogenically-driven hypoxia is becoming increasingly abundant in natural ecosystems. The sources of pollutants driving environmental hypoxia can include combined sewer systems (Zhu et al. 2008; Baker 2009), agricultural runoff (Bernhardt et al. 2008), and urbanization (Paul and Meyer 2008; Zhu et al. 2008). These factors typically increase nutrients (e.g., nitrogen and phosphorous), oxygen-depleting substances, or both, which lower dissolved oxygen (DO) in the aquatic environment. These low oxygen events can persist for weeks (Burton and Pitt 2002; Alp 2006), and can have negative impacts on fish populations, such as mass mortalities, extirpation, and population declines (Pollock et al. 2007). Because of this growing issue, it is becoming exceedingly important to understand hypoxia and its effects on aquatic communities.

The objective of this study was to quantify the impacts of extended exposure to low dissolved oxygen (3.0 mg/L) on the subsequent oxygen tolerance of largemouth bass. Largemouth bass were held at low oxygen for an extended period of time and then given a low oxygen challenge. Blood physiology parameters were quantified and compared to a control

group not held in a low oxygen environment. Results have important implications for understanding how fish respond to chronic hypoxia, and how these changes may provide a potential advantage(s) that enhance the survival of the animal.

Methods

Fish collection and husbandry

Largemouth bass (range 150-180 mm) were acquired from the Logan Hollow Fish Farm (Murphysboro, IL, USA) in October, 2011, and transported to the University of Illinois Aquatic Research Facility (Champaign, IL, USA). Upon arrival at the facility, fish were transferred into two 1,136 L plastic holding tanks where they were held for 48 h to recover from hauling and handling stressors. Largemouth bass were fed floating pelleted food (Aquatic Eco-Systems Inc., Apopka, FL, USA) *ad libitum* once per day, and solid wastes were removed from the tank by a siphon every other day. To remove nitrogenous waste from the tank, a combination of powdered ammonia remover (ProLine®, AquaticEcosystems, Apopka, FL, USA) and a container of activated carbon was used; ammonia levels did not exceed 1 ppm. A 10 L/min pump was placed inside the tank to ensure adequate mixing.

Acclimation treatments

Following the 48 h recovery period, each of the two tanks was assigned to one of two acclimation treatments: high dissolved oxygen (H) or low dissolved oxygen (L). The H tank was aerated continuously with air stones and a compressed air blower (Sweetwater Linear II, Aquatic Eco-Systems, Apopka, FL, USA), and a 10 L/min pump was placed in the tank to ensure water mixing. Dissolved oxygen (DO) was measured daily with a hand-held dissolved oxygen meter (YSI 550A, Yellow Springs Instruments, Yellow Springs, OH, USA) and remained at 9.0 mg/L ± 0.25 ($\bar{x}$ ± standard error, SE). The L treatment held fish at 3.3 mg/L ± 0.07 by bubbling

nitrogen gas (N_2) into the tank to displace oxygen, and a 10 L/min pump was again used to ensure homogeneous oxygen concentrations throughout the tank. Dissolved oxygen concentrations were held constant by a 1-channel oxygen analyzer (OXY-REG) (Loligo Systems, Tjele, Denmark), which regulated oxygen levels using a DO probe connected to a solenoid switch, which, in turn, was attached to a tank of nitrogen gas. The oxygen analyzer was programmed such that, when DO increased above a threshold of 3.5 mg/L, nitrogen gas would diffuse into the tank. Once DO fell to a target concentration (~2.8 mg/L), the OXY-REG would shut off the solenoid, ending nitrogen gas diffusion. Dissolved oxygen for the L treatment was also quantified daily with the same hand-held meter. Each tank was held at these respective DO concentrations for 50 d as previous work with low oxygen acclimation has shown 6 weeks to be a sufficient duration of time to induce plastic physiological changes and cause acclimation to low oxygen conditions (Timmerman and Chapman 2004; Petersen and Gamperl, 2010). The temperatures in the L tank (15.0 ± 0.4 ℃) were not statistically different than the temperatures in the H tank (14.6 ± 0.28 ℃) during the 50 d acclimation period (t_{48} = 0.82, P = 0.42).

Oxygen shock experiment

Following the 50 d holding period, largemouth bass from both acclimation groups were subjected to an oxygen shock experiment to quantify the physiological response to prolonged holding at low oxygen. For this, 16 largemouth bass (8 from each oxygen group) were randomly assigned to a treatment (oxygen shock or control) and placed in individual, aerated, sensory-deprived chambers continuously supplied with fresh water from a central basin in a flow-through, closed system maintained at approximately 8.0 mg/L (Suski et al. 2006). Following a 24 h acclimation period to the chamber, 8 of the largemouth bass were subjected to a DO shock of 2.0 ± 0.01 mg/L for 6 h by pumping N_2 gas into the central basin to displace oxygen (Suski et

al. 2006). DO concentration in each individual chamber typically fell from 8.0 to 2.0 mg/L in < 3 min. Following this 6 h exposure to 2 mg/L dissolved oxygen, largemouth bass were sacrificed with an overdose of tricaine methanesulfonate [MS-222 (250 mg/L buffered with 250 mg/L sodium bicarbonate)], added directly to each chamber. Following cessation of ventilation, fish were measured to the nearest mm (total length, TL), weighed to the nearest g, then sampled for blood and tissues. Blood was extracted from the caudal vessel using a 1 mL syringe and a 21-guage needle rinsed with lithium heparin. A small volume of whole blood was placed in a capillary tube and spun for 2 min using a hematocrit spinner to quantify percent hematocrit (i.e., packed red cell volume). A second small aliquot of whole blood was placed in a 1.5 mL centrifuge tube for subsequent quantification of hemoglobin. The remaining whole blood was centrifuged for 2 min at $2000 \times$ gravity (g) to separate erythrocytes from plasma, stored in a 1.5 mL centrifuge tube, and flash frozen in liquid nitrogen until further processing. A section of epaxial muscle above the lateral line was excised with a razor blade, placed in a 1.5 mL centrifuge tube and stored in liquid nitrogen until further processing. Control fish remained in the individual, aerated chambers for 6 h (following the 24 h acclimation to the chambers) with no change in DO ($8.13 \pm .01$ mg/L O_2) and were sampled in a manner identical to fish exposed to low oxygen.

Quantification of physiological parameters

Cortisol concentrations were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Kit # 900-071, Farmingdale, NY, USA; Sink et al. 2008), while plasma glucose and plasma lactate concentrations were quantified enzymatically using methods described in Lowry and Passonneau (1972). Plasma sodium (Na^+) and potassium (K^+) concentrations were quantified using a flame photometer (Model 2655-00; Cole-Parmer

Instrument Company, Chicago, IL, USA), and plasma chloride (Cl⁻) was quantified

enzymatically with a commercially available kit (DICL-250, BioAssay Systems, Hayward, CA).

Whole blood hemoglobin concentration (Hb) was quantified using a commercially available kit

(DIHB-250, BioAssay Systems, Hayward, CA, USA). Mean cell hemoglobin concentration

(MCHC) was calculated as [(% Hb ÷ Hct) × 100] (Houston, 1990). Relative weight (W_r) was

calculated by: [W/W_s × 100], where W is the weight of the fish and W_s is a length-specific

weight standard (Anderson and Neumann, 1996). Muscle water content [(wet weight – dry

weight) ÷ 100] was quantified by obtaining wet weights and subsequent dry weights of the

muscle samples excised during sampling after being placed in a drying oven for 72 h.

Statistical analyses

A two-way analysis of variance (ANOVA) was used to quantify differences between the

two acclimation treatments. The main effects in the ANOVA were acclimation treatment (i.e.,

high or low dissolved oxygen group), experimental treatment (i.e., control or oxygen shock), and

their interaction. If the interaction was significant, or if the interaction was not significant but at

least one of the main effects was significant, a Tukey's post-hoc test was used to separate the

means. All tests were performed using JMP version 9.0 (SAS Institute Inc., Cary, NC, USA) and

significance was assessed at $\alpha = 0.05$.

Results

Exposure of largemouth bass to 6 h of water at 2.0 mg/L dissolved oxygen did not have a

significant effect on concentrations of plasma cortisol (acclimation: $F_{1,23} = 0.20$, $P = 0.66$;

treatment: $F_{1,23} = 1.83$, $P = 0.19$; interaction: $F_{1,23} = 2.35$, $P = 0.14$; Fig. 5a) or plasma glucose

(acclimation: $F_{1,23} = 1.14$, $P = 0.30$; treatment: $F_{1,23} = 0.25$, $P = 0.62$; interaction: $F_{1,23} = 3.17$, P

$= 0.09$; Fig. 5b). Plasma lactate increased threefold as a result of the exposure to the low oxygen

for both groups compared to controls ($F_{1,23}$ = 22.1, P = 0.0001; Fig. 5c), however, there was no significant effect of either acclimation or the interaction of acclimation and treatment (acclimation: $F_{1,23}$ = 0.63, P = 0.44; interaction: $F_{1,23}$ = 0.085, P = 0.77; Fig. 5c).

Prior to exposure to low oxygen, there were no significant differences in hematocrit values between largemouth bass in the H and L treatments (Fig. 6a). After 6 h exposure to water at 2.0 mg/L, however, hematocrit values for the L treatment fish were 22 % higher than the H treatment fish (Tukey-Kramer HSD, P = 0.03; Fig. 6a). Similarly, hemoglobin values for the L group treatment fish were 14% higher than H group treatment fish following 6 h exposure to water with 2.0 mg/L dissolved oxygen (Tukey-Kramer HSD, P = 0.046; Fig. 6b). MCHC, however, did not differ significantly across acclimation, treatment, or their interaction (acclimation: $F_{1,23}$ = 1.53, P = 0.23; treatment: $F_{1,23}$ = .17, P = 0.68; interaction: $F_{1,23}$ = 0.44, P = 0.51; Fig. 6c).

Plasma Na^+ and K^+ were not significantly affected by acclimation, the treatment, or their interaction (Na^+: acclimation: $F_{1,23}$ = 0.63, P = 0.44; treatment: $F_{1,23}$ = 0.25, P = 0.62; interaction: $F_{1,23}$ = 0.0078, P = 0.93; K^+: acclimation: $F_{1,23}$ = 0.06, P = 0.81; treatment: $F_{1,23}$ = 0.3, P = 0.60; interaction: $F_{1,23}$ = 2.3, P = 0.15). Plasma Cl^- increased significantly as an effect of the treatment after exposure to water at 2.0 mg/L dissolved oxygen ($F_{1,23}$ = 4.9, P = 0.038, Fig. 7c). Muscle water content was not significantly affected by the acclimation, treatment, or their interaction (acclimation: $F_{1,23}$ = 3.97, P = 0.060; treatment: $F_{1,23}$ = 0.11, P = 0.74; interaction: $F_{1,23}$ = 0.095, P = 0.76).

At the conclusion of the 50 d acclimation period, largemouth bass in the L group (L_{Tlow} = 172.8 mm ± 2.8; mean ± SE) were 8% longer than the H group fish (L_{Thigh} = 159.2 mm ± 2.5). Fish from the L group (T_{Wlow} = 77.4 g ± 3.7) were also approximately one-third heavier than in

the H group (T_{Whigh} = 62.4 g ± 3.3). Relative weights were similar across acclimations and treatments ($F_{3,25}$ = 0.55, P = 0.65).

Discussion

Exposure of largemouth bass to low dissolved oxygen challenge induced physiological disturbances caused by anaerobic metabolism. Following the oxygen shock treatment, largemouth bass from both treatment groups experienced an increase in lactate, as well as an increase in the concentration of chloride ions in plasma, relative to control values. In the absence of oxygen, ATP synthesis continues anaerobically, which results in an accumulation of lactate (Wright et al. 1989). Previous work has shown that quantifying lactate in plasma can measure the intensity and duration of the response to hypoxia in fish (Zhou et al. 2000; VanLandeghem et al. 2010; O'Connor et al. 2011). Previous exposure of largemouth bass to dissolved oxygen concentrations for similar durations of time resulted in increases of lactate comparable to those in the current study (Suski et al. 2006; VanLandeghem et al. 2010). Despite the changes observed in plasma Cl⁻, earlier work has shown that Cl⁻ does not play a role in increasing or decreasing the affinity for oxygen to bind to hemoglobin during a hypoxic challenge (Wells 2009). It is, therefore, not likely the changes in chloride concentrations played a significant role in any acclimation benefits or detriments.

Extended holding of largemouth bass at low dissolved oxygen induced an improved ability to transport oxygen in blood relative to fish held at higher oxygen concentrations. Concentrations of both Hct and Hb were significantly higher in largemouth bass held at low oxygen for 50 days relative to fish held at higher oxygen. Hct is the percentage of packed red blood cells relative to the whole volume of blood, but does not account for the size or number or erythrocytes. Hb is a quantification of the O_2 binding protein found in red cells, whereas MCHC

is a measure of the Hb in a given volume of packed erythrocytes (Houston 1990). Increased Hct

for fish in the L treatment relative to the H treatment is likely due to an increase in the

production of erythrocytes, swelling of the erythrocytes, or a combination of both. These

changes typically occur as a result of catecholamine releases that induce the release of

erythrocytes from the spleen (Jensen et al. 1993). Another factor that may influence the changes

observed in Hct and Hb may be acidosis in the blood, which can alter oxygen affinity of Hb (i.e.,

via the Bohr and/or Root effect), stimulating an increase in the oxygen capacity of erythrocytes

(Wells 2009). Differences in Hb and Hct concentrations between the low and high oxygen

groups during an oxygen challenge may be driven by the release of erythropoietin (the hormone

responsible for synthesizing erythrocytes) from the kidney and releases of erythrocyte stores

from the spleen. Increased production and release of erythrocytes in the low oxygen group

would increase the oxygen carrying capacity of the blood, imparting an advantage to low oxygen

over the H oxygen group. Rainbow trout (*Oncorhynchus mykiss*) subjected to sustained hypoxia

(maximum 216 h) had persistent increases in erythropoietin, as well as increased Hb levels in the

exposed trout (Lai et al 2006) thereby providing an improved ability for oxygen uptake.

Additionally, long-term exposure to hypoxia increases both Hb and Hct concentrations for

numerous species, both air and water-breathers (Scott and Rogers 1981; Tun and Houston 1986;

Petersen and Petersen 1990; Timmerman and Chapman 2004). These changes typically confer

an increase in oxygen binding affinity or increased surface area for oxygen binding on the

erythrocyte, with improved performance to fish in low oxygen conditions. We did not observe a

significant increase in basal Hct or Hb levels (i.e., Hct and Hb concentrations did not differ

across control treatments for the H and L group), largemouth bass held in a low oxygen

environment were able to increase those hematological variables relative to the high oxygen

group following a low oxygen challenge. These changes as a result of chronic exposure to a low oxygen environment likely confer a beneficial advantage to the low oxygen group with regard to hypoxia tolerance, due to an improved ability to transport oxygen via erythrocytes.

Many physiological parameters previously shown to increase following exposure to low oxygen in fishes did not change significantly for largemouth bass in the current study. Concentrations of plasma cortisol and plasma glucose did not significantly change as a result of the oxygen shock, and did not result in loss of cations from plasma. Cortisol is a hormone that is part of the primary stress response, and is released following the onset of a stressor, while glucose is a fuel for aerobic tissues such as gills and heart (Wendelaar Bonga 1997; Barton 2002). Previous studies examining the response of fishes to hypoxia has shown that exposure to 2.1 mg/L for 2.5 hours can cause a threefold increase in cortisol levels in the spotted wolfish (*Anarhichas minor*; Lays et al. 2009), and exposure of juvenile Atlantic sturgeon (*Acipenser oxyrhunchus*) to a 1 h hypoxia challenge of approximately 1.5 mg/L (10 mmHg) doubled levels of plasma glucose (Kieffer et al. 2011). Response of largemouth bass to hypoxia of this magnitude generated concentrations of plasma cortisol similar to those in the current study (VanLandeghem et al. 2010). Unlike VanLandeghem et al. (2010), we did not observe a significant increase in plasma glucose following exposure to 2.0 mg/L dissolved oxygen, likely due to cooler water temperatures (~15ºC here compare to 20ºC for VanLandeghem et al., (2010)). Fish exhibit lower activity rates in cooler temperatures with lower energetic demands that promote sustained aerobic respiration and reduce the need for aerobic fuels (Chabot and Dutil 1999). Gill permeability typically increases during exposure to low dissolved oxygen, which can lead to a decrease in plasma ion concentrations (Gonzalez and McDonald 1992). Due to the low cortisol response, coupled with a low glucose response, it is not likely that largemouth

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bass experienced increased gill surface area to improve oxygen uptake, which often drives ion loss at gills (Gonzalez and McDonald 1992). Combined, the results of this study suggest an oxygen shock for high and low acclimated fish at the temperatures we examined was not significant to elicit a range of stress responses.

Following 50 days exposure to hypoxia, largemouth bass in the high oxygen group were 8% longer and 24 % heavier than fish in the low oxygen group. Fish growth typically decreases in hypoxia, likely due to reduced feeding rates (Cech et al. 1984; Pichavant et al. 2001; Brandt et al. 2009). Larger sizes for largemouth bass following prolonged holding at low oxygen is likely due to a sampling effect and relatively small sample sizes (8 individuals per treatment), and not an actual increase in growth rates. More importantly, allometry likely did not have a role in the hematological differences observed between the two oxygen groups. Additionally, length and weight were added as a covariate in the statistical analyses for all parameters measured and were found to have no effect on any of the significant effects (Engqvist 2005). Previous studies have shown the response of largemouth bass to hypoxia can differ between smaller and larger individuals, however, these differences were observed over a much wider range of fish sizes (23-500g largemouth bass compared to 1000-3000g largemouth bass; Burleson et al. 2001).

In conclusion, we demonstrated hematological differences between largemouth bass acclimated to a low oxygen environment relative to control individuals following a low oxygen shock. These compensatory differences may be attributed to a beneficial acclimation of the low oxygen group to an increase in the number of erythrocytes (i.e., increased surface area for oxygen binding). The differences observed may have important implications for an understanding of how fish in the environment respond to chronic changes in environmental variables. Fish exposed to environments such as urban streams where low oxygen levels are

frequently observed (Burton and Pitt 2002) may utilize beneficial changes to their phenotypes to compensate when behavioral (e.g., movement away from low oxygen environments) mechanisms are not feasible. These long-term changes can be of benefit and are advantageous for the animal to survive in these environments.

Acknowledgements

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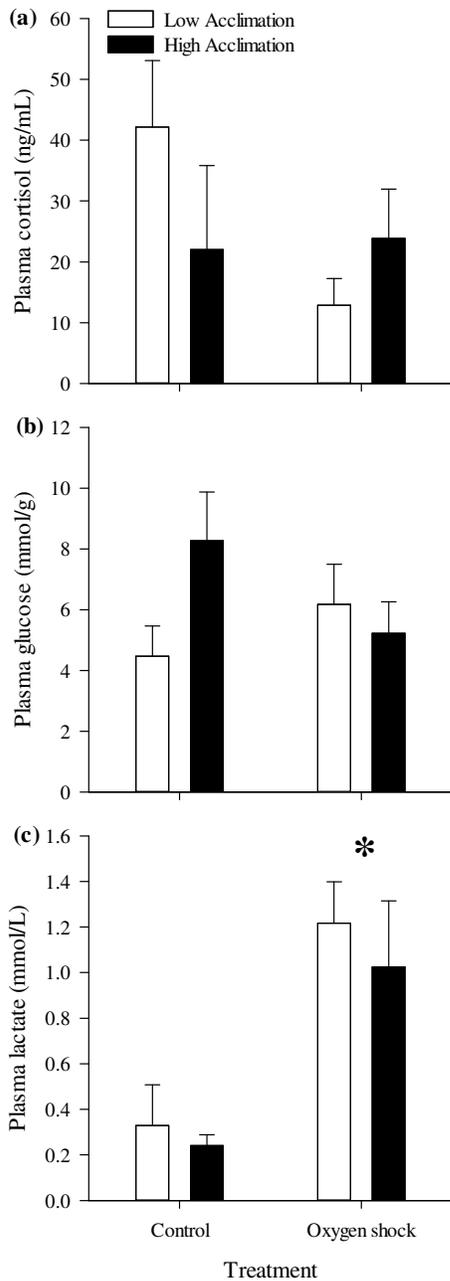   and reduced pollutant retention. J Contemp Water Res Educ 139:15-21

Fig. 5 Concentrations of plasma cortisol (a), plasma glucose (b), and plasma lactate (c) for largemouth bass held for 50 days at high dissolved oxygen (8.0 mg/L – H) or 50 days at low dissolved oxygen (3.0 mg/L – L) and then subjected to a low oxygen shock (2 mg/L for 6 h). Control fish for each group were held for 6 h at 8.0 mg/L. Error bars show ±1 SE and an asterisk (*) indicates a significant treatment effect.
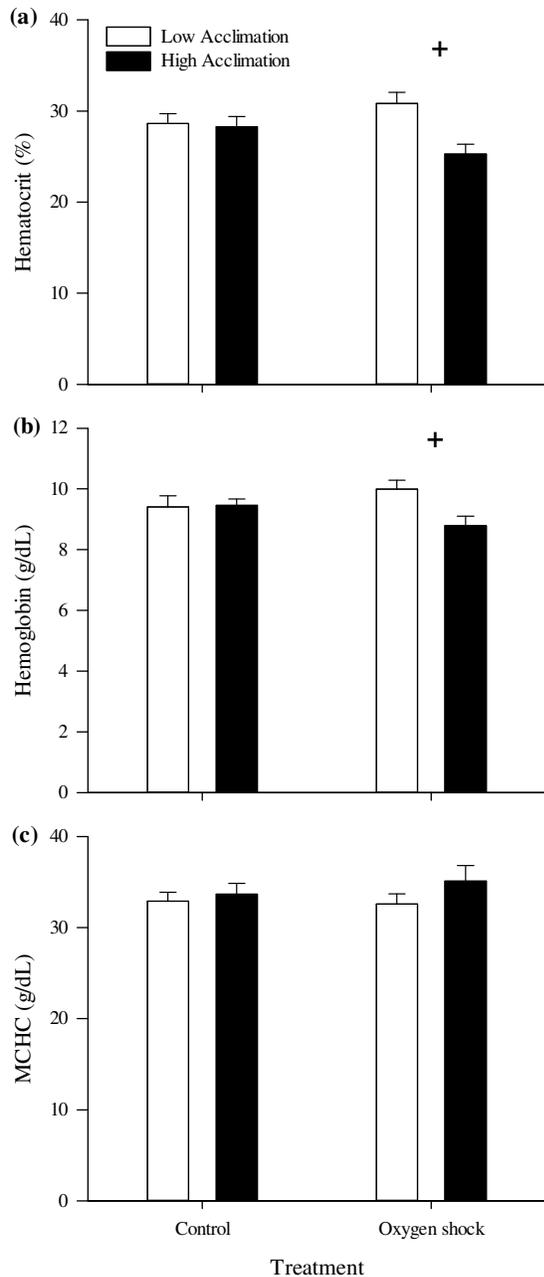
Fig. 6 Concentrations of hematocrit (a), hemoglobin (b), and MCHC (c) for largemouth bass held for 50 days at high dissolved oxygen (8.0 mg/L – H) or 50 days at low dissolved oxygen (3.0 mg/L – L) and then subjected to a low oxygen shock (2 mg/L for 6 hrs). Control fish for each group were held for 6 h at 8.0 mg/L. Error bars show ±1 SE and plus sign (+) indicates a statistically significant difference between low and high acclimation means after a low oxygen shock.
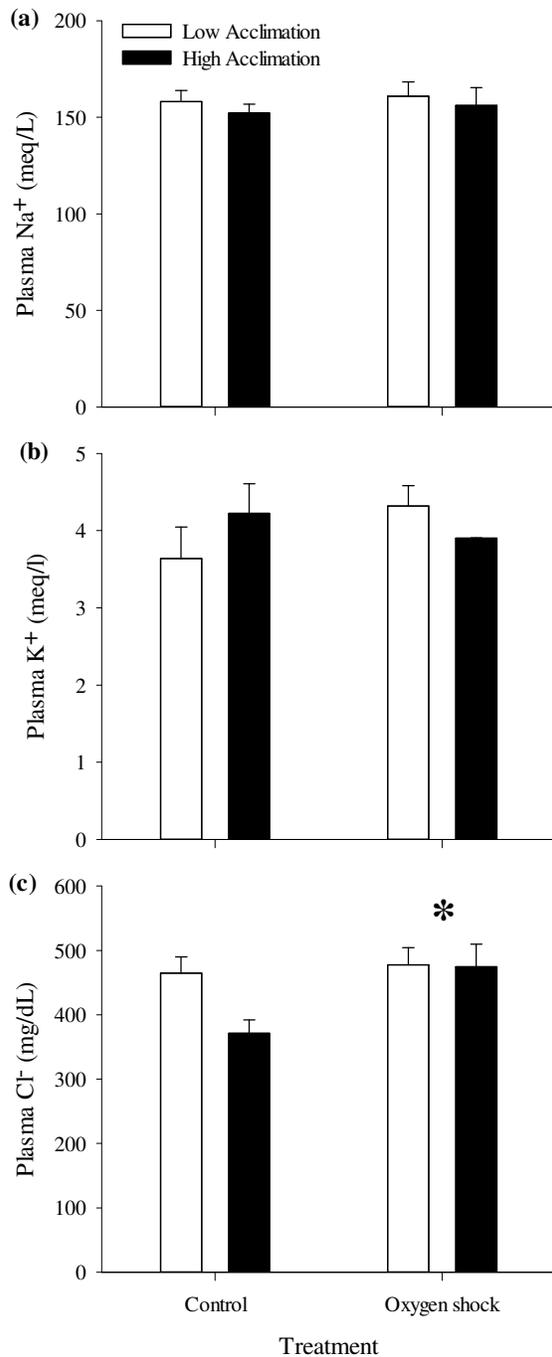
Fig. 7 Concentrations of plasma Na^+ (a), plasma K^+ (b), and plasma Cl^- (c) for largemouth bass held for 50 days at high dissolved oxygen (8.0 mg/L – H) or 50 days at low dissolved oxygen (3.0 mg/L – L) and then subjected to a low oxygen shock (2 mg/L for 6 h). Control fish for each group were held for 6 h at 8.0 mg/L. Error bars show ±1 SE and an asterisk (*) indicates a significant treatment effect.

CHAPTER 4: CONCLUSION

Understanding the effects of hypoxia on aquatic fauna is becoming an increasingly important topic as urbanization continues to grow throughout both the United States and globally. Studies such as these are important to both researchers and managers when crafting and creating guidelines meant to be protective of aquatic organisms. The two separate, yet complementary studies described in this thesis help to bridge the knowledge gap that exists concerning the behavioral and physiological response of largemouth bass to low dissolved oxygen levels, both in the laboratory and in the field.

The two chapters of this thesis demonstrate that while largemouth bass are relatively hypoxia tolerant, when exposed to chronic low oxygen levels, they are able to induce plastic changes to hematocrit and hemoglobin levels. Animals living in such environments are typically unable to seek more favorable refuge and understanding how these animals survive during these disturbances is becoming increasingly important. Places such as the Chesapeake Bay and the Mississippi River delta are two highly publicized areas of concern in relation to hypoxia. Understanding how aquatic species cope with these changing environments, especially the aforementioned, have important implications to further guide management practices and enacting policies that protect these species. Despite largemouth bass being relatively hypoxia-tolerant, the implications from this study can further the knowledge gap between hypoxia responses and other less-tolerant species. This study serves as a starting-point for future research to consider these potential mechanisms, and others, when studying the effect of chronic hypoxia on fish species. The findings in this thesis can further elucidate the mechanisms by which aquatic fauna cope with environmental stressors.

It is important for fisheries managers and policy-makers to promote guidelines and rules that are protective of aquatic species, all while not severely impacting human activities. While the results of this study suggest largemouth bass in the CAWS are not suffering chronic effects of the environments in which they reside, the species abundant in that area are severely skewed towards generalists, with more oxygen- and pollutant- sensitive species completely absent, indicating the system is in poor condition. Because the CAWS is being utilized more and more by fisherman and small craft enthusiasts, it seems imperative to restore fish diversity and richness, and overall improvements to water quality to a point where it becomes economically feasible to sustain a recreational fishery and aquatic environment. Because many sections of the CAWS still do not comply with DO regulations set by the EPA (e.g. remain above 4 mg/L year-round), and there appears to be no commitment to change this, a potential recommendation (yet highly unlikely scenario) would be to shutdown of the CSO system and restructure the sewage system in Chicago to handle the large quantities of waste water and sewage without discharging into the aquatic environment. A secondary step to improve the aquatic environment from the long-term damage would be a complete dredge of the substrate within the CAWS to remove the toxins and oxygen-depleting substances that are leading to the decline in fish diversity and abundance. Until drastic measures are taken, this system will continue to degrade, potentially to the point where it will be completely uninhabitable.